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MICROBIAL TARGETS OF THE HUMORAL IMMUNE RESPONSE IN PERIODONTAL DISEASE

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Thesis submitted for the degree of PhD to the Faculty of Medicine,
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Table of Contents

List of Tables IX

List of Figures XI

List of Abbreviations XVI

Acknowledgements XX

Declaration XXII

Summary XXIII

Chapter 1 General Introduction 1

1.1 Biological Structure of the Periodontium 1

1.1.1 Introduction 1

1.1.2 The gingiva 1

1.1.3 The oral epithelium 3

1.1.4 The sulcular epithelium 3

1.1.5 The junctional epithelium 3

1.1.6 Clinical features of the healthy gingiva 5

1.1.7 Clinical features of the diseased periodontium 5

1.2 The Classifications of Periodontal Disease 6

1.2.1 Normal gingiva 6

1.2.2 Gingival diseases 6

1.2.2.1 Plaque-induced gingivitis associated with local factors 7

1.2.2.2	Plaque-induced gingivitis associated with local factors and modified by specific systemic factors	8
1.2.2.3	Gingival diseases associated with systemic diseases	10
1.2.3	Periodontitis	10
1.2.3.1	Chronic periodontitis	11
1.2.3.2	Aggressive periodontitis	12
1.3	**The Microbiology of Periodontal disease**	**13**
1.3.1	Introduction	13
1.3.2	*Porphyromonas gingivalis*	20
1.3.3	*Actinobacillus actinomycetemcomitans*	21
1.3.4	*Prevotella intermedia*	23
1.3.5	*Bacteroides forsythus*	23
1.3.6	Spirochaetes	24
1.3.7	Microbial composition associated with health	25
1.3.8	Microbial composition associated with gingivitis	26
1.3.9	Microbial composition associated with periodontitis	27
1.4	**The Host Immune Response**	**27**
1.4.1	Introduction	27
1.4.2	The innate immune system	27
1.4.3	The inflammatory response	29
1.4.4	Complement	29
1.4.5	Prostaglandins	31
1.4.6	Cytokines in inflammation	31
1.4.6.1	Interleukin-1	32
1.4.6.2	Interleukin-6	33
1.4.6.3	Interleukin-8	33
1.4.6.4	Tumour necrosis factor-α	34
1.4.7	Adhesion molecules	35
1.4.7.1	The selectins	36
1.4.7.2	The integrins	37

1.4.7.3	The immunoglobulin superfamily	38
1.4.8	Polymorphonuclear leukocytes	42
1.4.9	The adaptive immune system	46
1.4.10	The humoral immune response	47
1.4.10.1	IgM	54
1.4.10.2	IgA	55
1.4.10.3	IgG	56
1.4.10.4	IgD	57
1.4.10.5	IgE	57
1.4.11	The role of the adaptive humoral immune response in periodontal disease	58
1.4.12	The local antibody response	58
1.4.13	The systemic antibody response	61
1.4.14	The cell mediated immune response	64
1.4.15	T cell functions	67
1.4.1 1.4.15.1	The role of $CD4^+$ T cells in periodontal disease	67
1.4.15.2	Gamma delta T cells	68
1.4.16	Natural killer cells	70
1.5	**Cytokines in Periodontal Disease**	**71**
1.5.1	Interleukin-1	71
1.5.2	Tumour necrosis factor	72
1.5.3	Interleukin-10	73
1.6	**Important Antigens in Periodontal Disease**	**76**
1.6.1	Introduction	76
1.6.2	Outer-membrane proteins	83
1.6.3	Fimbriae	86
1.6.4	Lipopolysaccharide	89
1.6.5	Heat shock proteins	92
1.6.6	Leukotoxin	95
1.6.7	Cysteine proteases	97
1.6.8	Superantigens	99

1.6.9	Other strategies for identifying immunodominant antigens	101
1.7	**Pathogenesis of Periodontal Disease**	**103**
1.7.1	Introduction	103
1.7.2	Gingivitis	104
1.7.3	Periodontitis	105
1.8	**Risk Factors**	**110**
1.8.1	Introduction to risk factors	110
1.8.2	Age	110
1.8.3	Low socio-economic status	110
1.8.4	Smoking	111
1.8.5	Systemic disease	111
1.8.6	Other possible risk factors	112
1.8.7	Genetics	113
1.9	**Aims and Objectives of Thesis**	**114**
1.9.1	Introduction	114
1.9.2	Aims of chapter 3: A comparison of the changes in the humoral immune response to antigens of periodontal pathogens following periodontal therapy	114
1.9.3	Aims of chapter 4: Immortalised B cells from periodontal disease patients	115
1.9.4	Aims of chapter 5: Analysis of the target of antibody secreting cells from diseased tissue of chronic periodontitis patients by ELISPOT	115
1.9.5	Aims of chapter 6: Detection of antibody-bearing plasma cells in periodontitis granulation and gingival biopsy tissue	115
1.9.6	Layout of the thesis	116
Chapter 2	**Materials and Buffers**	**117**
2.1	**Introduction**	**117**
2.2	**Materials**	**117**

2.2.1	A comparison of the changes in the humoral immune response to antigens of periodontal pathogens following periodontal therapy: materials	117
2.2.2	Immortalised B cells from periodontal disease patients: materials	120
2.2.3	Analysis of the target of antibody secreting cells from diseased tissue of chronic periodontitis patients by ELISPOT: materials	122
2.2.4	Detection of antibody-bearing plasma cells in periodontitis granulation and gingival biopsy tissue: materials	123
2.3	**General Buffers**	**124**
Chapter 3	**A Comparison of the Changes in the Humoral Immune Response to Antigens of Periodontal Pathogens Following Periodontal Therapy**	**129**
3.1	**Introduction**	**129**
3.2	**Methods Part 1: Enzyme Linked Immunosorbent Assay**	**136**
3.2.1	Subjects and blood samples	136
3.2.2	Antigen preparation	137
3.2.3	Analysis of sera samples by Enzyme Linked Immunosorbent Assay	139
3.2.4	Statistical methods	140
3.3	**Methods Part 2: Adsorptions**	**141**
3.3.1	Subjects and blood samples	141
3.3.2	Antigen preparation	141
3.3.3	Adsorption of samples	142
3.3.4	Analysis of sera samples by Enzyme Linked Immunosorbent Assay	142
3.4	**Results**	**143**

3.4.1	Results of part 1 of the study: Enzyme Linked Immunosorbent Assay	143
3.4.1.1	Relationship between treatment and antibody titres	164
3.4.1.2	Relationship of pocket depths and antibody titres	165
3.4.1.3	Other parameters	165
3.4.2	Results of part 2 of the study: Adsorptions	165
3.5	**Discussion**	**171**
Chapter 4	**Immortalised B Cells From Periodontal Disease Patients**	**180**
4.1	**Introduction**	**180**
4.1.2	Epstein-Barr transformation	183
4.1.3	The CD40 system	184
4.2	**Methods**	**186**
4.2.1	Subjects	186
4.2.2	Cell culture	186
4.2.3	The CD40 system	187
4.2.4	Lymphocyte preparation	187
4.2.5	Preparation of AET-treated sheep red blood cells	188
4.2.6	T cell depletion	188
4.2.7	Expansion of the B cell numbers by the Banchereau method	189
4.2.8	Enzymatic dissociation of granulation tissue	189
4.2.9	PEG fusion	190
4.2.10	Growth and preparation of bacteria	191
4.2.11	Analysis of hybridoma supernatants by the Enzyme Linked Immunosorbent Assay	192
4.3	**Results**	**192**
4.3.1	L-cell culture	192
4.3.2	CRL-1668 cell culture	192

4.3.3	The CD40 system	192
4.3.4	Cell fusion	196
4.4 Discussion		**196**
Chapter 5	**Analysis of the Target of Antibody Secreting Cells From Diseased Tissue of Chronic Periodontitis Patients By ELISPOT**	**201**
5.1	**Introduction**	**201**
5.2	**Methods**	**205**
5.2.1	Patients and tissue samples	205
5.2.2	Antigen preparation	205
5.2.3	Enzymatic dissociation of granulation tissue	206
5.2.4	Enumeration of specific immunoglobulin producing cells by ELISPOT	206
5.2.5	Statistical methods	207
5.3	Results	208
5.4	Discussion	218
Chapter 6	**Detection of Antibody-Bearing Plasma Cells in Periodontitis Granulation and Gingival Biopsy Tissue**	**223**
6.1	**Introduction**	**223**
6.2	**Methods**	**228**
6.2.1	Preparation of Fab fragments of goat anti-human IgG	228
6.2.2	Subjects and samples	229
6.2.3	Method 1	229
6.2.3.1	Bacteria preparation	229
6.2.3.2	Immunohistochemistry	230
6.2.4	Method 2	231
6.2.4.1	Antigen preparation	231
6.2.4.2	Immunohistochemistry	231

6.2.5	Method 3	232
6.2.5.1	Antigen preparation	232
6.2.5.2	Biotinylation of bacteria and bacterial sonicates	233
6.2.5.3	Biotinylation of purified antigens	234
6.2.5.4	Immunohistochemistry	234
6.2.6	Data collection and analysis	235
6.3	**Results**	**236**
6.4	**Discussion**	**260**
Chapter 7	**Methodological Considerations and General Conclusions**	**271**
7.1	**Methodological considerations**	**271**
7.2	**Conclusions**	**271**
7.3	**Suggestions for future research**	**276**
7.3.1	Examination of the effect of treatment on the humoral immune response	276
7.3.2	Further work on monoclonal antibody production	276
7.3.3	Detection of antibody-bearing plasma cells in granulation and gingival tissue	276
7.4 Concluding remarks		**277**
Bibliography		**278**
List of publications		**369**

List of Tables

Table	Title	Page
Table 1.1	Major Adhesion Molecules	41
Table 1.2	The Role of Cytokines in Periodontal Disease	75
Table 3.1	Polyvalent immmunoglobulin titres before and after treatment and the differences between these	144
Table 3.2	Polyvalent Immunoglobulin, IgG and IgA titres before and after treatment and comparisons of these for *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *B. forsythus* and *T. denticola*	152
Table 3.3	The correlation between change in pocket depth and change in antibody titre following treatment	155
Table 3.4	To show the correlation between pre-treatment pocket depth and pre-treatment antibody titre	158
Table 3.5	To show the correlation between length of treatment and change in antibody titre following treatment	161
Table 3.6	The median percentage reduction in antibody titre following adsorption compared to sham adsorbed sera	166
Table 5.1	To show the median number of ELISPOTs enumerated against the periodontal bacteria tested	215
Table 5.2	To show the median difference in the number of ELISPOTs enumerated for the two different cell concentrations	216
Table 6.1	Median figures for cells per field bearing antibody to the whole bacterial cells and purified antigens tested. Magnification x200 for granulation tissue sections taken from patients with chronic periodontitis	247

Table 6.2 Median figures for cells per field bearing antibody to the whole bacterial cells and purified antigens tested. Magnification x200 for gingival tissue sections taken from patients with chronic periodontitis 247

Table 6.3 Median differences in the number of cells per field for the micro-organisms and purified antigens tested in the granulation and gingival tissue samples. Magnification x200 for granulation and gingival tissue sections taken from patients with chronic periodontitis 250

Table 6.4 Mean figures for the percentage of the plasma cells per field specific for the antigens tested. Magnification x200 for granulation and gingival tissue sections taken from patients with chronic periodontitis 252

Table 6.5 Mean figures for the percentage of plasma cells per field specific for the whole bacterial cells and antigens tested for each of the patients individually. Magnification x200 for granulation and gingival tissue sections taken from patients with chronic periodontitis 254

List of Figures

Figure	Title	Page
figure 1.1	The structure of IgG1	48
figure 1.2	The pristine gingiva	107
figure 1.3	The healthy gingiva	107
figure 1.4	The early gingival lesion	108
figure 1.5	The established gingival lesion	108
figure 1.6	The advanced lesion	109
figure 3.1	Plot of polyvalent immunoglobulin titres pre- and post-treatment against *P. gingivalis*	146
figure 3.2	Plot of polyvalent immunoglobulin titres pre- and post-treatment against *P. gingivalis* OMP	146
figure 3.3	Plot of polyvalent immunoglobulin titres pre- and post-treatment against the β segment of RgpA	147
figure 3.4	Boxplot of the difference between pre- and post-treatment antibody titres against LPS	150
figure 3.5	Plot of IgG titres pre- and post-treatment against *P. gingivalis*	153
figure 3.6	Plot of IgA titres pre- and post-treatment against *P. gingivalis*	153
figure 3.7	Plot of change in pocket depth against change in antibody titre for *B. forsythus*	156
figure 3.8	Plot of change in pocket depth against change in antibody titre for *P. gingivalis*	156
figure 3.9	Plot of pre-treatment pocket depth against pre-treatment antibody titre for *A. actinomycetemcomitans*	159
figure 3.10	Plot of length of treatment against change in antibody titre for heat shock protein 60 of *P. gingivalis*	162
figure 3.11	Plot of length of treatment against change in antibody titre for *P. gingivalis*	162

figure 3.12 Plot of the median percentage reduction in antibody titre against *P. gingivalis* following adsorption against *P. gingivalis*, *A. actinomycetemcomitans*, *B. forsythus* and *P. intermedia* when compared to the titre observed against the sham adsorbed sera 167

figure 3.13 Plot of the median percentage reduction in antibody titre against *A. actinomycetemcomitans* following adsorption against *P. gingivalis*, *A. actinomycetemcomitans*, *B. forsythus* and *P. intermedia* when compared to the titre observed against the sham adsorbed sera 168

figure 3.14 Plot of the median percentage reduction in antibody titre against *B. forsythus* following adsorption against *P. gingivalis*, *A. actinomycetemcomitans*, *B. forsythus* and *P. intermedia* when compared to the titre observed against the sham adsorbed sera 169

figure 3.15 Plot of the median percentage reduction in antibody titre against *P. intermedia* following adsorption against *P. gingivalis*, *A. actinomycetemcomitans*, *B. forsythus* and *P. intermedia* when compared to the titre observed against the sham adsorbed sera 170

figure 4.1 L cells 194

figure 4.2 Heteromyeloma cells 194

figure 4.3 Proliferating cells at day 3 of culture 195

figure 4.4 Proliferating cells at day 11 of culture 195

figure 5.1a *P. gingivalis* ELISPOT 209

figure 5.1b Control 209

figure 5.2a *A. actinomycetemcomitans* ELISPOT 210

figure 5.2b Control 210

figure 5.3a *P. intermedia* ELISPOT 211

figure 5.3b Control 211

figure 5.4a *B. forsythus* ELISPOT 212

figure 5.4b Control 212

figure 5.5	Repeated measures boxplot for the addition of 10^8 B lymphocytes	213
figure 5.6	Repeated measures boxplot for the addition of $5x10^7$ B lymphocytes	213
figure 6.1a	Antibody-bearing cells specific for *P. gingivalis* in a granulation tissue sample	237
figure 6.1b	Control for *P. gingivalis*	237
figure 6.2a	Antibody-bearing cells specific for *A. actinomycetemcomitans* in a granulation tissue sample	237
figure 6.2b	Control for *A. actinomycetemcomitans*	237
figure 6.3a	Antibody-bearing cells specific for *P. intermedia* in a granulation tissue sample	238
figure 6.3b	Control for *P. intermedia*	238
figure 6.4a	Antibody-bearing cells specific for *B. forsythus* in a granulation tissue sample	238
figure 6.4b	Control for *B. forsythus*	238
figure 6.5a	Antibody-bearing cells specific for *T. denticola* in a granulation tissue sample	239
figure 6.5b	Control for *T. denticola*	239
figure 6.6a	Antibody-bearing cells specific for *E. coli* in a granulation tissue sample	239
figure 6.6b	Control for *E. coli*	239
figure 6.7a	Antibody-bearing cells specific for HSP60 (of *P. gingivalis*) in a granulation tissue sample	240
figure 6.7b	Control for HSP60 (of *P. gingivalis*)	240
figure 6.8a	Antibody-bearing cells specific for leukotoxin (of *A. actinomycetemcomitans*) in a granulation tissue sample	241
figure 6.8b	Control for leukotoxin (of *A. actinomycetemcomitans*)	241
figure 6.9a	Antibody-bearing cells specific for the β segment of RgpA (of *P. gingivalis*) in a granulation tissue sample	242
figure 6.9b	Control for the β segment of RgpA (of *P. gingivalis*)	242

figure 6.10 Plot of number of specific antibody-bearing cells per field against different concentrations of whole fixed bacteria used. Magnification x200 for granulation tissue sections from patients with chronic periodontitis 243

figure 6.11 Plot of number of antibody-bearing cells per field against different concentrations of purified antigen. Magnification x200 for granulation tissue sections from patients with chronic periodontitis 245

figure 6.12a Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the granulation tissue sample of patient 1 255

figure 6.12b Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the gingival tissue sample of patient 1 255

figure 6.13a Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the granulation tissue sample of patient 2 256

figure 6.13b Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the gingival tissue sample of patient 2 256

figure 6.14a Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the granulation tissue sample of patient 3 257

figure 6.14b Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the gingival tissue sample of patient 3 257

figure 6.15a Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the granulation tissue sample of patient 4 258

figure 6.15b Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the gingival tissue sample of patient 4 258

figure 6.16a Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the granulation tissue sample of patient 5 259

figure 6.16b Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the gingival tissue sample of patient 5 259

List of Abbreviations

Aa	*Actinobacillus actinomycetemcomitans*
A. actinomycetemcomitans	*Actinobacillus actinomycetemcomitans*
Aa OMP	*Actinobacillus actinomycetemcomitans* outer-membrane proteins
ADCC	Antibody-dependent cell mediated cytotoxicity
APC	Antigen presenting cell
AT	After periodontal treatment
ATCC	American Type Culture Collection
A. viscosus	*Actinomyces viscosus*
Bf	*Bacteroides forsythus*
B. forsythus	*Bacteroides forsythus*
Bf OMP	*Bacteroides forsythus* outer-membrane proteins
BOP	Bleeding on probing
BT	Before periodontal treatment
CB	Coating buffer
CD	Cluster of differentiation antigen(s)
CD3	Integral part of the T cell receptor complex
CD4	Marker for T-helper cell(s)
CD8	Marker for cytotoxic T cell
CD45RA	Marker for naive and resting memory T cell
CD45RO	Marker for activated memory T cell
C.I.	Confidence interval
CTL	Cytolytic T lymphocyte
C. rectus	*Campylobacter rectus*
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus

Ec	*Escherichia coli*
E. coli	*Escherichia coli*
ELAM-1	Endothelial Leukocyte Adhesion Molecule-1
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunospot
E. saburreum	*Eubacterium saburreum*
FAA	Fastidious anaerobe agar
FCS	Foetal calf serum
FDC	Follicular dendritic cell
F. nucleatum	*Fusobacterium nucleatum*
GC	Germinal centre
GCF	Gingival crevicular fluid
GlyCAM-1	Glycosylated Cellular Adhesion Molecule-1
HEL	Hen egg lysozyme
H. influenzae	*Haemophilus influenzae*
HIV	Human immunodeficiency virus
HPT	Hygiene phase therapy
HPRT	Hypoxanthine phosphoribosyl transferase
HRP	Horseradish peroxidase
HSP	Heat shock protein
HVJ	Hemagglutinating virus of Japan
ICAM (-1, 2, 3)	Intercellular Adhesion Molecule
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
INF-γ	Interferon gamma
kDa	Kilodalton

LAD	Leukocyte adhesion deficiency
LBP	Lipopolysaccharide binding protein
LFA (-1, 2, 3)	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
LOS	Lipooligosaccharide
LTX	Leukotoxin
M. avium	*Mycobacterium avium*
MAC	*Mycobacterium avium* complex
MadCAM-1	Mucosal Addressin Cellular Adhesion Molecule-1
MHC	Major histocompatability complex
mRNA	Messenger Ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NK cell	Natural killer cell
OMP	Outer-membrane protein
PALs	Periarteriolar lymphoid sheaths
P.D.	Probing pocket depth
PECAM	Platelet Endothelial Cell Adhesion Molecule
Pg	*Porphyromonas gingivalis*
P. gingivalis	*Porphyromonas gingivalis*
Pg OMP	*Porphyromonas gingivalis* outer-membrane proteins
Pi	*Prevotella intermedia*
P. intermedia	*Prevotella intermedia*
Pi OMP	*Prevotella intermedia* outer-membrane proteins
PGE_2	Prostaglandin E_2
PMN	Polymorphonuclear leukocyte
P. nigrescens	*Prevotella nigrescens*
RNA	Ribonucleic acid

rRNA	Ribosomal Ribonucleic acid
RTX	Repeats in toxin
SRBC	Sheep red blood cells
TCR	T cell receptor
Td	*Treponema denticola*
T. denticola	*Treponema denticola*
TH	T-helper cell subset
TNF-α	Tumour necrosis factor alpha
T. pectinovarum	*Treponema pectinovarum*
T. socranskii	*Treponema socranskii*
T. vincentii	*Treponema vincentii*
V. alcalescens	*Veillonella alcalescens*
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA-4	Very Late Antigen-1

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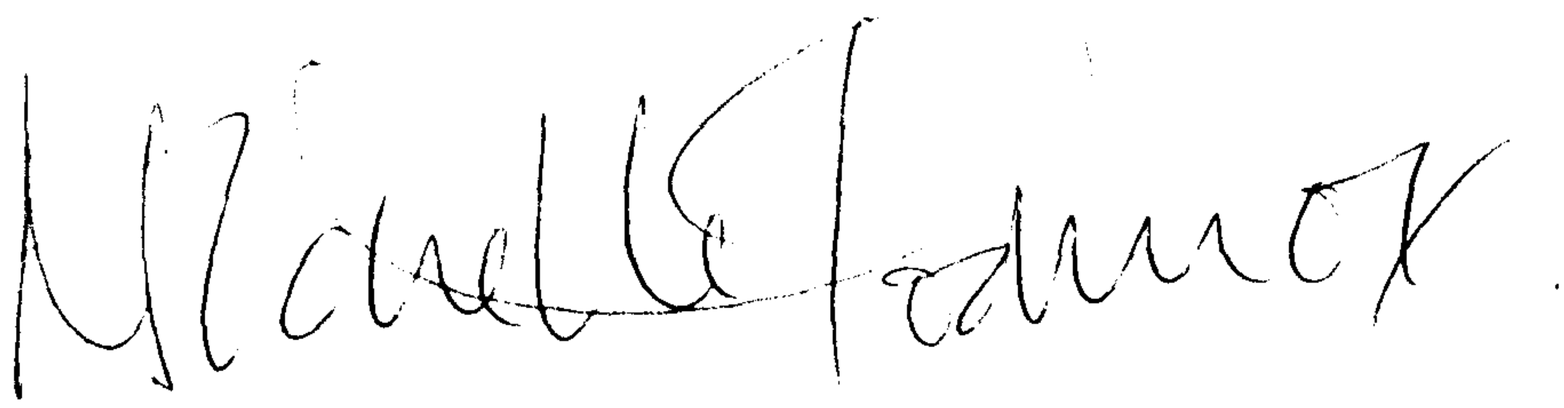
Declaration

This thesis is the original work of the author



Michelle Podmore BSc

Summary

Periodontitis is a ubiquitous infectious disease severely affecting 10% of the population. There are many areas of our knowledge regarding this disease that are incomplete. In particular, the microbial, inflammatory and immunological aetiology and pathogenesis of this disease remain the subject of many current investigations.

Substantial literature exists on the humoral immune response against micro-organisms present within periodontal pockets and tissues. However, the literature is complex and it is difficult to elucidate due to the large number of putative periodontal pathogens, the large array of immunogenic antigens present on the surface of these micro-organisms and the potential cross reactive nature of them. In addition, there is enormous variation in both individual immune responses and the timing of a serum sample collection with respect to treatment stage and disease course. Identification of the immunodominant antigen or antigens involved in the disease process of periodontitis would be extremely informative, as it would pin point the important pathogens relevant to periodontal disease and identify the specific bacterial antigens of importance. In addition, it may furnish us with new approaches to diagnosis, aid the selection of potential vaccine candidates and suggest new therapeutic possibilities.

This thesis reports on investigations carried out to identify the important antigens inducing a humoral immune response in periodontal disease. A number of different techniques were used. The first study examined the systemic immune response, specifically the effects of treatment on the immune response of patients with chronic periodontitis against a panel of periodontal pathogens and a large panel of antigen preparations from these bacteria. The results of the study indicated that there was no difference in antibody titre following treatment against any of the putative periodontal antigenic targets tested. These results conflict with many published reports, however, differences in factors such as length of treatment and antigen preparation often make the comparison of different studies futile.

An investigation to examine the phenomenon of cross-reactivity between antibodies induced against putative periodontal pathogens was the second part of this overall project. The results suggested a high proportion of cross-reactivity between the 4 periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus*. Results indicating such a high cross-reactivity between these micro-organisms prove that the issue of cross-reactivity is an important one that should be considered when carrying out any immunological and microbiological investigations in this field.

Following the above investigations of the systemic immune response the remainder of this thesis is a report of three studies examining the local immune response in periodontal disease. The study reported in chapter 4 was an attempt to produce human monoclonal antibodies from immortalised antibody-bearing cells. These were isolated from the granulation tissue of diseased sites and the peripheral blood of patients with chronic periodontal disease. The aim of this study was to produce monoclonal antibodies against periodontal pathogens to be able to examine the antigenic target of the antibodies produced in more detail and to assess which bacteria induced humoral responses. Sequencing of the antigen binding domain of a monoclonal antibody produced in this way could indicate which antigen on the surface of a micro-organism is immunogenic and the target of the immune response in a particular disease. The results were disappointing in terms of monoclonal antibody production, however, antibodies specific for putative periodontal pathogens were initially produced which were indicative that the technique has potential for this type of study.

An alternative approach was, therefore, undertaken to investigate the local immune response in periodontal disease. Chapter 5 describes the study that was carried out to examine the target of antibody-bearing cells isolated from granulation tissue removed from patients with chronic periodontal disease. This was investigated utilising Enzyme Linked Immunospot (ELISPOT), a technique which detects antibody-secreting cells directly. The results of the study indicate that there are antibody-bearing cells in diseased periodontal lesions specific to the pathogens tested, and there is no significant difference in the number of antibody-bearing cells detected against the 4 micro-organisms.

The final study set out to detect and enumerate antibody-bearing cells in tissue samples and this was accomplished by the development of an innovative and unique technique. The technique used was an immunohistochemical technique developed for this project. The results of this experiment were in agreement with those of the ELISPOT technique, that there was no significant difference in the number of cells detected against the antigenic targets tested. When the number of cells detected against the antigenic targets were compared in the granulation and gingival tissues sections there were significantly more cells detected against *P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia* in the granulation tissue sections. When the numbers of cells were expressed as percentages of the total number of infiltrating plasma cells, however, this difference was not seen. To have the ability to express the numbers of cells specific for a particular antigen as a percentage of the total infiltrate, and in particular the total plasma cell infiltrate, could have major effects in the determination of the importance of a particular micro-organism and antigenic target and in the overall magnitude of a specific response.

The future identification of the target of the humoral immune response in periodontal disease could have wide-ranging effects, not only in the progression of research and knowledge but also in terms of diagnostics and therapeutics. Numerous reports have suggested that elevations in humoral immune responses to micro-organisms are associated with disease severity (Genco *et al.*, 1980b; Mouton *et al.*, 1981; Tolo and Brandtzaeg, 1982; Listgarten *et al.*, 1981; Ebersole *et al.*, 1982b). Thus the discovery of the target of the humoral immune response is of utmost importance in diagnosis of disease activity and the possibilities of enhancing or modifying the humoral immune response to achieve treatment outcome benefits.

Chapter 1. General Introduction

1.1 Biological Structure of the Periodontium

1.1.1 Introduction

A definition of the periodontium is "the tissues investing and supporting the teeth" (Hassell, 1993). This includes the alveolar bone, root cementum, periodontal ligament, and the gingivae. Together these form a functional unit (Lindhe and Karring, 1997), which can be reversibly or irreversibly disrupted by the inflammatory mechanisms of disease. Periodontal disease is a general term for the inflammatory reactions affecting the periodontium. From the above description, it can be seen that there are a number of different tissue types from a number of different development origins making up the periodontium, thus disease affecting any one of these tissues is strictly speaking a disease of the periodontium (Kinane and Davies, 1990). In the context of this thesis the term periodontal disease will be restricted to denote gingivitis and periodontitis, as classified in section 1.2.

1.1.2 The gingiva

The gingiva is the part of the oral mucosa that covers the alveolar processes of the jaws and the cervical portions of the teeth. Anatomically, it is divided into three distinct areas; the marginal, attached and interdental areas. The tissues collectively termed the gingiva are said to belong to the oral mucous membrane and the periodontium (Schroeder and Listgarten, 1997). Clinically, the gingiva is regarded as a combination of epithelial and connective tissues. These make up the mucosa that is around the teeth of the complete deciduous or permanent dentition and is attached to both teeth and alveolar processes (Schroeder and Listgarten, 1997).

The gingiva is normally pink in colour, has a scalloped outline, a firm texture, stippling, and is demarcated apically from the oral mucosa by the mucogingival line. The colour of the gingivae may vary due to various amounts of melanin pigmentation.

The mucogingival line normally resides approximately 3-5mm apical to the level of the alveolar crest. Attached keratinsed gingiva extends coronally from the mucogingival line and is firmly bound to the underlying periosteum by collagen fibres. The corono-apical width of the attached gingiva can vary significantly from tooth to tooth. The free gingival margin, which is normally about 1.5mm in the corono-apical dimension, surrounds but is not attached to each tooth. In health the gingiva completely fills the embrasure space between the teeth, and this part is termed the papilla (Wennström, 1988).

The gingival sulcus consists of the tooth surface on one side and sulcular epithelium of the free gingiva on the other. It is a shallow crevice, and in fully developed teeth is lined coronally with sulcular epithelium, the non keratinised extension of the oral epithelium. The bottom of the sulcus is formed by the coronal surface of the junctional epithelium.

The attached gingiva is continuous with the free gingiva and its apical extension leads to the mucogingival junction. At this point it is continuous with the relatively loose and movable alveolar mucosa. The attached gingiva is tightly attached to the underlying alveolar bone and demonstrates comparative immobility relative to the underlying tissue. The width of the attached gingiva differs among different teeth (Bowers, 1963).

The interdental gingiva occupies the interproximal space beneath the area of tooth contact. The interdental gingiva can be pyramidal in shape i.e. one papilla's tip is immediately beneath the contact point, or have a 'col' shape i.e. a valley-like depression that connects a facial and a lingual papilla and conforms to the shape of the interproximal contact (Cohen, 1959).

The marginal gingiva is comprised of three areas of epithelium; the oral epithelium which faces the oral cavity, the sulcular epithelium which faces the tooth without any contact with the tooth surface and the junctional epithelium which is in contact with the tooth surface. The principal cell type of the gingival and oral epithelium is the keratinocyte, which constitutes more than 90% of the gingival epithelium. Other cell

types present include Langerhans cells (DiFranco *et al.*, 1985), Merkel cells (Ness Morton and Dale, 1987) and melanocytes (Schroeder, 1969a). Keratinisation is a process that involves a sequence of biochemical and morphologic events that occur in the cell during its migration from the basal layers towards the surface (Schroeder, 1969b). The gingival epithelium is able to express three types of surface differentiation; keratinisation, where the surface cells form scales of keratin and lose their nuclei, parakeratinisation, where the cells of the superficial layers retain their nuclei but there is no granular layer present, and non-keratinisation where the cells preserve their nuclei but all signs of keratinisation are absent.

1.1.3 The oral epithelium

The crest and the outer surface of the marginal gingiva and the surface of the attached gingiva are covered by the oral epithelium. This epithelium is made up of cells that are stratified squamous type, and exhibit keratinisation or parakeratinisation or both. The epithelium is bound to connective tissue by a basal lamina (Stern, 1965). In health the border between the oral epithelium and the connective tissue is characterised by deep epithelial ridges or rete pegs which are separated from each other by connective tissue papillae.

1.1.4 The sulcular epithelium

The sulcular epithelium lines the gingival sulcus and is a thin, non-keratinised stratified squamous epithelium displaying rete pegs.

1.1.5 The junctional epithelium

The junctional epithelium is also known as the dento-gingival epithelium. It is characterised by a collar-like band of stratified squamous epithelium and can range in length from 0.25 to 1.35mm. The junctional epithelium is attached to the tooth surface by a basal lamina (Listgarten, 1966). Unlike the oral and sulcular epithelium, the junctional epithelium lacks rete pegs (Lindhe and Karring, 1997).

The lamina propria consists of supra-alveolar fibre apparatus, blood and lymphatic vessels and nerves (Schroeder and Listgarten, 1997). The fibre apparatus is a dense network of fibre bundles which make up 55-60% of the connective tissue volume. The fibres are densely populated by fibroblasts and consist of collagen type I and III (Narayanan and Page, 1976). Mast cells are regularly found in this area and lymphocytes, monocytes and macrophages can also be evident. The supra-alveolar fibre apparatus plays an important role in attaching the gingiva to the teeth and bone, and also provides a dense framework that gives the rigidity and biochemical resistance to the gingiva (Schroeder and Listgarten, 1997). As well as providing this important rigid framework, the fibre apparatus also protects the cellular defences that are located at the dento-gingival interface (Schroeder, 1986).

The gingival lamina propria or connective tissue, is highly vascularised. Branches of the main arteries supplying the blood to the maxilla and the mandible reach the gingiva from 3 sites; the interdental septa, the periodontal ligament and the oral mucosa (Castelli, 1963, Saunders and Röckert, 1967). The blood vessels of the gingiva are arterioles, capillaries and small veins. The nutritional supply for the gingival epithelium is provided by thin capillaries that terminate immediately below the basement membrane. The lymphatic drainage of the periodontal tissues involves the lymphatics of the connective tissue papillae. The lymph progresses into the collecting network of the larger lymph vessels and is drained into the regional lymph nodes of the head and the neck, before entering the blood circulation. Lymphatics just below the junctional epithelium extend into the periodontal ligament and accompany the blood vessels. High endothelial venules are not present when the gingiva is healthy, however, even in the early stages of experimental gingivitis, high endothelial venules are seen. It is considered their presence may be induced by mediators of inflammation. The reason for their presence is not confirmed, however, it may indicate a process of selective homing and migration of lymphocytes to the gingiva on infection (Wynne *et al.*, 1988).

1.1.6 Clinical features of the healthy gingiva

In 'normal' appearance the gingiva is pink in colour, firm in consistency and the gingival margin has a clear scalloped outline. It does not bleed when gently probed and fills the entire space between adjacent teeth (Ainamo and Löe, 1966; Wennström, 1988). Characteristically in health, the depth of the gingival sulcus is minimal at the microscopic level, with the alveolar bone located 1mm apical to the cementoenamel junction (Eliasson *et al.*, 1986). The oral surface is covered by oral epithelium that is keratinised and fused with the junctional epithelium. The junctional epithelium is weakly attached to the tooth surface by hemidesmosomes. The oral and junctional epithelia are supported by the collagenous structures of the underlying connective tissue. Directly below the junctional epithelium is a microvascular plexus containing numerous venules (Egelberg, 1967).

1.1.7 Clinical features of the diseased periodontium

The discussion of the pathogenesis and histopathology of gingivitis and periodontitis can be found in section 1.7 of this thesis. As discussed there, the inflammatory and immune reactions that results from the hosts response to the plaque microflora and its products are visible both histopathologically and clinically in the affected periodontium.

Periodontal disease is broadly classified into two distinct entities; gingivitis and periodontitis. Gingivitis refers to the pathological changes which are confined to the gingivae. Clinically this disease entity is characterised by a change in colour from pink to red of the gingivae, as well as a change in texture and appearance of the gingivae as it swells. The gingivae becomes more prone to bleeding on gentle probing and gingival sensitivity may be experienced, however, this disease is largely reversible (Löe *et al.*, 1965). Periodontitis, affects the deeper attachment apparatus including the alveolar bone, periodontal ligament and the cementum, resulting in loss of periodontal support, and is largely irreversible. It is frequently associated with the presence of periodontal pockets, bleeding on probing, and bone loss is a pathognomonic feature.

1.2 The Classifications of Periodontal Disease

1.2.1 Normal gingiva

A normal healthy gingiva is characterised as described in 1.1.6. In histological terms a normal gingivae is free from inflammation and accumulation of inflammatory cells is not significant. As described by Kinane and Lindhe (1997), two different types of 'healthy' gingivae can be described. The 'pristine' gingiva has all the characteristics described above including little or no inflammatory infiltrate. This condition can only be achieved in humans by several weeks of experimental conditions requiring supervised and meticulous daily plaque control. The second type is the 'clinically healthy' gingiva, which again has the features as described above, but histologically an inflammatory infiltrate is seen. This picture of health is clinically seen in everyday situations.

1.2.2 Gingival diseases

The following classifications of periodontal diseases and conditions is based on the recent classification system developed at the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions.

To define all the various clinical entities that effect the gingiva as 'gingivitis' is considered too restrictive and confusing. The term 'gingival diseases' has, therefore, been chosen as it provides a more comprehensive and encompassing definition of all the different entities that effect the gingiva. The following characteristics are common to all gingival diseases: (i) the clinical signs are confined to the gingiva; (ii) bacteria is present and its role is in either initiation or exacerbation of the severity of the lesion; (iii) there are clinical signs of inflammation and these include enlarged gingival contours due to oedema or fibrosis (Mühlemann and Son, 1971; Polson and Goodson, 1985) colour transition from pink to a red and/or bluish-red hue (Mühlemann and Son, 1971; Polson and Goodson, 1985), elevated sulcular temperature (Haffajee *et al.*, 1992; Wolff *et al.*, 1997), bleeding on probing (Mühlemann and Son, 1971; Löe *et al.*, 1965, Greenstein *et al.*, 1981; Engelberger *et al.*, 1983) and increased gingival exudate

(Löe and Holm-Pedersen, 1965, Egelberg 1966, Oliver *et al.*, 1969; Rüdin *et al.*, 1970); (iv) the clinical signs are not associated with attachment loss, and are therefore, reversible when the aetiologies are removed.

Gingival diseases that are dependent on the presence of dental plaque for their initiation fall into two categories; plaque-induced gingivitis that is associated with local factors, and plaque-induced gingivitis that is affected by local factors and modified by specific systemic factors found in the host.

1.2.2.1 Plaque-induced gingivitis associated with local factors

Plaque-induced gingivitis is the result of bacterial presence. The aetiology of plaque bacteria was convincingly demonstrated by classical studies of experimental gingivitis in humans (Löe *et al.*, 1965; Theilade *et al.*, 1986). These studies have shown that in healthy individuals, gingivitis can develop when plaque bacteria accumulate, particularly at the gingival margin, and can be reversed by removing the plaque. Plaque-induced gingivitis has been shown to be prevalent at all ages in dentate populations (U.S. Public Health Service, 1965; U.S. Public Health Service, 1972; Stamm, 1986; U.S. Public Health Service, 1987; Bhat, 1991). This disease is considered to be the most common form of periodontal disease (Page, 1985).

Clinically this disease is an inflammation of the gingiva. The normal 'healthy' firm, regular contour of the gingiva appears swollen. In light skinned people the normal pink colour may appear red or blue-red (Mühlemann and Son, 1971; Polson and Goodson, 1985), however, in dark skinned individuals, this may not be so obvious. Within 10-20 days clinical signs of inflammation can be detected in most individuals, although this varies between individuals markedly depending on whether an individual is resistant or prone to the disease (Van der Weijden *et al.*, 1994).

The clinical changes in the early stages of gingivitis are usually subtle, although the intensity of the symptoms will vary amongst individuals as well as between sites within a dentition (Mariotti, 1999). Histologically the symptoms of gingivitis are quite marked. Many capillary beds are opened as alterations in the vascular network

occur. The tissues begin to take on the characteristic swollen appearance as exudative fluid, proteins and inflammatory cells move into the connective tissues subjacent to the junctional epithelium. The cellular infiltrate is comprised of mainly lymphocytes, macrophages and polymorphonuclear leukocytes (PMN). The lymphocytes and macrophages adhere to the collagen matrix and remain there, whereas the PMN tend to migrate into the gingival sulcus (Kinane and Lindhe, 1997). Other changes include proliferation of basal and junctional epithelium, which leads to apical and lateral cell migration. A marked reduction in the amount of collagen and fibroblasts occurs (Kinane and Lindhe, 1997). As previously mentioned, this disease is reversible, and radiographic analysis and examination of individuals with plaque-induced gingivitis do not reveal loss of supporting tissues (Mariotti, 1999).

1.2.2.2 Plaque-induced gingivitis associated with local factors and modified by specific systemic factors

It has been shown that gingival disease can be associated with many different factors.

Endocrinotropic gingival diseases

Studies undertaken in the eighteenth century revealed that an exaggerated gingival response can be seen during pregnancy (Eiselt, 1840; Pinard, 1877). Since these early reports, many others have followed indicating that sex hormones such as androgens, oestrogens and progestins can have an effect on the periodontal tissues. These types of gingival diseases are mainly seen in females at the time of distinct hormonal events, such as menstruation (menstrual-cycle-associated gingivitis) (Hugoson, 1971) and pregnancy (pregnancy-associated gingivitis) (Hugoson, 1971; Löe and Silness, 1963; Löe, 1965; Arafat, 1974). They can also be seen in both sexes during puberty (puberty-associated gingivitis) (Mariotti, 1994). It should be noted that although the sex hormones can modulate the tissues during this disease, plaque micro-organisms must also be present to produce the gingival response.

Drug influenced gingival diseases

Drug-induced gingival enlargement has a number of characteristics and there is a wide variation in inter-patient and intra-patient patterns (Hassell and Hefti, 1991; Seymour *et al.*, 1996). It is more often found on the anterior gingiva (Hassell and Hefti, 1991; Seymour *et al.*, 1996) and there is a higher prevalence of this disease in children (Esterberg and White, 1945; Rateitschak-Plüss *et al.*, 1983; Hefti *et al.*, 1994). Its onset is usually within 3 months (Hassell and Hefti, 1991; Hassell, 1981; Seymour, 1991; Seymour and Jacobs, 1992) and enlargement is first observed at the interdental papilla (Hassell and Hefti, 1991). A change in gingival contour is seen which leads to modification of its size, and there is a change in colour of the gingiva and an increased gingival exudate. Clinically bleeding on probing is seen and it is found in the gingiva with or without bone loss, but it is not associated with attachment loss (Hassell and Hefti, 1991; Seymour *et al.*, 1996). In this disease, as with all of the gingival diseases, there is a pronounced inflammatory infiltrate in response to the dental plaque present. Removal of the bacterial aetiologies can limit the severity of the lesion (Mariotti, 1999).

This disease is associated with anticonvulsants (e.g. phenytoin), immunosuppressants (e.g. cyclosporin A) and calcium channel blockers (e.g. nifedipine, verapamil, diltiazem, sodium valproate) (Hassell and Hefti, 1991; Seymour *et al.*, 1996).

Oral-contraceptive associated gingivitis

Use of oral contraceptive agents has been linked to the development of gingival overgrowth. Several reports have shown the development of this disease in patients that had previously healthy gingivae (Kaufman, 1969; Lynn, 1969; Sperber, 1969). The reports indicate that the disease is reversed when the patients discontinue the contraceptive, or the dose is reduced. However, it has been shown that this disease is developed in the presence of very little plaque.

1.2.2 3 Gingival diseases associated with systemic diseases

Diabetes mellitus-associated gingivitis

A number of studies have been carried out investigating a possible link between diabetes and diseases of the periodontium (Bacic *et al.*, 1988; Campbell, 1972). It has also been reported that diabetes mellitus-associated gingivitis seems to be a common feature of children with poorly controlled type I diabetes. The characteristics associated with this type of gingivitis are very similar to those of plaque-induced gingivitis, however, the control of the diabetes is much more important that control of plaque levels (Cianciola *et al.*, 1982; Gusberti *et al.*, 1983; Ervasti *et al.*, 1985).

Haematologic gingival diseases

One example of these diseases is leukaemia-associated gingivitis. Most of the studies on this disease type have noted an association between the acute forms of leukaemia (Lynch and Ship, 1967). The characteristics of the disease are inflammation of the gingivae which give it a swollen, glazed appearance and the tissues appear spongy and purple (Dreizen *et al.*, 1984).

Although most of the cases of gingival diseases seen are plaque-induced, non-plaque associated types of gingivitis do exist. These can be related to bacterial, viral or even fungal infections. They will not be discussed in this thesis.

1.2.3 Periodontitis

Periodontitis is distinguished from gingivitis by the inflammation which extends to the periodontal structures, leading to a loss of connective tissue attachment of the teeth (Ranney, 1991). It has been recognised that there are different forms of periodontitis, and classifications of the different types is based on the classification system developed at the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions.

1.2.3.1 Chronic periodontitis

Until the World Workshop on classification in 1999, this form of periodontitis was referred to as adult periodontitis. It was characterised as a form of periodontitis that has an onset after the age of 35. The point of contention over the name for this form of periodontitis comes from the fact that although the prevalence, extent and severity of the disease increase with age, it is very difficult to determine at what age exactly the onset of the disease will occur. Although the disease is seen most commonly in the adult population, it can also be found in children and adolescents. Therefore, for this reason it has been renamed chronic periodontitis.

Before periodontitis can occur there has to be a pre-existing gingivitis, and hence bacterial flora in association with the tissues. However, not all cases of gingivitis progress to periodontitis (Ånerud *et al.*, 1979; Listgarten *et al.*, 1985). Periodontitis in contrast to gingivitis is seen in a much smaller subset of the population. Results from studies suggest that relatively few subjects in each age group suffer from advanced periodontal destruction, and that site predilection may be evident in the individuals affected (Jenkins and Kinane, 1989; Löe *et al.*, 1965; Papapanou *et al.*, 1988).

Clinically, periodontitis is described by a number of features including clinical attachment loss, alveolar bone loss, periodontal pocketing and gingival inflammation (Flemmig, 1999). In addition to these features, enlargement and recession of the gingiva, bleeding on probing and increased mobility, drifting and tooth exfoliation may occur (Page and Schroeder, 1976).

The recent World Workshop on classification (1999) decided that chronic periodontitis can be further characterised by the extent and severity of the disease and can be designated either localised chronic periodontitis or generalised chronic periodontitis. A guide has been given that disease can be termed localised if <30% of the sites are affected and generalised if >30% of the sites are affected.

1.2.3.2 Aggressive periodontitis

This form of periodontitis was previously referred to as early onset periodontitis. Like adult periodontitis, there has been controversy over the name early onset periodontitis, as the disease is not confined to individuals under the chosen age of 35, which is used for diagnosis. It has therefore, been decided that diagnosis of the disease should not be based on the age at the which the patient presents his or her symptoms, but by clinical, radiographic, historical and laboratory findings, as this is a more reliable method (World Workshop Consensus Report, 1999).

Aggressive periodontal disease is identified by common features which include: (i) that apart from periodontal disease the patients are fit and healthy; (ii) rapid attachment loss and bone destruction must be present; and (iii) that there is familial aggregation. The 1999 World Workshop also decided that the disease, sometimes but not always, has other characteristics and have therefore, published a list of secondary features that may be useful in diagnosis. These include: (i) that the amount of microbial deposits seen are inconsistent with the severity of the tissue destruction; (ii) that there are elevated proportions of *Actinobacillus actinomycetemcomitans* (*A. Actinomycetemcomitans*), and in some populations *Porphyromonas gingivalis* (*P. gingivalis*); (iii) there are phagocyte abnormalities; (iv) there may be a hyper-responsive macrophage phenotype evident, including elevated levels of prostaglandin E_2 (PGE_2) and interleukin-1β (IL-1β); and (v) that the progression of attachment and bone loss may be self arresting (World Workshop Consensus Report, 1999).

There are two forms of aggressive periodontal disease; localised aggressive periodontitis and generalised aggressive periodontitis. Both of these are classified by the common features listed above, however, the disease entities may be subdivided by the following features. Localised aggressive periodontitis may have: (i) a circumpubertal onset; (ii) a robust serum antibody response to infecting micro-organisms; and (iii) the disease may be localised to a first molar/incisor. There should also be interproximal attachment loss on at least two permanent teeth, one of which is a first molar, and involving no more than two teeth, other than first molars and incisors.

Generalised aggressive periodontitis may be diagnosed by the following characteristics: (i) usually the individuals presenting with this form of the disease are under 35 years of age. However, as already discussed, age is a disputable factor to use for diagnostic purposes, because classification will change the diagnosis as the patient grows older. The time that a patient presents with the disease does not necessarily mean that it is the age of onset, and therefore, may be older than 35; (ii) that individuals with this form of the disease have a poor serum antibody response to infecting agents; (iii) the destruction of attachment and alveolar bone is episodic; and (iv) there is a generalised interproximal attachment loss affecting at least 3 permanent teeth, other than first molars and incisors.

1.3 The Microbiology of Periodontal Disease

1.3.1 Introduction

Although there are various environmental factors and host predispositions associated with the onset of periodontal disease, it is accepted that this disease is associated with microbial plaque, that is micro-organisms colonising tooth surfaces (Socransky and Haffajee, 1993).

The oral cavity is made up of a number of distinct habitats, all of which experience different ecological conditions. These different ecological niches all support different populations of bacteria, and have a microbial community distinctive to themselves (Marsh, 1989). This microbial colonisation of the oral cavity originates from birth and it is thought that the colonising bacteria are acquired from the mother. Evidence for this theory comes from studies on serotype tracing, bacteriocin patterns and more recently by restriction endonuclease mapping (Berkowitz and Jordan, 1975; Kulkarni *et al.*, 1989).

There are several ways of detecting and sampling oral micro-organisms. The most commonly used sampling techniques in the detection of bacterial species in periodontal samples are dental curettes and paper points (Baehni and Guggenheim, 1996). Quantification of oral micro-organisms can be difficult. One of the main

methods used in characterisation of the oral microbiota is that of *in vitro* culturing methods, and hence, culturing observations (Baehni and Guggenheim, 1996). When using this approach, the sample is plated out on to non-selective agar, and the 'predominant cultivable flora' detected and identified. Although an effective method, this approach can be technique-sensitive and very difficult to carry out. In addition it has been estimated that less than 50% of the total flora from subgingival samples can be cultivated (Loesche *et al.*, 1992b). Other molecular biology techniques have been developed over recent years including immunoassay and DNA probe methods.

Three different types of DNA probe method have been developed; whole-genomic probes, cloned probes and synthetic oligonucleotide probes (Dewhirst and Paster, 1991). The basis for this method is that DNA segments or sequences of nucleotides of 16S rRNA unique to each bacteria are isolated. DNA probes prepared from a representative strain of the target micro-organism will hybridise to these sequences, hence, the detection of these bacteria is due to the binding of the DNA probe to the complementary strands of nucleic acids in these unique regions (Baehni and Guggenheim, 1996).

Immunological methods have also been developed to detect different bacteria species (Tanner *et al.*, 1991). Immunoassays include immunoprecipitation, immunofluorescence, immunohistochemistry, flow cytometry and Enzyme Linked Immunosorbent Assay (ELISA). Most of these methods do not identify the bacteria directly. In general polyclonal or monoclonal antibodies against species-specific surface antigens can be used to identify target bacteria (Baehni and Guggenheim, 1996).

Research by taxonomists has identified 300-400 bacterial species as being present in the human oral cavity (Moore and Moore, 1994). Some species are recognised as being transient, however, most are permanent residents (Darveau *et al.*, 1997). These have fully adapted themselves to living in the environment offered to them by both the tooth surface and the surrounding conditions of the oral cavity and gingival epithelium (Darveau *et al.*, 1997). Most of the time a dynamic equilibrium exists between the plaque bacteria and the host. Even in health, there are large numbers of micro-

organisms present in the oral cavity. The bacteria have adapted themselves to be able to survive in the environment, and the hosts immune system, both innate and adaptive arms, normally limit the growth of these micro-organisms. As mentioned, in health these micro-organisms challenge the host to maintain an effective defence. Under select conditions that may involve acquisition of certain species, combinations of species or less than optimal host defence, these bacteria can cause destructive inflammation (Darveau *et al.*, 1997).

If a tooth is freshly cleaned, a bacterial coating occurs rapidly on its surface (Marsh and Bradshaw, 1995). Within the first few hours, on the tooth surface a pellicle forms that consists of proteins and glycoproteins, that are found in saliva and gingival crevicular fluid (GCF) (Marsh and Bradshaw, 1995). Numerous studies have indicated the role of the pellicle in providing specific receptors for bacterial attachment (Duan *et al.*, 1994; Gibbons *et al.*, 1991; Hsu *et al.*, 1994; Scannapieco, 1994; Scannapieco *et al.*, 1989; Scannapieco *et al.*, 1995). The bacterial attachment is enhanced by the formation of the pellicle in both initial bacterial colonisation, and also additional bacterial attachment (Skopek and Liljemark, 1994). The term co-aggregation (Whittaker *et al.*, 1996), is used to describe the phenomenon of two genetically different bacteria recognising and binding to one another. This is the mechanism by which the formation of dental plaque biofilm occurs (Whittaker *et al.*, 1996), and is based on specific interactions of a proteinaceous adhesion produced by one bacterium and a respective carbohydrate or protein receptor found on the surface of another bacterium (Darveau *et al.*, 1997). Streptococcus species and Fusobacterium are thought to play a major role in the supragingival biofilm formation (Whittaker *et al.*, 1996).

Once the bacterial biofilm has been established, bacterial growth will occur. Characteristically, biofilms contain areas of high and low bacterial biomass interlaced with aqueous channels of different size (Costerton *et al.*, 1995). The biofilm is designed to give each bacterial species its optimal environment for growth and gaining nutrients. There are two different types of dental plaque biofilms, each of which is composed of distinctly different species; supragingival plaque, which forms above the

gingival margin and subgingival plaque which forms below the margin (Darveau *et al.*, 1997).

Supragingival plaque resides above the gingival margin and is, therefore, found in the open space of the oral cavity. Due to its location it experiences constant disruption due to mastication and salivary flow, which restricts its net accumulation (Sissons *et al.*, 1995). Supragingival plaque is also exposed to the innate immune mechanisms found in the saliva of the host. Saliva firstly, flows around the oral cavity creating a 'washing' characteristic. It also contains numerous host defence components including secretary IgA, lactoferrin, lysosyme, and peroxidases (Shenkels *et al.*, 1995). Plaque accumulation occurs in the absence of oral hygiene.

Subgingival plaque resides in a more protected location and the space available for this bacterial growth is limited in periodontally healthy individuals (Darveau *et al.*, 1997). More space can be made however, by reduction of the epithelial cell attachment levels and by an increase in pocket depth, related to this and the gingival swelling. GCF is not always advantageous to the host. Although it contains the innate and adaptive components of the host defence (Cimasoni, 1983), it also contains nutrients for the biofilm. In terms of the studies carried out in this thesis, subgingival plaque plays a more pivotal role in periodontal disease and the immune response that is induced. The following discussion highlights the species of importance in this disease, the characteristics they possess that make them harmful and their role in the disease progression. The problem with a discussion of this type when considering periodontal disease, is that unlike most other diseases, where the presence of some bacterial species can indicate disease, prevalence does not always correlate with periodontal disease. It is known that dental plaque is a complex permanent community and that the presence of large numbers of an indigenous species alone is not enough to lead to periodontal disease. The balance of the species probably plays a role, as well as the characteristics of the teeth, the gingival crevice and the host itself (Liljemark and Bloomquist, 1996).

The bacteria resident in plaque could be advantageous to the host as well as making the host susceptible to disease. Firstly, it has been suggested that exposure to normal

microbial flora (commensal flora) may protect against colonisation by more pathogenic strains of the same species. Many indigenous microbes are thought to be closely related to their more virulent relatives and often possess common antigens (Liljemark and Bloomquist, 1996). Antibodies induced in an individual against a common antigen may provide a rapid immune response against a more pathogenic strain should the situation arise.

Evidence for the accepted dogma, that most periodontal diseases are caused by bacteria in dental plaque (Socransky and Haffajee, 1990; Socransky and Haffajee, 1991; Socransky and Haffajee, 1992), is strong and comes from several sources including: (i) Studies that show a correlation between most forms of gingivitis and periodontitis, and accumulated dental plaque; (ii) evidence suggesting that elimination of plaque micro-organisms leads to clinical improvement; and (iii) *in vivo* and *in vitro* reports indicating that different plaque micro-organisms have been shown to have virulent properties (Zambon, 1996).

The last 3 decades have seen the emergence of the concept of microbial specificity in the aetiology of periodontal disease (Loesche, 1976; Socransky, 1977). Until discussions began as to the relative importance of individual bacterial species within the dental plaque, it was believed that periodontal disease resulted from the gross accumulation of dental plaque. This theory, known as the non-specific plaque hypothesis, suggested that the plaque associated with periodontal disease causes the disease when the homogeneous mass accumulates to the point of exceeding the host defences. This hypothesis explained previous clinical experiences when investigators linked the universal presence of gingival inflammation and periodontal pocket formation with the presence of abundant plaque (Tanner, 1988). Loesche (1976) suggested that periodontal disease be considered as non-specific because: (i) the lack of bacterial invasion. Bacterial invasion has been demonstrated, but it does not appear, as yet, to be part of an acute phase of disease progression; (ii) their apparent non-specific nature; (iii) their chronicity; and (iv) their universality. This non-specific hypothesis was also supported by Löe *et al.* (1965), who demonstrated that cessation of oral hygiene is associated with the concominant development of

gingivitis, and that resumption of oral hygiene eliminated the accumulated plaque mass and resolved the marginal gingivitis.

This non-specific theory has been disregarded because the 300-400 bacterial species in the oral cavity show different characteristics and therefore, must play different roles (Dahlén, 1993). The non-specific hypothesis is contrasted by that of the specific plaque hypothesis. This states that the dental plaque micro-organisms isolated from periodontitis lesions are qualitatively different from those isolated from healthy sites (Loesche, 1976). Further evidence from cross-sectional and longitudinal studies of the predominant cultivable microflora, indicated that although periodontal diseases are polymicrobial infections, only a small number of species are associated with human periodontal disease (Moore *et al.*, 1983; Theilade, 1986; van Winkelhoff *et al.*, 1988a; Haffajee & Socransky, 1994).

The concept of bacterial specificity in periodontal disease has been further supported by clinical observations, by therapeutic effect of control of these bacteria, and by experimental models of periodontitis in both gnotobiotic and conventionally maintained animals. However, the continued finding of increased numbers of a range of species in periodontitis patients, the presence of suspected agents in inactive sites, the failure of "specific" antibiotics to stop disease progression, and the absence of these agents in some active sites indicates that the specific plaque hypothesis may be invalid (Tanner, 1988).

Therefore, while it is accepted that gingival inflammation can be associated with the build up of non-specific dental plaque micro-organisms, periodontitis may be associated with a much smaller number of bacterial species. The actual identification of these important pathogenic species remains elusive, but there are a number of bacteria which are considered possible candidates.

In an attempt to identify periodontal pathogens the approach has been to use the criteria known as Koch's postulates. Koch's postulates were originally developed when Robert Koch was attempting to identify the aetiology of monoinfections such as the importance of *Mycobacterium tuberculii* in tuberculosis (Zambon, 1996). They

are less useful in the quest for the identification of periodontal pathogens for a number of reasons, firstly, it is a polymicrobial infection. Further to this, the postulates cannot be fulfilled for organisms that cannot be grown in pure culture, that demonstrate a long incubation period, that express pathogenicity first after another infectious agent has weakened the host immune response and that exhibit a range that is restricted to humans or to animal species in which the human disease cannot be reproduced (Slots, 1999). Therefore, in recent years, Koch's postulates have been extended by periodontal research workers, to try and develop a set of postulates especially for the study of chronic infectious diseases such as periodontitis. As reported by Socransky (1977), the criteria used for identifying bacteria as being important in the aetiology of periodontal disease include: (i) compared to low numbers or absence in healthy or non-progressing sites there is a high number of the micro-organism in the periodontal lesion. The micro-organism is to be identified at numbers above a critical threshold prior to initiation of progressive disease, and statistical determination of the micro-organism as a risk factor; (ii) clinical improvement in the site should be seen on elimination of the micro-organism; (iii) host responses to the micro-organism should be seen. Indicators of this are high levels of specific antibody in the serum, saliva or GCF and a cell mediated immune response; (iv) clinical disease can be correlated with virulence factors; and (v) demonstration of tissue destruction in the presence of the micro-organism in appropriate animal models.

A small number of the 300-400 of species that inhabit the oral cavity have been implicated as periodontal pathogens. These species include; *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*), *Bacteroides forsythus* (*B. forsythus*), *Campylobacter rectus* (*C. rectus*), *Fusobacterium nucleatum* (*F. nucleatum*), *Prevotella intermedia* (*P. intermedia*), *Prevotella nigrescens* (*P. nigrescens*), *Porphyromonas gingivalis* (*P. gingivalis*) and treponemes (Zambon, 1996).

Longitudinal studies examining the periodontal pathogens in sites undergoing clinical attachment loss have provided much of the evidence implicating pathogens in periodontal disease. In brief, pocket depth, attachment levels, subgingival temperatures, bleeding on probing, suppuration, redness and plaque were all

monitored in a group of 67 patients with no prior evidence of destructive periodontal disease. In addition to these clinical indices, the levels of 14 subgingival species were determined in subgingival plaque samples. These included *A. actinomycetemcomitans* serotypes a and b, *B. forsythus*, *F. nucleatum ss vincentii*, *Peptostreptoccus micros*, *P. gingivalis*, *P. intermedia* homology groups I and II, *Streptococcus intermedius* and *C. rectus*, also four beneficial species *Capnocytophaga ochracea*, *Streptococcus sanguis* I and II and *Veillonella parvula*. The results of this particular study confirmed the role of certain suspected pathogens (Haffajee *et al.*, 1991). However, microbial species have been shown not to be evenly distributed from subject to subject or even from site to site, and even more so between different periodontal disease entities. A further problem connected with the clinical indices is that it is generally impossible to sample subgingival plaque bacteria at exactly the same time as clinically detectable connective tissue loss occurs. Therefore, speculation arises as to whether the resulting micro-organisms are the cause of the connective tissue loss or arrive secondary to it (Zambon, 1996).

Of the putative periodontal pathogens, stronger evidence exists for some more than others. The following discussion looks at the micro-organisms considered to be the more important species in periodontal disease. It is important to stress that these bacterial species are currently considered as putative periodontal pathogens, however, whether these pathogens are true aetiologic agents remains elusive.

1.3.2 *Porphyromonas gingivalis*

Members of the *Porphyromonas gingivalis* species have the characteristics of being non-motile, asaccharolytic, Gram negative and obligately anaerobic coccobacilli which exhibit smooth, raised colonies. The morphology of the colonies may vary from smooth to cauliflower shaped on blood agar plates. The normal habitat of *P. gingivalis* is the oral cavity and most likely the gingival sulcus (Olsen *et al.*, 1999). The organism is rarely found in supragingival plaque or outside the oral cavity (van Winkelhoff *et al.*, 1988a).

These species show excellent properties for evading the host immune responses due to the production of enzymes, proteins and end products of their metabolism, which are active against a large number of host proteins (Holt *et al.*, 1999). Many strains of *P. gingivalis* possess an external capsule. A strong correlation has been identified between the extent of encapsulation and several biological functions that have a significant effect on the biological properties of the bacteria (Holt *et al.*, 1999). Studies have suggested that there is a decreased tendency of the encapsulated strains to be phagocytosed, an important factor for host response evasion (Schifferle *et al.*, 1993, Sundqvist *et al.*, 1991). *P. gingivalis* has been shown to differ from other Gram negative bacteria, in that it does not have the capacity to directly stimulate the production of E-selectin by human endothelial cells, thus, hindering the migration of leukocytes into the extravascular compartment (Darveau *et al.*, 1995). Further to this, lipopolysaccharide (LPS) of *P. gingivalis* has also been shown to bind LPS binding protein (LBP) very poorly, leading to poor myeloid cell activation (Cunningham *et al.*, 1996). Both of these strategies by this micro-organism hinder the protective characteristics of the innate host response. In addition, the cysteine proteases of *P. gingivalis* appear to be of great physiological importance, and will be discussed later in section 1.6.7.

P. gingivalis is found rarely in health, however is often found in disease, particularly in periodontitis (Slots and Listgarten, 1988; van Winklelhoff *et al.*, 1988a; Moore *et al.*, 1991). It has also been shown that there is a higher load of *P. gingivalis* in active lesions compared with inactive lesions (Dzink *et al.*, 1988; Walker and Gordon, 1990). Evidence from studies investigating the effect of therapy on disease, indicate that levels of *P. gingivalis* remain high in sites that have responded poorly to therapy (van Winkelhoff *et al.*, 1988b; Choi *et al.*, 1990). High proportions of *P. gingivalis* were found in sites following breakdown of treatment, (Choi *et al.*, 1990) indicating that this species may play an important role in the pathogenesis of disease recurrence.

1.3.3 *Actinobacillus actinomycetemcomitans*

Actinobacillus is a member of the family of Pasteurellaecae. This family are a group of nonenteric, fermenting Gram negative rods. The term 'Actinobacillus' refers to the

internal star shaped morphology of its bacterial colonies on solid media, and to the short rod or bacillary shape of individual cells. Many of the family are found in animals, however, only *A. actinomycetemcomitans* is routinely cultured from humans (Lally *et al.*, 1996). *A. actinomycetemcomitans* has been associated with a number of disease entities in man including; infective endocarditis, brain abscesses, osteomyelitis, subcutaneous abscesses and several forms of periodontal disease (Block *et al.*, 1973; Page and King, 1966; Zambon *et al.*, 1983; Zambon, 1985).

Most periodontally healthy adults show low or no detectable levels of subgingival *A. actinomycetemcomitans* (Alaluusua and Asikainen, 1988; Gmür and Guggenheim, 1994). There are five known serotypes of *A. actinomycetemcomitans*, a-e, and studies have indicated that serotype c seems to be found predominantly in health and that serotype b is found more frequently in patients with periodontal disease (Asikainen *et al.*, 1991).

A. actinomycetemcomitans was first described to be found in increased numbers in patients with localised aggressive periodontitis (Slots *et al.*, 1980; Zambon, 1985). More recently, studies on localised aggressive periodontitis have shown *A. actinomycetemcomitans* to be higher in numbers in periodontal lesions when compared with non-diseased sites (van Winkelhoff *et al.*, 1994), and in active sites compared to inactive sites (Haffajee *et al.*, 1984; Mandell, 1984). Although, *A. actinomycetemcomitans* has been linked predominantly with aggressive periodontitis, it is still implicated in chronic periodontitis (Gmür and Guggenheim, 1990).

Further evidence of a pathogenic role for *A. actinomycetemcomitans* comes from studies investigating the effects of periodontal treatment on the microbiological parameters. Studies have reported a correlation between successful treatment and suppression of *A. actinomycetemcomitans*, below detectable levels and unsuccessful treatment and a failure to suppress numbers of this micro-organism (Christersson *et al.*, 1985; Mandell *et al.*, 1986). Given the virulence of *A. actinomycetemcomitans* a number of authors have suggested that elimination of this micro-organism should be the goal of any periodontal therapy. Failure to eliminate this bacteria can lead to continued attachment loss or reduced healing (Zambon, 1996).

This species produces a number of virulence factors, such as cell surface carbohydrates and leukotoxin. Leukotoxin has drawn much interest and may play an important role in periodontal disease. It will not be covered here as a more comprehensive discussion of the topic can be found in section 1.6.6.

1.3.4 *Prevotella intermedia*

Prevotella intermedia is a black pigmented, short, round-ended anaerobic rod. It is thought that *P. intermedia* has a number of virulence properties also exhibited by *P. gingivalis*. Recently two species of *P. intermedia* that show identical phenotypic traits have been separated into two species; *P. intermedia* and *P. nigrescens* (Shah and Gharbia, 1992).

P. intermedia has been associated with many forms of periodontal disease (Slots, 1982; Loesche *et al.*, 1982; Slots and Listgarten, 1988; Williams *et al.*, 1985). Levels of *P. intermedia* have been shown to be elevated in certain forms of periodontitis (Dzink *et al.*, 1983, Moore *et al.*, 1985; Tanner *et al.*, 1979). Both the frequency of detection and levels of *P. intermedia* are increased in active periodontitis lesions (Slots *et al.*, 1986; Dzink *et al.*, 1988; Moore *et al.*, 1991), and it has been detected attached to epithelial cells in increased numbers at diseased sites (Dzink *et al.*, 1989; Dibart *et al.*, 1998). *P. intermedia* is a likely candidate for a periodontopathic organism, however, it does not appear to be as prevalent or pathogenic as *P. gingivalis*.

1.3.5 *Bacteroides forsythus*

Bacteroides forsythus is a Gram negative, anaerobic, spindle shaped, highly pleomorphic rod. The last decade has seen the utilisation of new molecular biology techniques, such as detection of bacteria in plaque samples using immunofluorescence with polyclonal and monoclonal antibodies (Lai *et al.*, 1987, Gmür, 1988) and DNA probes (Moncla *et al.*, 1991). This work has led to suggestions that *B. forsythus* is a putative periodontal pathogen. Until these studies, *B. forsythus* was rarely reported to be one of the more predominant organisms in periodontal lesions. This was probably

due to the difficulties that occur when trying to culture *B. forsythus*. Culture of *B. forsythus in vitro* has many specific requirements (Wyss *et al.*, 1989) and often requires 7-14 days for minute colonies to develop. Growth of this organism in vitro has been shown to be enhanced by co-cultivation with *F. nucleatum*, which it has been shown to commonly occur in subgingival sites (Socransky *et al.*, 1988). *B .forsythus* has a requirement for N-acetylmuramic acid, and inclusion of this in media enhances growth of this micro-organism (Wyss *et al.*, 1989).

B. forsythus has been detected in the supragingival plaque of healthy patients (Gmür and Guggenheim, 1994). However, studies have shown *B. forsythus* to be at higher proportions in patients with gingivitis and periodontitis, than healthy subjects (Lai *et al.*, 1987; Gmür, 1988; Moncla *et al.*, 1991). Dzink *et al.* (1988) showed *B. forsythus* to be at increased proportions and with increased frequency in active sites compared to quiescent sites of periodontitis patients, and Lai *et al.* (1987) also showed increased numbers of the micro-organism in sites with periodontal breakdown.

1.3.6 Spirochaetes

Of the oral spirochaetes, the *Treponema* genera appears to be regarded as one of the more important. There are currently four named human species: *T. denticola*; *T. vincentii*; *T. pectinovarum*; and *T. socranskii*. The organism *T. denticola* has been consistently reported to be associated with periodontal disease (Simonson *et al.*, 1988; Haapasalo *et al.*, 1992; Zambon, 1996), and is the cultivable spirochaete which predominates in most patients with chronic periodontal disease (Moter *et al.*, 1998). Spirochaetes are not usually detected in healthy sites, but reach high levels in sites of disease. It is thought they average 30-50% of the microscopic counts in subgingival plaque samples recovered from periodontal pockets (Listgarten and Helldén, 1978). However, it has been difficult to ascertain whether the oral treponemes are present in large numbers in subgingival plaque from periodontitis sites and discover whether they are aetiologically relevant. Moter and colleagues (1998) recently suggested that the environment of the periodontal pocket is a very suitable niche, with its anaerobic environment and plentiful supply of host and microbial molecules for enriched growth of these species.

Spirochaete numbers are generally increased in patients with chronic periodontitis and can be found in very high prevalence (60-100%) (Loesche *et al.*, 1985; Savitt and Socransky, 1984; Riviere *et al.*, 1992; Riviere *et al.*, 1995). They have also been linked with increasing pocket depth, bleeding on probing and bone loss (Omar *et al.*, 1991; Tanner *et al.*, 1984; Savitt and Socransky, 1984).

A number of potential virulence factors have been reported for *T. denticola* and following the trend seen for other putative periodontal pathogens, potential significant antigens have been suggested. These include: a periplasmic flagella, which provides motility (Boehringer *et al.*, 1986); and two proteases (Ohta *et al.*, 1986; Grenier *et al.*, 1990). The latter could contribute to the pathogenesis of periodontal disease, through their activity on other bacteria and host proteins (Grenier, 1996). A 53 kDa porin (Olsen *et al.*, 1984; Haapasolo *et al.*, 1992; Egli *et al.*, 1993); some major outer-membrane proteins, which appear to function in attachment of the micro-organism to various host cells (Weinberg *et al.*, 1990); and cystalysin, which enzymatically produces H_2S and acts an hemolysin (Chu *et al.*, 1997). Difficulties encountered when working with these often uncultivable species, have contributed to minimal data detailing the host immune responses to the oral treponemes.

Further evidence indicating the importance of *T. denticola* is given in a study by Simonson *et al.* (1992) which shows a decrease in the proportion of *T. denticola* following successful treatment, however, MacFarlane *et al.* (1988) reported that spirochaetes are poor predictors of future disease activity.

1.3.7 Microbial composition associated with health

Further studies of the microbial composition of a healthy gingivae could provide important information regarding the distribution and numbers of bacteria present between health and disease. This could also lead to a clearer picture of micro-organisms that colonise and play a more important role in disease progression.

The nature of periodontal disease makes comparison with healthy gingiva difficult as mentioned earlier. Subgingival plaque resides in more protected locations and the

space available for bacterial growth is limited in periodontally healthy individuals (Darveau *et al.*, 1997). Much of the data regarding health has come from evaluation of healthy sites that have been selected as controls in other studies. The type of studies that this kind of data is obtained from includes; the starting point of experimental gingivitis (Listgarten *et al.*, 1975; Moore *et al.*, 1982; Syed and Loesche, 1978; Theilade *et al.*, 1966) and healthy sites in periodontally diseased subjects (Moore *et al.*, 1987; Moore and Moore, 1994; Tanner *et al.*, 1979). The literature suggests the bacterial load associated with health in young individuals is relatively low and the micro-organisms present are predominantly Gram positive, streptococci and actinomyces. Approximately 15% of the microbiota isolated seem to be Gram negative rod species (Moore *et al.*, 1982; Syed and Loesche, 1978; Tanner *et al.*, 1996). The situation appears to be a little different in older individuals. It has been reported that in healthy individuals, with no signs of previous gingivitis, up to 45% of the microbiota isolated from them appeared to be Gram negative bacteria (Newman *et al.*, 1978; Slots, 1977). Microbiota isolated from healthy sites of adult patients with periodontitis were also identified as being from a similar range of Gram negative microflora (Moore and Moore, 1994; Tanner *et al.*, 1979). The results discussed above suggest that colonisation of healthy sites with Gram negative bacteria occurs, however, it does not appear to result in disease alone.

1.3.8 Microbial composition associated with gingivitis

Although it is not the only predisposing factor for disease, colonisation by Gram negative bacteria is necessary for the onset and progression of disease. Many studies, both cross-sectional and longitudinal, have reported that gingivitis is associated with a change in the balance of Gram negative and Gram positive organisms and an increased microbial load. The microbial load has been suggested to change from approximately 10^2-10^3 in health to 10^4-10^6 in gingivitis and that there is an increase of 15-50% of Gram negative organisms isolated from gingivitis versus health (Gmür *et al.*, 1989; Lai *et al.*, 1987; Listgarten *et al.*, 1975; Moore *et al.*, 1987; Moore *et al.*, 1984; Moore *et al.*, 1982; Preus *et al.*, 1995; Riviere *et al.*, 1996; Slots *et al.*, 1978; Syed and Loesche, 1978; Tanner *et al.*, 1996; Theilade *et al.*, 1966).

1.3.9 Microbial composition associated with periodontitis

A further increase in microbial load is seen in periodontitis compared to health and gingivitis, and the load is reported to be in the range of 10^5-10^8 micro-organisms (Darveau *et al.*, 1997). A significant proportion of periodontal sites harbour periodontal pathogens at a high prevalence, and this prevalence is seen to increase in deep periodontal pockets (Wolff *et al.*, 1993). All of the above micro-organisms are found at high prevalence in disease sites of patients with periodontal disease.

1.4 The Host Immune Response

1.4.1 Introduction

The immune system can be divided into two broad categories; the innate response and the adaptive immune response. Innate immunity is present from birth and does not change or adapt in response to a particular antigen. The adaptive or acquired immune response is specific and is acquired during the lifetime of an individual. The following discussion highlights the role of these two arms of the immune response in the onset and progression of periodontal disease.

1.4.2 The innate immune system

The innate immune response was first described by the Russian immunologist Elie Metchnikoff (1905). Metchnikoff discovered that many micro-organisms could be engulfed and digested by phagocytic cells called macrophages. This non-specific defence against infection is inborn or innate since its action does not depend upon prior exposure to a particular antigen (Janeway and Travers, 1994).

The innate immune response is important as it is the first line of defence against infection. It can be divided into two clear phases. The first phase operates immediately, is constantly present and requires no induction. Physical barriers such as the skin and mucous membranes represent this component, which infectious agents must breach to gain access to the host. The washing action of fluids such as saliva,

tears, urine and GCF keeps mucosal surfaces clear of invading organisms. These secretions also contain bacteriocidal agents. The intact epithelial barrier of the gingival, sulcular and junctional epithelium usually prevents bacterial invasion of the periodontal tissues. The epithelial cell wall, secretes proteins and fatty acids which are toxic to many microbes. Salivary secretions provide a continuous flushing of the oral cavity as well as providing a continuing supply of agglutinins and antibodies. In addition, the GCF flushes the gingival sulcus and delivers all the components of the serum. Most epithelia of the body are associated with a normal flora of non-pathogenic bacteria which compete with pathogenic micro-organisms for nutrients and attachment sites on cells. The normal flora can also produce anti-microbial substances that prevent colonisation by other bacteria.

All of these physical and chemical barriers to infection may be considered to be part of the innate immune system since they are non-specific and do not become more effective after exposure to a particular pathogen. However, barriers can be breached. All surfaces of the host can be damaged by cuts, grazes and burns which allow pathogens entry, and some bacteria can break down the barriers with enzymes. Thus, a second line of defence is necessary.

Within seconds of a breach in the epithelial barrier, the second phase of the innate immune response is initiated. This is induced but still non-specific for the particular invading organisms, and does not generate immunological memory. One of the first signs of this phase of the immune response is inflammation. An inflammatory response can be triggered directly by pathogens, especially bacteria. The term itself is purely descriptive and was originally defined by the four Latin words *dolor*, *rubor*, *calor* and *tumor*, meaning pain, redness, heat and swelling. The quality of the host inflammatory response is critical to the disease process, although its purpose is protection and prevention of bacterial invasion, it can also be detrimental. It may also be an ineffective, chronic frustrated response which actually causes much of the tissue damage that occurs in periodontal disease. As the inflammatory immune response is non-specific and is induced against many different infectious agents all over the body, it seems reasonable to assume that it is not defective. Periodontitis patients, are not

typically susceptible to infections at other sites and hence it can be assumed that the inflammatory immune response that they induce, is effective.

1.4.3 The inflammatory response

The effects that occur due to the induction of an inflammatory response result from dilation of the local blood vessels. These vessels also become permeable and show expression of adhesion molecules which capture passing leukocytes. The reaction is accelerated by the first cells to arrive. These cells release molecules or inflammatory mediators which recruit more cells and which further enhance the reaction. The inflammatory response is mediated by an array of molecules including complement components, the contents of mast cell and basophil granules, and molecules secreted by macrophages. There is strong evidence that inflammatory mediators play a role in periodontal disease. In the periodontium, inflammatory mediators such as cytokines, proteinases, thrombin, histamine and prostaglandins are evident. These are produced and released from the activated infiltrating cells, plasma cells, resident fibroblasts and other connective tissue cells, and from activated complement and other plasma proteins (Page, 1991). They are produced in the blood plasma by the complement cascade and kinin system. Monocytes from individuals susceptible to or suffering from severe periodontitis, produce elevated amounts of mediators (Garrison and Nichols, 1989). Mediators are present within the inflamed gingiva and in the GCF of diseased sites in high concentrations. Concentrations of inflammatory mediators usually decrease following successful periodontal therapy (Offenbacher *et al.*, 1993).

1.4.4 Complement

Complement is a collective term for a series of about 20 serum proteins that circulate in the body fluids in an inactive form. When the first component of a complement cascade is activated, a complex reaction is set in motion. The reaction products of each step in the sequence activate the next component in the cascade. A complement cascade can occur due to both innate and adaptive immune responses. The final products of a complement cascade are known as the membrane attack complex. This is a cylindrical assembly of molecules that are inserted into the cell wall of the

microbe in question. The insertion of this complex, opens up a pore through which sodium ions and then water flood into the cell, hence causing death (Janeway and Travers, 1994).

The alternative pathway of the complement cascade can occur in the absence of specific antibodies when directly triggered by a microbial surface. In this way the same antimicrobial complement actions take place as seen in the classical pathway, but without the delay of 5-7 days that it takes to induce a specific antibody response. The necessary characteristics of a pathogens cell surface that allow direct triggering of the complement cascade are unknown. Endotoxins such as LPS from cell walls of Gram negative bacteria have been suggested as a possible trigger (Eley and Cox, 1998).

The protein C3 is abundant in the plasma and C3b is constantly produced by spontaneous cleavage. Although most of this will be inactivated by hydrolysis, some will bind covalently to the surface of host cells and pathogens. Binding of C3b leads to the binding of Factor B and the initiation of the cascade. The C3b that binds to the surface of host cells will not generally lead to the initiation of further activation steps. Cell surface proteins such as decay accelerating factor, membrane co-factor protein and the plasma protein factor H, prevent further activation.

Not only is the membrane attack complex of importance, but in addition the small fragments of C5 and C3, called C5a and C3a are peptide mediators of inflammation. These fragments play a role early on and mediate local inflammatory responses, recruiting leukocytes and protein to the site of infection. They also lead to the accumulation of fluid. C3a and C5a are also able to attach to receptor sites on mast and inflammatory cells. They bring about the release of histamine and other substances from mast cells and prostaglandins from inflammatory cells. These complement fragments are chemotactic for PMN. They also aid phagocytosis by attaching the antigen to the phagocyte through the C3 receptor on the surface of PMN, monocytes and macrophages (Rietschel *et al.*, 1984). Complement activation in the gingival crevice could provide the chemotactic stimulus for leukocyte recruitment and the release of leukocyte lysosomal enzymes, further increasing epithelial permeability.

Complement activation in the tissues itself can lead to increased vascular permeability and kinin activity (Nisengard, 1977). Complement studies of GCF from patients with periodontal disease suggest that complement is activated by the alternate pathway (Schenkein *et al.*, 1976). Although studies have detected the presence of some complement factors in both periodontal patients and in subjects taking part in experimental gingivitis studies (Pattres *et al.*, 1989), none of these factors appear to have any diagnostic value (Eley and Cox, 1998).

1.4.5 Prostaglandins

Prostaglandins play an important role in inflammation. PGE_2 particularly exhibits a broad range of proinflammatory effects and these effects are enhanced by synergism with other inflammatory mediators (Williams and Peck, 1977). *In vitro* studies have indicated that prostaglandins have numerous effects in periodontal disease. They mediate bone resorption (Klein and Raisz, 1970; Goldhaber, 1971; Goldhaber *et al.*, 1973; Gomes *et al.*, 1976). In terms of the inflammatory response, the production of prostaglandins from gingival tissues increases with inflammation (El Attar, 1976). In addition human monocytes have been shown to produce PGE_2 when stimulated with LPS from various periodontal pathogens (Garrison *et al.*, 1988). Gingival and periodontal ligament fibroblasts also secrete PGE_2 in response to both IL-1β and in culture to media conditioned with LPS (Richards and Rutherford, 1988).

1.4.6 Cytokines in inflammation

The term "cytokine" is derived from the Greek word kytos meaning cell and kinesis meaning movement. Cytokines are small soluble proteins that make up a large and diverse group of molecules. They have a vast range of potent biological functions. Cytokines were first discovered in the context of research into the mechanisms of pyrogenesis and cellular immunology and the first cytokines to be discovered were IL-1 and IL-2. The number of cytokines discovered over the last 20 years now exceeds 100. Cytokines are referred to as mediators because they are molecules which direct and regulate inflammation and wound healing. As a rule, the synthesis of cytokines is inducible, although some factors are known to be produced constitutively.

Cytokine regulation is achieved through several mechanisms. Control can take place at the gene activation level, during secretion and circulation and at the cell receptor level (Bendtzen, 1994). Although good control mechanisms exist, cytokine production is usually short lived and self limiting (Howells, 1995). In addition, many cytokine receptors exist in soluble forms, which may be cleaved from the target cells allowing them to bind and neutralise cytokines extracellularly (Bendtzen, 1994).

Cytokines can be broadly split into two groups, those involved in inflammatory and immune reactions, and those involved in tissue growth and repair. The first group can be further sub-divided into; the interleukins that transmit information between leukocytes, chemokines or chemotactic cytokines that are involved in cell recruitment, and interferons which are involved in influencing lymphocyte activity.

1.4.6.1 Interleukin-1

In humans, there are two distinct gene products that have been cloned IL-1α and IL-1β. These molecules are distinct but structurally related (Tatakis, 1993), they have similar proinflammatory properties, although IL-1β is more potent (Stashenko *et al.*, 1987). Sources of IL-1 production include macrophages, monocytes, lymphocytes, vascular cells, brain cells, skin cells, fibroblasts (Alexander and Damoulis, 1994), keratinocytes (Luger *et al.*, 1981), endothelial cells (Miossec *et al.*, 1986) and osteoblasts (Hanazawa *et al.*, 1987). IL-1 is a multifunctional cytokine and is one of the key mediators of the body's response to microbial invasion, inflammation, immunologic reactions and tissue injury (Alexander and Damoulis, 1994).

IL-1 is a major mediator in periodontal disease. It has been found in the GCF of patients with the disease (Reinhardt *et al.*, 1993) and also both as a protein (Jandinski *et al.*, 1991) and mRNA transcripts (Matsuki *et al.*, 1993) in inflamed gingival tissues. Production of IL-1 is induced by LPS (Manthey and Vogel, 1994) and other bacterial components, including cell surface carbohydrates (Takada *et al.*, 1993) and porins (Galdiero *et al.*, 1993).

IL-1 is known to stimulate the proliferation of keratinocytes, fibroblasts and endothelial cells, and also to enhance fibroblast synthesis of type I pro-collagen, collagenase, hyaluronate, fibronectin and PGE_2. From these functions it is obvious that IL-1 is a critical component in the homeostasis of the periodontal tissues (Okada and Murakami, 1998). However, when the inflammatory response becomes chronic and there is unrestricted production of IL-1, it can become harmful and promote tissue damage.

1.4.6.2 Interleukin-6

IL-6 is a multifunctional cytokine that regulates immune responses, acute phase reactions and haematopoiesis (Hirano, 1991). IL-6 is produced by macrophages, fibroblasts, lymphocytes and endothelial cells. The production is induced by IL-1, tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Shalaby *et al.*, 1989). The biological effects of this cytokine are similar and overlap with the properties of other cytokines including IL-1 and TNF-α.

There have been reports of the presence of IL-6 in the periodontal tissues (Kamagata *et al.*, 1989; Barthold and Haynes, 1991; Shimizu *et al.*, 1992), often at levels higher than those found in healthy controls. IL-6 is thought to be an essential cytokine for the terminal differentiation of activated B cells and may play a role in the induction of the elevated B-cell response seen in the gingival tissues of patients with chronic periodontitis (Fujihashi *et al.*, 1993).

1.4.6.3 Interleukin-8

There are 12 members in the human IL-8 family (Skerka *et al.*, 1993). IL-8 falls into the category of cytokines known as the chemokines. The chemokines are divided into two broad groups, α and β. They are distinguished by minor differences in structure and activation of different cell types. IL-8 is an α-chemokine and this group promotes the migration of PMN. IL-8 is a chemotactic factor for PMN, inducing them to leave the bloodstream and migrate into the surrounding tissues, in this case, the gingival

tissues. This process occurs in two stages. Firstly, IL-8 arrests the circulation of the cell. Subsequently it directs the migration of the cell along a gradient that increases in concentration towards the site of infection.

IL-8 is produced by monocytes, macrophages, fibroblasts and keratinocytes. The production of mature protein and expression of IL-8 mRNA was demonstrated by various cell types, including neutrophils, particularly following phagocytosis (Skerka *et al.*, 1993).

Takashiba *et al.* (1992), reported that human gingival fibroblasts expressed IL-8 mRNA when stimulated with IL-1α and IL-1β. In addition, these cells also produced biologically detectable IL-8 when stimulated with IL-1β or TNF-α. This study also showed an increase in chemotactic activity in the conditioned culture medium which was in parallel with IL-8 mRNA expression. It is hardly surprising that IL-8 should be found in the tissues of the periodontium since PMN are the first cells to arrive at the site of inflammation and infection.

1.4.6.4 Tumour necrosis factor-α

TNF-α and TNF-β are proinflammatory cytokines, produced mainly by macrophages and lymphocytes respectively (Manogue *et al.*, 1991). TNF-α and TNF-β show considerable sequence homology and bind to the same receptor (Aggarwal *et al.*, 1985). TNF-α is produced by macrophages in response to the recognition of microbial constituents, particularly LPS (Beutler *et al.*, 1985), and the local effects of TNF-α are extremely striking. TNF-α primarily initiates the inflammatory response. It increases the vascular diameter of the local blood vessels, leading to increased blood flow and vascular permeability and local accumulation of serum. TNF-α also has an effect on the endothelium, inducing the expression of adhesion molecules that bind to the surface of circulating monocytes and PMN. This greatly enhances the rate at which these cells migrate across capillary walls into the tissue. TNF has been reported to have similar effects to IL-1 and IL-6 (Manogue *et al.*, 1991).

Matsuki *et al.* (1992), reported that TNF-α mRNA was abundant in macrophages and T cells in the gingival tissues of patients with moderate to severe periodontitis.

1.4.7 Adhesion molecules

One of the effects of TNF-α is to act on the endothelium to induce the expression of adhesion molecules that bind to the surface of circulating monocytes and PMN. This binding increases the rate at which these cells migrate from the small blood vessels into the tissues. This process is called extravasation. It is now evident that adhesive interactions control inflammatory/immune reactions from the initial stages through to the end stage "effector" functions of host-response cells (Crawford, 1994).

Leukocyte adhesion deficiency (LAD), gives evidence of the beneficial role of cell adhesion molecules in inflammatory diseases. Patients suffering from this disease have an abnormal gene that codes for the family of adhesion molecules called the leukocyte integrins. Patients with this abnormal gene have a dramatically increased susceptibility to infections and suffer from a very severe form of periodontal disease that can lead to premature loss of deciduous and permanent teeth (Crawford, 1994).

The main classes of adhesion molecules known to have a role in inflammation and immunity are the selectins, integrins, members of the immunoglobulin superfamily and some mucin-like molecules. All of these groups share similar properties. They are all complex proteins that interact via heterophilic binding with one or more ligands on other cells or the extracellular matrix. They are all involved in signalling events across the cell membrane, are regulated by cytokines and their expression and activity is localised at the site of inflammatory lesions.

1.4.7.1 The selectins

Selectins are cell surface molecules and all members of this group have a similar core structure but they differ in the lectin-like domain in their extracellular portion. Lectins are molecules that bind to specific sugar groups, and each selectin binds to a cell surface carbohydrate molecule. The selectins have therefore, so been named

based on their selective distribution and lectin aminoterminal domain (Bevilaqua, 1989; Bevilaqua *et al.*, 1991). There are 3 selectins described to date; E-selectin, P-selectin and L-selectin. Selectins are particularly important for leukocyte homing to specific tissues, and can be expressed on either leukocytes (L-selectin) or on vascular endothelium (P- and E-selectins). Selectin expression is induced by C5a, histamine and TNF-α and these selectins can be considered as the initial binding molecules. They mediate leukocyte rolling on the endothelial cells, thus allowing the leukocyte to be exposed to the same soluble factors as the endothelium. They are expressed relatively early in the inflammatory process i.e. 3-6 hours after exposure to antigen *in vitro* (R&D Systems, 1994).

E-selectin was originally described as an activation antigen on endothelial cells in inflamed skin (Cotran *et al.*, 1986). It is expressed by endothelial cells at the site of PMN infiltration (Munro *et al.*, 1991), and constitutively by large blood vessels (Page *et al.*, 1992a). In the periodontal setting it has been shown that E-selectin is expressed very early on in the inflammatory process (Moughal, 1992) and is drastically down-regulated in the more established lesion (Gemmel *et al.*, 1994). It shows high affinity for PMN (Janeway and Travers, 1994) which explains its elevated expression in the inflammatory response when PMN numbers are high and decreased expression later on in the immune response when PMN numbers have declined (Kinane and Lindhe, 1997; Lappin *et al.*, 1999).

P-selectin is only expressed on the cell surface after activation with thrombin, histamine or phorbol esters (Larsen *et al.*, 1990; Toothill *et al.*, 1990). P-selectin is contained in Wiebel-palade bodies of endothelial cells and α-granules of platelets. It is released during clotting, and at times of platelet activation and mediating adhesion between leukocytes and platelets (Freemont, 1998).

L-selectin is the only selectin to be expressed constitutively at the cell surface. It is expressed by some lymphocytes, PMN, monocytes and other myeloid cells (Stoolman, 1989). L selectin has been termed the "homing receptor" and is involved in trafficking PMN into inflammatory lesions.

E-, P- and L-selectin all have the common ligand the Siayl-Lewis^x moiety. This is found in the sulphated form as part of the structures of mucin-like vascular addressins: CD34; Glycosylated Cellular Adhesion Molecule-1 (GlyCAM-1); and Mucosal Addressin Cellular Adhesion Molecule-1 (MadCAM-1) molecules. Each integrin also has other ligands (see Table 1.1) (Janeway and Travers, 1994; McMurray, 1996).

1.4.7.2 The integrins

The integrins comprise an extensive family of adhesion molecules and consist of a large α chain paired non-covalently with a smaller β chain. There are several subfamilies of integrins which are defined by their β chain. However, this concept is controversial since it is difficult to group integrins by individual α chains associated with one β chain, as one α chain can associate itself with more than one β chain (Crawford and Watanabe, 1994). Despite this, 3 distinct families of integrins have arisen based on β1, β2 and β3 chains. The β1 family, are involved in adhesion to connective tissue macromolecules such as fibronectin, laminin and collagens. The β2 family are involved in cell/cell interactions in inflammation and immunological reactions. The β3 family, are involved in binding to vascular ligands such as fibrinogen, van Willebrand factor, thrombospondin and vitronectin (Crawford and Watanabe, 1994) and are recognised as playing an important role in inflammation.

α1β2 (LFA-1) was the first integrin to be characterised and is expressed on all leukocytes, with the exception of some macrophages. Ligands of α1β2 include Intercellular Adhesion Molecule 1 (ICAM-1) (Marlin and Springer, 1987), ICAM-2 (Diamond *et al.*, 1990) and ICAM-3 (Vaseuax *et al.*, 1992). LFA-1 is thought to be the most important adhesion molecule for lymphocyte activation, as antibodies to LFA-1 inhibit the activation of naive and effector T cells. α2β2 (Mac-1), is expressed by PMN, monocytes and macrophages and α3β2 (gp150,95) is mainly expressed by monocytes and macrophages, and at a lower density on PMN.

1.4.7.3 The immunoglobulin superfamily

The immunoglobulin superfamily has a distinguishing feature from other adhesion molecules, they have a domain that is a compact region of the polypeptide chain enclosed by a disulphide bond. Members of this family include ICAM-1, ICAM-2, ICAM-3, LFA-3, and VCAM-1 (Simmons *et al.*, 1988; Staunton *et al.*, 1989; Osborn *et al.*, 1989).

ICAM-1 is expressed on vascular endothelium, thymic epithelial cells, fibroblasts, macrophages and germinal centre dendritic cells in lymphoid tissue, epithelial cells in the mucosa of tonsils (Dustin *et al.*, 1986) and Langerhans cells (De Panfilis *et al.*, 1990). ICAM-1 plays an important role in the inflammatory response, by being induced on endothelial cells and encouraging the migration of leukocytes into infected tissues.

ICAM-2 binds to LFA-1 but not Mac-1 (Diamond *et al.*, 1990). It is thought to be more important in re-circulation of lymphocytes, rather than in extravasation and their localisation into sites of infection. The reasoning behind this theory is that ICAM-2 is the major ligand for LFA-1 on resting endothelium (Defougerolles *et al.*, 1991)

ICAM-3 is expressed on unstimulated peripheral blood lymphocytes, monocytes and PMN (DeFougerolles and Springer, 1992; Fawcett *et al.*, 1992). ICAM-3 is homologous to ICAM-1.

V-CAM-1 is another adhesion molecule that is involved in the extravasation process of leukocytes into sites of infected tissue. During inflammation at peripheral sites, postcapillary venules express V-CAM-1 in response to cytokines such as IL-1 and IL-8 (Albeda *et al.*, 1994). As the periodontal lesion progresses there is increased expression of VCAM-1 and ICAM-1, which favours the migration of leukocytes expressing ligands for these molecules i.e. Very Late Antigen-4 (VLA-4/ $\alpha 4\beta 1$ integrin) and LFA-1 integrin respectively. VLA-4 is specific for T and B lymphocytes and is upregulated on these cells when in contact with activated endothelium

(McMurray, 1996), hence the increased proportions of lymphocytes in the more established lesion (Kinane and Lindhe, 1997; Lappin *et al.*, 1999).

Mucosal Addressin Cellular Adhesion Molecule-1 (MAdCAM-1) has recently been identified on the post-capillary venules of gut mucosa and high endothelial venules of lymphoid tissue and dendritic cells. It is considered to be a specific adhesion molecule for the mucosal tissue and is an important controlling factor determining whether a mucosal or non-mucosal immune reaction occurs due to the cell type it recruits (Briskin *et al.*, 1997). MAdCAM-1 has been shown to have strong homology with VCAM-1 and ICAM-1 in certain domains and its principle ligand is the $\alpha4\beta7$ integrin on T and B lymphocytes which can bind to MAdCAM-1 or VCAM-1, probably via the common $\alpha4$ sub-unit (Leung *et al.*, 1996). L-selectin is also a known ligand for MAdCAM-1. The role of MAdCAM-1 in the periodontal disease immune response is still not clear.

The migration of leukocytes from blood vessels into the tissues is called extravasation. The whole process can be described in four different steps. The first step involves weak adhesion of leukocytes to the vessel wall. Circulating leukocytes are seen "rolling" along the area of endothelium which is involved in the inflammatory response. These weak adhesions are mediated by the adhesion molecules P-selectin and E-selectin which are found on endothelium cells. P-selectin is expressed after a few minutes of exposure to TNF-α, and E-selectin after a few hours. All selectins bind leukocytes after recognition of specific carbohydrate epitopes on their surface; for example the Siayl-Lewis^{x} moiety on specific glycoproteins.

The second step of this migration process involves interactions between the immunoglobulin related molecule ICAM-1 and LFA-1 and Mac-1. ICAM-1 is induced by TNF-α on the surface of endothelial cells. LFA-1 and Mac-1 are always found on the surface of phagocytic cells, however, they do not have a high affinity for ICAM-1. IL-8 is a chemotactic factor for PMN inducing them to leave the bloodstream and migrate into the surrounding tissue, in this case the gingival tissues. IL-8 induces phagocytes to leave the blood vessels by greatly increasing the affinity of

LFA-1 and Mac-1 for ICAM-1. This causes the phagocytes to bind tightly to endothelium, and the rolling to arrest.

The third step involves the cell actually crossing the endothelial wall. This is due to adhesive interactions involving LFA-1 and Mac-1 found on the leukocytes and also PECAM or CD31, an immunoglobulin related molecule found both on the leukocyte and also at the junctions of the endothelial cells. These interactions allow the leukocyte to cross the vascular endothelial cell wall by a process called diapedesis.

The final step, is the migration of leukocytes through the tissues to the site of infection. This occurs along a chemotactic gradient set up by chemotactic factors.

Table 1.1 Major Adhesion Molecules

NAME	SYNONYM (CD number)	LIGAND(S)	RECEPTOR EXPRESSION
SELECTINS *Initiate leukocyte to endothelium interaction*			
L-Selectin	LAM-1, Mel-14, (CD62L)	GlyCAM-1, MAdCAM-1, (CD34), Siayl Lewis^x moiety	T-cell, Monocyte, Neutrophil
E-Selectin	ELAM-1, (CD62E)	Siayl Lewis^x moiety	Endothelium
P-Selectin	GMP-140, PADGEM, (CD62E)	PSGL-1, Siayl Lewis^x moiety	Platelets, Endothelium
MUCIN LIKE VASCULAR ADDRESSINS *Bind to L-selectin to initiate leukocyte-endothelial interactions*			
CD34		L-Selectin	Endothelium
GlyCAM-1		L-Selectin	HEV Endothelium
MAdCAM-1 (Immunoglobulin supergene member also)		L-Selectin/α4β7/CS-1	Mucosal Lymphoid Tissue Venules
INTEGRINS *Bind cell adhesion molecules and extracellular matrix strongly*			
β1 (VLA)			
α4β1	VLA-4 (CD49d/29)	VCAM-1, Fibronectin, Peyer's Patches High Endothelial Venules	T-cell, B-cell
α5β1	VLA-5 (CD49e/29)	Fibronectin	T-cell, Endothelium, Epithelium, Platelets
β2			
αLβ2	LFA-1, (CD11a/18)	ICAM-1, ICAM-2	Leukocytes
αmβ2	MAC-1, CR3, CD11b/18)	ICAM-1, C3bi Fibronectin	Monocytes Neutrophils
β7			
α4β7	LPAM-1 (CD49d/-)	MAdCAM-1, VCAM-1	T-cell, B-cell
IMMUNOGLOBULIN GENE SUPER-FAMILY *Various roles-Target for Integrins*			
ICAM-1	(CD54)	MAC-1, LFA-1	All cells
ICAM-2	(CD102)	α1β2	Endothelium
PECAM-1	(CD31)	PECAM-1	Endothelium
VCAM-1	(CD106)	α4β1	Endothelium
LFA-2	(CD2)	LFA-3	T-cell

1.4.8 Polymorphonuclear leukocytes (PMN)

PMN are granulocytes and along with basophils, mast cells and eosinphils are short lived phagocytes which play an important role in the inflammatory immune response. Under normal conditions PMN comprise between 50% and 70% of the circulating leukocyte pool in man (Miller *et al.*, 1984). The half-life of the PMN in the peripheral circulation is brief, being 6-7 hours (Murphy, 1976).

The PMN plays a key role in the early stages of an inflammatory response and arrives at the site of infection within a few hours. As previously discussed, when functioning against an infectious agent, PMN leave the blood vessels and move across tissues towards a chemotactic source. Adhesion molecules necessary for the movement of PMN from vessels are expressed on endothelial cells and PMN (Rosales and Brown, 1993). The adhesion of PMN is controlled by upregulation of P-selectin, on endothelial cells of post capillary venules by thrombin or histamine (Staunton *et al.*, 1990).

PMN respond to many different chemoattractants, including N-formyl peptides, complement derived C5a-desarg, Leukotriene B4, IL-8, TNF and platelet activating factor (Havarth, 1991). Using time-lapse cinematography or other visual methods for following the tracks of cells, it has been shown that PMN can migrate in fairly straight paths towards the source of a chemical gradient (Zigmond, 1974; Allan and Wilkinson, 1978). Once PMN have been stimulated by chemoattractants they undergo morphological changes from their unstimulated spherical morphology. Within two or three minutes, the typical locomotor morphology changes to a ruffled anterior lamellipodium, tapered cell body, with a tail (Smith *et al.*, 1979; Shields and Haston, 1985; Keller *et al.*, 1983). As the cell moves forward, the most active movements are seen at its head, and contraction waves may pass down through the body of the cell to the tail (Haston and Shields, 1984). There is also evidence that some receptors of the PMN may redistribute to the head of moving cells (Wilkinson, 1990). The mechanism for this is unknown. One theory suggests that a signal from a ligand causes actin polymerisation. Thereafter, the newly formed cytoskeleton in the

pseudopod sweeps forward not only activating the receptor-ligand complex, but also other membrane proteins (Wilkinson, 1990).

Once at the site of the chemotactic stimulus and prior to attachment of the PMN to the invading organism, opsonisation takes place either in the presence or absence of complement and antibody. The presence of opsonins, as mediated by PMN membrane receptors, dramatically increases the rate of phagocytosis by PMN. Heat stable opsonins include all 4 classes of Immunoglobulin G (IgG) (Miller *et al.*, 1984) and the heat labile opsonin, attributed to complement cleavage protein C3b (Stossel, 1974). The products of C3 activation and IgG bind their respective PMN receptors, CR1 and CR3 for complement and FcγRII and FcγRIII for IgG. The only drawback of this mechanism is when the host is dealing with a capsular organism. Capsular organisms are able to evade this opsonisation process, and hence phagocytosis. This is due to the masking of the antigenic portion of their cell wall by the capsule which avoids the deposition of complement on their surface (Slots and Genco, 1984).

If antibody or complement are not available then alternative mechanisms of opsonisation of the bacteria come into play. Lipopolysaccharide binding protein (LBP) is an acute phase reactant which binds LPS, a cell wall component of Gram negative bacteria themselves. Opsonisation is achieved in this way, as the binding of LBP to LPS on bacteria enables ligation of bacteria to PMN through the CD14 receptor, thus enhancing phagocytosis (Wright *et al.*, 1989).

Following opsonisation, phagocytosis can take place. Phagocytosis is the process whereby the PMN takes up a particle. Adherence of the phagocytic cell to a pathogen triggers a system of contractile actin-myosin filaments inside the phagocyte. This enables the cell to "throw its arms" or pseudopodia around the target and enclose it in its membrane. The membrane surrounding the pathogen fuses with membranes surrounding lysosomes in the phagocytic cell. Lysosomes are small intracellular packets of powerful degradative enzymes and toxic molecules, which are emptied onto the engulfed pathogen with a destructive effect.

Once phagocytosed and engulfed by the PMN, there are two mechanisms for killing the target organism; the oxygen dependent and the oxygen independent pathways (Van Dyke, 1985). Oxygen dependent killing is activated when the PMN undergoes a respiratory burst coincident with phagocytosis. Nicotinamide adenine dinucleotide hydrogen phosphate (NADPH), a continuous supply of which is generated from glucose via the hexose monosulphate shunt in the phagolysosome membrane, is activated and reduces oxygen (O_2) to superoxide (O^{2-}) (Weiss *et al.*, 1982). Following the generation of hydrogen peroxide (H_2O_2) and hydroxyl ions (OH), a second enzyme system involving myeloperoxidase becomes activated. Myeloperoxidase is released in large quantities, however, on its own has little effect. In combination with H_2O_2, myeloperoxidase is capable of oxidising halides to reactive toxic compounds like $HOCl_3$ and chloramines (Weiss, 1989).

OH is considered the most toxic of all the free radicals. This reaction in vivo is limited by the availability of iron. Little free iron is actually available due to its binding to transferrin and lactoferrin extracellularly and apoferrin intracellularly (Sanchez *et al.*, 1992). In the phagocytic vacuole the pH is reduced and some iron may be lost from transferrin. Lactoferrin retains its ability to bind iron at low pH, thus protecting the cell and limiting the production of OH (Halliwell and Gutteridge, 1985).

Oxygen independent killing is based on the PMN granule network. Antimicrobial agents, which are independent of oxygen, are released into the phagolysosome during phagocytosis (Thomas *et al.*, 1988). These granule components, include bactericidal/permeability-increasing protein, chymotrypsin-like cationic protein and defensins (Weiss *et al.*, 1982) and those outlined above. They are strongly bactericidal and in most cases kill bacteria (Miyazaki *et al.*, 1997). In addition to killing bacteria, the proteolytic enzymes, including elastase and the metalloproteinases, have the potential to damage extracellular tissue.

Many investigators have addressed the issue of whether the presence of PMN in the sulcus indicates anything about the pathogenesis of periodontal disease. An experimental study was carried out by Löe (1961). This investigated healthy gingival

sulci in a group of dogs that were sealed at the margin. The results of the study indicated that neutrophils and epithelial cells clustered near the margin and had filled the sulci in 12-48 hours. This study demonstrated that the increase in trapped material was due to the continuing phenomenon of epithelial desquamation and migration of neutrophils that occurs in normal situations. It is generally accepted that PMN migrate into clinically healthy sulci in small numbers. One study which supports this theory, was carried out by Yamasaki *et al.* (1979). This report showed that PMN were routinely seen within the intercellular spaces of the junctional epithelium of germ free rats, at all levels of attachment. This investigation was carried out on germ free rats and therefore, indicted that the presence of PMN in the sulcus cannot be exclusively explained on the basis of the presence of micro-organisms. It may represent a physiological phenomenon specific to this tissue type.

There is a statistically significant increase in PMN in the junctional epithelium of humans in experimental gingivitis trials after 2-4 days, compared to clinically healthy gingiva (Payne *et al.*, 1975). In this study, as gingivitis developed, the number of leukocytes also increased, until oral hygiene measures were reinstated. Cell counts showed that 95-100% of the cells collected were PMN. Very few leukocytes were found in the crevices of healthy gingiva.

Animals have been used over the last few decades for immunological studies to provide evidence for the function and importance of PMN in the periodontal environment. Garant (1976) showed that in monoinfected rats, the PMN response to micro-organisms resulted in formation of a neutrophil wall or barrier between the plaque and host tissue. It has been suggested that this barrier may assist in preventing invasion of bacterial products into the tissues.

Further indications that PMN play a role in the inflammatory immune response, come from patients that suffer from periodontal disease, as well as other clinical manifestations, particularly deficient PMN function. Neutropenic states have been associated with rapidly progressive forms of periodontal disease (Miller *et al.*, 1984). In cyclic neutropenia, every 14-21 days, PMN disappear from the circulation and reappear after approximately 5 days. In health, PMN comprise 50% to 70% of the

circulating leukocyte pool, whereas in cyclic neutropenia they never comprise more than 50%. Recurrent ulcerations of the oral cavity are common, particularly on the mucosa, the lips and the throat, whenever the number of PMN fall. Radiographs have been taken in some cases, these have indicated involvement of the periodontal tissues, showing bone loss around the primary and secondary teeth (Miller *et al.*, 1984).

A high percentage of family members, with a background of localised aggressive periodontitis, have been reported to show abnormal chemotaxis (Van Dyke *et al.*, 1985). When 22 families with localised aggressive periodontitis were analysed, it was shown that in 19 of these families, the localised aggressive periodontitis patient and their family members all had neutrophil chemotaxis disorders. In all of the 22 families the chemotactic defect was shown in 50% of the siblings, indicating a dominant mode of inheritance. The cause of the defect remains controversial, being due to either intrinsic defects in the PMN itself or extrinsic factors in the sera is unknown (Agarwal *et al.*, 1996). If the neutrophil chemotactic defect is due to a major gene locus, heterogeneity exists as there is a significant number of patients and families that show no evidence of the defect (Kinane *et al.*, 1989a; Page and Beck, 1997)

1.4.9 The adaptive immune system

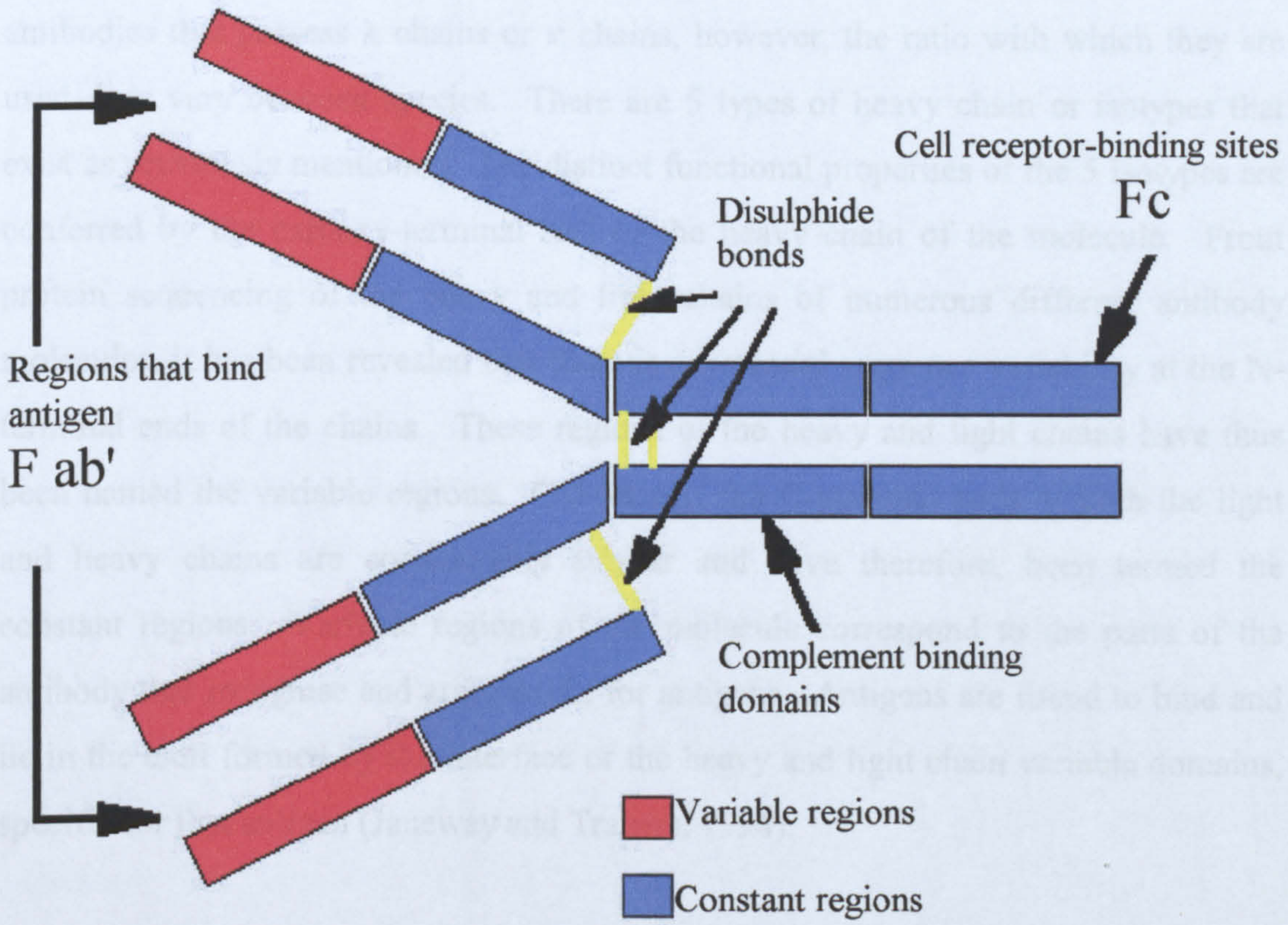
It is unclear how many infectious agents encountered by an individual, are dealt with and eliminated by our innate immune system. For the adaptive arm of the immune system to be triggered, infection has to elude the innate defence mechanisms and exceed the threshold dose of antigen required to initiate the adaptive immune response. The adaptive immune response can be divided into two distinct entities; the humoral immune response and the cell mediated immune response. Both of these arms of the immune system are adaptive, both involve the single most important principle in adaptive immunity, which is clonal selection of lymphocytes, and both lead to a long lasting protection. The divergent lifestyles of different pathogens lead to the requirement of different mechanisms for their recognition and elimination.

1.4.10 The humoral immune response

The antibody was the first product of the specific immune response to be identified. It was found in plasma of blood, and at that point body fluids were called humors, so the antibody immune response became known as the humoral immune response (Janeway and Travers, 1994). B lymphocytes are small lymphocytes that give rise to antibody-producing cells. They are called B lymphocytes or cells because they emanate in the bursa of Fabricius. This is a central lymphoid organ for B cell development, and found only in birds and the bone marrow in mammals. B cells are stimulated by antigen to produce and secrete antibodies, however, at the point when antibodies are produced, the B cells have differentiated into plasma cells. The regulation of immunoglobulin synthesis can be divided into two different processes; the control of the maturation of stem cells into B cells, and the regulation of the terminal maturation of B cells into immunoglobulin secreting plasma cells, a process which is under the control of a complex network of regulatory T cells (Waldmann, 1983). Plasma cells differ from B cells, as they have cytoplasm characteristic of an active secretary cell. It is dominated by layers of rough endoplasmic reticulum and a prominent golgi apparatus. The interaction between an antibody and an antigen comes about solely on the basis of specific antigen recognition (Janeway and Travers, 1994).

Antibodies of all specificity's belong to a family of plasma proteins called immunoglobulins (Igs). Five distinct classes of immunoglobulin or isotypes exist; IgM, IgD, IgE, IgA and IgG (McGhee and Kiyono, 1999). These different classes of immunoglobulin can be distinguished biochemically and functionally. Each antibody is a 'Y' shaped molecule whose arms form two identical antigen binding sites, that are highly variable between one molecule and another. An example can be seen in figure 1.1. The stem of the 'Y' shaped molecule has limited diversity and is known as the constant region. The structure of the 'Y' shaped molecule has been determined using x-ray crystallography.

Figure 1.1 Structure of Immunoglobulin IgG1



Adapted from the Department of Immunology, University of Glasgow web site.

The immunoglobulin molecule is made up of 3 equally sized globular domains and these are held together by a hinge region, that is a stretch of polypeptide chain. Each arm of the 'Y' is formed by the association of a light polypeptide chain of approximately 25 kDa, with the amino terminal half of a heavy polypeptide chain of approximately 50 kDa. There are 2 types of light chain that exist. These have been termed lambda (λ) and kappa (κ). No functional difference has been found between antibodies that possess λ chains or κ chains, however, the ratio with which they are used does vary between species. There are 5 types of heavy chain or isotypes that exist as previously mentioned. The distinct functional properties of the 5 isotypes are conferred by the carboxy-terminal half of the heavy chain of the molecule. From protein sequencing of the heavy and light chains of numerous different antibody molecules, it has been revealed that there is substantial sequence variability at the N-terminal ends of the chains. These regions of the heavy and light chains have thus been named the variable regions. Conversely, the C-terminal ends of both the light and heavy chains are considerably similar and have therefore, been termed the constant regions. Variable regions of the molecule correspond to the parts of the antibody that recognise and are specific for antigens. Antigens are found to bind and lie in the cleft formed by the interface of the heavy and light chain variable domains, specific for that antigen (Janeway and Travers, 1994).

Regions of antigens recognised by antibodies are called antigenic determinants or epitopes. When an epitope is recognised by a specific antigen binding portion of an immunoglobulin an interaction is made. A number of environmental factors can disrupt interactions, examples of which are extremes of pH, high salt concentrations and detergents. Interactions are held together by non-covalent forces the sum total strength of which is called the avidity. Hence, the above mentioned environmental factors can disrupt the interaction and decrease the avidity.

As previously mentioned, differentiation from a B cell into an immunoglobulin secreting plasma cell, requires the co-operation of other cell types. The current theory indicates the requirement of B cells, T cells and macrophages, implying that a B cell requires more than one signal for activation, as well as the essential recognition signal of an antigen. The first contact between antigen and the B cell evokes a primary

antibody response. This has the characteristically long lag period between stimulus and detection of specific antibodies, an exponential increase in antibody before it reaches equilibrium after some days, followed by a steady decline (Virella, 1990). In the primary antibody response, the first antibody class to be synthesised is usually IgM. The activation of these small naive resting B cells is a multistep process. The B cell leaves the bone marrow expressing on its surface both IgM and IgD, which are receptors specific to that B cell. They also express major histocompatability complex (MHC) II molecules and CD45 (Snow and Noelle, 1993). After leaving the bone marrow the naive B cells circulate around the body, between the blood and the lymphatics, before they either converge with their specific antigen or die. Most peripheral B cells have a life span of less that two weeks (Freitas *et al.*, 1986; Udhayakumar *et al.*, 1988).

During the humoral immune response, B cells are activated through the recognition and binding of their immunoglobulin receptor with that of an antigen. This activation leads to a differentiation process which results in the production of either plasma cells or long lived memory cells (Berek, 1992). With some bacterial antigens, for example surface polysaccharides, the process of plasma cell differentiation is independent of T cell help. The activation of B cells by protein antigens however, induces a more complex set of reactions (Berek, 1992).

For activation to occur, a B cell must interact with a T cell also specific for the antigen, as well as with the antigen itself (Vitetta *et al.*, 1989; Noelle and Snow, 1990). This mechanism may have arisen because the regulation of B cells is not as stringent as for T cells. Surface immunoglobulin specificity is generated through random gene rearrangements. This makes the existence of B cells expressing surface immunoglobulin reactive with self antigens possible. T cells, however, have to undergo extensive selection in the thymus, to try and ensure all the self-reactive T cells are removed. Therefore, to activate a B cell, an antigen also has to be processed and recognised by a T cell. Consequently, the lack of self-reactive T cells can directly limit B cell responses to self antigens (Myers, 1991).

The B cell receptor, which as previously discussed is the immunoglobulin molecule, recognises epitopes on the native antigen and has the same specificity as the antibody that is eventually secreted by the cell (Vitetta *et al.*, 1989). However, before most antigens can be presented and recognised by T cells, they have to be processed (Allen, 1987). Protein antigens can be processed by B cells and other antigen presenting cells. T and B cells, both with specific receptors for the antigen can collaborate by cross-linking of the immunoglobulin and T cell receptor antigen receptor complexes (Reth, 1992; Weiss and Littman, 1994). The interaction of T cells with antigen-activated B cells and antigen presenting cells initiates a cascade of reactions which lead to the maturation of the immune response and development of memory cells (Berek, 1992).

There seem to be two discrete developmental pathways following primary antigenic exposure of B cells (Maclennan and Gray, 1986). The first pathway involves a rapid clonal expansion and plasma-cell differentiation of the cells in the T-cell rich areas of the secondary lymphoid organs. These clonally expanded plasma cells thus, carry out the immediate effector function, the clearing of target antigens (McHeyzer-Williams and Ahmed, 1999). The second pathway involves extensive clonal expansion within the specialised microenvironment of the germinal centre (GC). GCs are sites of intense B-cell proliferation. A GC reaction is observed only where T helper cells are involved (Kroese *et al.*, 1990).

Therefore, B cells first bind antigen specific to their immunoglobulin receptors, and are activated by helper T cells in the T cell areas of lymphoid tissues. This area is known as the periarteriolar lymphoid sheath, or PALS (Kelsoe, 1996). At this point B cells or centrocytes, as they are known, proliferate in the PALS and then either develop locally into foci of antibody secreting plasmacytes, or migrate to adjacent lymphoid follicles to initiate the GC reaction (Kelsoe, 1996). The majority of early primary antibody is produced by these plasmacytic foci. They are able to secrete unmutated IgM or IgG over the following 10-12 days, thus providing an early source of circulating antibodies. These cells eventually undergo apoptosis and disappear (Jacob *et al.*, 1991a; Jacob *et al.*, 1991b; Jacob and Kelsoe, 1992; Smith *et al.*, 1996).

Other activated B cells migrate into the primary follicles and form GCs. Primary follicles contain resting B cells clustered around a dense network of follicular dendritic cells (FDC), a specialised cell type in this area. The activated B cells accumulate amongst the FDC which bear antigen-antibody and antigen-complement complexes on their surface (Kelsoe, 1996). The activated B cells rapidly proliferate and acquire new phenotypic characteristics (Kelsoe, 1996). Over the period of the following 14 days, these B cells give rise to a GC developing into at least two regions: a dark and a light zone (Maclennan, 1994).

Hypermutation of the variable region of the immunoglobulin molecules and selection of high affinity variants occurs in the GC. This is of key importance to the maturation of the immune response in this environment (Berek and Ziegner, 1993). Somatic hypermutation affects all the rearranged variable-regions in a B cell, allowing the generation of variability (Janeway and Travers, 1994). This is due to a molecular mechanism that introduces point mutations into V regions. The recruitment of a B cell into the maturation process is dependent on the affinity of its receptor for antigen (Berek and Ziegner, 1993). One view is that the initial selection of these cells is based upon their continuous ability to bind antigen from the surface of the FDC, as an antigen-antibody complex (MacLennan *et al.*, 1990; MacLennan *et al.*, 1992; Liu *et al.*, 1992). Efficient affinity selection is only thought to occur when cells that have a higher affinity for antigen than the antibody within the FDC immune complex are stimulated (MacLennan and Gray, 1986). This makes sense considering the memory B cell response is of higher affinity than the primary response (Gray, 1993). Following this positive selection in the GC reaction, affinity matured B cells can either exit the GC as memory cells or sometimes re-enter the dark zone of the GC for a further round of mutation and selection (Berek and Ziegner, 1993)

It is generally accepted that the cells produced in the GC are memory cells, which then migrate into the circulation to be stimulated on a second encounter with the same antigen (Hanna *et al.*, 1968; Jacobson and Thorbecke, 1968; Wakefield and Thorbecke, 1968a; Wakefield and Thorbecke, 1968b). Studies have shown that cells produced from the GC are not likely to be involved in antibody production, since there

is no correlation between the production of cells in the GC and serum levels of antibody (Buerki *et al.*, 1974).

At the beginning of the humoral immune response, the predominant antibody isotype is IgM (Snow and Noelle, 1993). Later on in the immune response, the B cells are able to undergo isotype switching and other immunoglobulin isotypes are seen. This phenomenon is important to the humoral immune response, as each different isotype possess unique capabilities that improve the effector capabilities of the organism against pathogens (Snow and Noelle, 1993). Isotype switching occurs during the GC reaction (Kraal *et al.*, 1982; Butcher *et al.*, 1982; Apel and Berek, 1991).

Antibodies have different functions, all of which are centred around protecting the host from extracellular pathogens and their products. Antibodies have a number of ways of providing protection.

Neutralisation is a process whereby antibody binds to the surface of a pathogen itself, or to a toxin or product produced by the pathogen. Intracellular bacteria and viruses survive and reproduce inside cells. However, in order to increase in number they need to be transferred from cell to cell and to accomplish this, these bacteria and viruses bind to cell surface molecules of other host cells. Specific antibodies are able to bind to the bacteria or virus particles and neutralise them, preventing cell binding and spread. Many bacteria cause devastating effects by secreting molecules called bacterial toxins. The toxins bind to a molecule that acts as a receptor, on the surface of a host target cell. Antibodies specific for the receptor portion of the toxin, can block binding to a host cell and protect it from toxic attack. Although neutralisation prevents adherence of pathogens and their products to host cell surfaces, it does not remove the pathogens from the host altogether. Furthermore, many pathogens are not neutralised by antibodies. They have developed strategies whereby they can avoid this defence mechanism.

Other bacteria and pathogens reproduce and survive extracellularly. These pathogens, along with those that cannot be neutralised, need to be removed. Antibodies protect

against these by facilitating their uptake into phagocytic cells, such as PMN, by a process called opsonisation. There are two ways in which this can occur.

Opsonisation can occur when an antibody binds to a micro-organism by its Fab portion. The Fc portion of the antibody is recognised and bound by a receptor found on PMN. The micro-organism then becomes engulfed by the phagocyte as receptors for Fc and the Fc regions on the antibodies continue to combine, leading to final engulfment and destruction of the micro-organism. The second way in which opsonisation can occur is by activation of the complement cascade and binding of phagocytes to complement components found on the surface of the micro-organism.

1.4.10.1 IgM

The first antibodies to be produced in an immune response are IgM. Antibodies of this isotype are produced before B cells have undergone somatic hypermutation and affinity maturation, and hence tend to be of low affinity. However, IgM molecules are usually found as pentamers with 5 immunoglobulin molecules and 10 antigen binding sites. This compensates for the low affinity binding of these antibodies, as it allows simultaneous binding of antibody to repetitive sites, which are often found to be expressed by bacterial cell wall polysaccharides. This binding to multimeric antigens can confer high avidity.

IgM is a large molecule and the pentamer is formed in association with a J chain and for this reason it does not pass through the placenta. The monomers of IgM are cross-linked to each other by disulphide bonds and also to the J chain. Due to the large size of the IgM molecule, it is usually confined to the blood, however, IgM can be found in tissues at sites of infection that are undergoing an inflammatory response. Elevated levels of IgM usually indicate either recent infection or exposure to antigens.

One of the most important functions of IgM is that it is very potent at activating the complement cascade. Activation of these plasma proteins is extremely important, especially at the beginning of an infection as it helps recruit and activate phagocytes and can directly destroy pathogens (Janeway and Travers, 1994). IgM antibodies are

not particularly versatile, are poor toxin-neutralising antibodies and are not efficient in the neutralisation of viruses.

1.4.10.2 IgA

IgA is the major mucosal immune system isotype made by plasma cells and has a half-life of 5.5 days. The primary site of IgA synthesis is at the epithelial surfaces of the body and its main role is thought to be to protect epithelial cells from infectious agents. IgA achieves this by preventing attachment of bacteria and toxins to epithelial cells, and is involved in the neutralisation of harmful toxins. IgA provides the first line of defence for these surfaces. IgA is a poor opsonin and does not contain receptors for complement, and thus is not a complement-activating or complement-fixing immunoglobulin. This is not surprising as IgA is usually found in areas which are lacking in accessory cells.

The lamina propria (connective tissue), that is found beneath the basement membrane of many surface epithelia, hosts a large number of IgA secreting plasma cells (Heremans, 1974). Active transport or transcytosis of IgA occurs from the lamina propria, where it is produced, to the external surface of the epithelium. Secretary IgA, or IgA that undergoes transport is predominantly of the polymeric form (Heremans, 1974). Polymeric IgA is usually found as a dimer with a molecular weight of 400 kDa. Two IgA molecules are held together by disulphide bonds and are both also bound to a J chain, found also in pentameric IgM. The J chain, produced by plasma cells has been implicated as a component for the polymerisation of IgA and IgM (Koshland, 1975).

There are 2 subclasses of IgA; IgA1 and IgA2. IgA1 and IgA2 are distributed differently in the body fluids; 80% or more of serum IgA belongs to the IgA1 subclass, whereas 50-74% of the IgA found in external secretions is of this subclass (Vaerman *et al.*, 1968; Grey *et al.*, 1968; Delacroix *et al.*, 1982). IgA2 is found in a higher percentage in external secretions. The reason for this is unknown, but it could be due to the preferential transport of IgA2, or that increased numbers of IgA2 secreting cells are found at secretary surfaces.

IgA antibodies are secreted in breast milk and transferred to the gut of new born infants. Their specialised defence of mucosal surfaces provide protection of the new born until they can provide their own.

1.4.10.3 IgG

IgG molecules are small and able to diffuse out of the blood and into the tissues. IgG molecules are always monomeric and are the principal isotype in the blood and extracellular fluids.

There are 4 different subclasses of IgG; IgG1, IgG2, IgG3 and IgG4. Except for IgG3 which has a rapid turnover, the half-life of IgG is the longest of all the isotypes at about 23 days. Functional differences can be noted amongst the different isotypes. IgG2 subclass is more frequently found to be produced in response to polysaccharide antigens, IgG1 and IgG3 are involved in defence against protein and viral antigens, and IgG4 is mainly associated with responses that can lead to allergic type reactions.

IgG1, IgG2 and particularly IgG3 play an important role in the activation of the classical pathway of the complement cascade. IgG1 and IgG3 are the only subclasses that are found to bind to macrophages and other phagocytes, and hence play an important role in the process of opsonisation. IgG are high affinity antibodies, as they are produced by B cells that have undergone affinity maturation and isotype switching in the GC reaction. They also have the ability to cause neutralisation of toxins and agglutination.

IgG molecules play an important role in antibody-dependent cell mediated cytotoxicity (ADCC). This process involves binding of the Fab portion of the antibody with the target cell, and the Fc portion binds with specific receptors found on natural killer (NK) cells. The target cell is then killed, not by opsonisation but by specific release of harmful molecules produced by NK cells.

IgG is the only antibody that is transported across the placenta. It is actively transported directly into the bloodstream of the foetus by a specific IgG transport protein. The small size and high affinity nature of the IgG molecule makes it an ideal isotype for this function. IgG2 is the only isotype of IgG that does not cross the placenta (Janeway and Travers, 1994).

1.4.10.4 IgD

IgD has a half-life of 2.8 days and is found on the surface of B cells at different stages of their maturation. The function of IgD remains elusive.

1.4.10.5 IgE

IgE has a half-life of 2 days, which is the shortest of all the immunoglobulins. It is also found at the lowest concentration in the serum. IgE cannot cause agglutination or neutralisation, and has very specific functions in the immune surveillance of the body.

IgE antibodies are thought to play an important role in parasitic infections and hypersensitivity reactions. IgE antibodies are able to bind directly to mast cells and basophils by a receptor. The receptor is found on the surface of these cells and is specific for the Fc portion of the IgE molecule. Cross-linking of IgE to an antigen via its Fab portion, and a basophil or mast cell via its Fc portion, leads to activation of these leukocytes. Activated mast cells and basophils release the contents of their granules which include; histamine, heparin and leukotrienes and these can trigger hypersensitivity reactions (Benjamini *et al.*, 1996). The Fc receptors found on mast cells and basophils, which are designated FcεI receptors, bind the Fc portion of IgE with very high affinity.

Degranulation occurs within seconds of cross-linkage. First the vasoactive amines, histamine and serotonin are released which leads to the initiation of an inflammatory immune response. There is increased vasodilation and vascular permeability, which quickly leads to the accumulation of immune cells and exudative fluids at the site of infection. Serotonin and histamine are very short lived, but the inflammatory response

is sustained by the subsequent release of other mediators by the mast cells and basophils such as leukotrienes and other metabolites of archidonic acid (Janeway and Travers, 1994).

1.4.11 The role of the adaptive humoral immune response in periodontal disease.

Two distinct humoral effector systems that contribute to the defence in the oral cavity have been postulated by Brandtzaeg (1972, 1973). Salivary IgA has been suggested to be the first line of defence, perhaps playing a role which involves trapping of the antigen in a mucin layer with subsequent disposal of the antigen (Brandtzaeg, 1972). The second involves the local production of IgG which is protective in nature and found in inflamed gingival and periodontal tissues (Brandtzaeg, 1973). These early ideas are still postulated in publications on this topic, and are often referred to as the local systemic immune responses. The importance of these different fluid types and what they actually indicate is still to be confirmed. In addition, it is not fully understood whether the local levels, such as that of saliva and GCF, are reflections of the serum antibody levels or whether production of the antibodies found in such fluids is distinct and unique.

1.4.12 The local antibody response

In terms of the local immune response, saliva is known to contribute to maintaining oral health, and secretary IgA is found in large quantities in saliva. It has been suggested that an individual with high levels of IgA in their saliva, which is directed against micro-organisms such as *A. actinomycetemcomitans*, *P. gingivalis* and *Streptococcus mutans*, are more likely to be protected against gingivitis (Shenk *et al.*, 1993). Another study carried out by Sandholm *et al.* (1987), found elevated levels of salivary IgG in patients with chronic and aggressive periodontitis against *A. actinomycetemcomitans.*

Various studies have reported an increase in antibody levels in the GCF (Reinhardt *et al.*, 1989; Wilton *et al.*, 1993) and in the gingival tissues (Smith *et al.*, 1985). Further studies have detected specific antibody activity in the GCF of patients with

periodontal disease (Ebersole and Cappelli, 1994; Ebersole *et al.*, 1985a; Ebersole *et al.*, 1985b; Smith *et al.*, 1985). Through clinical observation of diseased tissues taken from periodontitis patients, it is known that they are populated by a large number of B lymphocytes and plasma cells, thus suggesting local antibody production (Ranney, 1991; Sims *et al.*, 1991). These cells are seen to differentiate and proliferate in this area, and in susceptible subjects this area is also populated by an increased number of TH2 cells that have selectively homed to the gingiva to aid in B-cell expansion (Seymour *et al.*, 1993). More evidence for local antibody production comes from reports indicating the presence of activated complement components in GCF from both the classical and alternate pathways (Niekrash and Patters, 1985; Schenkein and Genco, 1977). This further indicates the possibility of local IgG synthesis in the tissues.

Many of the studies observing the presence and function of antibody in GCF have been particularly involved in looking at antibodies directed against *A. actinomycetemcomitans* and *P. gingivalis*. A study carried out by Ebersole and Cappelli (1994) investigated antibodies directed against *A. actinomycetemcomitans* and the presence of different subclasses of antibody in GCF of patients with periodontal disease. They compared the findings with those from subjects in health. The frequency and distribution of antibody in the GCF indicated a potential role for local antibody directed against *A. actinomycetemcomitans* in relation to colonisation and clinical presentation. The results also suggested a relationship between the distribution of infection and the levels of antibody directed against *A. actinomycetemcomitans* in GCF.

The results gleaned from studies observing antibodies directed against *P. gingivalis* give varying reports. For example, Baranowska *et al.* (1989) reported no difference in antibody levels against *P. gingivalis* in the GCF of periodontitis patients compared with health. However, Suzuki *et al.* (1984), indicated there was an increase in the local production of antibodies directed against this micro-organism in patients with chronic periodontitis as compared with patients with localised aggressive periodontitis.

Although the results from a lot of the work carried out in this area indicate that there is a local immune response due to the micro-organism in question providing antigenic stimulation at a local site, there is also a systemic serum antibody response to the local infection. A relationship between the local and systemic antibody levels and specificity is thought to exist. Theories have been put forward trying to link the local and the systemic immune responses. Three main theories that have been suggested by Kinane *et al.* (1999): (i) local antibody is produced due to continuous antigenic challenge, however, this antibody leaves the tissues via the lymphatics and enters the circulation becoming systemic; (ii) a systemic response is induced due to a breakdown in the tissue integrity of the gingivae, allowing bacteria and antigenic components to leave, migrate to the lymph nodes in the circulation, and induce a systemic response; (iii) local antigen presenting cells pick up and process antigens in the tissue before leaving and homing to systemic lymphoid tissues, where they induce a systemic antibody response.

Studies have reported GCF levels of antibody to be greater than levels found in the serum (Ebersole *et al.*, 1985a; Kinane *et al.*, 1993; Tew *et al.*, 1985a). As well as being a product of the local immune response, it has also been suggested that serum antibody specificity may reflect a local gingival response. This has been suggested following studies observing the response to *A. actinomycetemcomitans* in periodontitis patients (Ebersole, 1990; Ebersole *et al.*, 1992).

IgG, IgM and IgA classes of antibodies have been described in GCF (Brill and Brönnestam, 1960; Brandtzaeg, 1965) and in the gingival tissues (Brandtzaeg and Kraus, 1965; Thonard *et al.*, 1966; Genco *et al.*, 1974). Although these and serum antibodies, against individual plaque and Gram negative bacteria have been shown to be present in humans with varying degrees of periodontal disease, their specific antigenic target remains on the whole elusive. Different subclasses of antibody, particularly IgG, carry out various functions, and in general are directed against different antigenic molecules. Therefore, a number of studies have been carried out to look at the different subclasses of antibody, with the aim of providing further information on the nature and target of the immune response.

A study carried out by Ebersole and Cappelli (1994) investigated the different subclasses of IgG directed against *A. actinomycetemcomitans* in the GCF of patients with periodontal disease. This study showed that IgG3 and IgG4 subclasses were particularly increased as compared with the levels seen in the serum. The patterns of subclass distribution were quite distinct. In GCF samples, where the antibody levels were below those found in the serum, IgG2 levels were found to be high. In GCF samples that had antibody levels in the same range as those found in the serum, IgG1 was strikingly increased. In GCF samples that had elevated levels of antibody compared with serum, elevated levels of IgG3 and IgG4 were found overall. Results showed that IgG3, IgG4, IgG1 and IgG2 antibodies were elevated by 58%, 35%, 25% and 25% respectively. The correlation between elevated levels of IgG4 antibody to *A. actinomycetemcomitans* and the presence of the micro-organism in the plaque was positive.

Antibodies in the oral cavity have been detected against non-oral bacteria (Berglund, 1971; Mallison *et al.*, 1989). Some of the antibodies may be cross-reacting antibodies induced to another bacteria in a different part of the body. However, even if this is so for a percentage of the antibody response, it is generally accepted that there is a specific humoral immune response, with specific antibody produced, that plays a role in protection against periodontal disease.

1.4.13 The systemic antibody response

The majority of aggressive and chronic periodontitis patients mount a systemic humoral immune response to antigens of the infecting bacteria (Ishikawa *et al.*, 1997). As discussed previously there are several theories on how a systemic response is induced.

One of the first experiments on this topic was carried out by Genco *et al.* (1980a). This study indicated that there were antibodies directed against sonicate preparations of *A. actinomycetemcomitans* in the sera of patients with localised aggressive periodontitis, but not in healthy subjects. Many studies that have been carried out

since these early experiments have tended to target the Gram negative anaerobes *A. actinomycetemcomitans* and *P. gingivalis* because of their marked association with periodontal disease (Socransky and Haffajee, 1990). Systemic antibodies to a large number of Gram negative bacteria have been investigated. *F. nucleatum* appears to be a regular resident in plaque. Although serum antibodies directed against this bacteria seem to be detectable in patients with periodontitis, the titres are not elevated in all periodontitis patients (Farida *et al.*, 1986a; Suzuki *et al.*, 1984; Tolo *et al.*, 1981). Elevated levels of serum antibody have been detected in selected periodontitis patients against *E. corrodens* (Ebersole *et al.*, 1987; Ebersole and Holt, 1988) and elevated IgG antibody levels against *C. rectus* have been shown in all periodontitis patients compared with health (Nisengard *et al.*, 1980).

Most of the studies attempt to assess the antibody responses to *A. actinomycetemcomitans* and *P. gingivalis*. A strong correlation has been shown between increased levels of antibody to *A. actinomycetemcomitans* and localised aggressive periodontitis. It is now accepted that this micro-organism is important in the causation of aggressive periodontitis. This is because of its high prevalence in the disease and the elevated antibody levels in these patients against this bacteria (Ebersole *et al.*, 1980; Ebersole *et al.*, 1989; Genco *et al.*, 1980a; Farida *et al.*, 1986; Genco *et al.*, 1980b; Tew *et al.*, 1985a). Although in many diseases there appears to be considerable variation in responses seen between different populations, global results agree that there are elevated levels of serum antibodies to *A. actinomycetemcomitans* in patients with aggressive periodontal disease, particularly the localised form. The results of these different geographical studies seem to suggest, that the predominant serotype of *A. actinomycetemcomitans* varies (Gunsolley *et al.*, 1988; Gmür and Baehni, 1997; Çelénligil and Ebersole, 1998).

Elevated serum antibody titres to *P. gingivalis* have also been identified in patients with aggressive periodontal disease. A large study carried out by Ebersole indicated that of the 62 generalised aggressive periodontitis patients observed, 37% of these had elevated antibody titres to *P. gingivalis*, compared with only 25% in control subjects (Ebersole *et al.*, 1982a). Similar results were obtained from a study carried out by

Mooney and Kinane (1994) who showed that 25% of the generalised aggressive periodontitis patients tested had elevated antibody levels.

In terms of chronic periodontal disease, *A. actinomycetemcomitans* does not appear to be uniformly linked. Many studies have not found any elevated levels of serum antibodies to *A. actinomycetemcomitans* in patients with chronic periodontitis compared with healthy controls (Doty *et al.*, 1982; Gmür *et al.*, 1986; Listgarten *et al.*, 1981). Results from investigations by Ebersole *et al.* (1991a; 1994), have suggested that increased titres of serum antibodies are seen, in some but not all, of patients with chronic periodontitis. It is thought there is a group of individuals that are more susceptible to infection by this bacteria than others, and that in this 'at risk' group, *A. actinomycetemcomitans* is capable of initiating disease.

Although serum antibody titres specific for *A. actinomycetemcomitans* may not be elevated in patients with chronic periodontitis, many studies have reported increased antibody levels in these patients against *P. gingivalis* (Ebersole *et al.*, 1982a; Mansheim *et al.*, 1980; Socransky and Haffajee, 1994). In addition there have also been reports indicating a correlation between levels of IgG antibody to *P. gingivalis* and periodontal destruction (Gmür *et al.*, 1986), disease severity (Naito *et al.*, 1987), and alveolar bone loss in an elderly population (Wheeler *et al.*, 1994).

Many studies have tried to evaluate the different subclasses of IgG in the serum of periodontitis patients. This information may give an indication of the target of the antibody response due to the functional differences noted amongst the different subclasses. A number of studies by Ebersole *et al.* (1985c; 1989) investigated the IgG subclass distribution in the serum directed against *A. actinomycetemcomitans* and *P. gingivalis* in patients with localised aggressive, generalised aggressive, chronic periodontitis and also in healthy subjects. The results showed elevated levels of IgG3 and IgG1 to *A. actinomycetemcomitans* in patients with localised aggressive periodontitis and elevations in IgG3, IgG1 and IgG4 in patients with generalised aggressive periodontal disease. Studies have also been carried out to investigate levels of IgG2, a subclass that is found frequently in response to polysaccharide antigens (Kinane *et al.*, 1999a). Results from these studies indicated that aggressive

periodontitis patients seem to have elevated levels of serum IgG2, directed against LPS of *A. actinomycetemcomitans*, and that these antibodies are opsonic for this micro-organism (Gmür and Baehni, 1997; Wilson *et al.*, 1995; Wilson and Hamilton, 1995).

Elevated levels of IgG2 antibodies have also been found in the serum of chronic periodontitis patients against the LPS of *P. gingivalis* (Schenk and Michaelsen, 1987). Moderate amounts of IgG1, IgG3 and IgG4 were also found in the serum of these patients. Other studies have shown there to be elevated levels of IgG2, IgG1 and IgG4 to *P. gingivalis* in patients with generalised aggressive and chronic periodontitis (Ebersole *et al.*, 1985c; Farida *et al.*, 1986).

Evidence exists which may explain why high levels of IgG2 against *P. gingivalis* and *A. actinomycetemcomitans* are seen in patients with periodontal disease. Both of these micro-organisms are Gram negative bacteria containing both LPS and a capsular polysaccharide. As discussed in section 1.6.4 LPS is an immunogenic molecule and as IgG2 is predominantly induced in response to polysaccharide antigens it is expected that there would be elevated levels of IgG2 in patients mounting a response. In respect to the distribution of the other subclasses of IgG, there appears to be enormous variation between different populations and between the different forms of the disease.

1.4.14 The cell mediated immune response

The principal components of the cell mediated arm of the immune system are T cells. These lymphocytes are capable of recognising antigens, that are processed and presented by antigen presenting cells (APC), in the context of the MHC (Babbitt *et al.*, 1985; Buus *et al.*, 1987). The MHC is a set of polymorphic genes encoding class I and class II cell-surface glycoproteins whose function is to present antigenic peptides to $CD8^+$ and $CD4^+$ T cells respectively (Accolla *et al.*, 1995).

Effector T cells fall into 3 functional classes that detect antigens derived from different types of pathogens, presented by the two different classes of MHC molecule.

Antigens derived from pathogens that multiply in the cytosol are carried to the cell surface by MHC class I molecules and presented to $CD8^+$ cytotoxic T cells. Histologically different cell types may be differentiated by cell-surface molecules. These can be identified by a specific group of monoclonal antibodies. These molecules are known as cluster of differentiation antigens and are designated CD.

Cells infected with viruses or bacteria that reside in the cytosol are eliminated by $CD8^+$ cytotoxic T cells. The function of these cells is to kill infected cells. Naive $CD8^+$ T cells can only differentiate into cytotoxic cells. They require more co-stimulatory activity to activate them than do $CD4^+$ T cells. This is possibly because they are so destructive. Cytotoxic T cells principally act by, release of secretary granules onto the surface of a target cell on recognition of an antigen (Janeway and Travers, 1994) and the production of IFN-γ.

Antigens derived from extracellular bacteria and toxins are processed within the APC. They are carried to the surface by MHC class II molecules and presented to $CD4^+$ T cells. There are two distinct subsets of $CD4^+$ T cells; TH1 and TH2. The division of $CD4^+$ T cells into subsets is based upon cytokine production (Mossman and Coffman, 1989). TH1 cells secrete both IL-2 and IFN-γ when activated by certain types of T-dependent antigens so they can enhance cell-mediated responses. The TH2 subset produces IL-4, IL-5, IL-10 and IL-13, and thereby promote the humoral immune response. TH0 cells have also been identified and these are thought to secrete IL-4 and IFN-γ. Their role is yet to be confirmed, however, it is thought they may act as a precursor cell to T-helper cells yet to have differentiated into either TH1 or TH2 cells (Modlin and Nutman, 1993). Several factors can influence the development of the TH subsets and these include the antigen itself, the antigen concentration, the route of antigen administration and the antigen presenting cells (Williams *et al.*, 1991; Gajewski and Fitch, 1991; Bretscher, 1991). TH1 cells, called inflammatory $CD4^+$ T cells, are specialised to activate macrophages to have a greatly increased ability to kill intracellular bacteria. TH2 cells, called helper $CD4^+$ T cells activate antigen-binding B cells to differentiate into antibody-secreting cells (Janeway and Travers, 1994).

Although much of the literature discusses the importance of the humoral aspect of the immune response in periodontal disease, models used in earlier years suggested that the initial periodontal lesion is composed mainly of T lymphocytes, with B cells and plasma cells predominating at a later stage (Mackler *et al.*, 1977; Seymour *et al.*, 1982; Seymour *et al.*, 1983). Studies have also reported differences in CD4:CD8 ratios in lesions and peripheral blood of periodontitis patients (Okada *et al.*, 1982; Taubman *et al.*, 1984), compared to healthy controls. This indicates that the cell mediated immune system is potentially as important in a discussion of this disease as that of the humoral immune response.

It appears that the immune response in periodontitis takes the following pattern, the antigen is picked up by an APC, such as a Langerhans cell at the site of infection. It is then carried to a primary lymphoid tissue where presentation to a circulating naive T cell takes place. This leads to antigen-specific clonal activation, where some activated cells become effector cells and others remain in the circulation as memory cells. Naive and memory T cells may be recognised by the markers they express. Naive T cells express CD45RA and memory cells CD45RO.

CD45 is a transmembrane tyrosine phosphatase with 3 variable exons that encode part of its external domain. In naive T cells, high molecular weight isoforms CD45RA are found. In memory T cells, the variable exons are removed by alternate splicing of CD45 RNA and this isoform is known as CD45RO. General dogma indicates that memory T cells ($CD45RO^+$) greatly out number ($CD45RA^+$) T cells in periodontitis lesions (Gemmel *et al.*, 1992; Yamazaki *et al.*, 1993; Kinane *et al.*, 1999; Lappin *et al.*, 1999). However, it has been shown that a proportion of these cells are $CD45RA^+$, which suggests reactivation of the memory cell population by specific but as yet unidentified antigens in the tissues. It has been reported that memory T cells adhere to vascular endothelial cells and augment their permeability to macromolecules (Damle and Doyle, 1990). This functional ability of memory T cells may be a dominant factor that contributes to the preferential migration of memory T cells into sites of chronic inflammation (Pitzalis *et al.*, 1988), such as the diseased gingival tissues.

The realisation that T lymphocytes are present in large numbers in the diseased tissues of aggressive periodontitis patients and not in health, has led to investigations of the numbers, ratios and functionality of T cells in both the peripheral blood and diseased tissues of patients with periodontal disease. Studies of peripheral blood T lymphocyte subsets in aggressive periodontal disease have revealed a wide variety of contradictory results with either depressed $CD4^+$:$CD8^+$ ratios (Kinane *et al.*, 1989b), or a mixture of increased and depressed proportions of $CD4^+$:$CD8^+$ cells (Katz *et al.*, 1988) or even no correlation with disease (Engel *et al.*, 1984). Studies have also looked at the distribution of T cell subsets in tissues. One study reports that the $CD4^+$:$CD8^+$ ratio in the tissues of aggressive periodontitis patients and even their families tend to be increased (Syrjanen *et al.* 1984). A study by Stoufi *et al.* (1987) and one by Celenligil *et al.* (1993), however, clearly states that the ratio is decreased in patients with this disease. What is obvious, and further indicated by a study investigating juvenile periodontitis (Meng and Zheng, 1989), is that the ratios are highly variable between different patients with the same disease, making generalisations and conclusions very difficult. What the latter study did suggest however, was that high $CD4^+$:$CD8^+$ ratios were seen in those patients with a greater degree of inflammatory cell infiltration, while the low $CD4^+$:$CD8^+$ ratio tissues were less inflamed.

When diseased gingival tissue is removed and the cells extracted, both $CD4^+$ and $CD8^+$ lymphocytes are present in large numbers (Stoufi *et al.*, 1988; Taubman *et al.*, 1984). However, $CD4^+$ cells although prominent, were found at lower levels than in the peripheral blood. Overall ratios of $CD4^+$:$CD8^+$ were not found to be altered in aggressive periodontitis and chronic periodontitis patients (Marthur and Michalowicz, 1997), however, in one study these ratios were found to be depressed in patients with localised and generalised aggressive periodontitis (Kinane *et al.*, 1989b).

1.4.15 T cell functions

1.4.15.1 The role of $CD4^+$ T cells in periodontal disease

T cell functions in periodontal granulation and gingival tissues can be elucidated by the cytokine synthesis profile (Yamamura *et al.*, 1991) (see Table 1.2 for

characteristics of cytokines that play an important role in the progression of periodontal disease). Although the cytokine profile is a good tool for T cell subset investigations, the results reported are often conflicting causing confusion. Often the results cannot assess the relative importance of the TH1 and TH2 subsets.

Fujihashi *et al.* (1996), investigated TH1 and TH2 cytokine mRNA expression by $CD4^+$ T cells from diseased gingival tissues. They detected IFN-γ, a TH1 cytokine, but failed to detect the TH2 cytokine IL-4, thus indicating a more dominant role for TH1 cells. Other groups indicated a more dominant role for TH2 cells after detection of IL-4, IL-5, IL-6 (Yamazaki *et al.*, 1995; Aoyagi *et al.*, 1995; Manhart *et al.*, 1994) and IL-10 (Gemmell *et al.*, 1994). One of the current theories of the TH1/TH2 paradigm is that both cells have an important but different role. Gemmell *et al.* (1997), have suggested that TH1 cells remain tightly localised at sights undergoing active destruction. Whereas, TH2 cells are more widely distributed throughout the tissue and typify a more quiescent stage of the disease. In other words the immune response in periodontal disease is predominantly TH2 driven with active phases of the disease leading to foci of TH1 activity.

The role of these cell types is that of help for the immune response. TH1 cells, characterised often by their production of IFN-γ, are important in microbial killing and are known to augment cytotoxic T cell functions through their production of IL-2 and IFN-γ. TH2 cells, however, inhibit much of the work done by TH1 cells. The production of IL-4 and IL-10 by these cells inhibits the actions of IFN-γ, and hence has anti-inflammatory characteristics. The production of IL-4, IL-5 and IL-6 elicits and helps maintain the humoral immune response.

1.4.15.2 Gamma delta T cells

$CD4^+$ and $CD8^+$ T cells usually express the αβ T cell receptor (TCR). In contrast to this, cells expressing the γδ TCR are typically $CD3^+$and $CD4^-$ $CD8^-$. T cells bearing γδ TCRs are a distinct lineage of cells of unknown function. The ligand for this receptor is also unknown although attempts to identify the ligands have focused on

MHC class-I like proteins, mycobacteria, and heat shock proteins (Haas *et al.*, 1990). There has been growing suspicion over the last decade that γδ T cells do not recognise antigens in the classic T cell:MHC complex way and that this T cell subset may recognise antigens presented by non-classical MHC proteins or members of the CD1 family of proteins (Haas *et al.*, 1990).

In peripheral lymphoid tissues, 1-5% of $CD3^+$ cells express the γδ TCR. However, in epithelial tissues a much higher percentage of the T cells express this receptor (Janeway and Travers, 1994). γδ T cells do not appear to have a great diversity in their receptors. If γδ T cells found in surface epithelium are examined it can be seen that the receptors are essentially homogenous in any one epithelium. One explanation for this is that these T cells are part of the non-adaptive process of the immune response. Because they have a single receptor and do not re-circulate, could be the reason why they recognise alterations on the surface of epithelial cells infected with a pathogen, rather than specific features of the pathogen (Janeway and Travers, 1994).

The role of γδ T cells is still in question. They have been reported to lyse various cellular targets including chicken erythrocytes, cells treated with mycobacteria or staphylococcal enterotoxin A, and various leukaemic cells (Haas *et al.*, 1990). Data seems to suggest that these T cells play a protective role in infectious diseases. Abnormal cell proportions of γδ T cells have been reported in diseases caused by intracellular pathogens, such as leprosy and leishmania (Marthur and Michalowicz, 1997). High numbers of γδ T cells have been reported in leprosy lesions (Bloom *et al.*, 1992), rheumatoid arthritis (Réme *et al.*, 1990), Behçet's syndrome (Fortune *et al.*, 1990), and in the peripheral blood of patients with measles infections (Haas *et al.*, 1990).

The role of γδ T cells in periodontal disease is still not well understood. It has been reported that the mean percentage of γδ T cells in the peripheral blood of patients with periodontal disease, is similar to the numbers found in healthy controls. However, when the data is observed more closely it can be seen that although the mean values

are similar, patients with periodontal disease appear to have abnormally high or low proportions of γδ cells (Nagai *et al.*, 1993).

γδ T cells have been routinely identified in diseased periodontal tissues (Lundqvist and Hammarström, 1993; Gemmel and Seymour, 1995; Prabhu *et al.*, 1996). They have generally been shown to constitute about 1-3% of the $CD3^+$ cells residing in the diseased tissues (Gemmel and Seymour, 1995; Kawahara *et al.*, 1995). The numbers of these cells have been shown to increase significantly with disease, however, remain reasonably low in an environment of moderate to severe inflammation (Gemmel and Seymour, 1995). γδ T cells from diseased tissues have been shown to express either CD4 or CD8 (Lundqvist *et al.*, 1994), mRNA for IFN-γ, TNF-α, TGF-β and IL-6. However, the cytokine profile is dependent on whether the γδ cell expresses CD4 or CD8.

A number of contradictory studies have been reported on the presence of γδ T cells in healthy tissues. Lundquist and Hammarström (1993), reported that greater than 30% of all leukocytes separated from healthy tissues were $\gamma\delta^+$. They described these cells as being $\gamma\delta^+$, $CD4^-$, $CD8^-$ and $CD45RA^+$, hence naive. Contradictory to this, Kawahara *et al.* (1995) reported that when using immunohistochemistry, $\gamma\delta^+$ cells were rarely seen in healthy tissues.

The role of γδ T cells in periodontal disease remains elusive. One theory suggested for function of these cells in this disease, is that they may ligate with heat shock proteins (HSP) produced by stressed or damaged cells. This could be by binding to and destroying damaged or infected epithelial cells (Lundqvist *et al.*, 1994) or even stressed bacterial cells. However, whether these cells have a more important role in the acute rather than the chronic lesion or vice versa is unknown.

1.4.16 Natural killer cells

Natural Killer cells (NK cells) are also said to constitute a large part of the cell mediated immune system, although they are classed as a component of the innate

immune system. These cells are large granular lymphocytes which are often detected by the marker CD16. This makes actual numbers detected very difficult to report since this marker is also found on other peripheral blood lymphocytes and monocytes. In contrast to $CD8^+$ cytotoxic T cells, which kill infected targets cells in an antigen-specific manner, NK cells kill infected target cells or tumour cells in an antigen-non-specific manner (Marthur and Michalowicz, 1997).

The role of NK cells in periodontal disease at present is unknown. However, there are reports of an increase in the numbers of NK cells in the peripheral blood of patients with aggressive periodontitis (Celenligil *et al.*, 1990; Watanabe *et al.*, 1993; Kopp, 1988). Conflicting evidence comes from other studies that have reported no increase in the numbers of NK cells found in the peripheral blood of diseased patients when compared with health (Zafiropoulos *et al.*, 1990). In the tissues of diseased patients an increase of NK cells is seen. This is not surprising since in health very few NK cells are present in the gingival tissues. The numbers of NK cells in the tissues are seen to increase from health to gingivitis to periodontitis (Wynne *et al.*, 1986; Cobb *et al.*, 1989; Fujita *et al.*, 1992). However, the proportion of these cells relative to total lymphocyte numbers actually decreases (Cobb *et al.*, 1989).

The activity of NK cells is increased by soluble mediators such as IL-2, IFN-α, IFN-β and IFN-γ. The interferons α and β are antiviral proteins synthesised and released by leukocytes, fibroblasts and virally infected cells and IL-2 and IFN-γ are released by activated T cells (Benjamini *et al.*, 1996). Surface LPS from Gram negative bacteria appears to provide the major activation signal for NK-cell-mediated cytotoxicity in periodontal disease (Lindemann, 1988). This leads to NK cell cytotoxic activity against host cells.

1.5 Cytokines in Periodontal Disease

1.5.1 Interleukin-1

In the periodontal tissues IL-1β is predominant over IL-1α, and compared with healthy and stable sites is found at higher levels in active disease (Jandinski *et al.*,

1991; Stashenko *et al.*, 1991a). Variations have been noted in the concentrations of IL-1 between sites in the same individual, this coupled with variations in serum IL-1β levels between chronic periodontitis patients and healthy controls, suggests the production of this cytokine is at the local level rather than the systemic (Tatakis, 1993; Chen *et al.*, 1997). Macrophages, PMN, B cells and fibroblasts secrete IL-1β, and activated keratinocytes and Langerhans cells produce both IL-1α and IL-1β (Hillmann *et al.*, 1995; Jandinski *et al.*, 1991; Gemmel and Seymour, 1998; Takahashi *et al.*, 1995).

The production of IL-1 stimulates certain cells to express adhesion molecules. These include; fibroblasts, endothelial cells, monocytes and granulocytes (Takahashi *et al.*, 1994). The expression of adhesion molecules aids the migration of immune cells from the capillaries into the inflamed tissues. It has been shown that numerous periodontal pathogens can stimulate host cells to produce IL-1 (Gemmel *et al.*, 1992; Gemmel and Seymour, 1998).

IL-1 can induce the production of plasminogen activator, IL-6, matrix metalloproteinases and PGE_2 by gingival fibroblasts. These cells are involved in tissue destruction, turnover and remodelling (Richards and Rutherford, 1988). IL-1 stimulates osteoclasts to resorb bone (Horton *et al.*, 1972), whilst inhibiting bone formation (Stashenko *et al.*, 1987). IL-1-mediated bone resorption can be induced by periodontopathogens (Ishihara *et al.*, 1991).

1.5.2 Tumour necrosis factor

TNF-α, IL-1α and IL-1β are all known as pro-inflammatory cytokines and have all been identified at higher levels in periodontitis lesions from patients with chronic periodontitis compared with healthy sites (Matsuki *et al.*, 1992; Stashenko *et al.*, 1991b). However, cells containing IL-1β were found in much greater numbers than those producing TNFα and IL-1α (Stashenko *et al.*, 1991b). In contrast to IL-1, only very low concentrations of TNFα have been demonstrated in GCF from both chronic

and aggressive periodontitis patients (Rossomando *et al.*, 1990; Yavuzyilmaz *et al.*, 1995).

High levels of TNFα in periodontal lesions appear to represent an established inflammatory and immune response. Salvi and co-workers (1998) examined GCF IL-1β, TNFα and PGE_2 levels in type I diabetes mellitus patients with periodontal disease. They found that diabetics had significantly increased levels of PGE_2 and IL-1β but not TNFα, as compared with non-diabetic controls with similar periodontal status. In addition, diabetics with moderate to severe disease had almost two-fold higher levels of PGE_2 and IL-1β compared with diabetics suffering from gingivitis or mild periodontitis.

PGE_2 causes increased vascular dilation and permeability. It also stimulates macrophages to secrete matrix metalloproteinases and has been shown to trigger bone resorption *in vitro* (Birkedal-Hansen, 1993), thus acting synergistically with IL-1 and TNFα. Furthermore it upregulates complement and Fc receptors on the surface of monocytes and PMN (Offenbacher, 1996). In inflamed periodontal tissue, prostaglandins and leukotrienes are mainly produced by activated macrophages, although they can also be produced by fibroblasts. Prostaglandins, especially PGE_2, comprise the primary pathway of alveolar bone destruction in periodontitis. Leukotrienes, especially leukotriene B4, are potent chemoattractants for PMN.

1.5.3 Interleukin-10

A role for IL-10 in the progression of chronic periodontitis was suggested by Gemmell and Seymour (1998). This study indicated that T cell lines from both the peripheral blood of periodontal patients and their gingival tissue, secreted IL-10, but clones derived from the peripheral blood of an individual with gingivitis did not. It has also been suggested by Stein and co-workers that IL-10 may contribute to auto-immune reactions against gingival tissues (Stein and Hendrix, 1996; Stein *et al.*, 1997). Gemmell and Seymour (1998) have demonstrated a significantly reduced number of IL-10^+ $CD8^+$ cells from chronic periodontitis lesions, than from healthy/gingivitis sections. They suggested that in gingivitis, IL-10 might suppress inflammation by

decreasing macrophage activity, thereby preventing progression to periodontitis. No differences were found in the percentage of IL-10$^+$ CD4$^+$ cells between the two disease categories. However, some individuals were found to have higher numbers of IL10$^+$ T cells, regardless of disease category. It appears that the overall variation between the two diseases entities in IL-10$^+$ CD8$^+$ cells may have been due to individual variation in IL-10 secretion patterns, rather than differences in pathogenesis between the two disease categories. Further studies are needed to clarify the role of IL-10 in periodontal disease.

In summary, IL-1, IL-6 and TNF are pro-inflammatory cytokines which are detected and may be modulated within the periodontium. They are important aspects of the host defence system, operating in the periodontal tissues during health, destruction and healing phases. Further research may lead to a greater understanding of the roles of other members of the cytokine network, in particular IL-10 and various soluble and cell-bound receptors and inhibitors of cytokine function, in periodontal disease. These molecules are inter-related and are part of the immune, inflammatory, breakdown and repair homeostasis ongoing within the periodontium.

Table 1.2 The Role of Cytokines in Periodontal Disease

Cytokine	Produced by	Role
Interleukin-1 (IL-1)	large quantities produced by macrophages	• proinflammatory properties • mediator of tissue destruction in periodontal disease (Alexander & Damoulis; 1994)
Interleukin-4 (IL-4)	activated T cells	• inhibits production of IL-1, TNF and IL-6 • an absence of IL-4 in the periodontal tissues has been suggested to trigger disease progression (Fujihashi *et al.*, 1993, Hirano *et al.*, 1991)
Interleukin-6 (IL-6)	lymphoid and non-lymphoid cell types. Production is triggered by IL-1, TNF and IFN-γ	• mediates inflammatory tissue destruction • thought to be an important cytokine in B cell differentiation, and hence play a role in the induction of the elevated B cell response in patients with periodontal disease (Fujihashi *et al.*, 1993)
Interleukin-8 (IL-8)	mononuclear monocytes and many of the tissue cells	• attracts and activates neutrophils into the tissues of the periodontium, particularly as it is produced by gingival fibroblasts (Takashiba *et al.*, 1992)
Interleukin-12 (IL-12)	monocytes, macrophages, B cells and accessory cells	• Pleiotrophic effects on NK cells and T cells in the tissues • induces IFN-γ production and is necessary for TH1 induction
Tumor Necrosis Factor α and β (TNF-α, TNF-β)	macrophages and lymphocytes respectively	• similar effects to IL-1 and IL-6
Interferon γ (IFN-γ)	activated T cells	• potent inhibitor of IL-1, TNF-α and TNF-β

1.6 Important Antigens in Periodontal Disease

1.6.1 Introduction

The following section is a discussion of immunodominance and the existence of immunodominant antigens. The aim of the studies presented in this thesis is to focus on the target of the humoral immune response in periodontal disease, and in particular the importance of immune response inducing antigens. The subject of immunodominance is a controversial one, and the following section will be devoted to discussing evidence in the literature for immunodominant antigens in a number of diseases.

There is substantial literature on the humoral immune response detected against micro-organisms present within periodontal pockets. However, the significance and specificity of the immune response in periodontal disease has proved difficult to elucidate. This is due to the large number of putative pathogens in the plaque biofilm, and the apparent commensal nature of many of these opportunistic organisms. Identification of the immunodominant antigens involved in the disease process would be informative especially with respect to: (i) the relative importance of the implicated pathogens; (ii) new approaches to immunological diagnosis; (iii) specific bacterial virulence determinants; (iv) natural protective responses; and (v) the selection of potential candidate antigens for vaccine development.

The concept of immunodominance is often misused by investigators. Their interpretation of data is that specific antigens or epitopes of a micro-organism or macromolecule are 'immunodominant'. However, little explanation as to the basis of the finding and importantly, its significance is provided.

The phenomenon of 'immunodominance' was initially described by Sercarz and colleagues (Sercarz, 1989) to provide a framework for explaining the genetic control of antibody responses to hen egg lysozyme (HEL). HEL is a globular protein with a known 3-dimensional structure. These investigators were able to define antigenic epitopes using peptides derived from cyanogen bromide cleavage of this protein.

Specifically, immune responses of H-2^b mice to HEL were restricted to 3 amino terminal residues of the molecule. Wicker *et al.* (1984), reported that if these 3 residues were cleaved, 50% of the primary response antibody molecules bound poorly if at all to the HEL, and the immunogenicity of the protein was drastically effected. The term 'immunodominance' was used to describe the predominant immune recognition of these 3 amino terminal residues. Sercarz (1989) suggested that for efficient and regulated activity of the immune response to a particular antigen, there should be a small number of immunodominant epitopes on an antigen.

The primary, secondary, and tertiary structures of a protein antigen offer a vast array of potential epitopes. However, the number recognised by the immune system appears to be considerably smaller than the full antigenic repertoire of the immunogen (Laver *et al.*, 1990). Some of these antigenic epitopes will dominate the resulting specificity of the immune response. This can vary from species to species, and even among individuals within a species. They will not only induce a more pronounced immune response, but may also suppress the primary immune response to other antigenic determinants. Recent studies using a similar experimental system have demonstrated the broad heterogeneity of proliferative responses to the immunodominant determinants within HEL. The heterogeneity of response was suggested to relate to the competitive and regulatory nature of the interaction between various factors. These include different MHC molecules, determinant capture by antigen presenting mechanisms, and the available T cell repertoire. The authors suggested that these results may have important implications for studies of autoimmunity, infection and vaccine design in human populations, where heterozygosity is the characteristic of the population (Moudgil *et al.*, 1998).

The basis for the existence of immunodominant epitopes is not well understood. Recent findings have shown that peptide competition for MHC binding may not represent the most important event in processes leading to immunodominance (Lo-Man *et al.*, 1998). T lymphocyte responses to a protein antigen are restricted to a limited number of determinants and not to all peptides capable of binding to MHC class II molecules.

Focusing of the T cell immune response is also defined as immunodominance, and has been observed with numerous antigens. Recent investigations by Gapin *et al.* (1998) have suggested that dendritic cells play a major role in the immune response against a few antigenic determinants. Conversely, B lymphocytes may diversify the T cell response by presenting a more heterogeneous set of peptide-MHC complexes. Moreover, this T cell focusing has been extended to cellular immune responses, which are directed against a narrow set of immunodominant peptides derived from complex antigens. The existence of epitopes that are hidden or infrequently targeted by immune responses (cryptic epitopes) has also been identified.

Although the identification of immunodominant epitopes is important for vaccine development, understanding immunological reactivity to these cryptic epitopes may be important in the development of autoimmunity. Blum *et al.* (1997) have suggested that targeting exogenous antigens into specialised processing compartments within antigen presenting cells appears to be important in epitope selection and immunodominance.

The final outcome of the immune response may be an immunochemical phenomenon which is related to the protein structure or the host's immunoregulatory mechanisms. However, it also appears to be effectively used by various pathogens, as a host evasion tactic. This occurs when the microbial immunodominant epitopes are sterically close to antigens which have the capacity to demonstrate antigenic diversity and are capable of antigenic variation. Thus, they help to conceal the micro-organism from the host protective responses (Nara *et al.*, 1998).

In the field of periodontal research the term 'immunodominant antigen' and the concept of 'immunodominance' have been used imprecisely, are poorly defined and often misrepresented. There are multiple reports in the literature, purportedly characterising immunodominant antigens of oral micro-organisms, yet no clear definition of immunodominance is included. It is not known whether these antigens express an epitope that is the focus of the primary immune response, and which is essential for the initiation of the response to the antigen and micro-organism (Wicker

et al., 1984). Due to the chronic nature of periodontal infections, these subjective measures may only be defining the gravimetric quantity of a particular antigen, rather than describing a function of its unique antigenicity.

Thus, the original definition of immunodominance has been distorted to describe a microbial antigen to which a predominance of antibody is detected when compared to the myriad of other antigens presented by the micro-organism and recognised by the host. Investigators should be clearer when reporting results. They should indicate whether they have described an antigen to which there is a 'detectable antibody response' rather than an 'immunodominant antigen'.

Immunodominance and immunodominant antigens have been difficult to study in periodontal disease due to the large number of species comprising the microbial biofilm at sites of health and disease. Other factors including proposed genetic predisposition, behavioural variables such as oral hygiene levels, and environmental factors (e.g. smoking and socio-economic factors) have not made the problem any simpler. In addition, there is controversy about the nature of the humoral immune response itself. Whether the antibody response in this disease is protective or contributes to tissue destruction indirectly is unknown. It is reasonable to assume that disease activity would be abrogated if the antibody response was effective at eliminating or controlling the growth of the micro-organisms present. In contrast the antibody response may be harmful to the host if it protects certain bacteria or actually causes pathology itself (e.g. hypersensitivity). A study by Mooney *et al.* (1995), reported that patients with periodontal disease who possessed high avidity antibodies to oral pathogens, have a better prognosis than patients with low avidity antibodies, regardless of whether the latter have a high titre of antibodies or not. This study appears to indicate that an effective antibody response is protective. However, numerous reports speculate on a possible autoimmune aspect to periodontal disease (Brantzaeg and Tolo, 1977; Kristofferson and Tonder, 1973; Hirsch *et al.*, 1988; Anusakasathien and Dolby, 1991). There is no conclusive evidence that high levels of antibody are protective or indicative of disease. An important factor which must be considered is that, patients with periodontal disease are not found to be more susceptible to other bacterial infections. They also do not show severe pathology

linked to any other diseases. In addition, autoantibodies have not been found to predominate in the periodontal lesion, although antibodies to host components, in particular collagen (Ftis *et al.*, 1986; Peng, 1988; Hirsch *et al.*, 1988) have been reported. Until evidence is found to support the conclusion that the immune response is destructive it should be assumed that it is protective. However, the pathology seen in this disease is not only due to the direct harmful effects of micro-organisms. Production of high levels of pro-inflammatory cytokines (IL-6, IL-8, TNF) has been reported, (Tsai *et al.*, 1995; Yavuzyilmaz *et al.*, 1995) which could result in considerable tissue destruction.

A further consideration in periodontal disease, is that critical clinical features of the disease are often identified long after the biologic mechanisms have initiated the disease process.

When addressing our current knowledge of the antigens to which a detectable immune response in periodontal disease may be directed, one must consider that within a complex microbial biofilm, such as the subgingival plaque, it appears that certain micro-organisms elicit greater antibody levels (i.e. dominate the immune response) than others (Zambon 1996). This characteristic of the host response does not appear to depend entirely on the absolute numbers of an individual micro-organism. It may be related more to their structural and antigenic components or their pathogenic significance (virulence), as well as their distribution within the biofilm and/or host tissues. An understanding of the antigens, which elicit a detectable antibody response, should contribute to a better understanding of specific bacterial virulence determinants, natural protective responses, and even possible strategies for vaccine development, or new approaches to immunological screening and diagnosis of the disease.

A survey of the scientific literature on immunodominant antigens in medical infectious diseases provides an indication of the significance of this concept for identifying potential virulence, diagnostic, or vaccine candidates. In the following discussion, selected studies are reviewed to provide examples of the variation in the use of the terminology. In addition, the potential limitations of equating detectable

antibody response with immunodominant antigen and protective immune responses are highlighted.

Examples of reports identifying immunodominant and therefore, candidate diagnostic antigens in parasitic infections include studies on *Neospora canium* (Howe *et al.*, 1998), *Theilera parva* (Katende *et al.*, 1998), *Trypanasoma cruzi* (Pereira *et al.*, 1998) and *Schistosoma mansoni* (Chen and Boros 1998). It is clear from the literature that an antigenic relationship to similar antigens from other parasites and prevalence of the response during infection, is often used as evidence to speculate on the importance and function of potentially "important" antigens during infection. Identification of important antigens from these types of studies can also lead to a higher degree of sensitivity and specificity in diagnosis. Some studies have even uncovered information on the disease pathology and progression. For example, Chen and Boros (1998) identified that the immunodominant T cell epitope, P38, of *Schistosoma mansoni*, elicited the pulmonary granuloma formation, the disease associated hypersensitivity lesion of this infection.

There have also been a variety of similar studies which focused on dominance in bacterial or fungal infections. While the mortality rate for systemic candidosis remains high, the diagnosis is somewhat difficult and there has been considerable interest in developing a reliable serodiagnostic test. Mathews *et al.* (1988) identified the 47 kDa component of *Candida albicans* as an 'immunodominant antigen' in the serology of systemic candidosis. The 47 kDa antigen appears to be immunodominant and 92% of patients with candidal antibodies produced a response to an antigen of 47 kDa. It seems that this antigen is conserved in structure between strains, and an assay detecting this antigen would be the basis for a sensitive and specific diagnostic test (Mathews *et al.*, 1984, 1987).

Further interesting and important knowledge has been gained from studies involving immunodominant antigens and the quest to identify them. The *Mycobacterium avium* complex (MAC) consists predominantly of *Mycobacterium avium* (*M. avium*) and *Mycobacterium intracellulare* (Inderlied *et al.*, 1993). MAC members are ubiquitous environmental micro-organisms and infection in healthy individuals is rare. However,

MAC infection is a major cause of bacterial morbidity and mortality in AIDS patients. A recent report by Triccas *et al.* (1998) suggested, following molecular and immunological analyses, that a 35 kDa protein of *M. avium* is a homologue of the 35 kDa immunodominant antigen of *Mycobacterium leprae* (Triccas *et al.*, 1996). The 35 kDa protein of MAC, was confirmed to contain T-cell and B-cell epitopes recognised by human immune responses following mycobacterial infection. However, it was discovered that immunologically there is an inability to distinguish between MAC and *Mycobacterium tuberculosis*, even though the 35 kDa protein is not recognised by tuberculosis patients. Although this is an example of a study where an antigen eliciting detectable antibody response is identified, it does not identify a potential vaccine candidate or any other markedly positive conclusion. For both tuberculosis and mycobacterial infections, early diagnosis is crucial for the prognosis of the patients. Therefore, this emphasises a situation where using a detectable antibody response for differential diagnosis is of critical importance.

Studies investigating dominant antigens, even if they are not primarily designed to do so, can answer crucial questions. Otitis media is a common problem associated with high morbidity, in which a subset of children experience repeated episodes caused by nontypeable *Haemophilus influenzae* (*H. influenzae*) (NTHI). Murphy and Kyungcheol (1997) showed that the principal antibody response in animals to an NTHI strain was directed at an immunodominant epitope of the P2 molecule, which is the major outer-membrane protein of NTHI. However, this group hypothesised that the otitis prone children develop an antibody response to an immunodominant region of the P2 molecule, which is strain specific. Therefore, the child's protective response is directed exclusively against the infecting strain. These children will experience recurrent episodes of otitis media since they have no protection against a new strain. The discovery of the dominant antigen associated with this infection has further important implications. It contributes to the understanding of the recurrent nature of otitis media and provides useful data on a potential vaccine design for this disease.

As mentioned in the preceding section, the importance of T cell immunodominant epitopes is clear and must be distinguished from those eliciting a dominant B cell response. *Listeria monocytogenes* secretes proteins associated with its virulence

inside infected cells. These are degraded by host cell proteasomes, processed into peptides and bound to MHC class I molecules. These antigen epitopes are effectively presented to cytolytic T lymphocytes (CTL). Results of Pamer *et al.* (1997) indicated that immunodominant T cell responses cannot be predicted by the prevalence of antigens or epitopes. In fact, one of the least prevalent epitopes primes for the immunodominant CTL response in mice.

Above are all examples of the purported significance of identification of immunodominant antigens and the potential benefits in medically important infections. Utilisation of the antigen(s) to create diagnostic tools, and develop vaccines attaches greater value and relevance to the search for these types of antigen.

The need to identify periodontal pathogens as true aetiological agents in the disease process still exist. Many of these candidate pathogens may be merely opportunistic and depend on the biofilm "food web" to create the conditions or niche within which they can colonise, obtain nutrients and replicate. Thus, these micro-organisms would not classically be considered as the true pathogens against which host defence mechanisms should be directed. In spite of the potential microbial complexity of this disease, a number of candidate species have emerged which are thought to play a role in the pathogenesis of periodontal disease. These micro-organisms generally fulfil the modified Koch's postulates as described by Socransky and Haffajee (1992).

Examination of numerous periodontal pathogens has revealed these micro-organisms to possess a variety of potential virulence factors and structures that may be crucial to their pathogenicity. In the quest for the identification of the important bacterial antigens in periodontal disease, a number of different candidate antigens have been suggested.

1.6.2 Outer-membrane proteins

Much of the periodontal literature applicable to this topic covers studies on the outer-membrane proteins (OMP) of periodontal pathogens. OMP of Gram negative bacteria may reside on the inner or outer layer, or can span the entire outer-membrane. Gram

negative bacteria possess multiple OMP which provide important biological functions, such as phage receptors or receptors for uptake of nutritional substrates. Various specific microbial antigens have been identified from the outer-membranes of oral pathogens to play a role in periodontal disease. However, many studies have identified outer-membrane antigens of the same micro-organisms, which remain uncharacterised and may be of importance in protection afforded by the adaptive host immune response (Watanabe *et al.*, 1989; Califano *et al.*, 1989; Wilson *et al.*, 1991; Califano *et al.*, 1992; Page *et al.*, 1992b). These studies have tended to focus on either sonicate antigens or OMP which can be sheared from the surface of the bacteria (Nakagawa *et al.*, 1995). Since these two preparation methods differ, one could identify both antibodies to intracellular antigens, as well as to the surface antigens, however, similar results might be expected. It seems unlikely that patients would produce an array of antibodies to "inaccessible" intracellular proteins, and more likely that antigens eliciting a detectable antibody response would be expressed on the microbial surface or as secreted proteins. The majority of the outer-membrane components of putative periodontopathogens remain uncharacterised with respect to their metabolic or structural functions or their activity as virulence determinants. In general, patients with strong responses to these individual antigens often tend to be those who have a strong response to the whole bacterial cells.

Nakagawa *et al.* (1995) reported detection of antibodies to apparent 'immunodominant antigens' of *P. gingivalis* in the sera of chronic periodontitis patients. These were specific for antigens of molecular weight 75 and 31 kDa from outer-membrane preparations, and a 46 kDa antigen from sonicated extracts of the micro-organism. Further studies identified 75, 55, and 43 kDa bands in whole cell sonicates of *P. gingivalis* to be 'immunodominant antigens' in rapidly progressive periodontitis patients (Chen *et al.*, 1995). A similar approach was utilised by Ebersole and Steffen (1994). They documented 'immunodominant antigens' in outer-membrane preparations, from various strains of *P. gingivalis*. The results identified some similarities in antibody recognition of antigen bands across strains, however, a striking variability was noticed between the strains. Additionally, variations in response patterns were noted to be associated with high and low response patients, irrespective of the clinical disease classification.

Ebersole *et al.* (1995) and others (Bolstad *et al.*, 1990; Watanabe *et al.*, 1989; Sims *et al.*, 1991) have used Western immunoblot approaches to detect antibody responses specific for antigens in the outer-membranes of *A. actinomycetemcomitans*. These studies revealed a wide variation in responses among individual patients, thus adding to the difficulty regarding the identification and/or importance of an antigen(s) that dominates throughout a patient population. Alternatively, Flemmig *et al.* (1996) demonstrated IgG/IgA antibody activity in aggressive periodontitis (65%/70%) and chronic periodontitis (45%/55%) patients reactive with a 110 kDa OMP of *A. actinomycetemcomitans*. In contrast, control subjects had no IgG and only 5% had any IgA antibody activity. This approach exemplifies a strategy whereby a high frequency and or level of antibody is detected to a particular antigen in patients versus controls, which is then suggested to be an immunodominant antigen. Each of these studies aimed to identify immunodominant antigens as macromolecules which may play an important role in pathogenesis, and the response to which correlated with disease resistance. In fairness to the authors, many of these studies probably identified the molecular weight of antigens which elicited a detectable antibody response in this disease. However, none of the mentioned studies obtained sufficient evidence to describe an immunodominant antigen.

Importantly, the OMP are uniformly immunogenic and many demonstrate antigenic diversity (heterogeneity) (Holt and Bramanti, 1991; Holt and Ebersole, 1991; Mitchel, 1991). Moreover, environmental changes altering the growth of numerous micro-organisms, such as *Bordetella pertussis*, have been shown to result in antigenic modulation of OMP (Robinson *et al.*, 1986). Genco and colleagues (1991) presented the concept of the potential importance of antigenic heterogeneity, as a virulence strategy for oral micro-organisms. Recent studies by Ebersole and colleagues (1995) with *A. actinomycetemcomitans,* Chen *et al.* (1995) with *P. gingivalis*, and Sims *et al.* (1998) with *B. forsythus* have supported antigenic diversity of these pathogens within infected patients. However, there remains minimal information on its potential role in virulence within the periodontal microbiota.

The previously mentioned studies give examples of strategies which have been used and provide examples of some potentially interesting targets. However, they only "scratch the surface" in providing an understanding of the antigenic epitopes which activate the regulatory T cells. These T cells effectively stimulate regional B lymphocytes which could provide a functional host immunity in periodontal disease. Current genome sequencing of these pathogens will allow us to visualise the organisation of these protein genes. In addition it will enhance studies of genetic and antigenic heterogeneity within the population. Sequencing, purification and functional studies of these antigens will encourage evaluation of their significance in well characterised patient and control populations, as well as in *in vitro* and *in vivo* models of disease. More importantly, from the perspective of this review it is not clear that the concept of a ''dominant antigen'' is critical to the ultimate goals of this research. As mentioned previously, high antibody levels or raised titres to an antigen do not necessarily define immunodominance of the antigen. They may instead delineate a dominant antibody response. If this response is directed against a 'flak antigen', (an antigen capable of significant diversity or variation, or having little contribution to the pathogenicity of the micro-organism), the detection of a dominant antibody response does not inherently describe an immunodominant antigen nor provide insight into immune protective responses.

1.6.3 Fimbriae

Fimbriae or pili as they are often termed because of their functional and molecular homology to pap pili of *E. coli* (Krogfelt, 1991), are curled single stranded filaments with a diameter of approximately 5nm (Ishikawa *et al.*,1997). They are cell surface structures important in adherence and are possibly virulence factors in medically significant pathogens.

Gilsdorf *et al.* (1989) reported the fimbriae of *H. influenzae* as being immunogenic. Pichichero *et al.* (1982) detected anti-pilin antibodies in human serum following *H. influenzae* infection. However, many investigators have suggested that fimbriae of *H. influenzae* are not pathogenic when tested in animal models (Kaplan *et al.*, 1983; Stull *et al.*, 1984; Mylotte *et al.*, 1985).

Early ultrastructural studies clearly identified fimbriae-like structures on the surface of numerous oral micro-organisms, including *A. actinomycetemcomitans*, *Actinomyces* spp., *P. gingivalis* and *Prevotella* spp. (Cisar *et al.*, 1979; Woo *et al.*, 1979; Scannipieco *et al.*, 1983; Rosan *et al.*, 1988; Leung *et al.*, 1989). Therefore, as identified for other pathogens, these structures may be of importance in the pathogenesis of periodontal disease. The principle work in this area has involved detailed studies of these structures in *P. gingivalis*.

P. gingivalis has been identified in gingival tissues, which suggests that this micro-organism is able to successfully colonise the microbial biofilm in the gingival sulcus and cross the epithelial barrier into deeper tissues (Gibson *et al.*, 1964; Saglie *et al.*, 1988; Sandros *et al.*, 1996). Fimbriae appear crucial for the adherence of micro-organisms, such as *P. gingivalis*, to other members of the microbial plaque and to host gingival tissue surfaces. Supportive evidence arises from studies examining afimbriate variants of *P. gingivalis* with diminished capacity to adhere to epithelial cells (Genco *et al.*, 1994). Hamada *et al.* (1994) reported that a *fimA* mutant of *P. gingivalis*, which lacked surface fimbriae, showed deficient adherence to both cultured human gingival fibroblasts and epithelial cells. They concluded that this protein structure is essential for the micro-organism to interact with gingival tissues (Hamada *et al.*, 1994).

The functional importance of fimbriae has further been supported by Madianos *et al.* (1997), who described the adhesive and invasive properties of *P. gingivalis* strains on the KB oral epithelial cell line. In this study, a population of a fimbriated strain of *P. gingivalis* demonstrated 50% adherence to the epithelial cells. In addition 10% of the bacteria were internalised within 90 minutes of infection of the monolayer. In contrast, a mutant strain lacking fimbriae displayed a significantly decreased adherent capacity (10%), only 1.5% of the bacteria were internalised. The available literature supports the theory that fimbriae of this pathogen are a target of the humoral immune response in periodontal patients. However, a lack of antibodies reactive with fimbriae has not been identified as increasing disease risk. Condorelli *et al.* (1998) recently

reported detection of IgA antibodies in the GCF of patients with acute recurrent periodontitis. The antibodies were specific for the purified 43 kDa fimbrial antigen.

Malek *et al.* (1994) used *P. gingivalis* with an insertional mutation in the gene encoding the fimbrillin subunit. They showed that the mutant was unable to adhere to salivary pellicle coated tooth surfaces and could not induce bone loss in a gnotobiotic rat model. Additionally, an anti-fimbrial monoclonal antibody has been shown to block the adherence of *P. gingivalis* to salivary pellicle-coated tooth surfaces (Genco *et al.*, 1994). These two experiments suggest that an absence of antibodies to fimbriae may encourage the adherence/colonisation of *P. gingivalis* and enhance the risk of disease onset.

There is generally no evidence supporting the role of fimbriae as an immunodominant antigen in periodontal disease, or other diseases. These studies have described dominant humoral immune responses. They do not establish that fimbriae are immunodominant antigens. Fimbriae seem to be extremely important in mediating adherence to human mucosal surfaces (Beachey, 1981). Therefore, they must be considered an attractive candidate for a future vaccine. A study by Brant *et al.* (1995) reported a novel approach to induce protection against different oral micro-organisms, that was to define the immunodominant region of fimbrillin and then use synthetic peptides to mimic this epitope in a vaccine. This was a novel and theoretically excellent approach. However, with hindsight the lack of reports in dental literature about human antibody titres directed against fimbriae, coupled with the difficulty of extrapolating results from animal models, indicate that there are problems with this concept. The utilisation of adhesins as vaccine candidates is not a new idea. Clark *et al.*, (1985) developed a model to evaluate the role of fimbriae in the prevention of colonisation of teeth by *Actinomyces viscosus* (*A. viscosus*). After mice had been immunised with both type 1 and type 2 fimbriae, antibodies of isotype IgG and IgA were detected. Further challenge with *A. viscosus* resulted in a significant reduction in the number of mice colonised. The results of this early study, indicated that fimbrial vaccination may modulate colonisation of oral bacteria.

In conclusion fimbriae do not appear to be immunodominant although they do appear to be important. The available literature supports the theory that fimbriae of *P. gingivalis* are a target of the humoral immune response in periodontal disease patients. This has been delineated in various disease populations, with a general finding of increased levels and frequency of serum antibody to the fimbriae or fimbrillin subunits (Okuda and Takazoe, 1988; Ogawa *et al.*, 1989a; Genco *et al.*, 1991). Recently, Condorelli *et al.* (1998) reported the detection of IgA antibodies in the GCF of patients with acute recurrent periodontitis. The antibodies were specific for the purified 43 kDa fimbrial antigen. Thus, it is clear that both a local and systemic humoral immune response is generated to this antigen in many periodontitis patients. However, evidence that fimbriae are immunodominant antigens in periodontal disease or in other infectious diseases is generally lacking.

1.6.4 Lipopolysaccharide (LPS)

A major structural feature of all Gram negative bacteria is the LPS macromolecule comprising the outer-membrane of these prokaryotes. LPS exhibits a broad spectrum of pathophysiological properties. It is a potent immunostimulator and adjuvant, as well as being a principal virulence determinant of most Gram negative pathogens. It elicits these effects via induction of local and systemic inflammatory reactions.

The LPS macromolecule is composed of 3 distinct regions; the O polysaccharide-chain, the core region, and the lipid part, termed lipid-A. The O-Chain anchors LPS to the bacterial membrane (Luderitz *et al.*, 1982). This portion of the molecule shows enormous structural variability within prokaryotes. It is generally characteristic for a Gram negative bacterial species and often represents an antigenically diverse surface component of these micro-organisms (Zahringer *et al.*, 1994). The core region of LPS is organised into an outer core and an inner core (Holst *et al.*, 1992). The outer core region contains neutral sugars and the inner core often contains rare glycose residues. Moderate inter-bacterial variability has been suggested to occur in this region. The lipid A portion of LPS is linked to the polysaccharide core of the molecule. It is the least variable component of LPS (Rietschel *et al.*, 1984) and is generally considered to be responsible for the endotoxic, inflammatory and tissue destructive effects of LPS.

LPS from oral micro-organisms can penetrate calcified tissue (Aleo *et al.*, 1974) and gingival connective tissue (Aleo *et al.*, 1980). Studies have also suggested that LPS from oral bacteria may contribute directly to tissue damage and bone resorption characteristic of periodontal disease (Hausmann *et al.*, 1970). This early work and subsequent studies are based on *in vitro* cell cultures and therefore, may not necessarily apply to the *in vivo* situation. Nevertheless, proposing a pathogenic role for LPS is reasonable since it has been detected in periodontal tissues, even in the absence of identifiable bacteria. Large numbers of blebs (e.g. outer-membrane vesicles) have been observed associated with several Gram negative oral bacteria. These membrane bound, diffusable liposomal-like structures could enhance the transfer of LPS to sites distant from the plaque biofilm, such as the periodontal tissues (Nisengard and Newman, 1994).

Numerous studies have demonstrated that the serotype determinants of *A. actinomycetemcomitans* are associated with a high molecular weight LPS-associated antigen (Califano *et al.*, 1989) or carbohydrate smear antigen (Califano *et al.*, 1989; Sims *et al.*, 1991). In aggressive periodontitis patients with the highest serum antibody levels to *A. actinomycetemcomitans* the predominant antibody specificity is to LPS (Okuda *et al.*, 1986; Wilson *et al.*, 1991; Califano *et al.*, 1992; Wilson and Hamilton, 1995). LPS is generally thought to exhibit immune stimulating characteristics which are T cell independent, and thus principally induce an IgM response. However, it is clear that aggressive periodontitis patients produce substantial levels of IgG antibody to this antigen from *A. actinomycetemcomitans* (Lu *et al.*, 1993). The level and characteristics of this dominant antibody response suggests that an anti-LPS response is a significant marker of *A. actinomycetemcomitans* infection and existing periodontitis. However, whether LPS represents a virulence determinant, a true "dominant antigen", or an antigen to which the adaptive immune response can provide protective immunity remains to be proved.

The literature is replete with studies reporting high titres of serum IgG antibodies to *P. gingivalis* in periodontitis patients compared with healthy controls. However, the temporal acquisition of the antibody response relative to colonisation and/or infection

has not been clearly described. Thus, animal models have been developed to examine the acquisition and specificity of potential protective immunity. A recent study by Vasel *et al.* (1996) reported that immunisation of monkeys with a vaccine containing a monkey isolate of *P. gingivalis*, induced protection against alveolar bone destruction. This might be considered surprising since a number of pathogens are considered to be associated with the periodontopathic microbial ecology (Socransky and Haffajee, 1992; Zambon, 1996). In addition, in many periodontitis sites in humans, *P. gingivalis* is undetectable (Mombelli *et al.*, 1991). Further investigation of immune and pre-immune sera obtained from monkeys, used immunological analyses including ELISA and Western immunoblotting (Vasel *et al.*, 1996). After immunisation the sera of the monkeys contained increased antibody levels reactive not only with antigens of *P. gingivalis,* but also with antigens of *B. forsythus*. Detailed analyses suggested that the binding of serum antibodies was a specific, adaptive response to the LPS in the immunogen, since little reactivity was detected in the pre-immune sera. Thus, the antibodies induced by LPS from *P. gingivalis* appeared to be cross-reactive with the LPS of *B. forsythus*, and could contribute to a more general modification of plaque ecology.

These observations are similar to those obtained by other investigators examining LPS in non-oral Gram negative bacteria. For example, Seifert *et al.* (1996) reported the generation of a human monoclonal antibody that recognised a conserved epitope shared by LPS molecules of different Gram negative bacteria. This indicated a high degree of cross-reactivity of antibody across different bacterial species. The epitope recognised by the human monoclonal IgM antibody (i.e. LPD5H4) was located in the lipid A portion of the LPS molecule. Importantly, the monoclonal IgM antibody was shown to partially neutralise LPS activities of *Salmonella minnesota*, *Salmonella typhimurium*, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Neisseria meningitidis*. The binding specificity of LPD5H4 is only a small step in the search for the existence of immunodominant epitopes in the conserved regions of LPS. However, it does provide an indication of the potential for broad cross-reactivity of antibodies against LPS molecules of different bacterial genera. This phenomenon should be considered when documenting the role of LPS as an antigen in periodontal disease.

Preston *et al.* (1996) have reported that although many would assume LPS to be the major glycolipid in the cell wall of Gram negative bacteria, in fact lipooligosaccharide (LOS) is. LOS and LPS both share lipid A in their structure (Griffiss *et al.*, 1995). Therefore, if lipid A is the immunodominant portion of these glycolipids it could result in even broader cross-reactivity. Nevertheless, polyclonal anti-*E. coli* lipid A antibodies have been shown to cross-react with a large variety of free lipid A molecules. However, antibodies to lipid A do not cross-react with intact LPS *in vitro*, indicating that either the cross-reacting lipid A epitopes are not expressed or that the epitopes are cryptic (Zahringer *et al.*, 1994). These reports often evaluated monoclonal antibodies of murine or human lineage. A cautionary note was proved by Freudenberg *et al.* (1989) who suggested that ELISA determinations of relatively adhesive antibodies, such as IgM, to amphiphilic molecules, such as lipid A, can often lead to false positive results interpreted as cross-reactivity. It has also been shown that LOS from pathogenic *Neisseria* and *Haemophilus* species contain terminal tetrasaccharides that are homologous to paragloboside (Preston *et al.*, 1996). Thus, it is feasible that LOS of oral bacteria could be recognised similarly by the host, and present shared epitopes as a strategy to evade the immune response.

With certain periodontopathogens, the detectable antibody response to these types of antigens may provide some useful diagnostic information. The validity of targeting LPS from oral pathogens as an immunodominant antigen is still fraught with unknowns. Studies need to be carried out in the future to identify if naturally protective responses to LPS exist. If they do, the information may be of importance in prevention or intervention through vaccine implementation.

1.6.5 Heat shock proteins

Heat shock proteins (HSP) or stress induced proteins are produced by both prokaryotic and eukaryotic cells in response to various insults, such as a sudden increase in temperature (Kaufmann, 1990). HSP function to protect the cell during these adverse environmental changes. As well as being molecular chaperones (Georgopoulos,

1992), it has recently been suggested that they are important in host immune responses and the pathogenesis of disease (Panchanathan *et al.*, 1998).

Immunoreactivity of HSP was initially recognised by the homology between a highly immunoreactive 65 kDa protein of *Mycobacterium* and a 60 kDa HSP of *E. coli* (Shinnick *et al.*, 1988; Thole *et al.*, 1988). It was suggested that this protein was also related to a strongly immunoreactive antigen found in virtually all Gram negative bacteria. Immunoreactive proteins in many prokaryotes have been identified which show homology to HSP, especially the HSP90, HSP70, HSP60 and low molecular weight families (Shinnick *et al.*, 1991). The HSP70 family is highly conserved, with the amino acid sequence of the human HSP70 having 50% identity with the *E. coli* and 73% with the *Drosophila* HSP70 family. There have been reports demonstrating that some bacterial HSP are immunogenic proteins (Buchmeier *et al.*,1990; Wu *et al.*, 1994; Perez-Perez, 1996).

Tang *et al.* (1997) studied the immunodominant nature of *Salmonella typhi* HSP60 (GroEL). This report showed that HSP of pathogens are "immunodominant" antigens and targets of the host immune response. These results were in agreement with other studies and were confirmed by substantial production of antibody to these antigens. Further studies indicating the importance, and purported immunodominance of HSP include investigations of GroEL and GroES of *Camphylobacter jejuni* (Wu *et al.*, 1994) and *Mycobacterium leprae* (Mehra *et al.*, 1992). These reports supporting an important role for HSP and host responses to these antigens in other infectious diseases, naturally lead to an evaluation of the potential role of these macromolecules in periodontitis.

A recent study by Ando *et al.* (1995) examined the production of HSP by the periodontal pathogens: *A. actinomycetemcomitans*; *Eikenella corrodens*; *Fusobacterium nucleatum; P. intermedia*; *Prevotella nigrescens*; *Prevotella melaninogenica* and *Treponema socranskii.* All of the HSP produced by these bacteria reacted with anti-*Yersina enterocolitica* HSP60 antibodies and/or monoclonal antibodies to the 65 kDa HSP of *Mycobacterium.* Additionally, these authors investigated soluble HSP in inflamed gingival tissue of chronic periodontitis patients.

Gingival homogenate samples from 3 of 4 patients reacted with anti-human HSP60 and anti-bovine brain HSP70. However, none of the homogenate samples reacted with antibodies against bacterial HSP.

Maeda *et al.* (1994), cloned a HSP60 (GroEL) homologue from *P. gingivalis* using the *E. coli* GroEL gene as a probe. The sequence of this *P. gingivalis* GroEL HSP showed about 59% amino acid identity with *E. coli* GroEL and the 65 kDa *Mycobacterium tuberculosis* antigen, as well as with the human HSP60 sequence. An immunogen immunoblot analysis, using purified *P. gingivalis* GroEL, revealed that it was recognised by antibody in 8/10 sera from periodontitis patients, and in 3/9 healthy controls. These findings are consistent with the association of *P. gingivalis* with periodontitis. In addition these antibodies are present in response to a low level, continuous or transient exposure of the healthy subjects to *P. gingivalis* GroEL and GroEL homologues from many other micro-organisms, or even host molecules.

Hinode *et al.* (1998), attempted to address the cross-reactive potential of HSP. They evaluated the N-terminal amino acid sequences of GroEL (HSP60 family) and DnaK (HSP70 family) like proteins from *P. gingivalis*, *A. actinomycetemcomitans* and *B. forsythus*, in relation to potential cross-reactivity of antibodies. Antibodies specific for DnaK-like proteins appeared to be primarily induced against non-conserved epitopes of the protein. The DnaK-like proteins from *P. gingivalis* and *B. forsythus* reacted only very weakly with antibodies specific for the DnaK of *E. coli*. However, a clear cross-reactivity between antibodies raised to GroEL-like proteins of *P. gingivalis, A. actinomycetemcomitans* and *E. coli* was detected. This indicated that a significant portion of the antibodies induced against GroEL-like proteins are directed at epitopes conserved across bacterial genera. Why antibodies are produced in response to conserved epitopes of one HSP and to primarily non-conserved epitopes of another HSP is unknown. This could be a strategy adopted by the pathogens to increase their virulence. It may be a subversion of host responses by alteration of the efficiency of antigen processing and presentation by the host immune system.

A summary of the literature with respect to the role of HSP in the host response of the periodontium suggests two main opinions; One view proposed by Ando *et al.* (1995)

is that periodontopathic bacterial HSP antigens form immune complexes with local antibodies, which activate or exacerbate the inflammatory response, enhancing tissue damage. However, the lack of detection of bacterial HSP in gingival homogenate samples, and evidence of host derived HSP emphasises the potential for chronic inflammation to stress local tissues during destructive periodontitis (Kaufmann, 1990). The second view is that antigenic cross-reactivity poses a potential problem in evaluating HSP as immunodominant antigens of these pathogens. The term "common antigens" is indicative of their highly conserved nature. As discussed previously with LPS it may be difficult to ascertain if the detection of a host immune response documents: (i) the induction of elevated antibodies to a particular HSP of the oral bacterial pathogen under investigation; or (ii) cross-reacting antibodies elicited by other commensal or pathogenic bacteria, or even autoantibodies to human HSP. Bangsborg *et al.* (1989) reported the problems in documenting dominant serological reactions using HSP, as they induce antibodies that are strongly cross-reactive. However, antibodies against HSP have been implicated as "immunodominant" in various autoimmune diseases including Systemic Lupus Erythematosus (Jarjour *et al.*, 1991) and rheumatoid arthritis (Van Eden, 1990).

This is clearly an exciting area of investigation in the microbial pathogenesis of periodontal disease. Nevertheless, creative experimental designs and approaches will be required to identify the functional importance of these immune responses. In addition, the significance of HSP to oral bacterial virulence strategies, and the justification for considering these immunogens in vaccine development will need to be evaluated.

1.6.6 Leukotoxin

A number of Gram negative bacteria produce an array of diffusable protein molecules which belong to the repeats-in-toxin (RTX) family of bacterial exotoxins. These exotoxins are cytolytic proteins which are generally heat labile, induce tissue necrosis and can be used to stimulate protective immune responses (e.g. tetanus toxoid) (Nisengard and Newman, 1994). Members of the RTX exotoxin family are involved in a variety of different bacterial infections and target different cell types and tissues

specific for the associated disease entities. There are a number of features that are possessed by all members of this family, including: (i) the genetic organisation; (ii) certain structural features; and (iii) functional features such as haemolytic, leukotoxic and leukocyte-stimulating activities.

Of the oral micro-organisms associated with periodontitis, *A. actinomycetemcomitans* produces a leukotoxin which is a member of the RTX family. This is heat labile, soluble and was initially reported because of its ability to destroy PMN (Baehni *et al.*, 1979; Baehni *et al.*, 1980). However, recent studies indicate that it activates apoptotic processes in a variety of cell types (Mangan *et al.*, 1991; Kato *et al.*, 1995; Korostoff *et al.*, 1998). At high concentrations, leukotoxin appears able to bind to a PMN target cell membrane, to form pores in the membrane and to kill the cell through osmotic lysis. This releases intracellular lysosomal products and exacerbates tissue damage. Initial studies of antibody responses to *A. actinomycetemcomitans* sonicate antigens and leukotoxin in humans suggested that the characteristics of the responses generally paralleled each other (Ebersole, 1990). Importantly, patients with *A. actinomycetemcomitans* infections generally demonstrated extreme elevations in antibody to this antigen (Ebersole *et al.*, 1991a; Califano *et al.*, 1989; Gunsolley *et al.*, 1991), suggesting that it was a strong immunogen. This has been confirmed in rodent models (Shenker *et al.*, 1993). The human response showed elevated leukotoxin antibody consistently in localised aggressive periodontitis patients (McArthur *et al.*, 1981; Tsai *et al.*, 1981; Ebersole *et al.*, 1983). With regard to the issue of immunodominance, a crucial point is that the antibody to the leukotoxin was not a dominant antibody response in these patients. As mentioned previously, the main response was to LPS and serotype determinants of *A. actinomycetemcomitans*. It was concluded that LPS is an immunodominant antigen. Therefore, the response to this molecule, should be the focus of efforts to immunologically interfere with the pathogenicity of *A. actinomycetemcomitans*. The biologic relevance of leukotoxin as a virulence factor has not been clearly demonstrated *in vivo*. Nevertheless, recent genotypic studies support the concept that *A. actinomycetemcomitans* isolates which have an altered promoter region, resulting in enhanced leukotoxin production, are most frequently associated with disease sites in selected populations (DiRenzo *et al.*, 1994; Haubek *et al.*, 1995). Recent data suggests that leukotoxin antibody levels may

be more discriminatory than antibody to the whole bacteria (Califano *et al.*, 1997). Furthermore, one report has suggested that leukotoxin challenge of a human immune system reconstituted into mice, induced antibodies which correlated with some protection from lesion formation (Shenker *et al.*, 1993). To conclude, the reactivity of humans to leukotoxin cannot be considered a 'dominant' response. However, there appear to be 'dominant epitopes' on this antigen which can consistently elicit antibody in infected subjects which appears to ameliorate the effects of this toxin. Consequently, the use of antibody to leukotoxin as a biomarker of an immunological target in periodontal disease seems worthy of continued investigation.

1.6.7 Cysteine proteases

The cysteine proteases of *P. gingivalis* clearly contribute to the physiological function of this asaccharolytic bacterium. They are of great importance in the nutrition and growth of the micro-organism (Kesavalu *et al.*, 1997). They also appear to play a crucial role in the survival of the bacteria through proteolytic elimination of opsonins at the bacterial cell surface (Cutler *et al.*, 1993; Schenkein *et al.*, 1995), via degradation of host response communication molecules including cytokines and chemokines (Steffen *et al.*, 1999). These enzymes also appear to correlate with pathogenicity of the bacteria (Curtis *et al.*, 1993; Feuille *et al.*, 1996; Kesavalu *et al.*, 1998). They aid the adherence of *P. gingivalis* to erythrocytes (Nishikata *et al.*, 1991; Curtis *et al.*, 1993; Pike *et al.*, 1996), neutrophils (Lala *et al.*, 1994), fibronectin (Lantz *et al.*, 1991a), fibrinogen (Lantz *et al.*, 1991b), collagenous substrata (Naito *et al.*, 1988), as well as to other bacteria (Ellen *et al.*, 1992). These proteases can also directly contribute to the induction and alteration of the inflammatory response (Potempa and Travis, 1996).

Curtis *et al.* (1996) studied 3 types of cysteine proteases associated with *P. gingivalis*, each demonstrating a specificity for arginine containing peptide bonds (RgpA-1, RgpA-1A and RgpA-1B). Similar reports have provided evidence of additional cysteine proteases with varied specificity, which are produced by this pathogen (Bedi, 1994; Chen *et al.*, 1992; Pavloff *et al.*, 1995). In order to illustrate the relationship

between these proteases and the host response to *P. gingivalis*, there follows a review of the antigenic features RgpA-1.

RgpA-1 or HRgpA is a heterodimer of approximately 50 kDa, which is composed of an α and β segment or chain. RgpA-1A is a monomer of a free α segment and RgpA-1B appears to be a post-transcriptionally modified form of the α segment (Curtis *et al.*, 1996). One study by Curtis *et al.* (1993), used polyvalent rabbit antiserum raised against RgpA-1A. They demonstrated a recognition of all 3 protease types, indicating that the α chains are immunochemically related. However, 4 of 5 representatives from a panel of monoclonal antibodies recognised only RgpA-1. This indicated specificity for the β segment portion of this protease, which is the non-catalytic part of the enzyme. The α segment bears the protease active site. The β segment has a primary sequence similar to that of adhesins and haemagglutinins of other molecules and micro-organisms. Why the immune response is directed towards the seemingly non-destructive portion of the enzyme remains unknown. The β segment appears important in adherence. Therefore, the host may target antibodies towards this portion to block attachment and access of the protease to the host cells or proteins. Alternatively, the β segment may have other unknown functions which also increase its immunogenicity. In contrast, this may be a deliberate ploy of the bacteria to increase its virulence potential by diverting antibody responses to a less-relevant portion of the enzyme.

Nevertheless, these proteases, are implicated in periodontal disease, and induce the production of specific IgG in humans. As mentioned above, *P. gingivalis* appears to be effective in neutralising humoral elements of host defence. It uses trypsin-like proteases which degrade all isotypes of immunoglobulins (Gregory *et al.*, 1992) and many complement factors (Schenkein, 1988). Additionally, these proteases have been shown to give *P. gingivalis* the ability to degrade many human proteins (Kilian, 1981). Thus, clarifying the relationship between the systemic response to this antigen(s) and disease progression would appear useful, since this appears to be an important virulence factor enabling this bacteria to avoid host immune defences. Moreover, other reports have demonstrated the effective binding of IgG to virulent bacteria such

as *P. gingivalis*, has a crucial role in complement activation and also in the opsonisation and phagocytosis of this organism (Cutler *et al.*, 1991a,b). Increased antibody levels to a trypsin-like protease produced by *P. gingivalis* was identified in early studies of humoral immune responses in periodontitis patients (Ismaiel *et al.*, 1988). Aduse-Opoku *et al.* (1995), recently characterised a cell surface or extra-cellular arginine-specific protease antigen (i.e. related to the trypsin-like protease activity). This group also characterised this protease as a functionally important target of the host immune response (Curtis *et al.*, 1996). It has recently been demonstrated that active immunisation of non-human primates with a cysteine protease (e.g. porphypain-2) elicited significant antibody responses in the animals (Mortiz *et al.*, 1998). It also altered the microbial ecology in disease states, and significantly affected the progression of bone loss in the animals. Booth and Lehner (1997) have also produced a murine monoclonal antibody to *P. gingivalis* which retarded re-colonisation of deep pockets by this pathogen in periodontitis patients. Interestingly, this antibody inhibited the hemagglutination of red blood cells, which is related to certain functions of the cysteine proteases of *P. gingivalis*.

Importantly these relatively few human studies have generally shown an increased prevalence of antibody to these proteases in periodontitis patients. However, the antibody levels were clearly not dominant in the response to this pathogen. This finding highlights the potential uncertainty in the field when identifying critical antigens relative to antibody levels and functional capacity to protect the host. However, this aspect of the host-parasite interaction will remain a high profile research area with the goal of evaluating pathogenic mechanisms of *P. gingivalis*, as well as a potential immunologic targets.

1.6.8 Superantigens

Increased knowledge of the molecular mechanisms for cell co-operation and the cell surface molecules required for adaptive immune induction, has revealed that not all antigens which bind to the MHC class II complex are presented as peptides in the peptide binding groove. Hence, they do not result in the development of a specific immune response by the conventional method. One such class of antigens, identified

within various bacterial and viral protein repertoires, has been termed superantigens (Janeway and Travers, 1994).

Superantigens are not processed by antigen presenting cells and are not presented by MHC molecules in the conventional manner. Instead of binding to the peptide binding groove of the MHC molecule, superantigens bind directly to the lateral surface of both the MHC class II molecule and the Vβ region of the TCR. The superantigen is recognised solely by the V region of the β chain of the TCR (Kappler *et al.*, 1989). In contrast, to conventional antigen processing and presentation, the α chain V region and the DJ junction of the β chain have little or no effect on superantigen recognition. This recognition process gives superantigens the capability of stimulating a large number of T cells (4-5% of the T cell population) in a non-specific manner (Kappler *et al.*, 1989). This reaction with a large number of T cells gives rise to the characteristics associated with superantigens: (i) pyrogenic effects in excess of those produced by LPS; (ii) inhibition of immunoglobulin synthesis; and (iii) interference with LPS clearing by the liver causing a massive increase in the lethality of bacterial LPS (Daniel and Van Dyke, 1996).

Superantigens are known to be produced by a number of pathogenic micro-organisms, including some found in the oral cavity. However, to date no clear role for superantigens in periodontal disease has been demonstrated. Mathur *et al.* (1995) investigated superantigen like activities of putative periodontopathogens in both periodontally healthy and diseased subjects. Co-culturing peripheral blood mononuclear cells with *P. intermedia* resulted in an increased expression of $V\beta2^+$, $V\beta5^+$ and $V\beta6^+$ cells in both healthy and diseased subjects. However, similar treatment with either *P. gingivalis* or *A. actinomycetemcomitans* showed no changes in the Vβ TCR repertoire. Studies by Nakajima *et al.* (1996) and Zadeh and Kreutzer (1996) which involved profiling gene expression in gingival tissues, suggested that expression of the Vβ family in tissue borne T cells was not random. However, limiting an explanation for this finding to superantigen processes does not account for a skewing towards particular Vβ receptor families. This could be due to the presence of bacteria in the subgingival plaque biofilm selectively stimulating cells of a

particular Vβ receptor family (Mathur *et al.*, 1995). Alternatively, homing of cells to the diseased tissue may be mediated, in part, by Vβ expression (Taubman *et al.*, 1994). In addition, retention of specific T cells in the tissues may be due to the characteristics of their Vβ receptors and the presence of specific antigens. Therefore, our knowledge reflects a number of ill-defined and poorly understood processes, especially as related to the existence and importance of superantigens in periodontal disease. A more detailed evaluation of the available studies provides equally conflicting results. Vβ8 cells are over expressed (Zadeh and Kreutzer, 1996) or under expressed (Nakajima *et al.*, 1996). Alternatively, Petit and Stashenko (1996) demonstrated that extracts of periodontopathic bacteria do not stimulate T cells at all in a superantigenic manner when tested in a murine model. It is not clear if these conflicting reports are due to different types of superantigens, different sampling strategies, or even just variation in patient populations. The disparate findings need to be clarified in order to define the utility of the Vβ chains as markers of superantigen activity within the host-parasite interactions, in periodontal disease.

However, one might reconsider whether there is an *a priori* case for emphasising the contribution of superantigens in periodontal disease. This disease has been described as a chronic infection involving an ongoing humoral immune response, shown by the presence of predominantly antibody secreting cells in the periodontal lesion (Kinane & Lindhe, 1997). In contrast, infections in which the pathogens present superantigens as part of their virulence strategy, are generally acute, with a rapid onset of symptoms, and immunoglobulin production is usually suppressed. This profile is not observed in periodontitis and systemic pyrogenic activity (another characteristic of infections with superantigen possessing micro-organisms) is an exceptional occurrence in periodontitis patients. Therefore, at present it is questionable whether a role for superantigens is consistent with the characteristics of this disease.

1.6.9 Other strategies for identifying immunodominant antigens

A recent study (Kawai *et al.*, 1998) attempted to identify an immunodominant antigen in chronic periodontitis using monoclonal antibodies. Mouse monoclonal antibodies

were raised against crude antigen preparations of *P. gingivalis*. The antigenic specificity of the monoclonal antibodies were then compared with those of serum antibodies detected in chronic periodontitis patients and healthy subjects. Binding of one monoclonal antibody was inhibited by serum antibody molecules present in all chronic periodontitis patients. This serum antibody was absent in the polyclonal antibody population in the control subjects however. The inference was that the *P. gingivalis* antigenic epitope identified by this murine monoclonal antibody could represent an important immunogen in humans infected with this pathogen. The antigen recognised by this monoclonal antibody was demonstrated on the cell surface of *P. gingivalis*, and the epitope was composed of carbohydrate residues independent of the LPS antigen. Thus, new technological and conceptual advances will continue to challenge existing paradigms of immunodominant antigens in periodontal disease, and provide additional tools for addressing this important concept.

As mentioned previously, the aim of this text is to provide an overview of the approaches and strategies which have been used in the past and those which are currently being employed in periodontal infections, to examine the concept of immunodominant antigens. It appears there is no clear way ahead for the concept of immunodominance in periodontal disease research. This polymicrobial infection is complex due to the intertwining of the relationships within the microbial ecology. The plethora of host and microbial variables may make successful exploitation of any interventive strategy, based on the immunodominance concept, difficult to implement in this disease. This concern is exemplified by the breadth of studies supplying disparate findings regarding the antigenic specificity of dominant host responses to the putative pathogens. The identification of immunodominant antigens may be relevant if additional supportive knowledge is gained in the future. In this case it is suggested that utilisation of these critical antigens for screening and diagnosis of the disease, as well as using them as targets for vaccine development, will not only be feasible, but desirable.

1.7 Pathogenesis of Periodontal Disease

1.7.1 Introduction

The normal pink in colour and firm in consistency healthy gingiva should be free from histological evidence of inflammation (figure 1.2). But this is rarely the case, and however healthy an individuals gingiva may be, when seen through the microscope they are always slightly inflamed due to the ever present plaque bacteria (figure 1.3). The inflammatory cells which have infiltrated the healthy gingiva are predominantly PMN that are associated with the junctional epithelium, and lymphocytes that are found in the subjacent connective tissue (Page and Schroeder, 1976). All the characteristics of the inflammatory response, as previously described, are seen in the gingiva even in health. These include; increased vascular permeability and accumulation of fluid, plasma proteins and leukocytic cells into the gingival crevice region, to create GCF. The reason for the migration of cells into the junctional epithelium as well as the crevice is due to the production of cytokines and various other products by the host and bacteria. This subject is discussed in more detail in section 1.4.

A further indication that inflammation is present in a 'healthy' gingiva is that upregulation of the adhesion molecules ICAM-1 and ELAM-1 have been shown in health (Moughal *et al.*, 1992; Kinane *et al.*, 1991).

Although, as discussed above, an inflammatory response is thought to occur even in health, a balance has to be maintained for the gingiva to remain 'healthy'. Although bacterial plaque is present and the host responds to it by initiating an inflammatory response, the micro-organisms and the host are probably in equilibrium with each other. However, the gingiva stops being classified as 'healthy', and gingivitis follows when the micro-organisms in the plaque initiate a substantial inflammatory response.

1.7.2 Gingivitis

Gingivitis can occur after 10-20 days of plaque accumulation. It is detected due to redness and swelling of the tissues which have an increased tendency to bleed on probing. Histologically, there has been a massive influx of fluid and cells into the tissues. Dilation of arterioles, capillaries and venules increases hydrostatic pressure within the microcirculation leading to increased intercellular gaps between adjacent capillary and endothelial cells. The cells that migrate into the tissues are mainly lymphocytes, macrophages and PMN. Experimental work, particularly studies involving dogs (Lindhe and Rylander, 1975) have shown that when there is a cellular infiltrate, lymphocytes are found to adhere to the collagen matrix as they are possibly primed to periodontally relevant antigens, and therefore, remain in the tissues (Kinane and Lindhe, 1997). The PMN which infiltrate the tissues tend to migrate into the gingival sulcus and do not remain in the tissues.

In addition to the influx of inflammatory cells into the tissues, there is increased flow of plasma proteins, antibodies, complement proteins and protease inhibitors into the area (Payne *et al.*, 1975; Schroeder *et al.*, 1975). The flow of GCF also increases, so that toxins and substances released from microbes are washed out of the tissues and the gingival crevice. As the inflammatory response to the plaque bacteria increases there is also a marked expression of adhesion molecules to assist the migration of leukocytes out of the blood vessels, and into the tissues and crevice (Kinane and Lindhe, 1997).

After approximately 7 days the lesion is referred to as the early gingival lesion (Page and Schroeder, 1976) (figure 1.4). In the early gingival lesion, lymphocytes and PMN are the prominent infiltrating cells and very few plasma cells are noted at this time (Listgarten and Ellegaard, 1973; Payne *et al.*, 1975; Seymour *et al.*, 1983; Brecx *et al.*, 1987). At this stage the inflammatory cell infiltrate comprises approximately 15% of the connective tissue volume. However, due to fibroblast degeneration and collagen destruction that occurs in the tissue, further inflammatory cell infiltration can occur as space is created.

It is not known how long the gingivitis lesion remains like this or when it returns to health or progresses to an increased inflammatory state. There appears to be a significant variation between individuals however, probably based on individual predispositions and susceptibility.

If the plaque remains present, the inflammatory state continues and becomes exacerbated. Continuation of the inflammatory response sees the influx of further exudative fluid, inflammatory cells and plasma proteins. The tissues become even more swollen and oedematous (Löe and Holm-Pedersen, 1965), however, at this stage there is no bone loss nor apical epithelial migration evident. According to the classification determined by Page and Schroeder (1976) the lesion is termed the established lesion (figure 1.5). The main difference between the early and established lesion is not only the progression of the inflammatory immune response, but the established lesion contains many more mature plasma cells. Prominent infiltration of PMN is also observed in the junctional and sulcular epithelium (Brecx *et al.*, 1988; Lindhe *et al.*, 1980; Okada *et al.*, 1983). The latter may proliferate and migrate into the infiltrated connective tissue, along the root surface and extend deeper into the connective tissue. The gingival sulcus deepens and the coronal portion of the junctional epithelium is converted into 'pocket lining' epithelium. This is not attached to the tooth surface and has a heavy leukocyte infiltrate, predominantly made up of neutrophils which eventually migrate across the epithelium into the gingival crevice or pocket.

It is thought that two different types of established lesion exist. One is thought to be a more stable lesion that can remain this way for long periods of time, and the second is much more aggressive and converts to progressive destructive lesions (Lindhe *et al.*, 1975; Page *et al.*, 1975).

1.7.3 Periodontitis

The advanced gingival/periodontal lesion is the final stage of the disease. The advanced lesion differs from the established lesion by alveolar bone loss being

evident. There is also evidence of extensive fibre damage and apical migration of the junctional epithelium (Lindhe *et al.*, 1980; Listgarten and Helldén, 1978; Seymour and Greenspan, 1979; Seymour *et al.*, 1979). The inflammatory immune response continues to progress, and the inflammatory cell infiltrate extends laterally and apically into the connective tissue. Epithelium layers are broken down, re-growing at a more apical location. Pocket formation occurs, bone is resorbed and granulation tissue forms, which is heavily vascularised and full of antibody producing plasma cells (Kinane and Lindhe, 1997). Plasma cells are the predominant cell type in the advanced lesion (Garant and Mulvihill, 1972) (figure 1.6).

The progression of the inflammatory response continues in the presence of plaque bacteria and the microbes that have continued to inhabit the niches that have been created through tissue damage and the frustrated inflammatory response. When no oral hygiene improvements are made or treatment pursued the situation is worsened. Microbes will continue to produce noxious substances, the host inflammatory response will be perpetuated and tissue damage will continue. This is seen by continuous deepening of pockets, extending granulation tissue lesions, continuous bone and ligament loss and eventually loss of the teeth (Kinane and Lindhe, 1997).

The progression from gingivitis to periodontitis varies in time between individuals. Brecx *et al.* (1988) suggested that at least 6 months is required for gingivitis to progress to periodontitis in the normal situation. The reason some individuals go on to develop periodontitis and others do not and the length of time this takes in different patients is thought to be multifactorial. Seymour *et al.* (1979) suggested that it is a shift from B cell to T cell dominance that causes the transition from gingivitis to periodontitis. This theory has been contested by a number of authors (Gillett *et al.*, 1986; Page, 1986) and it is generally accepted that an advanced lesion is predominantly a plasma cell lesion (Garant and Mulvihill, 1972; Kinane and Lindhe, 1997).

Figure 1.2 The pristine gingiva

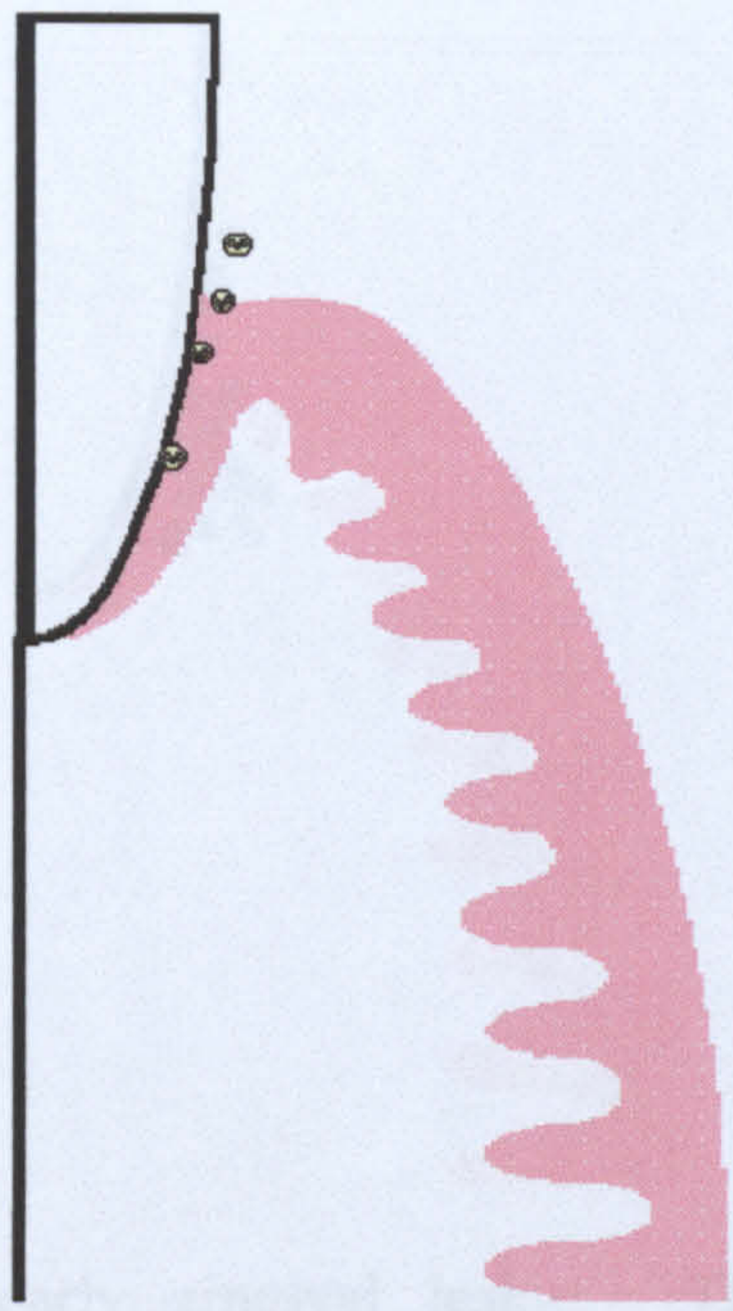


Figure 1.2 shows a pristine gingiva which is almost free from histological evidence of inflammation. This is rarely seen in health.

Figure 1.3 The normal healthy gingiva

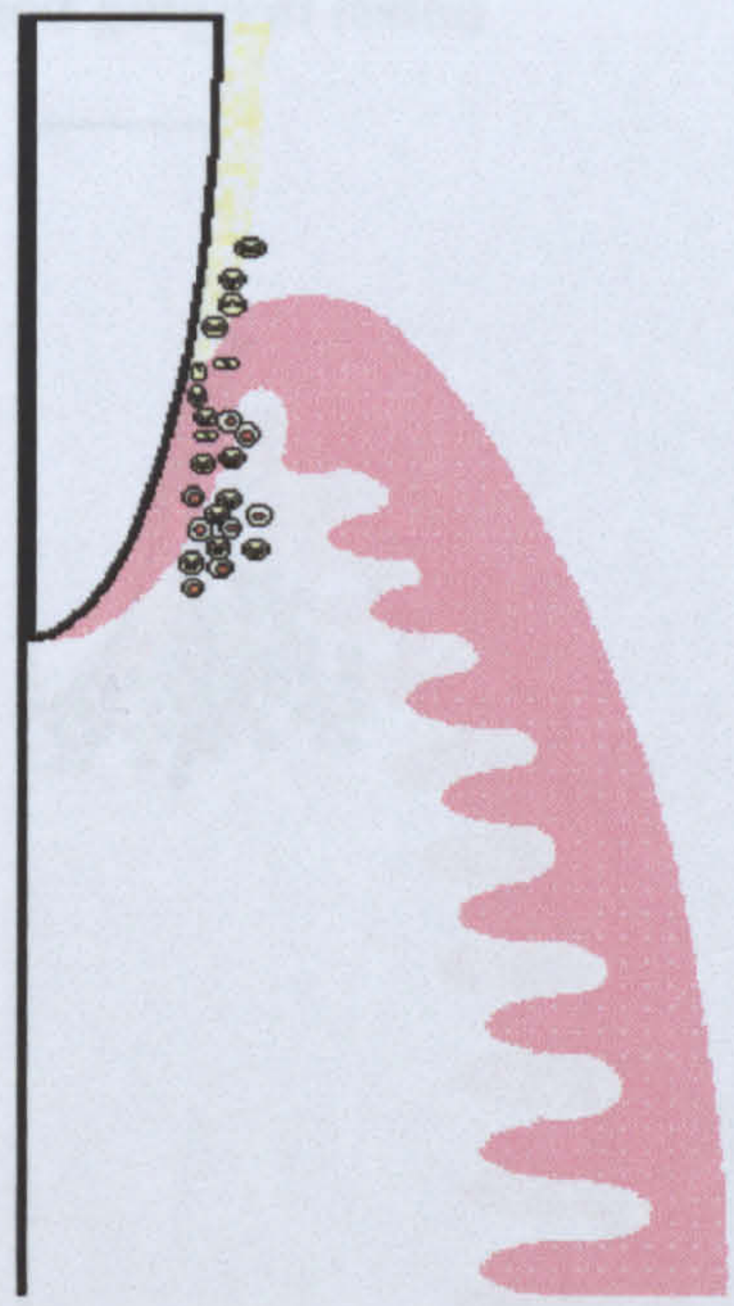


Figure 1.3 shows a normal healthy gingiva. This picture of microbial colonisation due to ever present plaque bacteria is usually seen in health. Inflammatory cells have begun to infiltrate the junctional epithelium and connective tissue. These are mainly PMNs , monocytes, macrophages and lymphocytes.

Figure 1.4 The early gingival lesion

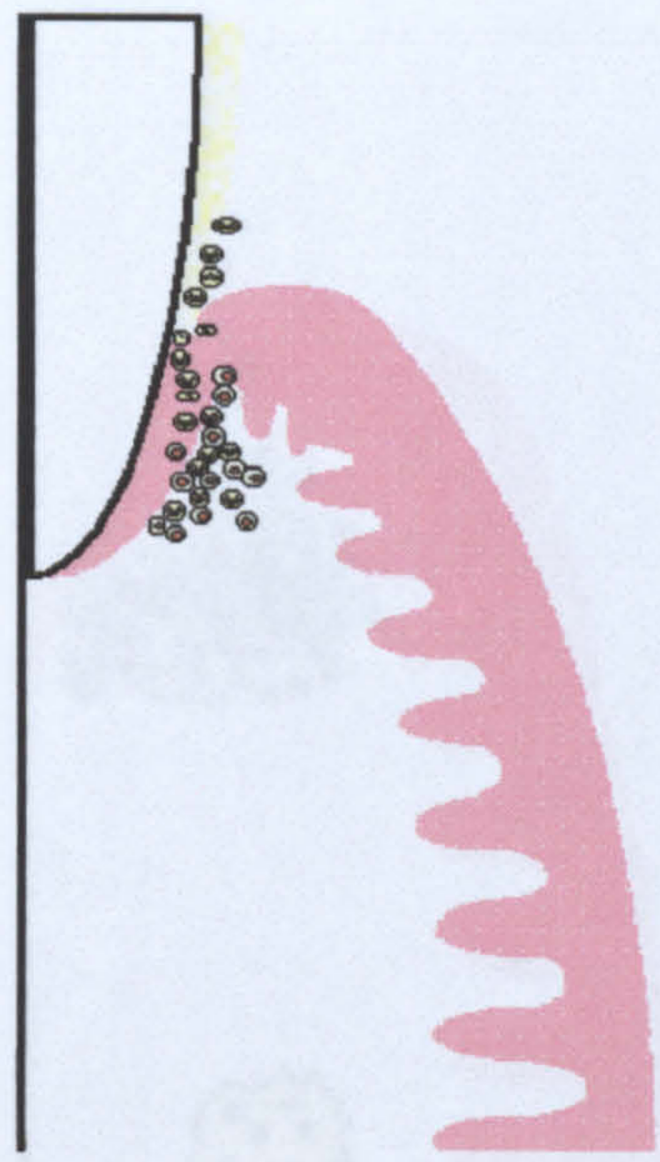


Figure 1.4 shows the early gingival lesion. This is thought to occur after approximately 7 days. The number of infiltrating inflammatory cells has increased and comprises about 15% of the connective tissue volume. The infiltrate is mainly PMNs, macrophages, monocytes and lymphocytes. Very few plasma cells are seen.

Figure 1.5 The established gingival lesion

Figure 1.5 shows the established gingival lesion. The inflammatory immune response has progressed, and the connective tissue is comprised of 10-30% plasma cells. Clinically marked proliferation of the junctional epithelium is seen, however, bone loss and apical epithelial migration are not evident.

Figure 1.6 The advanced lesion

Figure 1.6 shows the advanced lesion seen in periodontitis. The inflammatory cell infiltrate has progressed laterally and apically and is comprised of predominantly plasma cells. Clinically there is evidence of extensive fibre damage, apical migration of the junctional epithelium and bone loss. Figures adapted from Kinane and Lindhe (1997).

1.8 Risk Factors

1.8.1 Introduction

The term "risk factor" can be defined as "an aspect of personal behaviour or lifestyle, an environmental exposure, or an inborn or inherited characteristic, which on the basis of epidemiological evidence is known to be associated with a health related condition" (Last, 1988). Therefore, the presence of a risk factor implies that there is an increased chance of the disease occurring.

Periodontal disease is not a consequence of pathogens alone, and these are not sufficient on their own to cause the disease (Socransky and Haffajee, 1992). The presence of micro-organisms is a crucial factor, but the progression of the disease is related to host based risk factors.

1.8.2 Age

There can be little doubt that the prevalence of periodontal disease increases with age (Salvi *et al.*, 1997). However, it is not clear if age leads to increased susceptibility or that there are cumulative factors which control the onset of periodontitis. Horning *et al.* (1992), reported that age is a risk factor for periodontal disease. However, other authors (Holm-Pederson *et al.*, 1975; Machtei *et al.*, 1994) have indicated that this is not the case before the age of 70 years, and until this age the rate of periodontal destruction is the same throughout adulthood.

1.8.3 Low Socio-economic status

Low socio-economic status is generally associated with more severe periodontitis and data from the U.S. public health service (1965, 1979) indicates this. However, in studies where periodontal status is adjusted for oral hygiene and smoking, the associations between lower socio-economic status and more severe periodontal disease are not observed (Grossi *et al.*, 1994; Grossi *et al.*, 1995).

1.8.4 Smoking

There has been a substantial amount of literature reporting the detrimental effect of smoking on periodontal health. There is a consistent indication that smoking is a risk factor for periodontal disease and there is a positive correlation between attachment loss and smoking (Arnø *et al.*, 1959; Ah *et al.*, 1994; Bergström, 1989; Haber, 1994; Haber and Kent, 1992; Haber *et al.*, 1993; Horning *et al.*, 1992; Preber and Berström, 1990). In many of the studies on this subject, multivariate analyses are carried out to ensure that smoking is an independent risk factor for periodontal disease, and that any results showing a correlation are not due to poor oral hygiene, plaque, socio-economic status or any other risk factor. The risk for periodontitis is considerable if an individual uses tobacco products, with estimated ratios being of the order 2.5 to 7.0 or even greater for smokers compared with non-smokers (Salvi *et al.*, 1997).

The mechanisms by which smoking affects periodontal status is not very well understood (Haber, 1994). Studies have indicated that the occurrence, relative frequency, or combinations of micro-organisms associated with destruction do not differ between smokers and non-smokers (Preber *et al.*, 1992). However, perhaps smoking has a direct effect on the inflammatory and immune mechanisms. On this theme, smokers with periodontal disease have less clinical inflammation (Feldman *et al.*, 1983) and gingival bleeding (Bergström and Floderus-Myehed, 1983) compared with non-smokers. The biological effect on the inflammatory immune response due to smoking could be as a result of the by-products of tobacco such as nicotine, which causes local vasoconstriction reducing blood flow, oedema and clinical signs of inflammation.

1.8.5 Systemic disease

There is strong evidence that periodontal disease enhances the risk for many systemic diseases, including atherosclerosis, heart disease, stroke and the births of infants with low birth weight (Beck *et al.*, 1996; Offenbacher *et al.*, 1996). There is also evidence that certain systemic diseases can adversely effect host defence systems and, therefore, act as a risk factor for gingivitis and periodontitis.

Neutropenic states have been associated with rapidly progressive forms of periodontal disease (Miller *et al.*, 1984). Radiographs taken in some cases have indicated involvement of the periodontal tissues, showing bone loss around the primary and secondary teeth (Miller *et al.*, 1984).

There is an association between the disease leukocyte adhesion deficiency (LAD) and periodontal disease. Patients suffering from this disease have an abnormal gene that codes for the family of adhesion molecules called the leukocyte integrins. Patients with this deficiency have a dramatically increased susceptibility to infections and suffer from a very severe form of periodontal disease that can lead to premature loss of deciduous and permanent teeth (Crawford, 1994).

Diabetes Mellitus has been linked to an increased risk of periodontal disease (Bacic *et al.*, 1988; Bartolucci and Parkes, 1981; Belting *et al.*, 1964; Grossi *et al.*, 1994; Hugoson *et al.*, 1989). Type I diabetes mellitus is also known as insulin dependent diabetes mellitus, and commonly develops before 30 years of age. Type II, or non-insulin dependent diabetes mellitus is usually found to develop after the age of 40 years. A strong correlation has been found between aggressive periodontitis and type I diabetes mellitus, especially in patients with raised blood glucose levels (Bissada *et al.*, 1982; Cutler *et al.*, 1991; Manouchehr-Pour *et al.*, 1981a; Manouchehr-Pour *et al.*, 1981b). Type II diabetes has also been linked with periodontal disease (Beck *et al.*, 1990; Grossi *et al.*, 1994). However, differences in oral health between type I and type II diabetes occur and may be related to differences in glycaemic control, age, duration of disease or periodontal susceptibility (Salvi *et al.*, 1997). Well-controlled diabetics are more likely to be like non-diabetics in their periodontal status, and those with poorly controlled diabetes are likely to be more susceptible to disease (Mealey, 1996).

1.8.6 Other possible risk factors

Preliminary studies have indicated an association between stress, distress and coping behaviour (Moss *et al.*, 1996). The rationale behind many of the studies investigating

a link between periodontal disease and the psychoemotional state are that these factors can lead to depression of immune responsiveness to putative periodontopathogens.

1.8.7 Genetics

Many studies have been reported over the past few years regarding a possible genetic basis for periodontal disease, and many of these studies have been reviewed (Hart, 1994; Hart, 1996; Hart and Kornman, 1997; Michalowicz, 1994; Aldred and Bartold, 1998). The generally accepted dogma at present is that the aggressive forms of periodontitis have a heritable pattern with a single gene disorder (Aldred and Bartold, 1998). With regards to chronic periodontitis, there is a split in opinion, with a general surmise being that there is little genetic influence in the clinical manifestation of this disease opposing recent evidence that suspects a genetic susceptibility associated with the disease (Aldred and Bartold, 1998).

The exact genetic link with both aggressive and chronic forms of periodontal disease have yet to be identified. More work has been carried out investigating the link with aggressive periodontal disease, and although many studies have been undertaken the conjectures made from these have not been fully accepted. Specific chromosomal anomalies have been investigated, and the outcomes appear to indicate that even with a small disease entity such as localised aggressive periodontal disease, there is probably considerable genotype heterogeneity even if the clinical manifestations appear to be very similar. This can be observed by the fact that different groups carrying out genetic linkage studies have found such varying results (Boughman *et al.*, 1986; Roulsten *et al.*, 1985; Hart *et al.*, 1993; Wang *et al.*, 1996).

Studies have also been carried out to investigate a link between periodontal disease and genetic markers of a more general nature. Again, most reports made are controversial on this issue. A number of studies have associated HLA antigens with generalised aggressive periodontitis (Katz *et al.*, 1987; Reinholdt *et al.*, 1977; Shapira *et al.*, 1994), however, others have found no association at all (Cullinan *et al.*, 1980; Saxén and Koskimies, 1984).

Other genes have been suggested to be linked with periodontal disease including; the gene responsible for IgG2 production (Marazita *et al.*, 1994; Marazita *et al.*, 1996), FcγRIIa receptor (Wilson and Kalmar, 1996), prostaglandin endoperoxidase synthase 1 and other components of the cyclooxygenase pathway (Hart and Kornman, 1997) and IL-1 (Kornman *et al.*, 1997).

1.9 Aims and Objectives

1.9.1 Introduction

Many microbial pathogens have been proposed as playing a role in the induction of the immune response in periodontal disease. The aim of the thesis was to investigate the target of the humoral immune response in patients with chronic periodontitis. This was achieved by a number of studies following a logical progression. The first study investigated the systemic immune response by examination of the target of serum antibodies in patients with chronic periodontitis. The project then developed to investigate the target of the local immune response and to achieve this 3 different techniques were utilised: hybridoma production; ELISPOT; and immunohistochemistry.

1.9.2 Aims of Chapter 3 - A Comparison of the changes in the humoral immune response to antigens of periodontal pathogens following periodontal therapy

The aims of this part of the overall project were to:

1) investigate the effect of periodontal treatment in patients with chronic periodontitis on polyvalent immunoglobulin, IgG and IgA titres against whole fixed periodontal pathogens;
2) Investigate the effect of periodontal treatment on antibody titres to a large panel of antigenic targets including whole fixed bacteria, outer-membrane protein preparations and purified antigens;
3) Compare clinical and immunological parameters in chronic periodontitis patients before and after periodontal treatment;

4) Investigate the phenomenon of cross-reactivity by examining cross-reacting antigens to 4 periodontal pathogens.

1.9.3 Aims of Chapter 4 - Immortalised B cells from periodontal disease patients

The aims of this part of the overall project were to examine the feasibility of creating monoclonal antibodies from humans with periodontal disease by the:

1) Expansion of B cells numbers by utilisation of the CD40 system;
2) Fusion of human peripheral blood B lymphocytes and tissue plasma cells with a suitable partner to obtain hybridomas;
3) Successful culture and cloning of antibody secreting hybridomas;
4) Screening of monoclonal antibodies specific for periodontal pathogens.

1.9.4 Aims of Chapter 5 - Analysis of the target of antibody secreting cells from diseased tissue of chronic periodontitis patients by ELISPOT

The aims of this section of the project were:

1) Examination of the target of antibody secreting cells isolated from tissue taken from chronic periodontitis patients;
2) Comparison of the number of specific cells detected against 4 periodontal pathogens.

1.9.5 Aims of Chapter 6 - Detection of antibody-bearing plasma cells in periodontitis granulation and gingival biopsy tissue

In this part of the thesis the aim was to:

1) Develop a technique to determine the specificity and numbers of plasma cells present in tissue sections;
2) Examine the specificity of antibody bearing plasma cells in sections of diseased tissue taken from patients with chronic periodontitis;
3) Compare the number and specificity of plasma cells in granulation and gingival tissue samples detected using a panel of whole fixed periodontal pathogens, sonicates and purified antigens.

1.9.6 Layout of the thesis

The following chapter covers the source of the equipment and chemicals used in the various projects undertaken in this thesis. It also describes the basic methodology and buffers used. Following chapter 2, the thesis has been divided into 4 further chapters which describe individual studies: Comparison of the changes in the humoral immune response to antigens of periodontal pathogens following periodontal therapy (chapter 3); Immortalised B cells from periodontal disease patients (chapter 4); Analysis of the target of antibody secreting cells from diseased tissue of chronic periodontitis patients by ELISPOT (chapter 5); and Detection of antibody-bearing plasma cells in periodontal granulation and gingival biopsy tissue (chapter 6). In each of these chapters the relevant introduction and literature review, methodology, results and discussion of the results are presented. The organisation of this thesis is intended to clarify the separate studies for the reader, as they are fundamentally different and if grouped together could be confusing. These chapters are followed by a brief final chapter (chapter 7) which summarises the findings of this thesis and discusses them in the context of the aims of the overall project and the available literature on this subject.

Chapter 2. Materials and Buffers

2.1 Introduction

The following chapter describes the materials and methodology for making the buffers used routinely during the experiments presented in this thesis. Methods which are specific to the individual chapters are found detailed in the methods section of the relevant chapter.

2.2 Materials

The following list comprises the consumables used in the experiments presented in this thesis and their sources.

2.2.1 Chapter 3 materials

PCP-12 Probe - Hu-Friedy Mfg Co., Chicago, U.S.A.

Florida Probe - Florida Probe Corporation, Florida, U.S.A.

Non-heparinised 7ml vacutainers - Becton Dickinson Vacutainer Systems Europe, Meylan, France.

Fastidious anaerobe agar - LAB M, Bury, U.K.

Tryptic soy agar - Mast Diagnostics, Merseyside, U.K.

Horse blood - E & O Laboratories, Bonnybridge, U.K.

N-acetylmuramic acid - Sigma Chemical Company Limited, Poole, U.K.

Columbia blood agar - Mast Diagnostics, Merseyside, U.K.

Sodium chloride (NaCl) - BDH Limited, Poole, England, U.K.

Potassium dihydrogen orthophosphate (KH_2PO_4) - BDH Limited, Poole, England, U.K.

Di-sodium hydrogen orthophosphate 2-hydrate ($Na_2HPO_4.2H_2O$) - BDH Limited, Poole, England, U.K.

Potassium chloride (KCl) - BDH Limited, Poole, England, U.K.

Ethylenediaminetetracetic acid (EDTA) - BDH Limited, Poole, England, U.K.

Sodium hydroxide (NaOH) - BDH Limited, Poole, England, U.K.

Formal saline - BDH Limited, Poole, England, U.K.

Sodium azide (NaN3) - Sigma Chemical Company Limited, Poole, U.K.

Tris base - Sigma Chemical Company Limited, Poole, U.K.

Phenol red - Sigma Chemical Company Limited, Poole, U.K.

Sodium lauryl sarcosinate (SDS) - Sigma Chemical Company Limited, Poole, U.K.

BCA protein assay kit - Pierce & Warriner, Chester, U.K.

Immunlon 1B 96 well plates - Dynex Technologies, VA, USA.

Sodium carbonate (Na_2CO_3) - BDH Limited, Poole, England, U.K.

Sodium hydrogen carbonate ($NaHCO_3$) - BDH Limited, Poole, England, U.K.

Hydrochloric acid (HCl) - BDH Limited, Poole, England, U.K.

Polyoxyethylene sorbitan monolaurate (Tween 20) - Sigma Chemical Company Limited, Poole, U.K.

Lyophilised bovine serum albumin (BSA) - Sigma Chemical Company Limited, Poole, U.K.

Skimmed milk powder - Marvel, Premier Beverages, Stafford, UK.

Biotinylated goat-anti-human immunoglobulin A - Sigma Chemical Company Limited, Poole, U.K.

Biotinylated goat-anti-human immunoglobulin G - Sigma Chemical Company Limited, Poole, U.K.

Biotinylated Goat-Anti-Human Polyvalent Immunoglobulin (IgA, IgG, IgM): Sigma Chemical Company Limited, Poole, U.K.

Extravadin peroxidase - Sigma Chemical Company Limited, Poole, U.K.

TMB peroxidase substrate - Kirkegaard & Perry Laboratories, Maryland, U.S.A.

MRX II plate reader - Dynex Technologies, VA, U.S.A.

N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) - Sigma Chemical Company Limited, Poole, U.K.

L-1-Chloro-3-[4-tosylamido]-7-amino-2-heptanone-HCl (TLCK) - Sigma Chemical Company Limited, Poole, U.K.

Leupeptin - Sigma Chemical Company Limited, Poole, U.K.

2.2.2 Chapter 4 materials

Citrate treated 7ml vacutainers - Becton Dickinson Vacutainer Systems Europe, Meylan, France.

L-cells - Schering-Plough, Dardilly, France

CRL-1668 cells - American Type Culture Collection, VA, U.S.A.

Foetal calf serum (FCS) - Gibco Life Technologies Limited, Paisley, U.K.

Penicillin - Gibco Life Technologies Limited, Paisley, U.K.

Streptomycin - Gibco Life Technologies Limited, Paisley, U.K.

L-Glutamine - Gibco Life Technologies Limited, Paisley, U.K.

Sodium pyruvate - Gibco Life Technologies Limited, Paisley, U.K.

RPMI 1640 medium - Gibco Life Technologies Limited, Paisley, U.K.

G-418 - Gibco Life Technologies Limited, Paisley, U.K.

Iscoves medium - Gibco Life Technologies Limited, Paisley, U.K.

Mitomycin-C - Sigma Chemical Company Limited, Poole, U.K.

Crystal violet concentrate - Pro lab Diagnostics, Merseyside, U.K.

Acetic acid - BDH Limited, Poole, England, U.K.

6 well plates - Gibco Life Technologies Limited, Paisley, U.K.

Histopaque-1077 - Sigma Chemical Company Limited, Poole, U.K.

Falcon tubes - Becton-Dickinson, Cowley, U.K.

Glacial acetic acid - BDH Limited, Poole, England, U.K.

Nigrosin - Sigma Chemical Company Limited, Poole, U.K.

Sodium azide - Sigma Chemical Company Limited, Poole, U.K.

Sheep red blood cells - Scottish Antibody Production Unit, Carluke, U.K.

2-aminoethylisothiouronium bromide (AET) - Sigma Chemical Company Limited, Poole, U.K.

Sterile saline - BDH Limited, Poole, England, U.K.

Anti-human CD40 - Ancell Corporation, Bayport, MN, U.S.A.

Human interleukin-4 - ICN Biomedicals Limited, Oxon, U.K.

Dispase - Gibco Life Technologies Limited, Paisley, U.K.

Jockliks MEM medium - Gibco Life Technologies Limited, Paisley, U.K.

41μm nylon net filters - Millipore (U.K) Limited, Watford, U.K.

Neubauer haemocytometer - Sigma Chemical Company Limited, Poole, U.K.

Polyethylene glycol 1500 (PEG 1500) - ICN Biomedicals Limited, Oxon, U.K.

HAT concentrate - Sigma Chemical Company Limited, Poole, U.K.

Transferrin - Sigma Chemical Company Limited, Poole, U.K.

Insulin - Sigma Chemical Company Limited, Poole, U.K.

Ouabain - Sigma Chemical Company Limited, Poole, U.K.

HT concentrate - Sigma Chemical Company Limited, Poole, U.K.

Cell culture flasks (various sizes) - Gibco Life Technologies Limited, Paisley, U.K.

2.2.3 Chapter 5 materials

Multiscreen HA membrane-bottomed plates - Millipore Limited, Watford, U.K.

Tetanus toxoid - Evans Medical Ltd., Leatherhead, U.K.

Fungizone - Gibco Life Technologies Limited, Paisley, U.K.

HRP-conjugated avidin-D - ICN Biomedicals Limited, Oxon, U.K.

3-amino-9-ethylcarbazole (AEC) tablets - Sigma Chemical Company Limited, Poole, U.K.

N, N-Dimethylformamide - Sigma Chemical Company Limited, Poole, U.K.

Sodium acetate ($CH_3COONa.3H_2O$) - BDH Limited, Poole, England, U.K.

0.20μm filter - Millipore (U.K) Limited, Watford, U.K.

2.2.4 Chapter 6 materials

Paraffin wax - Bayer PLC., Newbury, U.K.

Silane-coated glass slides - BDH Limited, Poole, England, U.K.

Haematoxylin stain - BDH Limited, Poole, England, U.K.

Aluminium potassium sulphate ($AlK(SO_4)_2$) - BDH Limited, Poole, England, U.K.

Sodium iodate ($NaIO_3$) - BDH Limited, Poole, England, U.K.

Citric acid ($C(OH)(COOH)(CH_2COOH)_2.H_2O$) - BDH Limited, Poole, England, U.K.

Choral hydrate ($CCl_3CH(OH)_2$) - BDH Limited, Poole, England, U.K.

Calcium chloride ($CaCl_2$) - BDH Limited, Poole, England, U.K.

Eosin - BDH Limited, Poole, England, U.K.

Xylene - Genta Medical, Leeds, U.K.

Ethanol - BDH Limited, Poole, England, U.K.

3% Hydrogen peroxide - Sigma Chemical Company Limited, Poole, U.K.

Methanol - BDH Limited, Poole, England, U.K.

Goat serum - Scottish Antibody Production Unit, Carluke, U.K.

$F(ab)_2$ fragments of goat anti-human IgG - Sigma Chemical Company Limited, Poole, U.K.

Papain - Sigma Chemical Company Limited, Poole, U.K.

Biotin labelled fab fragments of goat anti-human IgG - Sigma Chemical Company Limited, Poole, U.K.

Biotin avidin HRP complex - Vector laboratories Inc., Burlingame, U.S.A

Developing solution - Vector laboratories Inc., Burlingame, U.S.A

Glycergel - Dako, Ely, Cambridgeshire, U.K.

Biotinamidocaproate N-Hydroxysuccimimidine ester (Biotin) - Sigma Chemical Company Limited, Poole, U.K.

2.3 General Buffers

Phosphate Buffered Saline (PBS): 8g NaCl, 0.2g KH_2PO_4, 1.44g $Na_2HPO_4.2H_2O$, 0.2g KCl, dissolved in 800ml of distilled H_2O. This was then made up to 1 litre and stored at 4°C for a maximum of 1 week (pH 7.4).

Phosphate Buffered Saline containing 1mM disodium EDTA (PBSE) : 8g NaCl, 0.2g KH_2PO_4, 1.44g $Na_2HPO_4.2H_2O$, 0.2g KCl, were dissolved in 800ml of distilled H_2O. 371mg EDTA was then added and the solution was made up to 1 litre and stored at 4°C for a maximum of 1 week. The pH was adjusted when necessary with 5M NaOH to pH 7.4.

0.1M Sodium Carbonate Buffer containing 0.02% NaN_3: 1.36g Na_2CO_3, 7.35g $NaHCO_3$, 200mg NaN_3 were dissolved in 950ml of distilled H_2O. The pH was adjusted to 9.2 using 1M NaOH.

Tris Buffered Saline (TBS): 8g NaCl, 0.2g KCl, 3g Tris base were dissolved in 800ml distilled H_2O. 0.015g of Phenol red was added and the pH of the mixture was

adjusted to 7.4 with 1M HCl. Distilled H_2O was then added to make the volume up to 1litre and the solution was stored at room temperature.

0.5% Sarkosyl: 0.5g w/v sodium lauryl sarcosinate (SDS) was dissolved in distilled H_2O.

Coating Buffer (CB): 1.59g Na_2CO_3, 2.93g $NaHCO_3$ was dissolved in 800ml distilled H_2O. The pH was adjusted to 9.6 at just under 1 litre, by adding 1M HCl up to 1 litre in a volumetric flask. It was stored in a sterilised bottle at 4°C for a maximum of 1 week.

Incubation Buffer (IB): 8g NaCl, 0.2g KH_2PO_4, 1.44g $Na_2HPO_4.2H_2O$, 0.2g KCl, 0.5g Tween 20 dissolved in 800ml of distilled H_2O. 1g BSA was layered on the surface and allowed to dissolve slowly. The volume was adjusted to 1 litre with distilled H_2O, then mixed and stored at 4°C for a maximum of 1 week (pH 7.4).

Wash Buffer (WB): This was prepared at 10 times the concentration of incubation buffer (nil BSA) and stored at room temperature. It was diluted 1/10 immediately before use.

N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) buffer: 229.2g TES, 372.24g EDTA and 0.2g (w/v) SDS were dissolved in 100ml distilled H_2O. The buffer was stored at 4°C for a maximum of 1 week.

Complete Medium: 50ml foetal calf serum (FCS), 5ml penicillin (10000IU), 5ml streptomycin (10000μg/ml), 5ml L-Glutamine (200mM), 5ml sodium pyruvate (1mM), RPMI 1640 to 500ml.

CRL 1668 culture medium: 100ml FCS, 10ml L-Glutamine (400mM), G-418 at a final concentration of 200μg/ml, Iscoves medium to 500ml.

Phosphate Buffered Saline containing 1mM disodium EDTA (Versene): 8g NaCl, 0.2g KH_2PO_4, 1.44g $Na_2HPO_4.2H_2O$, 0.2g KCl, dissolved in 800ml of distilled H_2O. 3.71g EDTA was then added and the solution was made up to 1 litre and stored at 4°C for a maximum of 1 week. The pH was adjusted when necessary with 5M NaOH to pH 7.4.

White cell counting fluid: 0.05% crytstal violet stain dissolved in 1% acetic acid.

Nigrosin in 7% acetic acid: 7ml glacial acetic acid, 0.05g nigrosin, 0.1g sodium azide dissolved in saline made up to a volume of 100ml. Filtration through filter paper.

0.143M 2-aminoethylisothiouronium bromide (AET) solution: 0.402g of AET dissolved in 10ml of distilled water, to pH 9.0 with 5M NaOH.

HAT ($5x10^{-3}$M hypoxanthine, $2x10^{-5}$M aminopterin, $8x10^{-4}$M thymidine) selection medium (HAT): 1 vial HAT concentrate dissolved in 10ml of RPMI 1640 medium, 100ml Foetal Calf Serum (FCS), 5ml glutamine (200mM), 5ml penicillin (10000IU), 5ml streptomycin (10000μg/ml), 1.25ml transferrin (2mg/ml) 1.25ml insulin (2mg/ml), Ouabain to final concentration 1mM, then addition of RPMI 1640 to 500ml.

HT ($5x10^{-3}$M hypoxanthine, $8x10^{-4}$ M thymidine) medium (HT): 1 vial HT concentrate , dissolved in 10ml of RPMI 1640 medium, 100ml Foetal Calf Serum (FCS), 5ml glutamine (200mM), 5ml penicillin (10000IU), 5ml streptomycin (10000μg/ml), 1.25ml transferrin (2mg/ml), 1.25ml insulin (2mg/ml), then addition of RPMI 1640 to 500ml.

Phosphate buffered saline containing 0.1% tween-20 (PBS-T): 8g NaCl, 0.2g KH_2PO_4, 1.44g $Na_2HPO_4.2H_2O$, 0.2g KCl, 0.5g Tween 20 dissolved in 800ml of distilled H_2O, and made up to 1 litre and stored at 4°C for a maximum of 1 week (pH 7.4).

Phosphate buffered saline containing tween and foetal calf serum (PBS-T-FCS): 8g NaCl, 0.2g KH_2PO_4, 1.44g $Na_2HPO_4.2H_2O$, 0.2g KCl, 0.5g Tween 20 dissolved in 800ml of distilled H_2O, and made up to 1 litre. 5% FCS was then added. The buffer was used on the day that it was prepared only.

0.1M sodium acetate buffer (pH 4.8-5.0): 40.81g $CH_3COONa.3H_2O$ was dissolved in 800ml distilled H_2O. The pH was then adjusted with glacial acetic and the volume made up to 1 litre with distilled H_2O.

Chromogen substrate: 1 tablet of 3-amino-9-ethylcarbazole was dissolved in 2ml N, N-Dimethylformamide. 60ml of 0.1M sodium acetate buffer, (pH 4.8-5.0) was then added and the solution was filtered through a 0.20μm filter.

10% Buffered formaldehyde: 1 part buffered formaldehyde concentrate: 4 parts distilled H_2O.

Mayers Haematoxylin: 2.5g of haematoxylin stain was dissolved in 2500ml of distilled H_2O using gentle heat. 125g of aluminium potassium sulphate was then added and dissolved. 0.5g sodium iodate, 2.5g citric acid and 125g chloral hydrate was then added, the solution was heated to boiling point for 5 minutes and then cooled and filtered.

1% Eosin: 2.5g $CaCl_2$ was dissolved in 500ml of distilled H_2O. 5g of eosin was then added and dissolved.

Blocking serum: 120μl goat serum was added to 5ml of PBS.

Biotin avidin HRP complex mixture: 2 drops of solution A added to 5ml PBS, mix, then 2 drops of solution B were added, mix. It is imperative that this solution is prepared 30 minutes before use.

Developing solution: 2 drops of buffer added to 5ml of distilled water, mix, 4 drops of DAB, mix, 2 drops of H_2O_2, mix.

100mM phosphate buffer: 5.3g NaCl, 0.13g KH_2PO_4, 0.96g $Na_2HPO_4.2H_2O$, 0.13g KCl, dissolved in 800ml of distilled H_2O. This was then made up to 1 litre and stored at 4°C for a maximum of 1 week (pH 7.4).

Chapter 3. A Comparison of the Changes in the Humoral Immune Response to Antigens of Periodontal Pathogens Following Periodontal Therapy

3.1 Introduction

The existence of a humoral immune response in periodontal disease is a generally accepted fact. Numerous studies have been carried out over the last few decades, suggesting that elevations in the humoral immune response are associated with increasing severity of disease (Genco *et al.*, 1980b, Mouton *et al.*, 1981, Tolo and Brandtzaeg, 1982, Listgarten *et al.*, 1981, Ebersole *et al.*, 1982b, Naito *et al.*, 1984, Tew *et al.*, 1985a). These studies suggest a relationship exists between specific periodontopathic microbiota and antibody levels to the bacteria in both serum and GCF.

The existence of a systemic humoral immune response to a variety of oral bacteria is well documented in subjects with different forms of periodontal disease (Taubman *et al.*, 1982). This is probably simply because microbial challenge generally induces antibody production and high titres indicate current or previous infection. One of the major difficulties in evaluating the antibody response in periodontal disease has been the antigenic complexity of periodontopathic bacteria. If investigations could lead to the identification of major immunogenic proteins and antigens, and could characterise the immune responses elicited more clearly, there may be a better understanding of the host parasite interactions in periodontal disease.

The immune response has been studied in different forms of periodontal disease, various age groups, and against a range of pathogens and antigens, and attempts have been made to draw conclusions or detect differences. An important consideration is the interaction between periodontal pathogens and the immune response of the host, which is a dynamic one. Hence, temporal relationships and alterations may exist in systemic antibody levels and relate to infection, treatment and active episodes of disease. Indeed, epidemiological investigations have suggested that only a subset of

the population is susceptible to severe periodontitis, and while in some of these individuals the disease appears to proceed as a chronic progressive destruction, in others the disease presents itself in phases which can be periods of active or inactive disease, or even remission (Socransky *et al.*, 1994).

Although the nature of the humoral immune response is not fully understood, the host response may provide a sensitive indicator of the flora found in the oral cavity, particularly in the gingival sulcus (Tew *et al.*, 1985b, Tew *et al.*, 1985c, Vandesteen *et al.*, 1984). As well as providing information about the residing bacteria, the humoral immune response has been suggested as a means of differentiating between distinct periodontal disease states. High correlations between disease activity and antibody titres have been reported for a number of micro-organisms. A high degree of association has been reported for *A. actinomycetemcomitans* and localised aggressive periodontitis (Altman *et al.*, 1982, Astemborski *et al.*, 1989, Ebersole *et al.*, 1982b) and *P. gingivalis* and chronic periodontitis (Mouton *et al.*, 1981). Thus, diagnostic differentiation of the periodontal disease forms has been suggested for both periodontal microbiology and humoral immunology.

Due to the nature of periodontal disease, it is virtually impossible to obtain a serum sample to investigate antibody targets and titres pre-disease. As a result, it is very difficult to draw conclusions and identify associations between disease severity, disease forms, antibody titres and micro-organisms. Even if a high correlation between disease severity and antibody titre is identified, numerous subjects possess antibody titres that fall above or below the ranges that identify health and disease, and quite often this is irrespective of their periodontal health status. Antibody titres of individuals not affected by periodontal disease ("normal") vary enormously. This difficulty is compounded by the fact that most of the putative periodontal pathogens can be found in healthy subjects (Loesche and Syed, 1978, Loesche *et al.*, 1985) and antibody titres against pathogens can be identified even in the absence of periodontal disease (health) (Doty *et al.*, 1982). Furthermore, it is often difficult to identify what point in the disease process individuals are at, especially if they do not present with disease until late on in life.

Due to all of these sources of variation between subjects, a number of studies have investigated relative changes in antibody titres against various micro-organisms, by assessing the success of a particular stage of periodontal therapy. These studies, discussed below, attempt to monitor the microbial flora of periodontal patients by assessing the host response. However, there are conflicting reports about the change in antibody level after treatment.

Aukhil *et al.* (1988) observed serum antibody titres of patients with chronic periodontal disease before and after periodontal treatment, against a number of oral micro-organism sonicate preparations. They reported a general reduction in the mean antibacterial titres following treatment. However, the intervals during which the decreases in antibody titres occurred were not the same for each micro-organism studied. Naito *et al.* (1987), reported a decrease in the serum antibody titre of periodontally diseased patients to *P. gingivalis* post-treatment. A decrease in titre was also reported by Mouton *et al.* (1987). This study reported the existence of two subgroups within the periodontitis subjects studies. The distinction between the subgroups was a difference in antibody reactivity against *P. gingivalis*. One subgroup were IgA negative and had IgM and IgG titres not significantly higher than healthy subjects. The second subgroup were IgA positive and showed IgM and IgG levels much higher than those of the first subgroup. The latter group showed a 55% decrease in antibody titres 5-7 months after treatment. Sandholm and Tolo (1987), also observed serum antibody levels to 4 periodontal pathogens pre- and post-treatment in aggressive periodontitis. Although they observed decreased antibody titres post-treatment in 25% of the subjects tested, in the remaining 75%, they reported unaltered antibody levels. More recently Horibe *et al.* (1995) reported a study on the effect of periodontal treatments on IgG titres against 7 different periodontopathic bacteria. Of the 7 bacteria tested, this group showed a significant decrease in antibody titres post-treatment against *P. gingivalis* and *P. intermedia*. However, no difference in titres between pre- and post-treatment were reported against *Prevotella loescheii*, *F. nucleatum*, *A. actinomycetemcomitans*, *Eikenella corrodens* and *Caphocytophga ochracea*.

The majority of the above studies report a decrease in antibody titre following treatment, however, some results indicated no difference in titre pre- and post-treatment against some of the bacteria tested. The studies reported are often carried out for different lengths of time and are difficult to compare. Ebersole and Holt (1991) noted that treatment can decrease serum IgG antibody levels to certain micro-organisms, in both aggressive and chronic periodontitis patients, however, they indicated that these changes were noted to take up to 8-10 months post-treatment and were quite specific for selected micro-organisms in individual patients.

In contrast to these studies, reports have been made of investigations that have indicated an increase in serum antibody titres to various micro-organisms following treatment. Ebersole and Holt (1991b) reported that 60% of periodontitis patients demonstrated increased titres against various periodontal pathogens post-treatment. Sjöström *et al.* (1994) examined IgG titres to *A. actinomycetemcomitans* in 30 patients with aggressive periodontitis. They reported a significant increase in titres at 6-12 months after beginning treatment. Mooney *et al.* (1995), also reported an increase in antibody titre post-treatment. However, this study is slightly different and cannot be directly compared with those above that suggest a decrease in titre following treatment, as it observed the dynamics of the immune response to *P. gingivalis* and *A. actinomycetemcomitans* in patients that were divided into subgroups. The group divisions were based on the subject's baseline IgG antibody levels to *P. gingivalis* or *A. actinomycetemcomitans*. The patients were labelled seropositive or seronegative. Being labelled seropositive for a micro-organism meant the subject had an IgG titre to an organism >2 times the control median. The study carried out by Mooney *et al.* (1995), along with a similar study by Chen *et al.* (1991), added an extra factor that included antibody avidity as well as antibody titre, and thus do not show antibody titres post-treatment alone. Chen *et al.* (1991) looked at antibody titres and avidities pre- and post treatment against *P. gingivalis* whole cell sonicates, LPS and protein fractions. Again, in this study there were two groups, sero-positive and sero-negative patients. The results indicated that following treatment, there was a decrease in antibody titre against the antigens in the seropositive group and an increase in titre in the sero-negative group. There was an increase in antibody avidity to the antigens

tested in both groups. Mooney *et al.* (1995), reported a significant increase in avidity of IgG to *P. gingivalis* in the seropositive group, but no increase in titre (i.e. no change). An increase in titre was reported for the sero-negative group. They also reported a significant increase in titre against *A. actinomycetemcomitans*, but again in the sero-negative group only. The studies reported by Mooney *et al.* (1995) and Chen *et al.* (1991) therefore, suggest that the characteristics of the humoral immune response to various pathogens may be quite different and depend on the immunological status of the patient prior to treatment, a factor which may not have been considered in many of the previously discussed studies. An increase in antibody titre in seronegative patients could be due to the 'inoculation' theory suggested by Ebersole *et al.* (1985d). Although the seronegative patients had a low antibody titre to the antigens prior to treatment, these antibodies may have been a low level presence of cross-reacting antibodies, and as a result of treatment, an antibody response may have been mounted following induction of the primary immune response to these Gram negative bacterial antigens. Chen *et al.* (1991) suggested that the increase in antibody avidity in both groups of patients could be due to the treatment having an effect on antibody responsiveness. They suggest that treatment leads to a reduction in antigenic load, which in turn leads to a selection of clones of B lymphocytes that produce antibodies of higher avidity.

The aim of the first part of the overall project was to investigate the humoral immune response in a group of chronic periodontitis patients against a panel of whole micro-organisms. In addition, responses to OMP and a sample of what are considered the more potentially important antigens from these micro-organisms. The micro-organisms used in this study were: *P. gingivalis; A. actinomycetemcomitans*; *P. intermedia*; *B. forsythus* and *T. denticola*. The OMP of *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus* were also used as well as the antigens: HSP60 (of *P. gingivalis*); LPS (of *P. gingivalis*); leukotoxin (of *A. actinomycetemcomitans*) and the β segment of RgpA protease (of *P. gingivalis*). The literature regarding these micro-organisms and antigens, and therefore, the reason why they have been selected has been discussed already in the introduction to this thesis. Although studies of this nature have previously been carried out, no study has yet observed the titres against all of these targets in one study group so as to permit

comparisons. The study has been performed on a group of patients scheduled for treatment for chronic periodontitis and antibody titres have been observed pre- and post-treatment.

The second part of this study was investigating the cross-reactivity between a number of these micro-organisms. Of the putative periodontal pathogens generally accepted to play a role in the pathogenesis of periodontal disease, all are Gram negative bacteria with the exception of the spirochaetes. Therefore, these different species will possess a certain amount of homology, with structural features of surface components being chemically very similar and possessing similar immunobiological properties. If these surface components are immunogenic and play a role in the induction of the immune response, perhaps there is some cross-reactivity occurring between antibodies induced against one pathogen and a structurally very similar surface component of another pathogen. Although numerous studies have been carried out to investigate serum antibody titres against individual periodontopathogens, there does not appear to be a large pool of literature regarding the question of cross-reactivity between the putative periodontopathogens.

Evidence can be seen in the literature however, to suggest that this idea is worth investigating. A study carried out by Kinane *et al.* (1993) observed specific immunoglobulin titres to *P. gingivalis* and *A. actinomycetemcomitans* in the sera and GCF of 20 patients with periodontitis. As part of the study they carried out adsorptions of the sera against *A. actinomycetemcomitans* and *Haemophilus aphrophilus* (*H. aphrophilus*). The adsorbed sera were then assessed for specific antibody to *A. actinomycetemcomitans*. The analysis gave a mean reduction in the anti-*A. actinomycetemcomitans* IgG of 68% and adsorption against *H. aphrophilus* gave a similar reduction in the anti-*A. actinomycetemcomitans* IgG of 63%. Thus the authors suggested that there are cross-reacting antibodies to these two species. Moreover, the results of the study suggested a strong correlation between serum IgG and IgM levels to these two bacteria, indicating cross-reactivity between antibodies directed against these pathogens.

A study by Vasel *et al.* (1996) reported immunisation of monkeys with a vaccine containing a monkey isolate of *P. gingivalis* that induced protection against alveolar bone destruction. These workers investigated immune and pre-immune sera obtained from the monkeys, using ELISA and Western immunoblotting. The results of the study indicated that following immunisation with killed *P. gingivalis*, the sera of the monkeys contained increased antibody levels reactive with *P. gingivalis* and also *B. forsythus*. Detailed analyses suggested that the binding of serum antibodies was a specific, adaptive response to the LPS in the immunogen, since little reactivity was detected in the pre-immune sera. Thus, the antibodies induced by LPS from *P. gingivalis* appeared to be cross-reactive with the LPS of *B. forsythus*.

Further to the above study other investigations have suggested cross-reactivity between antibodies against LPS in non-oral Gram negative bacteria. Seifert *et al.*, (1996) reported the generation of a human monoclonal antibody that recognised a conserved epitope shared by LPS molecules of different Gram negative bacteria, indicating a high degree of cross-reactivity of antibody across different bacterial species.

Hinode *et al.* (1998) reported a clear cross-reactivity between antibodies raised to GroEL-like proteins of *P. gingivalis, A. actinomycetemcomitans,* and *E. coli,* indicating that a significant portion of the antibodies induced against GroEL-like proteins are directed at epitopes conserved across bacterial genera.

Thus, there is evidence indicating cross-reactivity between antigens of different Gram negative bacteria. The aim of the second part of this serum antibody study was to investigate cross-reactivity between 4 periodontal pathogens, *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus*. Serum was used that had been taken from 9 individuals suffering from chronic periodontitis and was adsorbed against one bacteria before the antibody titres against all 4 pathogens was tested in an ELISA.

Previous adsorption studies that have been undertaken mainly involving only two micro-organisms (Vasel *et al.*, 1996; Kinane *et al.*, 1993; Seifert *et al.*, 1996). It was

felt that this only revealed part of the cross-reactivity story and that additional cross-reactivity testing would provide more information on *P. gingivalis*, *A. actinomycetemcomitans*, *B. forsythus* and *P. intermedia*. This investigation is therefore, of interest as the extent of cross-reactivity between more than two putative periodontopathogens is being investigated in a single study.

3.2 Methods Part 1: Enzyme Linked Immunosorbent Assay

3.2.1 Subjects and blood samples

Prior to the commencement of these studies, ethical approval was obtained from the Glasgow Dental Hospital Ethics Committee. Subjects participating in these studies were informed of the protocol and consent was obtained. All patients taking part in the investigation were free to withdraw from the study at any time. Twenty five subjects, 11 males and 14 females, with a mean age of 47.3 $\pm$ 7.6 years (mean $\pm$ S.D (range = 36-66 years), were enrolled in the study. Seven of the subjects were smokers, 18 were non-smokers. The subjects were patients attending Glasgow University Dental Hospital. The criteria for inclusion was that patients had advanced periodontal disease with probing depths (P.D.) of >5.0 mm in at least 2 non-adjacent sites per quadrant. Site selection was carried out at the screening visit, where full mouth periodontal pocket charting was performed using a PCP-12 periodontal probe. Wherever possible, one site in each quadrant over 5 mm in depth, without furcation involvement, was selected for inclusion in the study. These subjects had no history of systemic conditions which could influence the course of periodontal disease, had not received antibiotic treatment during the previous 3 months, and had received no prior periodontal treatment.

At the baseline appointment, P.D. and bleeding on probing (BOP) at these sites were assessed with the Florida probe (Gibbs *et al.*, 1988) and blood samples taken. Twenty one millilitres of venous blood were collected from the anterior cubital region using butterfly needles and 3 x 7 ml non-heparinised vacutainer tubes. The blood was allowed to clot overnight at 4°C. It was then centrifuged at 750 x g for 15 minutes and the serum was separated, aliquoted and stored at -80°C until required for ELISA

analysis. Patients were treated by an experienced periodontist and given a course of Hygiene Phase Therapy (HPT). This included oral hygiene instruction, quadrant scaling and root planing under local anaesthesia.

The subjects were reassessed with conventional probing charts after HPT. A blood sample was taken and P.D. and BOP measurements were recorded at the 4 pre-selected sites with the Florida probe 138.9 $\pm$55.4 days (mean $\pm$ S.D.), range = 56 - 280 days, after initial sampling.

3.2.2 Antigen preparation

1) Whole fixed Bacteria

P. gingivalis NCTC 11834 and *P. intermedia* ATCC 25611 were grown under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) on fastidious anaerobe agar (FAA). *B. forsythus* ATCC 43037 was also grown under anaerobic conditions in tryptic soy agar supplemented with 7% horse blood and 10μg/ml N-acetylmuramic acid. *A. actinomycetemcomitans* ATCC 29523 (serotype A) was grown in 5% CO_2 and 95% air on columbia blood agar. All bacteria were grown at 37°C. *A. actinomycetemcomitans* was harvested after 48 hours, *P. gingivalis* and *P. intermedia* after 5 days and *B. forsythus* after 7 days. *T. denticola* ATCC 35405 were cultured in medium OMIZ-pat + 1% human serum, and were a kind gift from Dr.C.Wyss, Institut fur Orale Mikrobiologie, Zurich, Switzerland (Wyss *et al.*, 1997).

All bacterial cells were harvested into phosphate buffered saline containing 1mM disodium EDTA (PBSE), washed by centrifugation at 200 x g for 10 minutes, and fixed for 1 hour at room temperature in 10% formal saline. The cells were then washed twice in PBSE and once in 0.1M sodium carbonate-bicarbonate buffer containing 0.02% NaN_3 at pH 9.6. Fixed cells were stored at 4°C until use.

2) Outer-membrane Protein Preparation

The preparation of the outer-membrane antigens was carried out using a method modified from that of Schryvers *et al.* (1988). *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus* were grown as described above. The bacteria were harvested into PBSE and centrifuged at 10,000 x g for 20 minutes. The pellet was then resuspended in tris buffered saline (TBS) and washed a further twice, as described above before being resuspended. The cells were sonicated for 10 pulses at 200W for 10 seconds, with a 2-minute cooling period between each pulse. Centrifugation was then carried out for 15 minutes at 8000 rpm at 4°C. The supernatant was removed and transferred into sterile ultracentrifuge tubes. Ultracentrifugation was then carried out for 1 hour at 40,000 rpm at 4°C and the pellet resuspended in 0.5% sarkosyl and retained at 4°C overnight. Ultracentrifugation was then carried out as above and the pellet resuspended in distilled water.

The protein concentrations were determined using the BCA protein assay kit.

3) Purified Antigen Preparation

Heat Shock Protein 60 of *P.gingivalis*

The heat shock protein 60 (HSP60) (of *P. gingivalis*) was purified according to Maeda *et al.* (1994), and was a kind gift from Dr. H. Maeda, Department of Periodontology and Endontology, Okayama University Dental School, Okayama, Japan.

Leukotoxin of *A. actinomycetemcomitans*

The leukotoxin (of *A. actinomycetemcomitans*) was purified in accordance with the protocol reported by Tsai *et al.* (1984) with modifications and was a kind gift from Dr. J. Korostoff, Leon Levy Research Center for Oral Biology, University of Pennsylvania, Philadelphia, USA.

Lipopolysaccharide of *P. gingivalis*

Lipopolysaccharide of *P. gingivalis* W50 was purified according to the method described by Darveau and Hancock (1983) and was a kind gift from Professor M.

Curtis, Department of Oral Microbiology, Medical research Council Pathogenesis Group, St. Bartholomews and The Royal London School of Medicine and Dentistry, London, UK.

The β segment of the RgpA protease of *P. gingivalis*

Domains of the RgpA protease of (*P. gingivalis*) were prepared as an N-terminal $(His)_6$ fusion protein as described by Slaney *et al.* (2000) and was a kind gift from Professor M. Curtis.

3.2.3 Analysis of sera samples by Enzyme Linked Immunosorbent Assay (ELISA)

Specific antibody titres were measured using enzyme-linked immunosorbent assay (ELISA) by the method of Ebersole *et al.* (1980).

The plates were pre-washed 3 times with coating buffer and coated overnight with the antigen at 4°C using 100μl per well. Immulon 1B plates were used because of their low protein-binding characteristics and low non-specific background. The antigens were coated onto the surface of the wells at a concentration which had been determined as optimum to coat these plates. These concentrations were *P. gingivalis* and *P. intermedia* whole cells; 0.05 at OD600, *A. actinomycetemcomitans* and *B. forsythus* whole cells; 0.02 at OD600, *T. denticola* whole cells; 0.001 at OD600, *P. gingivalis and A. actinomycetemcomitans* OMP; 10μg/ml, *P. intermedia* and *B. forsythus* OMP; 0.5μg/ml, lipopolysaccharide (*P. gingivalis*); 4μg/ml, leukotoxin (*A. actinomycetemcomitans*); 0.5μg/ml, HSP60 (*P. gingivalis*) and RgpA β segment (*P. gingivalis*); 2μg/ml. Control wells for each aspect of the ELISA process were arranged around the outside of the plates.

The ELISA plates were removed from the refrigerator and washed 4 times, then 4 more, 4 times again and lastly once more with wash buffer (4x4x4x1). The non-specific binding sites were blocked with 100μl per well of incubation buffer (IB) containing 5% skimmed milk powder for 1 hour at 37°C. The plates were then washed twice and once again (2x1) before addition of the sera.

Human sera collected from the study patients were diluted 1/200 using IB. 50μl of sera were added to each well and incubated at 37°C for 90 minutes. Each serum was tested in duplicate and the sera samples from the same patient at different time points were tested on the same plate. Following incubation with the sera the plates were washed 4x4x4x1. Afterward the plates were incubated at 37°C for 60 minutes with Biotin-goat-anti-human IgG at 1/2000 dilution in IB. The plates were then washed 4x4x4x1 and then incubated for 60 minutes at 37°C with 1/2000 dilution of extravidin peroxidase. After washing 4x4x4x1, the reactions were visualised using 100μl per well of TMB Peroxidase substrate and stopped after 5-10 minutes, depending on the speed of the reaction, using 50μl per well of 0.12M HCl.

The reactions were read using a Dynex Technologies MRX II plate reader at 630nm with a reference at 490nm and the results printed out. The duplicate results were averaged and the nonspecific background binding was subtracted. The final titre was expressed as O.D. units as has been done in numerous other studies (Suzuki *et al.*, 1984; Murray *et al.*, 1989; Sandholm and Tolo, 1987; Cridland *et al.*, 1994). The raw data was analysed in this study as the data being compared was obtained from the same 96 well plate in every case. Therefore, as all titres being compared were obtained at the same time point, it was not necessary to use a standard curve to correct for different variables. This study is comparative, rather than quantitative, therefore, the units are arbitrary. As such, converting the data into ELISA units would be an unnecessary data processing step.

3.2.4 Statistical methods

The data obtained from this study revealed that the values were adequately normalised by blotplots, although in a number of cases there were a few outliers. As the data was normalised, analyses of mean responses were undertaken using the statistical package Minitab, and parametric tests were used to assess relationships. Means, standard deviations and ranges are presented.

Mean values for pocket depths of the subjects were used. Significance levels for all tests were $p \leq 0.05$, unless otherwise stated.

3.3 Methods Part 2: Adsorptions

3.3.1 Subjects and blood samples

Prior to the commencement of these studies, ethical approval was obtained from the Glasgow Dental Hospital Ethics Committee. All patients taking part in the investigation were free to withdraw from the study at any time. Nine Subjects, 5 males and 4 females, were enrolled in this study. The subjects were patients attending Glasgow University Dental Hospital and they had a mean age of 45.2 $\pm$ 11.3 years (mean $\pm$ S.D.) (range = 29-60 years). The criteria for inclusion was as previously reported 3.2.1.

The blood was collected and stored as reported in 3.2.1.

3.3.2 Antigen preparation

P. gingivalis NCTC 11834, *P. intermedia* ATCC 25611, *B. forsythus* ATCC 43037 and *A. actinomycetemcomitans* ATCC 29523 (serotype A) were grown as described in 3.2.2.

All bacterial cells were harvested into PBSE, washed by centrifugation at 200 x g for 10 minutes. The bacterial pellet was then resuspended in PBS containing TLCK at 50μg/ml and Leupeptin at 0.1mg/ml. The cells were sonicated on ice using a Microson Ultrasonic Cell Disrupter for a 1 minute period, with a 1-minute cooling period following. This was repeated 10 times. The cell suspension was then microcentrifuged for 10 minutes at 13,000 rpm, before the supernatant was discarded and the cell pellet was resuspended in PBS again containing TLCK and leupeptin, at the same concentrations as described above.

3.3.3 Adsorption of samples

The plates were pre-washed 3 times with TES buffer and coated overnight with the antigen at 4°C using 100μl per well. Immulon 1B plates were used because of their low protein-binding characteristics and low non-specific background. Sonicates of *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus* were coated onto the surface of the wells at a concentration of 10μg/ml, which had previously been reported to be optimal (Vasel *et al.*, 1996). The sonicate preparations were diluted in CB.

The following day the plates were washed with WB before the addition of human serum diluted 1/100 in TES buffer. The plates were incubated for 1 hour with agitation at 37°C, before the serum was transferred to coated wells that had not been used and incubated for a further hour at 37°C. This was repeated 5 times. The serum was then moved to a fresh batch of pre-coated wells and the plate was incubated at 4°C overnight.

The following day, the serum was again moved to a fresh batch of pre-coated wells and incubated for 1 hour at 37°C with agitation, this was repeated 4 times to give a total of 10 x 1 hour incubations.

The serum was then removed from all of the wells and stored at 4°C until use.

The whole experiment was carried out in duplicate but instead of coating the plates with antigen they were coated with CB alone. This was so all of the serum samples could be sham absorbed as well as absorbed against the antigens.

3.3.4 Analysis of sera samples by Enzyme Linked Immunosorbent Assay (ELISA)

Serum antibody titres were measured using ELISA as described in 3.2.3. Each sample was assayed in triplicate.

The antigens were coated onto the surface of the wells at a concentration of 10μg/ml. Controls for each aspect of the ELISA process were arranged in the wells around the outside of the plates.

Human sera, both absorbed and sham-absorbed were used at 1/100 dilution.

The triplicate results were averaged and the final titre expressed as O.D units.

3.4 Results

3.4.1 Results of part 1 of the study: Enzyme Linked Immunosorbent Assay

The mean age of the subjects was 47.3 ± 7.6 years (mean ± S.D.). The length of treatment that each individual experienced varied quite extensively. The mean length of time between the taking of the first and second samples was 138.9 ± 55.4 days (mean ± S.D.), however, the range was 56 to 280 days, which makes it difficult to use the length of treatment parameter in statistical analyses.

Table 3.1 Polyvalent immunoglobulin titres for before and after treatment and the differences between these (O.D. units)

	Mean BT	S.D.	Mean AT	S.D.	Mean Difference (BT-AT)	S.D.	95% CI of Difference (BT-AT)	p-value*
PD	5.99	1.00	4.37	1.20	**1.62**	**0.98**	**(1.22, 2.02)**	**<0.001**
Pg	0.89	0.27	0.90	0.27	0.00	0.09	(-0.04, 0.04)	0.87
Aa	0.32	0.22	0.34	0.23	0.02	0.14	(-0.08, 0.04)	0.47
Pi	0.82	0.25	0.79	0.24	0.03	0.10	(-0.01, 0.07)	0.14
Bf	0.23	0.10	0.22	0.10	0.01	0.11	(-0.04, 0.06)	0.63
Td	0.28	0.12	0.25	0.13	0.03	0.10	(-0.01, 0.07)	0.16
Pg OMP	0.52	0.19	0.51	0.17	0.02	0.07	(-0.01, 0.05)	0.18
Aa OMP	0.72	0.14	0.73	0.15	0.01	0.07	(-0.03, 0.02)	0.55
Pi OMP	0.73	0.24	0.73	0.22	0.00	0.10	(-0.04, 0.04)	0.91
Bf OMP	0.46	0.15	0.46	0.12	0.00	0.10	(-0.04, 0.05)	0.82
Aa LTX	0.75	0.39	0.75	0.38	0.00	0.11	(-0.04, 0.04)	1.0
Pg LPS	0.25	0.14	0.25	0.12	0.01	0.06	(-0.02, 0.03)	0.63
Pg HSP60	0.24	0.12	0.23	0.09	0.01	0.06	(-0.01, 0.04)	0.39
Pg RgpA	0.18	0.10	0.16	0.12	0.02	0.10	(-0.02, 0.06)	0.34

*p-value for hypothesis test of no difference between before treatment and after treatment (paired t-test).

BT - before periodontal treatment, AT - after periodontal treatment, PD - pocket depth, Pg - *P. gingivalis*, Aa - *A. actinomycetemcomitans*, Pi - *P. intermedia*, Bf - *B. forsythus*, Td - *T. denticola*, Ec - *E. coli*, OMP - outer-membrane proteins, Aa LTX - Leukotoxin (of *A. actinomycetemcomitans*), Pg LPS - Lipopolysaccharide (of *P.*

gingivalis), Pg HSP60 - Heat shock protein 60 (of *P. gingivalis*), Pg RgpA - β segment of RgpA protease (of *P. gingivalis*).

Figure 3.1 Plot of polyvalent immunoglobulin titres pre- and post-treatment against *P. gingivalis*

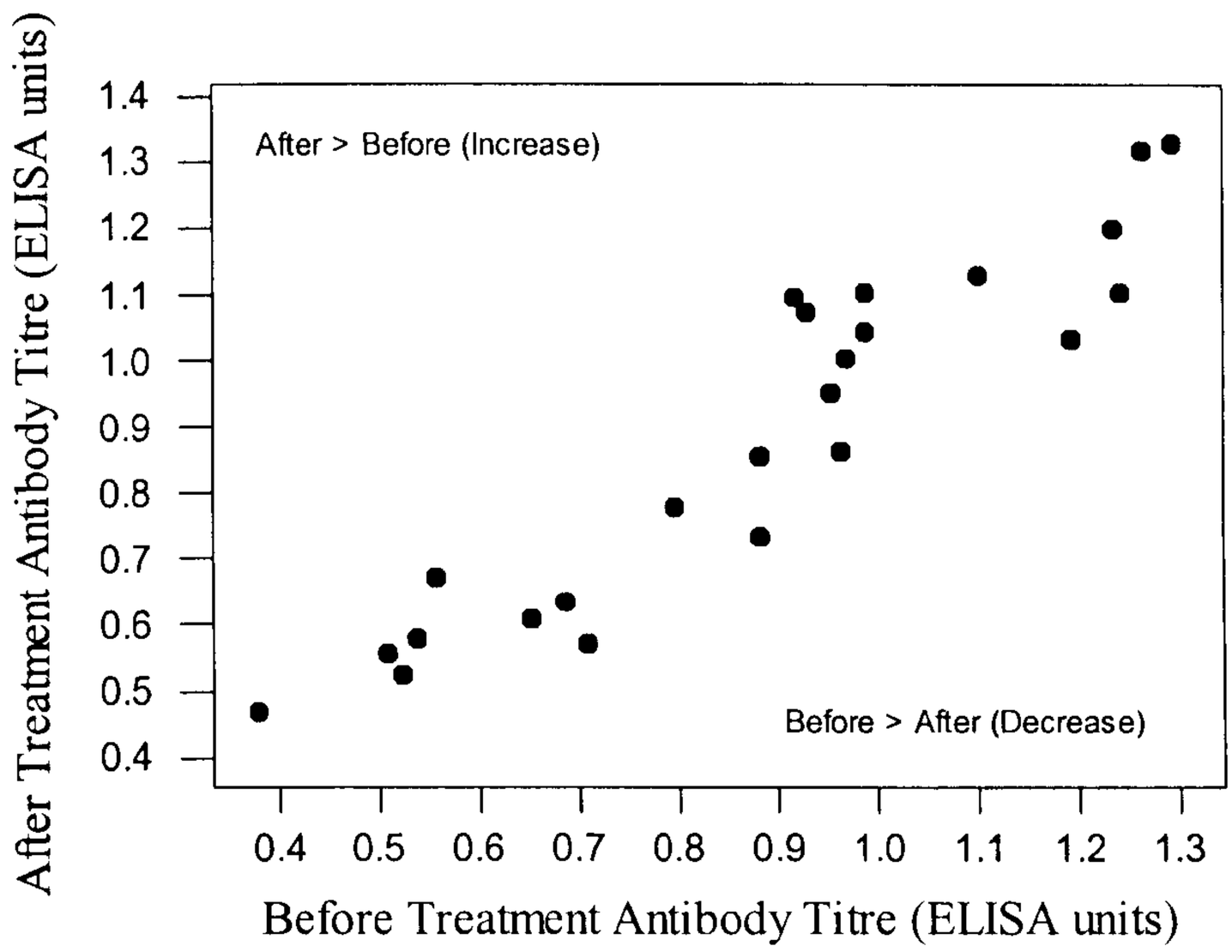


Figure 3.2 Plot of polyvalent immunoglobulin titres pre- and post-treatment against *P. gingivalis* OMP

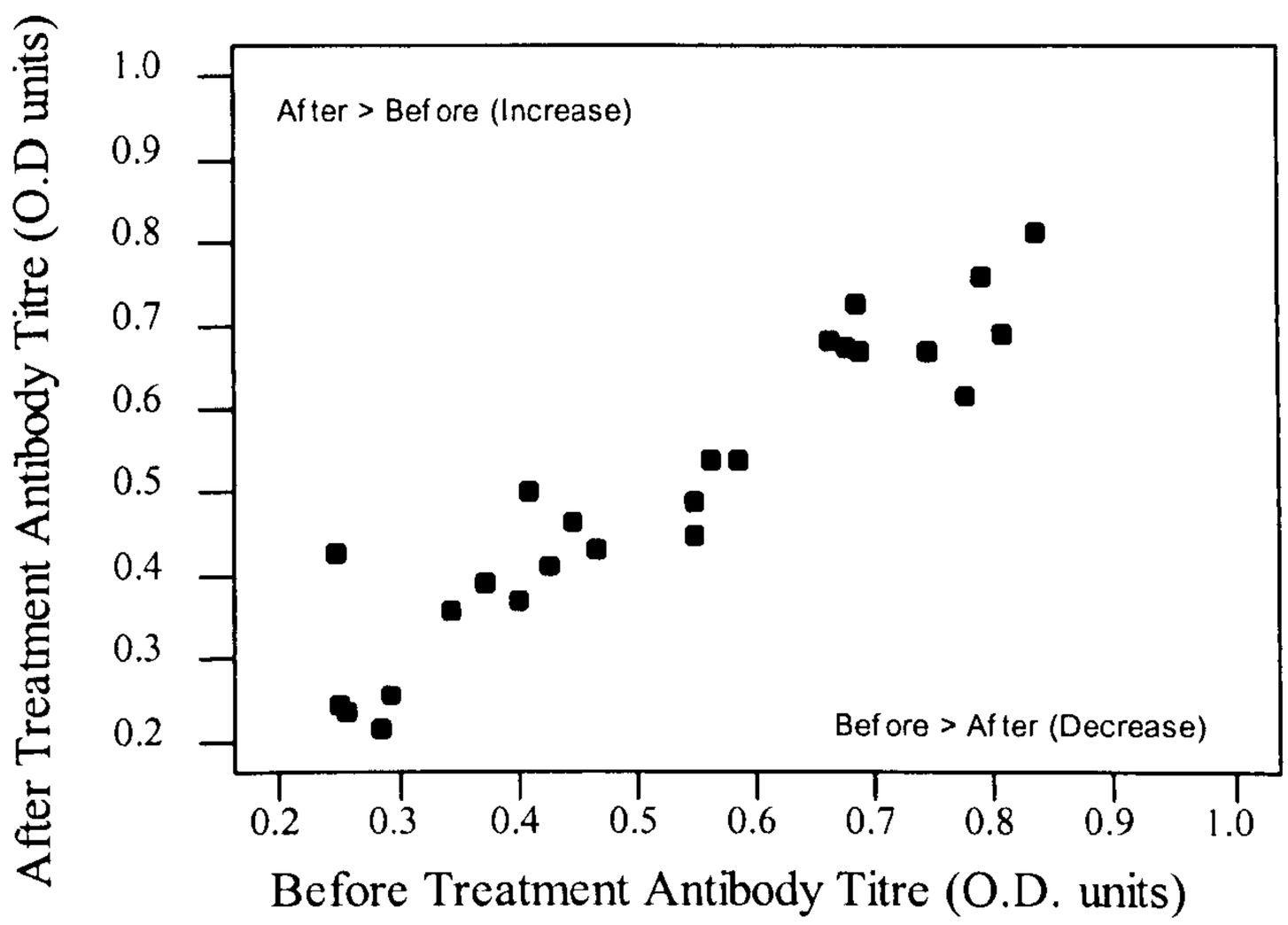


Figure 3.3 Plot of polyvalent immunoglobulin titres pre- and post-treatment against the β segment of RgpA (of *P. gingivalis*)

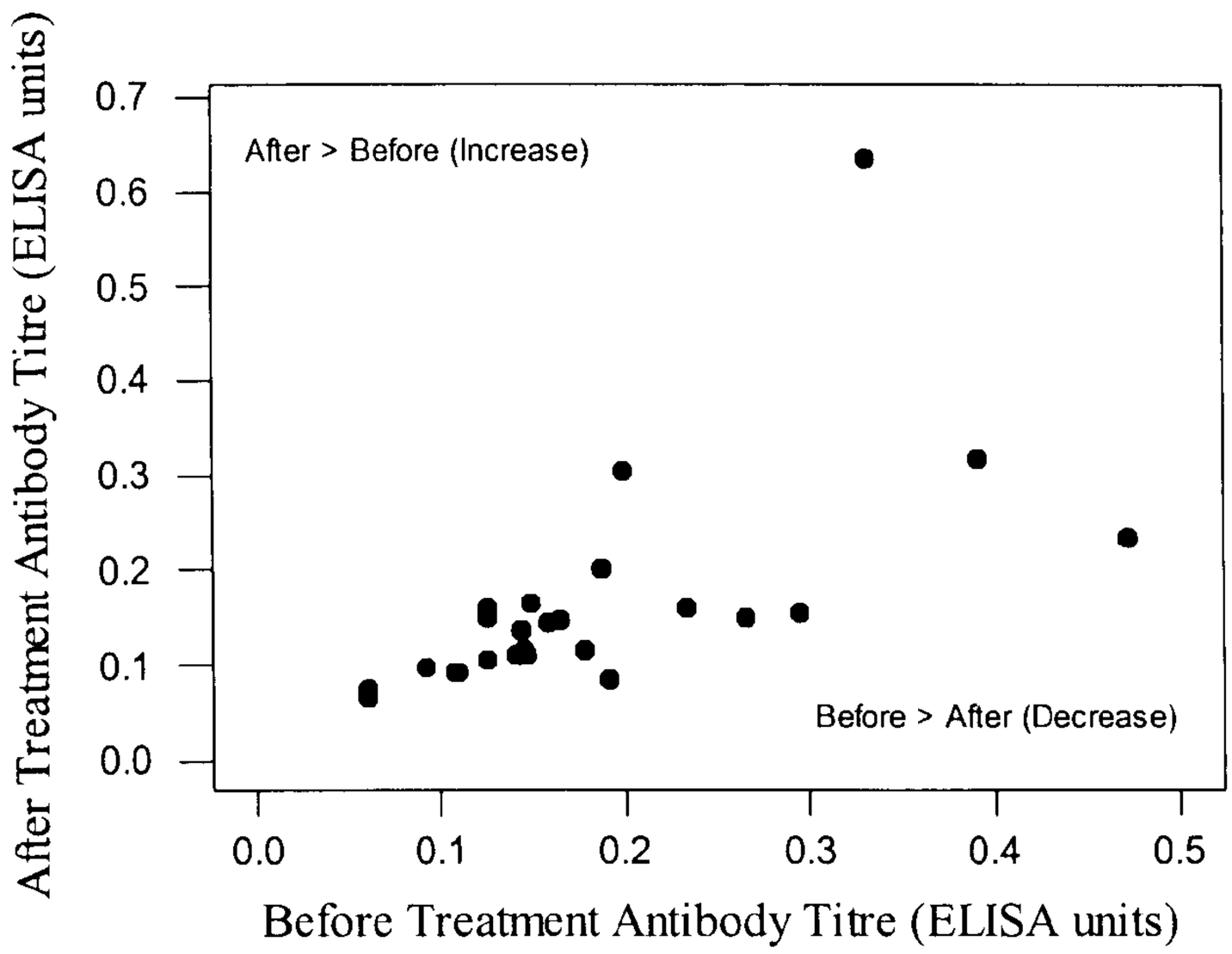


Table 3.1 shows the data collected for the pocket depths recorded pre- and post-treatment and the changes in these following treatment. Again the ranges of pocket depths were quite large both pre-and post-treatment, however, statistical analysis shows that there is a highly significant decrease in pocket depth following treatment.

Table 3.1 also shows the data collected following analysis of the serum by ELISA against the panel of micro-organisms antigen preparations before and after treatment. The polyvalent immunoglobulin titres (IgA, IgG and IgM) are expressed in O.D. units. Statistical analyses of these results indicate that there was no significant change in antibody titre following treatment for antibody levels against any of the micro-organisms or antigens tested. When the data is analysed in graphical form for each of the micro-organisms and antigens for a number of the targets outliers can be observed.

Figure 3.1 shows the plot of polyvalent immunoglobulin titres pre- and post-treatment against *P. gingivalis*. For each of the different analyses carried out, the graph for antibody titres for *P. gingivalis* will be used to show a representative sample of results. Other graphs are only shown to indicate a point of interest or a significant result.

Figure 3.2 shows the plot of polyvalent immunoglobulin titres pre- and post treatment for the 25 patients against the OMP of *P. gingivalis* and it can be seen that there are two outliers, patients 12 and 18. If these two patients are excluded from the statistical analysis, there is a significant decrease in antibody titre following treatment against this antigen preparation (mean difference = 0.021, S.D. = 0.046, 95% CI = 0.00, 0.04, p-value = 0.04). However, there seems to be no valid reason either clinically or experimentally, to exclude these 2 patients from the analysis and hence, the overall results indicate that there is no significant change in antibody titre following treatment.

Figure 3.3 which shows the plot polyvalent immunoglobulin titre pre- and post-treatment against the β segment of RgpA (of *P. gingivalis*), also indicates an outlier. When this subject is removed from the statistical analysis, a significant decrease in titre can be seen following treatment (mean difference = 0.03, S.D. = 0.07, 95% CI =

0.00, 0.06, p-value = 0.03), however, again there is no valid reason to exclude this subject from the analysis, and hence the overall data suggests no change in antibody titre against this antigen following treatment.

Figure 3.4 Boxplot of the difference between pre- and post-treatment antibody titres against LPS

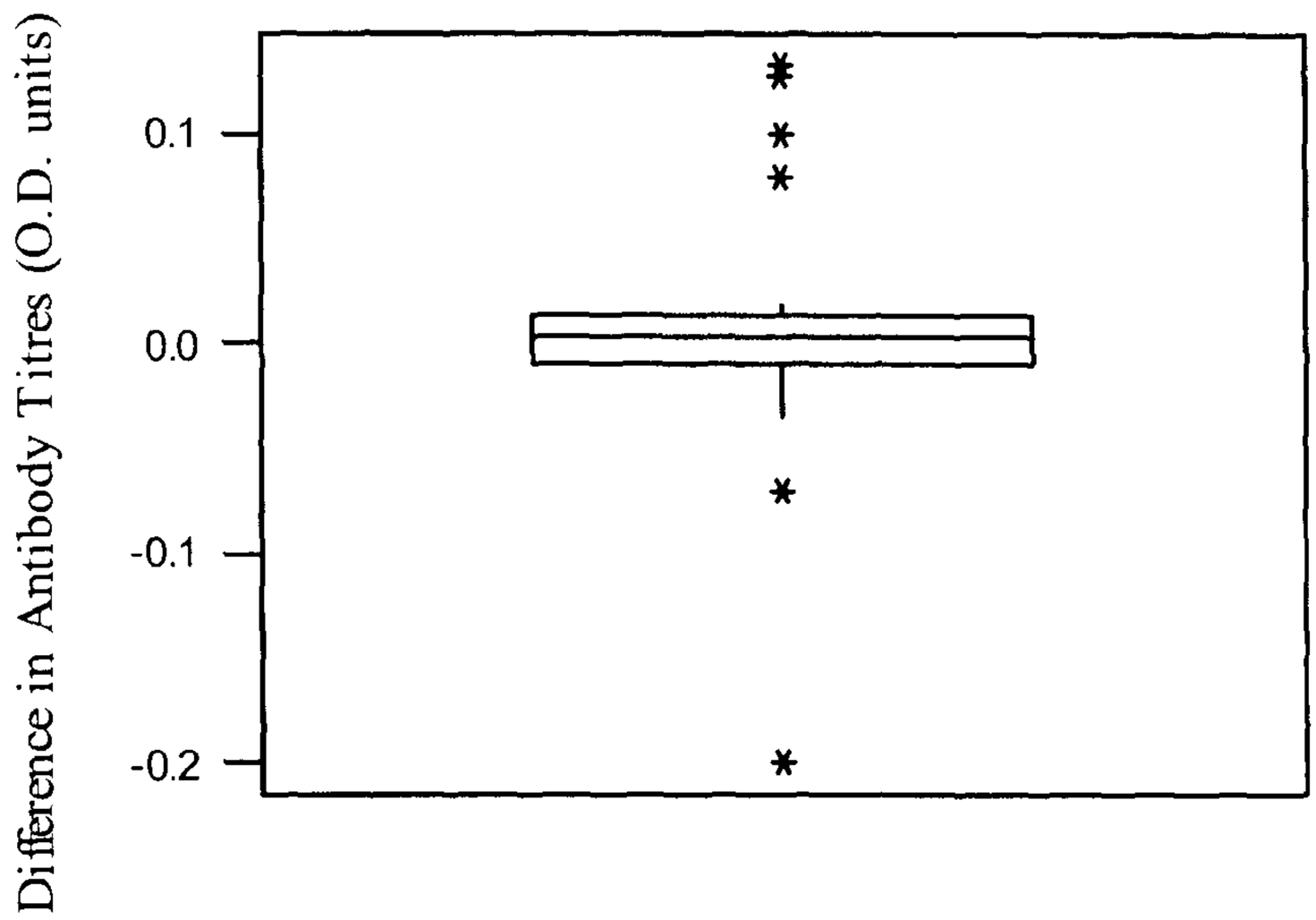


Figure 3.4 shows a box plot for the difference in antibody titres against LPS before and after treatment. It can be seen that 6 outliers appear to be slightly skewing the data. When all 6 of the subjects are removed from the analysis, there is still no significant difference between pre- and post treatment antibody titres. On removal of the 6 outliers the p-value for the difference in titres becomes larger. For a few of the micro-organisms and antigens, the boxplots showed a number of outliers, however, removal of them did not lead to a significant result, except for the incidents of *P. gingivalis* OMP and RgpA as discussed previously.

Patient 1 appeared to be an outlier on analysis of antibody titre change against a number of different micro-organisms and antigens. This patient always showed a decrease in antibody titre following treatment. The reason for this is not clear. It is noted, however, that patient (number) 1 had a pre-treatment pocket depth of 5.65mm and a final pocket depth of 5.45mm indicating that any decrease in pocket depth and improvement was insignificant. However, no conclusions can be made from this individual alone, and there was no significant correlation between change in pocket depth and change in antibody titre following treatment when all subjects were observed together (see table 3.3 below).

Table 3.2 Polyvalent immunoglobulin, IgG and IgA titres before and after treatment and comparisons of these for *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *B. forsythus* and *T. denticola*

	Mean BT	S.D.	Mean AT	S.D.	Mean Difference (BT-AT)	S.D.	95% CI of Difference (BT-AT)	p-value *
Pg								
Poly Ig	0.89	0.27	0.90	0.27	-0.01	0.09	(-0.04, 0.04)	0.87
IgG	1.03	0.35	1.05	0.36	0.02	0.08	(-0.05, 0.02)	0.35
IgA	0.34	0.29	0.35	0.28	-0.01	0.07	(-0.03, 0.02)	0.74
Aa								
Poly Ig	0.32	0.22	0.34	0.23	0.02	0.14	(-0.08, 0.04)	0.47
IgG	0.32	0.28	0.35	0.31	0.03	0.10	(-0.01, 0.07)	0.11
IgA	0.06	0.06	0.06	0.06	0.00	0.03	(-0.01, 0.01)	0.89
Pi								
Poly Ig	0.82	0.25	0.79	0.24	0.03	0.10	(-0.07, 0.01)	0.14
IgG	0.86	0.30	0.85	0.28	0.01	0.10	(-0.05, 0.03)	0.65
IgA	0.16	0.10	0.16	0.10	0.00	0.07	(-0.03, 0.03)	0.85
Bf								
Poly Ig	0.23	0.10	0.22	0.10	0.01	0.11	(-0.06, 0.04)	0.63
IgG	0.14	0.08	0.14	0.08	0.00	0.07	(-0.03, 0.02)	0.76
IgA	0.06	0.05	0.06	0.04	0.01	0.04	(-0.02, 0.01)	0.50
Td								
Poly Ig	0.28	0.12	0.25	0.13	0.03	0.10	(-0.01, 0.07)	0.16
IgG	0.20	0.09	0.17	0.08	0.03	0.09	(-0.07, 0.01)	0.15
IgA	0.07	0.05	0.06	0.03	0.01	0.04	(-0.03, 0.01)	0.31

* p-value for hypothesis test of no difference between before treatment and after treatment (paired t-test).

Figure 3.5 Plot of IgG pre- and post-treatment against *P. gingivalis*

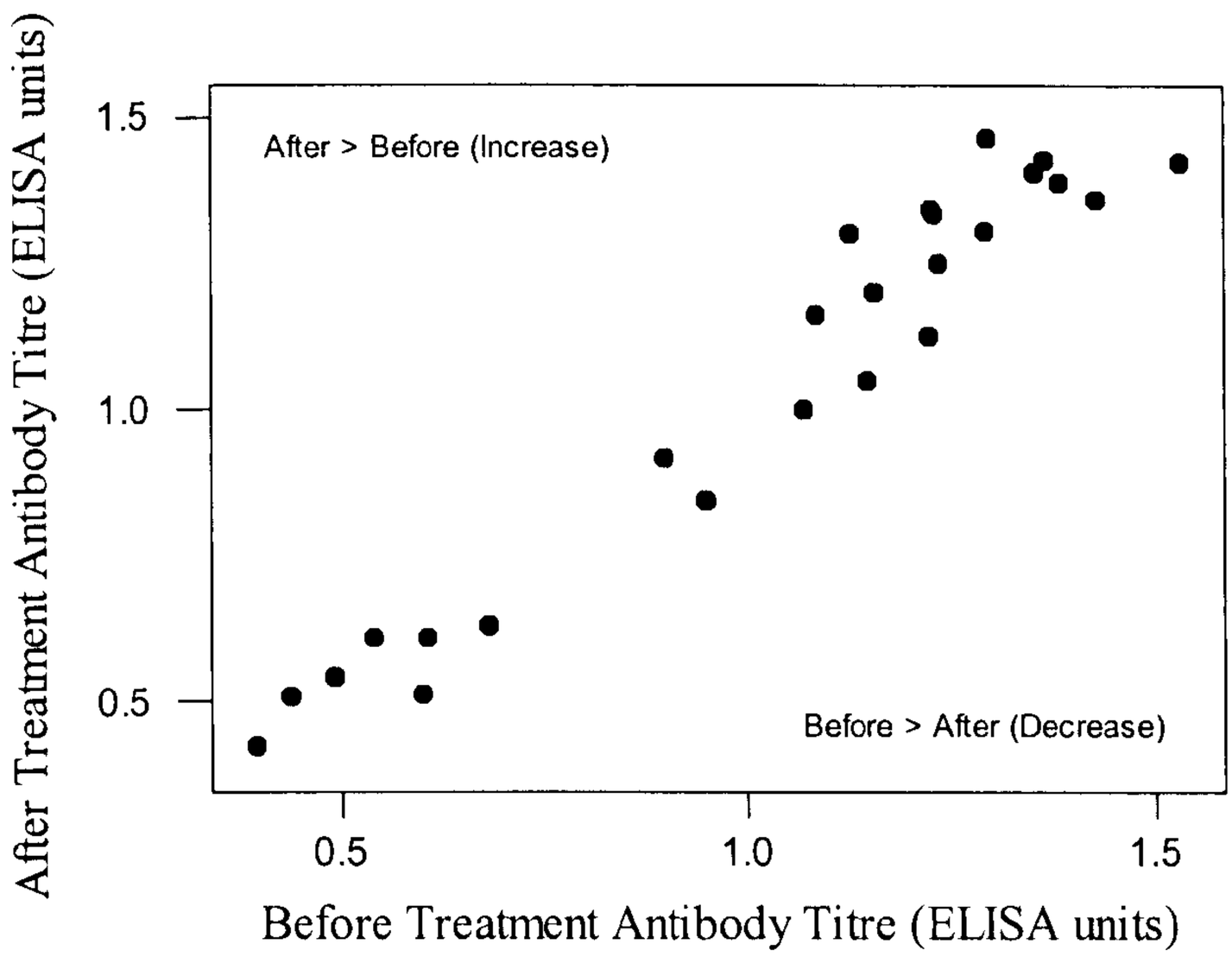


Figure 3.6 Plot of IgA titres pre- and post-treatment against *P. gingivalis*

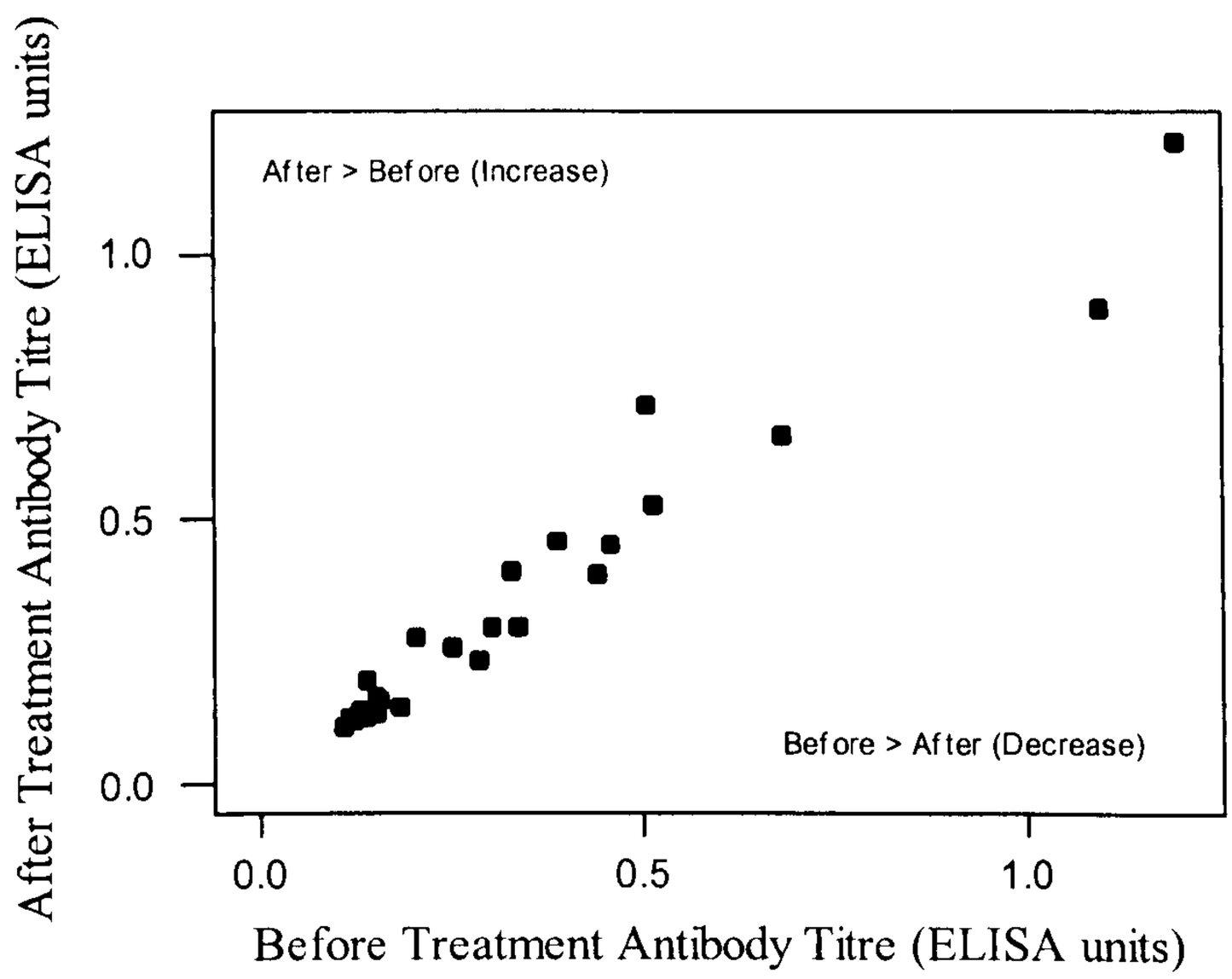


Table 3.2 shows the results for the titres of polyvalent immunoglobulin, IgG and IgA against *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *B. forsythus* and *T. denticola* pre- and post-treatment. The mean highest antibody titres were either polyvalent or of the isotype IgG, and the lowest IgA in most cases. No significant differences between any of the isotypes (polyvalent, IgG and IgA) were noted following treatment. Figure 3.5 shows the plot of the IgG titres pre-and post-treatment against *P. gingivalis*. Figure 3.6 shows the plot of the IgA titres pre- and post-treatment against *P. gingivalis*.

Table 3.3 The correlation between change in pocket depth and change in antibody titre following treatment

Correlation between change in pocket depth (mm) and change in antibody titre (O.D. units) against:	Correlation coefficient	95% CI for correlation coefficient	p-value*
Pg	-0.073	(-0.46, 0.33)	0.728
Aa	0.300	(-0.11, 0.62)	0.146
Pi	-0.099	(-0.48, 0.31)	0.639
Bf	**0.566**	**(0.22, 0.79)**	**0.003**
Td	-0.040	(-0.43, 0.36)	0.849
Pg OMP	0.063	(-0.34, 0.45)	0.766
Aa OMP	-0.059	(-0.44, 0.34)	0.780
Pi OMP	-0.122	(-0.49,-0.29)	0.562
Bf OMP	-0.197	(-0.55, 0.22)	0.346
Aa LTX	-0.204	(-0.55, 0.21)	0.328
Pg LPS	-0.105	(-0.48, 0.30)	0.616
Pg HSP60	0.133	(-0.28, 0.50)	0.527
Pg RgpA	0.245	(-0.17, 0.58)	0.239

*p-value for hypothesis test of no correlation/correlation coefficient equal to zero (Pearson product moment coefficient of correlation)

Figure 3.7 Plot of change in pocket depth against change in antibody titre for *B. forsythus*

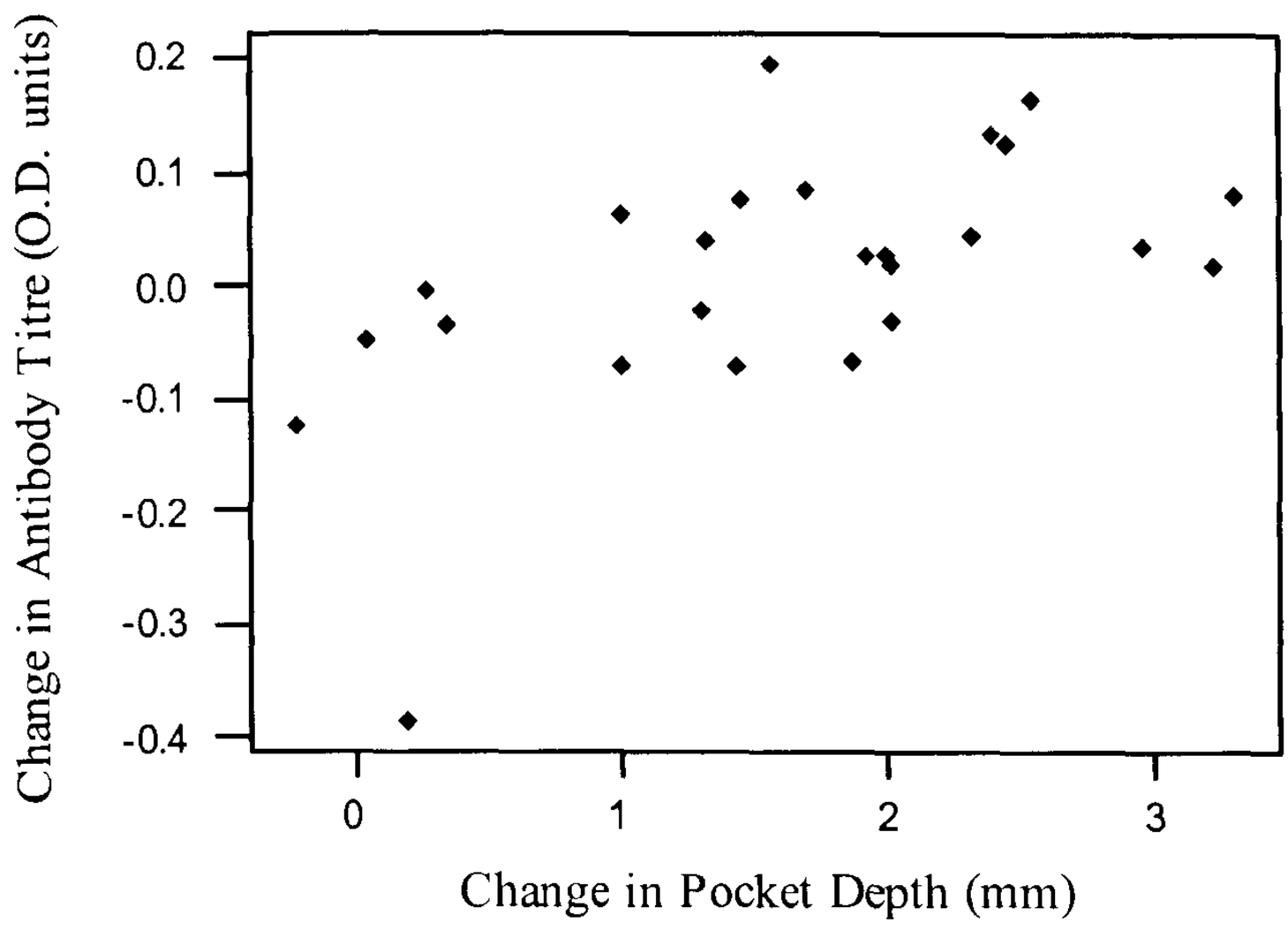


Figure 3.8 Plot of change in pocket depth against change in antibody titre for *P. gingivalis*

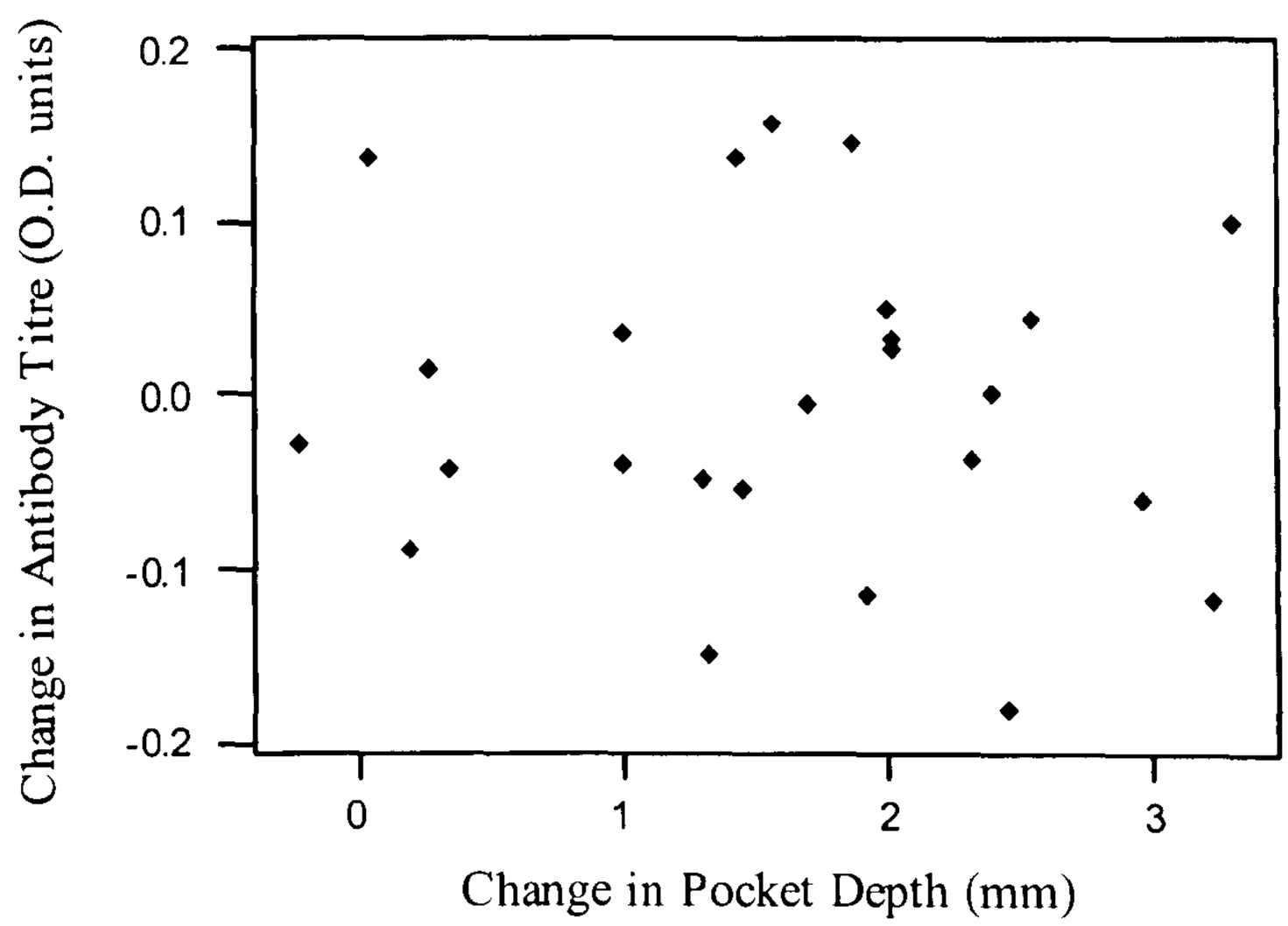


Table 3.3 shows the correlation between change in pocket depth and antibody titre against the micro-organisms and antigens tested following treatment. There is a significant correlation (non zero correlation) between change in pocket depth and antibody titre against *B. forsythus*, post-treatment. The correlation coefficient for this relationship is 0.566, implying that as the change in pocket depth increases, the change in antibody titre also increases. The graph suggests that the larger the decrease in pocket depth following treatment, the greater is the positive change in titre and hence the greater the increase in titre. Figure 3.7 shows that there is a correlation between change in antibody titre to *B. forsythus* and change in pocket depth. The graph is further evidence of a correlation. A positive correlation, although not significant can also be seen for change in pocket depth and change in titre against *A. actinomycetemcomitans*. Therefore, it is difficult to explain why there is a positively skewed correlation for *A. actinomycetemcomitans* and *B. forsythus* whole cells, but not for *A. actinomycetemcomitans* and *B. forsythus* OMP or *A. actinomycetemcomitans* leukotoxin.

One might remark that since 13 comparisons were made against pocket depth, i.e. one comparison for each antigen, some investigators might suggest the Bonferroni's approach (Glantz, 1981) to "correct" the individual p-values to ones more appropriate for the situation in which multiple, related tests are performed. Therefore, the only results that are significant in table 3.3 are those for which the uncorrected p-values are ≤ 0.015. As the only significant result of change in pocket depth against change in antibody titre to *B. forsythus* has a p-value of 0.003, the Bonferroni's approach has no effect in this case. Figure 3.8 shows the plot of change in pocket depth against change in antibody titre against *P. gingivalis*.

Table 3.4 To show the correlation between pre-treatment pocket depth and pre-treatment antibody titre

Correlation between pre-treatment pocket depth (mm) and pre-treatment antibody titre (O.D. units) against:	Pearson product Moment Correlation coefficient	95% CI for correlation coefficient	p-value*
Pg	0.465	(0.09, 0.73)	0.019
Aa	**0.573**	**(0.23, 0.79)**	**0.003**
Pi	0.149	(-0.26, 0.51)	0.477
Bf	-0.219	(-0.57, 0.19)	0.293
Td	-0.074	(-0.46, 0.33)	0.725
Pg OMP	0.333	(-0.07, 0.64)	0.104
Aa OMP	0.388	(-0.01, 0.68)	0.055
Pi OMP	0.054	(-0.35, 0.44)	0.799
Bf OMP	-0.026	(-0.42, 0.37)	0.900
Aa LTX	0.339	(-0.06, 0.65)	0.097
Pg LPS	0.332	(-0.07, 0.64)	0.104
Pg HSP60	0.057	(-0.35, 0.44)	0.786
Pg RgpA	-0.291	(-0.62, 0.12)	0.158

*p-value for hypothesis test of no correlation/correlation coefficient equal to zero (Pearson product moment coefficient of correlation)

Figure 3.9 Plot of pre-treatment pocket depth against pre-treatment antibody titre for *A. actinomycetemcomitans*

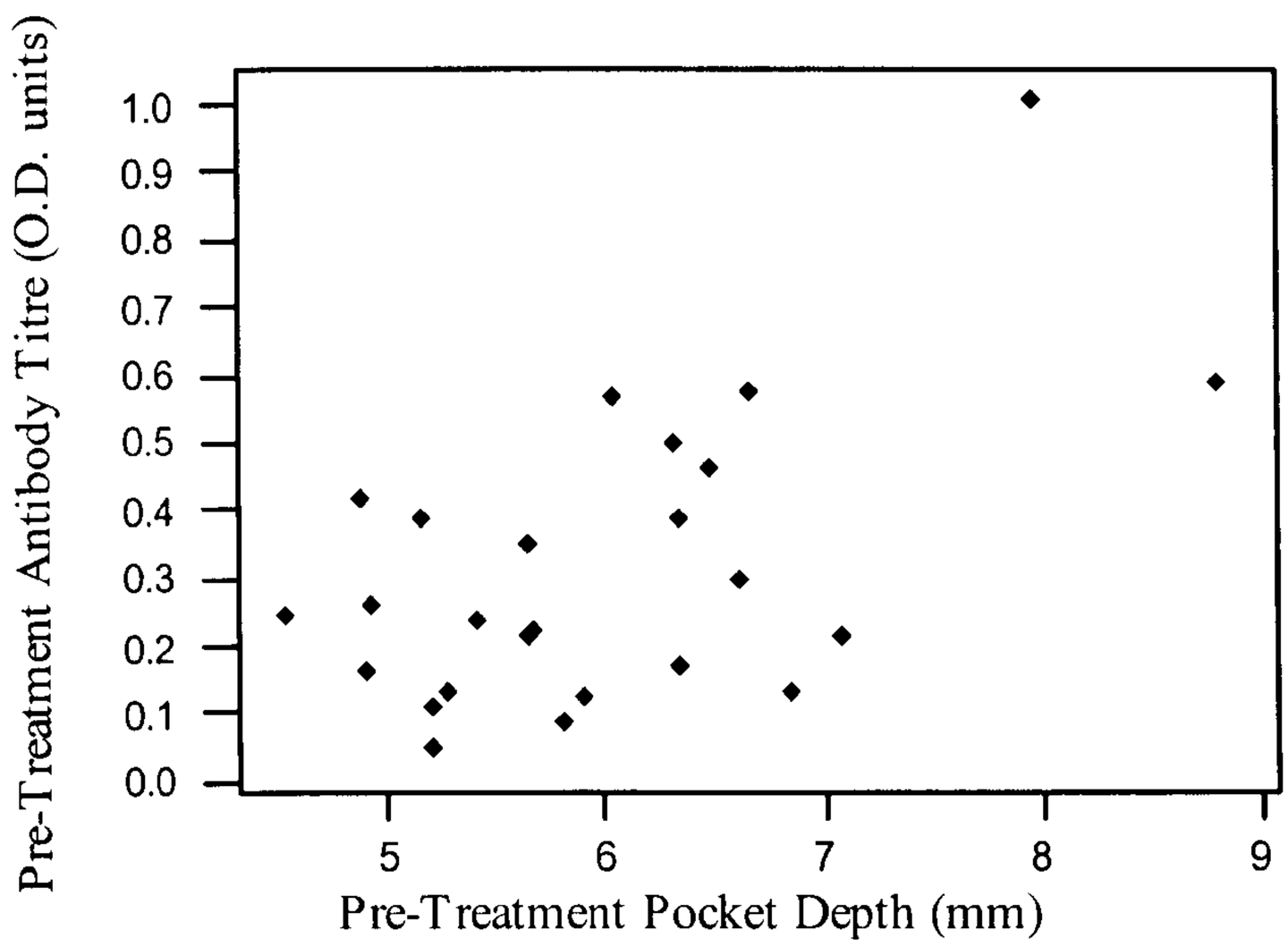


Table 3.4 shows the correlation between pre-treatment pocket depth and pre-treatment antibody titre. Statistical analysis has indicated there is significant correlation (non zero correlation) between pre-treatment pocket depth and pre-treatment antibody titres against *P. gingivalis*, *A. actinomycetemcomitans* and *A. actinomycetemcomitans* OMP preparation. The correlation coefficient for *P. gingivalis* is 0.465 and 0.573 for *A. actinomycetemcomitans*. Although, the p-values are significant (0.019 and 0.003 respectively), the correlation's are not strong. The correlation coefficient for pre-treatment pocket depth and pre-treatment antibody titre against *A. actinomycetemcomitans* OMP preparation is not significant. The correlation coefficient is 0.388 and the p-value is 0.055, however, the confidence interval is -0.01, 0.68. This indicates that there is a correlation, however, this is not significant. All 3 of the correlation's are positive, indicating that increased pre-treatment pocket depths can be correlated with increased pre-treatment antibody titres against *P. gingivalis*, *A. actinomycetemcomitans* and *A. actinomycetemcomitans* OMP preparation. Figure 3.9 shows the plot of pre-treatment pocket depth against pre-treatment antibody titre to *A. actinomycetemcomitans*. It can be seen from the graph that there are 2 outliers. When examined these patients are not young, i.e. there is no chance that they may be suffering from aggressive periodontitis instead of chronic periodontitis, and therefore, the reason for them being outliers is not clear.

Again some investigators may suggest that the Bonferroni's approach to "correct" the individual p-values would be appropriate in this situation. If it is used, then the p-value is ≤ 0.015 which means that only the correlation between pre-treatment pocket depth and antibody titre against *A. actinomycetemcomitans* is significant.

Table 3.5 To show the correlation between length of treatment and change in antibody titre following treatment

Correlation between length of treatment and Change in antibody titre following treatment against:	Correlation coefficient	95% CI for correlation coefficient	p-value*
Pg	-0.102	(-0.48, 0.31)	0.629
Aa	0.008	(-0.39, 0.40)	0.969
Pi	0.241	(-0.17, 0.58)	0.245
Bf	-0.201	(-0.55, 0.21)	0.335
Td	-0.032	(-0.42, 0.37)	0.879
Pg OMP	0.280	(-0.13, 0.61)	0.175
Aa OMP	0.218	(-0.19, 0.56)	0.296
Pi OMP	-0.005	(-0.40, 0.39)	0.980
Bf OMP	0.174	(-0.24, 0.53)	0.404
Aa LTX	0.092	(-0.31, 0.47)	0.663
Pg LPS	0.003	(-0.39, 0.40)	0.988
Pg HSP60	**-0.522**	**(-0.76, -0.16)**	**0.007**
Pg RgpA	-0.101	(-0.48, 0.31)	0.632

*p-value for hypothesis test of no correlation/correlation coefficient equal to zero (Pearson product moment coefficient of correlation).

Figure 3.10 Plot of length of treatment against change in antibody titre for heat shock protein 60 (of *P. gingivalis*)

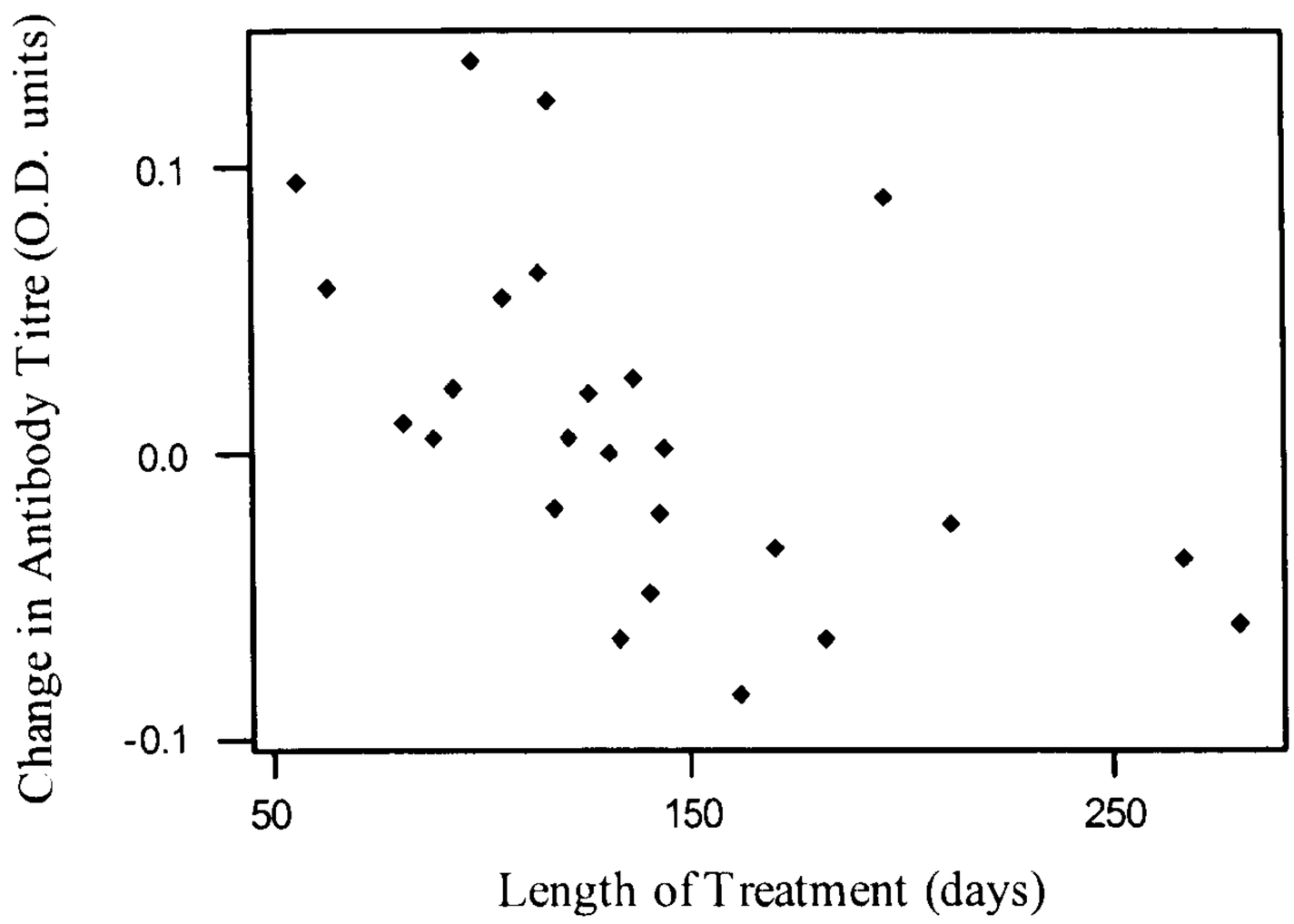
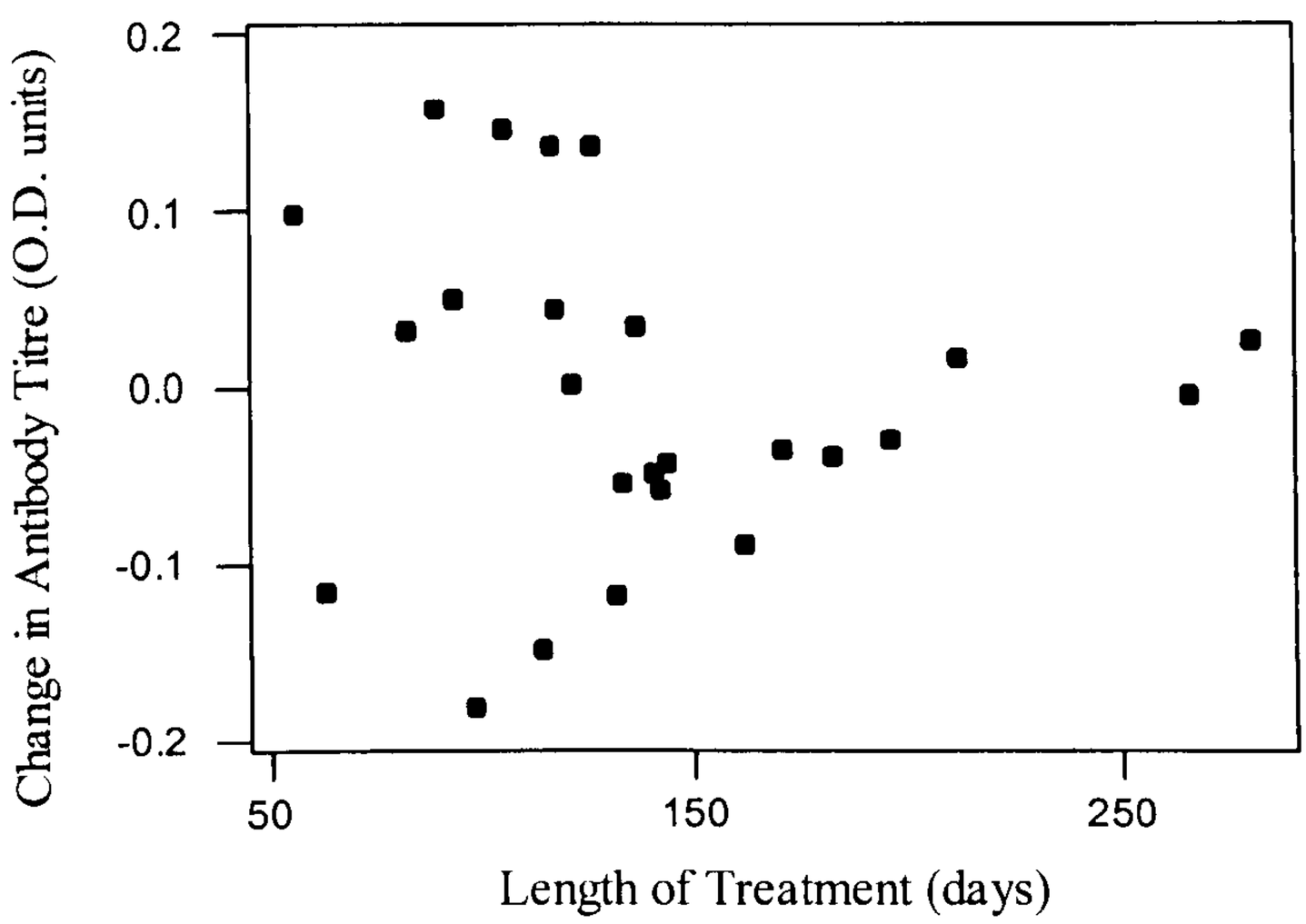


Figure 3.11 Plot of length of treatment against change in antibody titre for *P. gingivalis*



The correlation between change in antibody titre following treatment against various micro-organisms and antigens and the length of treatment undergone by the subjects is shown in table 3.5. There is a significant correlation recorded (non zero correlation) for the change in antibody titre following treatment against HSP60 (of *P. gingivalis*) and the length of treatment experienced by the subjects. The correlation coefficient is -0.522 implying that as the length of treatment increases, the change in antibody titre decreases. This relationship can be seen more clearly in figure 3.10. This figure shows the plot of length of treatment against the change in antibody titre to HSP60 (of *P. gingivalis*). It can be seen from the graph that there is a trend towards a negative correlation and that if the treatment is shorter than approximately 125 days, the change in antibody titre is positive (above zero) i.e. an increase in antibody titre following treatment. A period of treatment of above 125 days or more seems to lead to a change in antibody titre that is negative (below zero) i.e. a decrease in antibody titre following treatment. This correlation is still significant following application of the Bonferroni's approach. Figure 3.11 shows the plot of length of treatment against change in antibody titre to *P. gingivalis*.

3.4.1.1 Relationship between treatment and antibody titres

An analysis of the 25 patients before and after treatment can be seen in tables 3.1 - 3.6. The analyses looked at the effect of treatment on polyvalent immunoglobulin titres against a large panel of bacteria and antigens, and 3 different isotypes of immunoglobulin; polyvalent immunoglobulin, IgG and IgA against 5 whole putative periodontopathogen preparations. The analyses suggests that the treatment did not have an effect on antibody titres against the micro-organisms and antigens tested in this study and that there was no significant difference between antibody titres before and after treatment.

The data in table 3.1 shows the highest titres of polyvalent immunoglobulin were specific for *P. gingivalis* and *P. intermedia* whole fixed bacteria, and the antibody titres against purified antigens such as LPS, HSP60 and the β segment of RgpA were significantly lower.

Levels of 3 different isotypes of immunoglobulin were investigated before and after treatment for 5 whole fixed putative periodontopathogens. Analysis showed no significant difference between pre- and post-treatment titres for any of the isotypes against any of the micro-organisms. The IgA titres before and after treatment were extremely low when compared with the titres of polyvalent immunoglobulin and IgG. The levels of polyvalent immunoglobulin and IgG were very similar, and in some cases the IgG levels were slightly, but not significantly, higher than the levels of polyvalent immunoglobulin.

Although the results are not presented, analysis did show that the change in antibody titre against most of the micro-organisms and antigens tested correlated significantly with their corresponding pre-treatment antibody titre. The correlations were all positive, indicating that an increased pre-treatment titre against a particular antigen can be correlated with an increased change in antibody titre against that antigen following treatment.

3.4.1.2 Relationship of pocket depths and antibody titres

An analysis was performed to assess whether there was a correlation between change in pocket depth and change in antibody titre following treatment (table 3.1) and between pre-treatment pocket depth and pre-treatment antibody titre against the various micro-organisms and antigens (table 3.1). No strong correlations were found. Although the results are not shown, analysis was carried out to investigate a correlation between pre-treatment pocket depths and change in antibody titre against all of the micro-organisms and antigens. No significant correlations were found.

3.4.1.3 Other Parameters

Changes in antibody titre against the micro-organisms and antigens tested were analysed according to smoking status. The results indicate that there is no difference in the change in antibody titres for smokers and non-smokers.

The changes in antibody titre against the micro-organisms and antigens tested when analysed according to gender were also examined. The results indicated no significant difference in change of antibody titres following treatment between males and females.

Change in antibody titre following treatment, for any of the micro-organisms or antigens tested, was examined against the age of the subjects at the beginning of the study. Analyses were carried out to assess whether there were any correlations between age, change in pocket depth and length of treatment. No correlations were found to be significant.

3.4.2 Results of Part 2 of the study: Adsorptions

Table 3.6 The median percentage reduction in antibody titre following adsorption compared to the sham adsorbed sera

	Median Percentage Reduction in Antibody Titre **Against Pg**	Range
Sera Adsorbed against Pg	65.33	50.00 - 82.40
Sera Adsorbed against Aa	72.42	40.88 - 86.83
Sera Adsorbed against Bf	61.35	34.03 - 84.73
Sera Adsorbed against Pi	75.00	29.56 - 87.56

	Against Aa	
Sera Adsorbed against Pg	73.72	25.12 - 86.75
Sera Adsorbed against Aa	75.19	58.75 - 89.96
Sera Adsorbed against Bf	62.53	26.05 - 89.94
Sera Adsorbed against Pi	74.66	21.86 - 85.20

	Against Bf	
Sera Adsorbed against Pg	47.79	2.05 - 73.27
Sera Adsorbed against Aa	52.13	34.00 - 73.43
Sera Adsorbed against Bf	50.85	26.44 - 70.79
Sera Adsorbed against Pi	48.92	13.85 - 72.61

	Against Pi	
Sera Adsorbed against Pg	60.91	18.85 - 90.92
Sera Adsorbed against Aa	67.16	25.08 - 91.01
Sera Adsorbed against Bf	63.47	19.62 - 85.10
Sera Adsorbed against Pi	79.83	35.00 - 89.95

Figure 3.12 Plot of the median percentage reduction in antibody titre against *P. gingivalis* following adsorption against *P. gingivalis*, *A. actinomycetemcomitans*, *B. forsythus* and *P. intermedia* when compared to the titre observed against the sham adsorbed sera

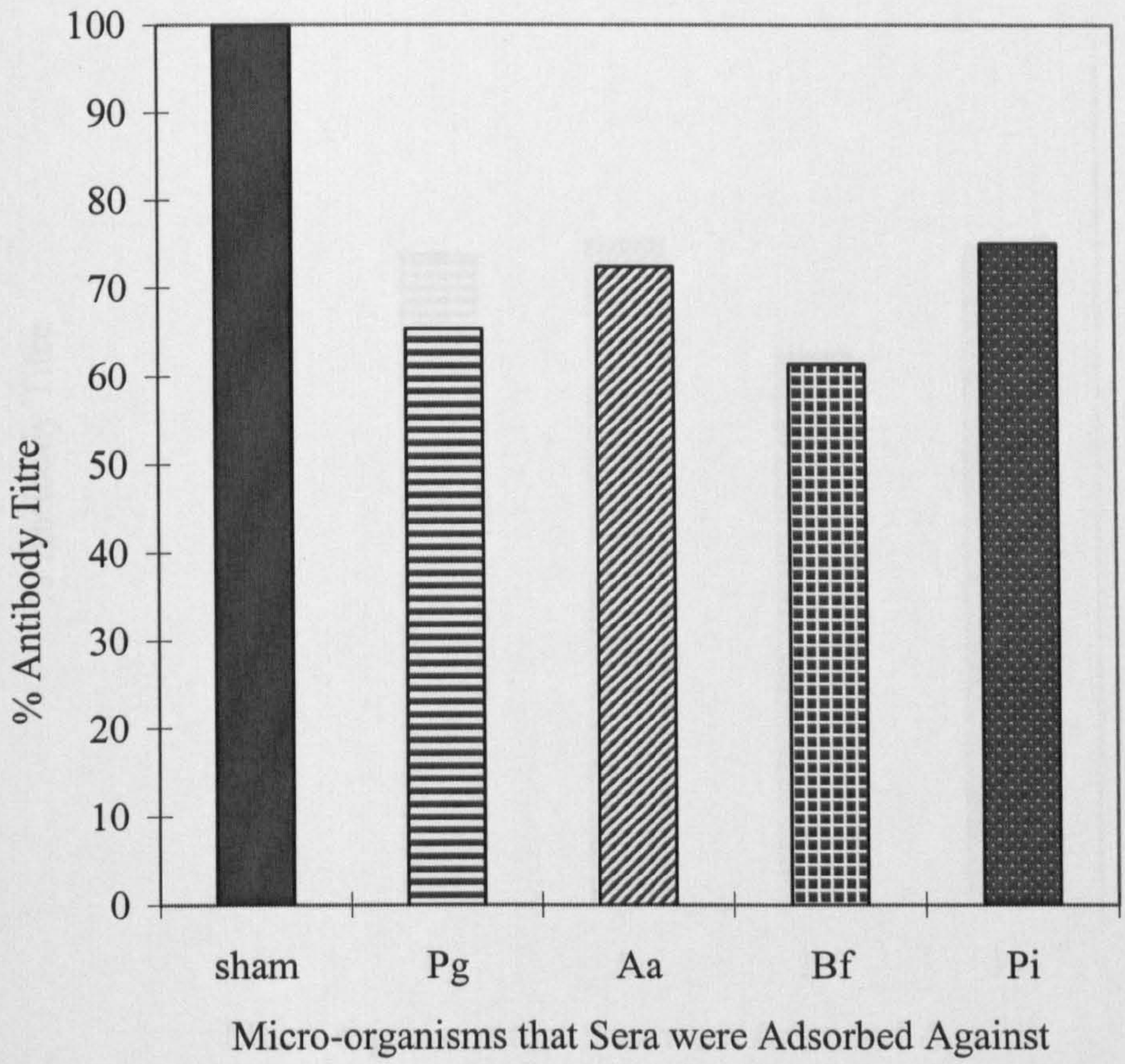


Figure 3.13 Plot of the median percentage reduction in antibody titre against *A. actinomycetemcomitans* following adsorption against *P. gingivalis*, *A. actinomycetemcomitans*, *B. forsythus* and *P. intermedia* when compared to the titre observed against the sham adsorbed sera

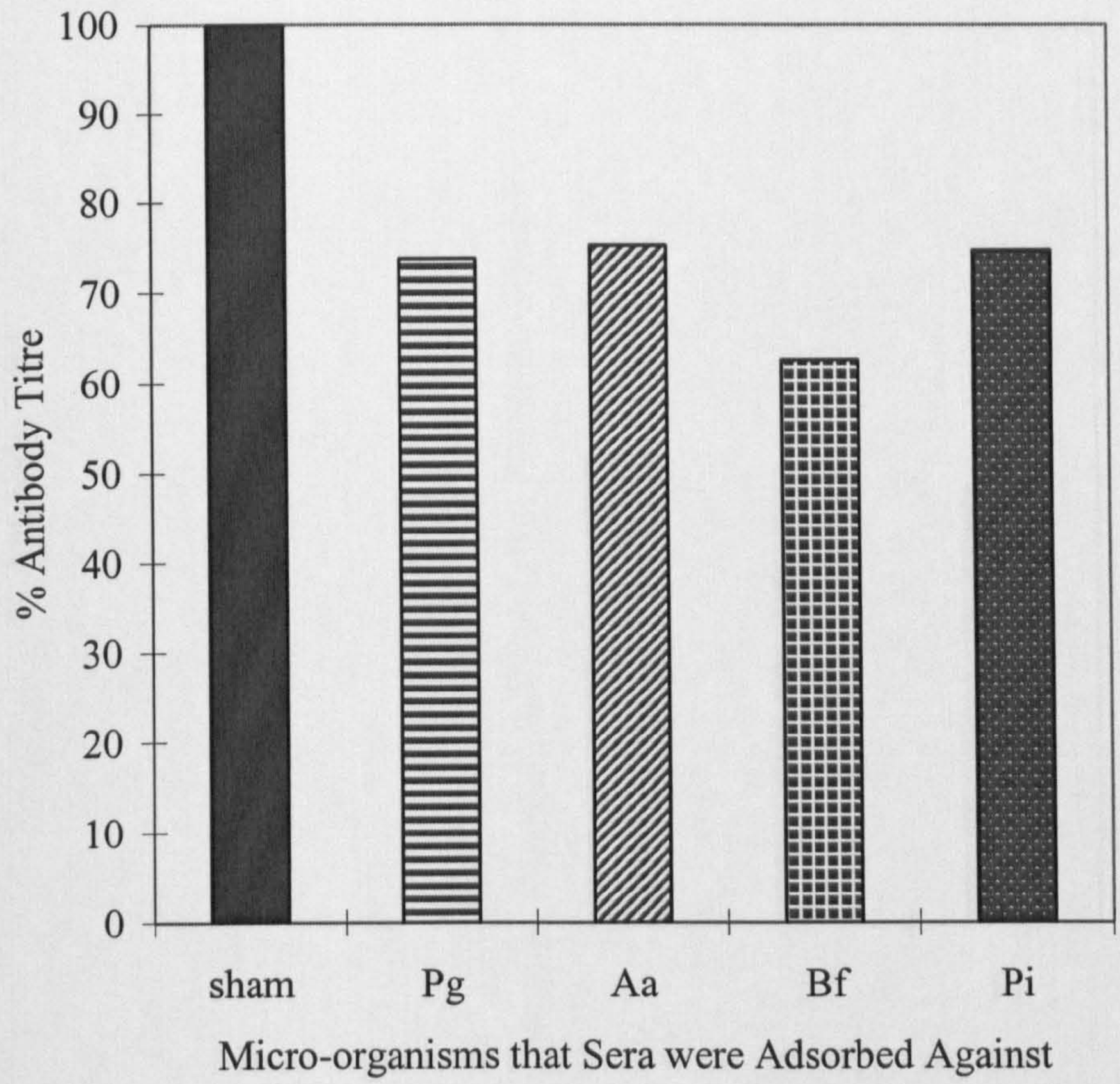


Figure 3.14 Plot of the median percentage reduction in antibody titre against *B. forsythus* following adsorption against *P. gingivalis*, *A. actinomycetemcomitans*, *B. forsythus* and *P. intermedia* when compared to the titre observed against the sham adsorbed sera

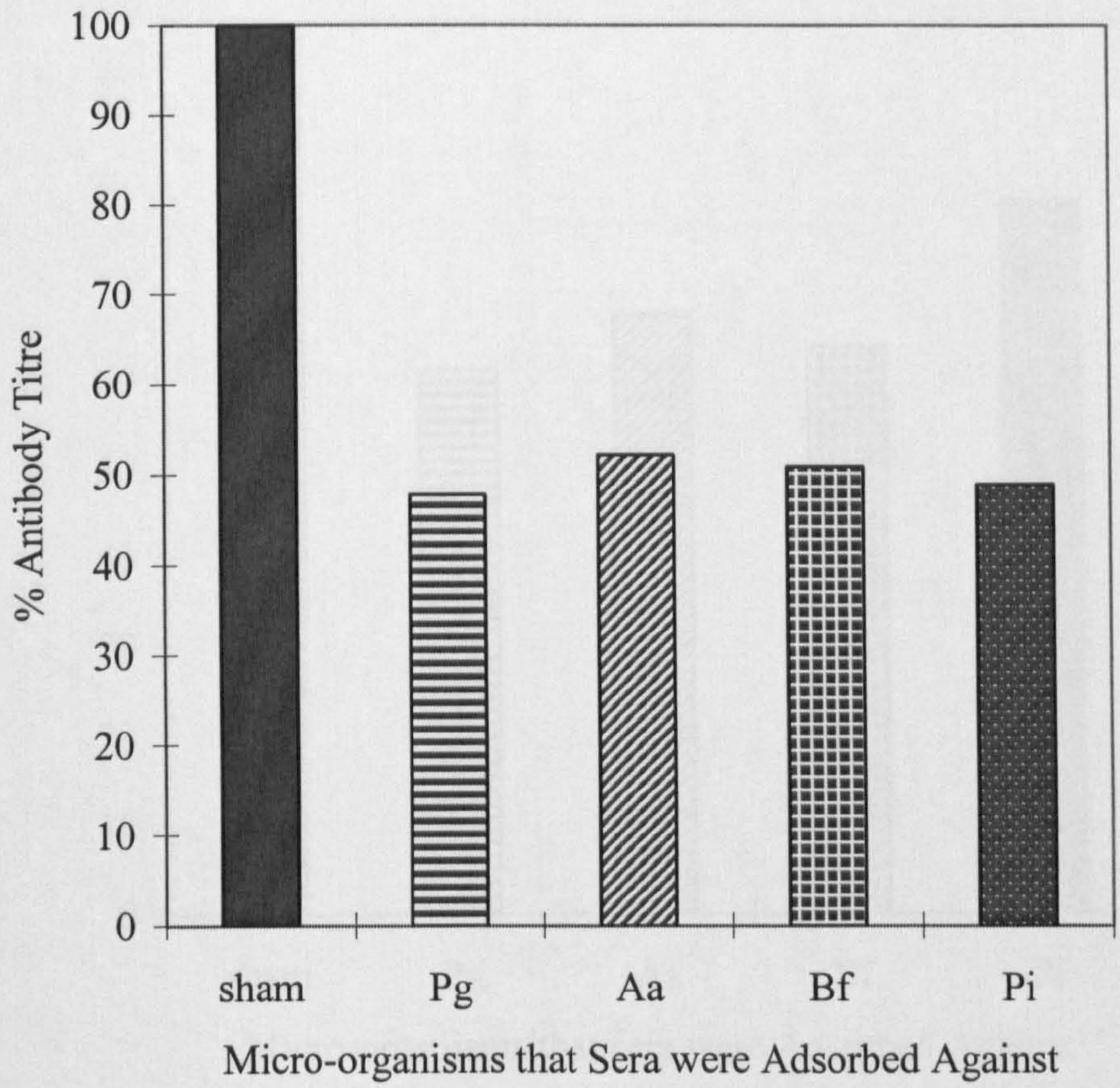


Figure 3.15 Plot of the median percentage reduction in antibody titre against *P. intermedia* following adsorption against *P. gingivalis*, *A. actinomycetemcomitans*, *B. forsythus* and *P. intermedia* when compared to the titre observed against the sham adsorbed sera

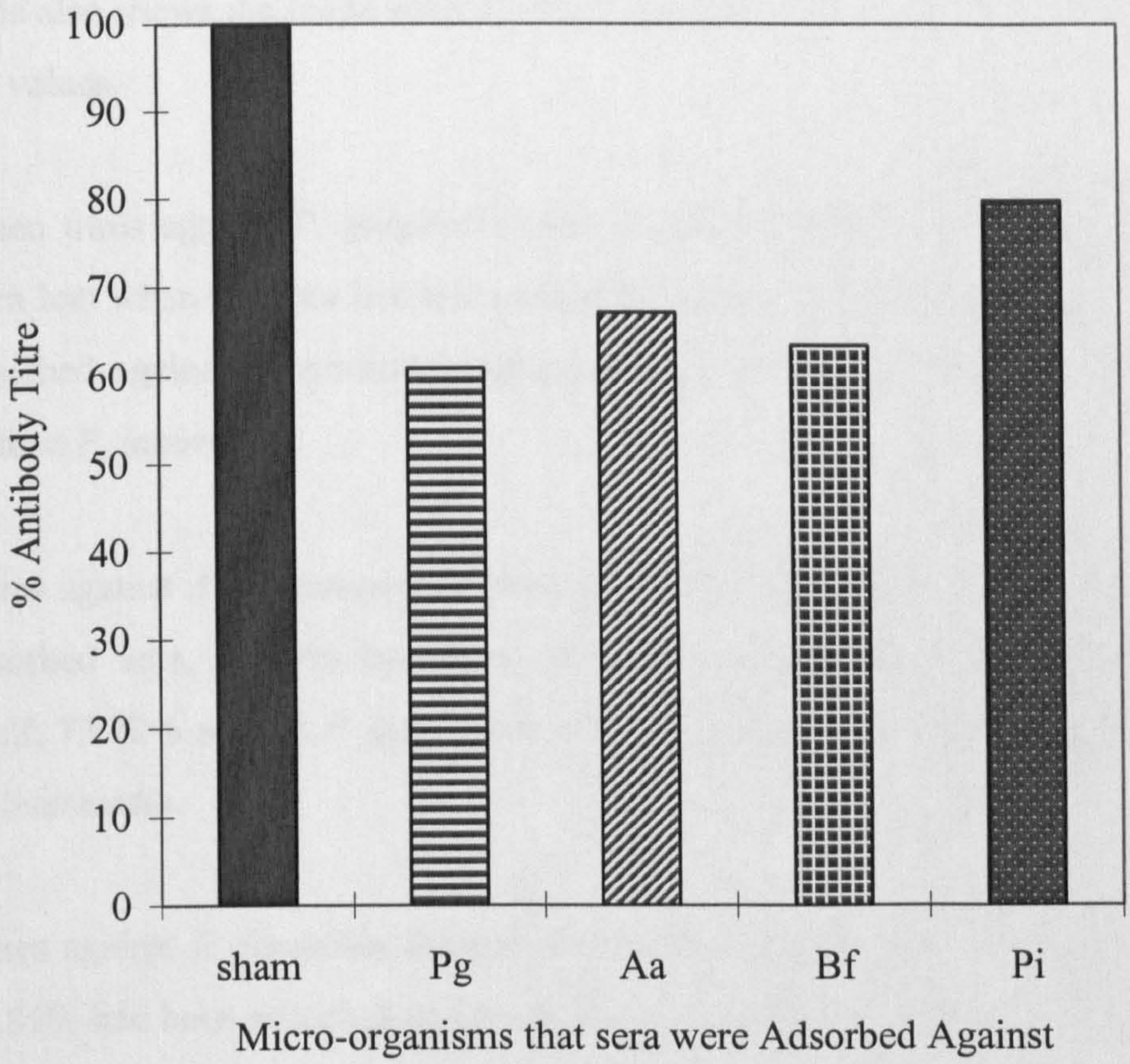


Table 3.6 shows the median percentage reduction in antibody titre against *P. gingivalis*, *A. actinomycetemcomitans*, *B. forsythus* and *P. intermedia* following adsorption. The median reduction is given as a percentage of the serum antibody titre for the sham adsorbed sera. The sham adsorbed sera gives a value of the antibody titre following the process of adsorption. No antibodies should have been lost due to specific binding, however, some may have been lost due to non-specific binding to the plastic wells of the plate. Sham adsorbed sera was designated to be 100%, and the median percentage reduction values were given as a percentage of this value. The table also shows the range values, which are wide, showing a lot of variation between the values.

When titres against *P. gingivalis* were measured, 65.33% of the antibody titre had been lost when the sera had been adsorbed against *P. gingivalis* itself, 72.42% when adsorbed against *A. actinomycetemcomitans*, 62.35% against *B. forsythus* and 75% against *P. intermedia*.

Titres against *A. actinomycetemcomitans* showed that when compared with the sham adsorbed sera, 75.19% had been adsorbed out against *A. actinomycetemcomitans* itself, 73.72% against *P. gingivalis*, 62.53% against *B. forsythus* and 74.66% against *P. intermedia*.

Titres against *B. forsythus* showed that when compared with the sham adsorbed sera 50.85% had been adsorbed against *B. forsythus*, 47.79% against *P. gingivalis*, 52.13% against *A. actinomycetemcomitans* and 48.92% against *P. intermedia*.

When titres were measured against *P. intermedia*, 79.83% was adsorbed out against *P. intermedia* itself, 60.91% was adsorbed against *P. gingivalis*, 67.16% against *A. actinomycetemcomitans* and 63.47% against *B. forsythus*.

3.5 Discussion

It is generally accepted that periodontal treatments, including scaling and root planing, are highly effective in the treatment of periodontal disease (Badersten *et al.*, 1981,

Lindhe *et al.*, 1982, Morrison *et al.*, 1980). Numerous reports have attributed the success of scaling and plaque control with qualitative and quantitative changes in the microflora associated with periodontal disease (Genco *et al.*, 1969, Socransky, 1970, Socransky *et al.*, 1982). Further to this, numerous reports have also considered the effect of treatment on immunological parameters, many of which are discussed below.

The first part of this study set out to examine the effects of periodontal treatment on the titres of serum antibodies specific for a range of bacteria, and antigen preparations. Titres were measured pre-and post-treatment in 25 patients with chronic periodontitis. The results presented here suggest that periodontal treatment does not have an effect on serum antibody titres against *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *B. forsythus*, *T. denticola*, the OMP of *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus* and the purified antigens; HSP60 (of *P. gingivalis*), LPS (of *P. gingivalis*), leukotoxin (of *A. actinomycetemcomitans*) and the β segment of RgpA protease (of *P. gingivalis*), within the time period measured in this study.

A study carried out by Murray *et al.* (1989) examined the humoral immune response against *P. gingivalis* in treated and untreated patients with chronic periodontitis. The results of this study suggested that serum IgG levels were significantly higher in subjects with chronic periodontal disease that had not received treatment, compared with subjects that had received treatment. Murray *et al.*, explained their results as supporting the notion that removal of antigen by subgingival root planing, i.e. those receiving treatment, results in a decline in serum antibody levels. This suggests that treatment has an effect on serum antibody titres against periodontal micro-organisms. Murray *et al.* (1989), therefore, appear to agree with numerous studies that have been reported in the literature, suggesting that periodontal treatment leads to a decrease in antibody titre. The problem with the study by Murray *et al.* (1989), is that by having 2 subject groups, one treated and one untreated it is difficult to know or say that the treated group had a lower antibody titre due to treatment, and that they did not have a lower antibody titre before they received any treatment. From the knowledge that every individual has their own distinctive immune repertoire, it is clear that the humoral immune responsiveness of patients will vary in any study. Therefore, it is

difficult to conclude anything from the study described above. For this reason in the present study, only one group of patients were examined, and the antibody titres for the one group of subjects were taken before and after treatment, to be sure that any effects seen, were due to treatment alone.

Another parameter that needs to be taken into account when considering the extensive literature on this theme is the length of treatment. The period of time that occurs between the first and second samples being taken varies enormously between studies. The average length of treatment, i.e. the period of time between the 2 samples being taken in the current study was approximately 5 months, with a range of 2-10 months. In terms of other studies on this topic this is quite a short period of time.

Aukhil *et al.* (1988) carried out a longitudinal study on 24 subjects with periodontal disease. The study was carried out to investigate the effect of treatment on serum antibody titres against 10 plaque micro-organisms, including *P. gingivalis*, *P. intermedia* and *T. denticola*. The antibody titres to the micro-organisms were measured at 5 different time points; pre-treatment, post hygienic phase, post-surgical phase, maintenance phase - 1 year, maintenance phase - 2 years. The results of this study, in general indicated that periodontal treatment leads to a decrease in antibody titre. However, many of the subjects were reported to be resistant to change in antibody titres. The time between treatment and occurrence of significant changes in antibody titres exceeded one year.

Mouton *et al.* (1987) also carried out a longitudinal study to investigate the effects of treatment on serum antibody titres against *P. gingivalis* in patients with chronic periodontitis. In this study samples were taken at various time intervals between 13 to 33 weeks post-treatment, and then at one year post-treatment. The overall conclusion was again that there was a decrease in antibody titres following treatment. Although as above, the year time point showed a very significant decrease in titre, a significant decrease in titre was seen approximately 5 months after treatment. The decrease was shown to be progressive. At 5 months after treatment, the results showed the serum antibody titres were reduced to 55% of the pre-treatment value and at 1 year post-treatment, the levels were reduced to 41% of the pre-treatment levels. It must be

noted, however, that even at a reduction to 41% of the pre-treatment levels, these titres were still 6 times higher than those of periodontally healthy subjects tested.

Tolo *et al.* (1982) also made a report on the antibody titres pre- and post periodontal treatment. This study investigated the effect of treatment on IgG, IgA and IgM levels in the serum against 7 periodontopathogens. Except for *P. gingivalis*, no significant changes in titres were recorded against any of the pathogens, when the serum titres were measured 1 year post-treatment. Successful treatment was said to be correlated with a significant decrease in titre against *P. gingivalis*.

Sandholm and Tolo (1986), looked at the effect of treatment on serum antibody levels in 12 aggressive periodontitis patients. The length of the treatment, i.e. the time between the first and second sample was 16-32 months. As already discussed, it is possible, that the length of time following treatment, (that is the time variation in taking the second sample) may be crucial with regards to measuring the effects of treatment on antibody titres. Therefore, with such a small number of subjects, 16-32 months is a very large time scale on which to be comparing parameters.

The results of the study reported by Sandholm and Tolo (1986), indicated that minimal alterations were observed in the levels of antibodies to *P. gingivalis*, *Capnocytophaga ochracea* and *Eubacterium saburreum*, however, a decrease in antibody titre against *A. actinomycetemcomitans* was recorded after treatment. This decrease was only seen in 3 of the 12 patients, admittedly a quarter of the subject population, but conclusions cannot really be made on the results from 3 subjects. Further to this, this study reports that 75% of the patients had low or not detectable levels of IgG antibodies to *P. gingivalis* and *E. saburreum*. If most of the patients had undetectable IgG levels, it is difficult to properly interpret this study, particularly as IgG is the most prominent immunoglobulin isotype in the serum, and IgA and IgM levels would always be expected to be extremely low in the serum of an individual with a chronic infection.

Horibe *et al.* (1995) compared the serum antibody titres against 7 periodontopathogens before and after treatment. The post-treatment sample was taken

6.9 months on average after the first sample. This study reported that titres against *P. gingivalis* and *P. intermedia* were significantly reduced following treatment, whilst the titre to the remaining 5 bacteria, showed no significant treatment-related change. It is very difficult to compare longitudinal studies of this kind. Many differences that cannot be controlled exist between studies, including patients compliance with the protocol, the timing of the study and length of treatment, as previously discussed. Further to this, variations in bacteria and antigen preparation could have an effect on the results seen. For example, examination of formalin-stable epitope recognition may provide very different results from those of "native" preparations.

In part 1 of the presently reported study antibody titres pre- and post-treatment were examined against a large panel of targets. The initial studies examined the antibody titres against 5 formalin fixed putative periodontal pathogens; *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *B. forsythus* and *T. denticola*. The polyvalent immunoglobulin, IgG and IgA titres were measured for these. None of the titres of immunoglobulin isotypes changed after treatment. For *P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia* there was no significant difference in the polyvalent immunoglobulin titres and the titres of IgG. For *B. forsythus* and *T. denticola* the IgG titres were slightly lower than the polyvalent immunoglobulin titres. The titres of IgA against all of the micro-organisms were very low, just above the background measurement. The low levels of IgA in the serum is not surprising given the mucosal nature and "local" arm of the IgA response. The titre of IgA was so low that no confident conclusions could be drawn, however, the analyses that were carried out indicated no change in titre post-treatment.

As the levels of IgA were so low, a large proportion of the antibodies measured against the antigenic targets were obviously of the IgG isotype. However, as mentioned above, the titres of IgG for most of the micro-organisms were not significantly different from the polyvalent immunoglobulin titres, or on a number of occasions were slightly lower. Therefore, polyvalent immunoglobulin titres were measured in this study, to ensure that any changes, which may be significant were caught.

The overall results suggest that there was no significant change in antibody titre after periodontal treatment, and therefore, do not agree with much of the literature discussed above. In many of the studies a change in titre was only found to be against 1 or 2 of the micro-organisms tested and the antibodies to the rest of the targets tested remained the same post-treatment. Although the overall results are of "no change", a few results obtained from this study were interesting and merit discussion.

Table 3.3 shows the correlation between change in pocket depth and change in antibody titre. There was a positive correlation when change in pocket depth was correlated with change in antibody titre against *B. forsythus* whole cells. The correlation co-efficient was 0.566 (p=0.003) indicating that the correlation is significant. The change in pocket depth following treatment is always negative i.e. a decrease in pocket depth, if the treatment is successful. Therefore, these results suggest that a relationship may exist between the success of treatment and the change in antibody titre against *B. forsythus*.

A number of studies have previously suggested that successful periodontal treatment, either hygiene phase therapy or surgery, are accompanied by a decrease in the frequency of microbiological detection of levels of *B. forsythus* (Haffajee *et al.*, 1995; Haffajee *et al.*, 1997; Socransky and Haffajee, 1993). In terms of previous studies regarding the effects of periodontal treatment on the immunological parameters of individuals with disease, there are very few reports. Studies of the humoral immune response to Gram negative organisms have tended to concentrate on *P. gingivalis* and *A. actinomycetemcomitans*. For this reason it is very difficult to hypothesise about a role for *B. forsythus* in chronic periodontal disease. However, the results of this study suggest that perhaps further studies into the effects of treatment on the humoral immune response against *B. forsythus*, may be of interest.

An interesting result was suggested from the analysis of the correlation between length of treatment and change in antibody titre to HSP60 (of *P. gingivalis*). The correlation appears to be significant and negative, indicating that the shorter the length of treatment the greater the change in antibody titre. As discussed in the results, it

seems that a treatment period of less than 125 days, leads to a positive change in antibody titre i.e. an increase in antibody titre following treatment, but a treatment period of 125 days or more leads to a decrease in titre. These results are interesting in terms of the inoculation theory suggested by Ebersole *et al.* (1985d). The results of their study indicated that scaling could lead to a significant increase in circulating antibodies to various periodontal pathogens and that the peak levels were seen 2 to 4 months following treatment. Therefore, the negative correlation seen between length of treatment and antibodies to HSP60 is possibly in agreement with the results of Ebersole *et al.* (1985d). This result suggests that perhaps we should be concentrating our attention on the time period of before 125 days after treatment, when an increase in antibody titre may indicate which pathogens are of importance.

Further studies regarding the length of treatment would be of interest. A longitudinal study observing the antibody titre of individuals every 2-4 weeks following treatment, up to 6 months would provide us with more knowledge of the effect of treatment on the immunological responses. Patient compliance may be difficult to achieve for such a study.

A large number of predominantly Gram negative bacterial species, possibly 5 to 7 or more (Schenk, 1985), have been implicated in the aetiology of periodontal disease. The rationale behind the second part of this study comes from the fact that many reports have suggested that there are shared antigens amongst the suspected pathogens. Hence it is of interest to investigate how much of the immune response in this disease is directed against these antigens. Studies that report an antibody titre measured against an individual bacterium often do not consider that some of these antibodies could in fact have been induced against a different bacterium and are cross-reacting.

A study by Vasel *et al.* (1996) investigated the cross-reactivity between antibodies against *P. gingivalis* and *B. forsythus*. The approach of their work was different to the study presented here, however, the results obtained suggest that cross-reactivity does exist. The study by Vasel *et al.*, investigated this theory using pre- and post-immune

serum taken from monkeys following immunisation with *P. gingivalis*, and demonstrated a clear cross-reactivity between *P. gingivalis* and *B. forsythus*.

A study by Kinane *et al.* (1993) has also shown cross-reactivity. Antibody titres against *A. actinomycetemcomitans* following adsorption against *A. actinomycetemcomitans* and *H. aphrophilus* were measured and the percentage mean reduction in the titre compared with the sham adsorbed sera. Sera adsorbed against *A. actinomycetemcomitans* showed a 68% reduction in titres against *A. actinomycetemcomitans* itself and sera adsorbed against *H. aphrophilus* showed a similar reduction of 63%. Therefore, most of the reactivity to *A. actinomycetemcomitans* could be removed by adsorption with both organisms, suggesting cross-reactivity between antibodies to these 2 different species.

This study suggests that there is cross-reactivity between *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus*. The results indicate that in general the same percentage of antibodies were adsorbed out against each of the different micro-organisms and this tended to be around 60-80% of the antibodies. The percentage of antibodies adsorbed out against *B. forsythus* seemed to be slightly lower than for the other 3 micro-organisms, around 40-50%. The range values for all of the results showed a large variation, however, making it difficult to note any real differences. The large variation seen in the values could have been due to the different antibody titres in the different patients, thus this fact makes it more difficult to compare the results as a group.

It was noted that for serum that had been adsorbed against *P. gingivalis* there was a higher percentage reduction in antibody titre against *P. intermedia* and *A. actinomycetemcomitans* than *P. gingivalis* itself. This seems impossible, as there cannot be a higher percentage of antibodies cross-reactive with *P. gingivalis*, found in the patient, than against *P. gingivalis* itself. Likewise, the same situation was seen when antibodies were adsorbed against *B. forsythus*. A higher percentage reduction in titre was seen against *A. actinomycetemcomitans* than *B. forsythus* itself. The reason for this is not clear, however, as previously mentioned the range of the values is very wide, and hence there is probably no significant difference between values. The

results of this study indicate that the immune response against all 4 of the periodontal pathogens tested is very similar and there appears to be a significant amount of cross-reactivity occurring. There appears to be approximately the same percentage of cross-reactivity between all combinations of the micro-organisms, except for possibly *B. forsythus* which appears to have a slightly lower percentage of cross-reacting antibodies against it.

The data was analysed to see if there were any correlations between the percentage reduction in antibody titre against the 4 periodontal pathogens and pocket depth of the patients at the beginning of the study, smoking status and age of the patients. No correlations were found. In addition, no groupings were noted between either antigens or in patients.

The apparent involvement of so many microbial agents in periodontal disease makes identification of the important ones so difficult. In addition, the situation is made more complex by the variations that exist between the immune repertoires of every individual. The significance of risk factors and the effects that they have on an individuals immune response is also unclear.

Chapter 4. Immortalised B Cells From Periodontal Disease Patients.

4.1 Introduction

The discovery, development and exploitation of human monoclonal antibodies has had a tremendous impact on medical research and now contributes greatly to diagnostics and therapeutics. Human monoclonal antibody production has permitted scientists to make huge advances in many areas including: the search for specific and therapeutic antibodies against human tumours; the production of well tolerated serum replacements such as anti-rhesus factor D, tetanus, hepatitis immunoglobulin and other antibodies against infectious diseases; our knowledge of the B cell repertoire in health and disease giving us significant insights into autoimmunity (Gigliotti *et al.*, 1984; Van Meel *et al.*, 1985; Stricker *et al.*, 1985; Atlaw *et al.*, 1985; Bron *et al.*, 1984).

The first description of monoclonal antibody secreting cell lines were reported by Kohler and Milstein (1975). From this first description, rodent monoclonal experiments progressed rapidly and have been applied to a wide variety of biological problems of importance. However, whilst the initial studies to produce human antibody secreting B cell lines was heralded with much enthusiasm, (Croce *et al.*, 1980; Olsson and Kaplan, 1980; Steinitz *et al.*, 1977) the available methods carry many technical problems that remain unsolved. Unfortunately, the generation of stable human B cell lines which secrete specific monoclonal antibodies is not yet as routine as the generation of murine monoclonal antibodies.

One aim, as mentioned above, for the production of monoclonal antibodies is to increase our knowledge of the B cell repertoire and its targets. The discriminating power of polyvalent serum antibody has limitations. An antigen usually has many epitopes leading to the production of anti-sera, which is made up of a mixture of antibodies all with varying specificity's for all the epitopes. Furthermore, immunisation with an antigen leads to the expansion of various populations of

antibody-forming lymphocytes. To observe and investigate these cells is difficult. These cells can only be maintained in culture for a short period of time, so it is impossible to grow normal cells and obtain clones that produce antibodies of a single specificity (Benjamini *et al.*, 1996). Kohler and Milstein (1975) saw an answer to this problem and it involved the use of a population of malignant plasma cells. Malignant cells are 'immortal' and can thus remain in culture for years. They took a malignant plasma cell population that was deficient in the enzyme hypoxanthine phosphoribosyl transferase (HPRT). This cell population would die unless they were introduced to HPRT. They then fused these cells with antibody producing cells that were able to produce HPRT. The nuclei of the two cell types fused, giving the hybridoma cells the characteristics of both. Hence, these hybrids were able to manufacture immunoglobulins as well as survive in culture. The hybrids were grown in a specific cell culture media, that malignant cells would not survive in without the HPRT enzyme. Thus allowing the separation of the hybrids from dead malignant cells. When utilising this method for production, screening is then carried out to identify hybrids synthesising a specific antibody, which are then cloned.

Cell hybridisation methods are mainly classified into three different categories: (i) hemagglutinating virus of Japan (HVJ); (ii) Polyethylene glycol (PEG) fusion; and (iii) electrofusion.

HVJ can agglutinate cells that have HVJ receptors on their cell membrane and cause efficient cell fusion. HVJ is not, however, really used for preparing hybridomas because of the possibility that the virus genome may influence hybrid cells. It is also a very complex procedure. The method was originally reported by Okada *et al*, (1957) and showed that animal cells could efficiently be fused using HVJ.

Polyethylene glycol (PEG) is a widely used method because of its simplicity. The action of PEG is complex, but in brief, it causes localisation of membrane proteins, allowing direct interaction of lipid bilayers of both cells, making cell fusion easier (Shirahata *et al.*, 1998).

The electrofusion methodology was developed by Zimmerman (1982). Due to electronic pulses the cell membrane structures are disturbed allowing fusion. The cells have to be brought into close proximity of each other to be successful, therefore, to establish close cell-cell adherence, dielectrophoresis of the cells is performed using sign waves. This causes the cells to align according to the waves and adhere to each other. Cell fusion is then triggered by the application of electronic pulses. Electrofusion has an advantage over the popular PEG method in that a much smaller number of cells are required, often a limiting factor when working with human cells, however, electrofusion apparatus is expensive and complex (Shirahata *et al.*, 1998).

As mentioned previously, many problems arise in the production of human monoclonal antibodies. One of the limitations experienced is the choice of lymphoid tissue for fusion. Animal work tends to use murine tissue and thus the experiments are not limited by the availability of immune lymphocytes. In murine work, if antigen substances are available in excess, are non-toxic and sufficiently immunogenic, immunisation strategies can be optimised to ensure sufficient immune cells are obtained for fusion (James and Bell, 1987). Obviously, when dealing with human subjects, the amount and characteristics of the immunogen that can be injected are much more stringently monitored and there is much more difficulty with safety and ethical issues.

Another difficulty encountered in human work is deciding what the correct time period is to harvest the cells following immunisation. This is important as it can influence their state of differentiation and proliferation (James and Bell, 1987), however, this is difficult to study due to practical reasons. Studies have indicated that the optimum time for harvesting peripheral blood cells is 6-7 days (Bogard *et al.*, 1985) and 3 days post-boosting for murine spleen cells (Schwaber *et al.*, 1984). It must be noted however, that monoclonal antibodies have been produced using lymphocytes harvested 1-3 months post boosting (Boyd *et al.*, 1984; Tiebout *et al.*, 1984; Tiebout *et al.*, 1985; Thompson *et al.*, 1986).

As mentioned previously, human hybridoma production is difficult due to the low numbers of specific B cells available for fusion, particularly when working with

peripheral blood B lymphocytes. In un-immunised patients, the B cells are known to be in low frequency and often also in a resting state which is incompatible with successful fusion. It is known that most fusion partners fuse efficiently only with proliferating B lymphocytes at an undefined stage of differentiation (Johnson and Rao, 1970; Andersson and Melchers, 1978). Therefore, in an attempt to increase the efficiency of fusion a number of methods have been developed. In vitro immunisation has been attempted (Borrebaeck, 1989; Borrebaeck *et al.*, 1988), but the rarity of antigen-specific B cells is still a problem, and is not feasible in the case of molecules which are poor immunogens.

4.1.2 Epstein-Barr transformation

Human lymphocytes can be immortalised by fusion with a myeloma cell line as already discussed. They can also be immortalised by a second completely different method called viral transformation. The virus used in this technique is Epstein-Barr virus (EBV) and it is usually obtained from the supernatant of the marmoset cell line B95-8 in culture (Millar and Lipman, 1973). EBV is a herpes virus and the receptor for EBV on human lymphocytes is the CR2 complement receptor (C3d) (Frade *et al.*, 1985). Binding of EBV to the CR2 receptor on human B lymphocytes can lead to insertion on the EBV genome thus causing transformation of the human cells. EBV is a potent polyclonal activator for human B cells, it is thought that EBV mediated activation also induces proliferation and clonal expansion of autonomous cell lines and differentiation into immunoglobulin secreting cells (Rosen *et al.*, 1977; Campling *et al.*, 1987; Gordon and Graeme, 1988). EBV transformed cells, therefore, can provide a continual source of immortalised cells for studying B cell-related phenomena.

EBV can bind to and penetrate all B cells, however, not all B cells are transformed. Also it has been shown that activation of B cells can be achieved using killed EBV or by addition of antibody directed against the CR2 receptor, thus activation and transformation are obviously two different events (James and Bell, 1987). There has been some confusion regarding the population of cells that are susceptible to transformation. Aman *et al.* (1984) suggested that the population of cells that are

susceptible to transformation are a small high density resting population that give rise to IgG and IgA secreting populations. Opposing this view Chan *et al.* (1986) have suggested that the population of cells susceptible to transformation is an activated large cell population that are destined to secrete IgM. Which one of these theories is correct is unknown, perhaps both are. Many studies suggest a bias towards IgM secreting cells, agreeing with the theory of Chan *et al.* (1986), however IgG and IgA is still found to be secreted by some cell lines. It is thought that only 0.1-5% of peripheral blood lymphocytes infected with EBV are activated to secrete immunoglobulin, and this tends to be predominantly of the IgM isotype. Furthermore, often cells transformed with EBV grow poorly and are low rate immunoglobulin secretors (Niedbala and Stott, 1998).

Experimental work carried out by Kozbor *et al.* (1982), combined the techniques of cell transformation with fusion to obtain hybridomas secreting antibodies against tetanus toxoid. It is often suggested that fusion of peripheral blood lymphocytes is unsuccessful because they are not in an actively dividing state. When the cells are not actively dividing the nuclei of the peripheral blood lymphocytes do not fuse with the nuclei of the myeloma cells (Burnett *et al.*, 1985). However, following EBV treatment, even if transformation does not occur, the cells become activated increasing their chance of successful fusion. The antibodies secreted following fusion are thought to be representative of the antibodies being produced by the transformed cell lines at the time of fusion.

4.1.3 The CD40 system

The CD40 system allows expansion of the B cell population obtained, and further isolation of individual proliferating, differentiating B cell clones (Banchereau *et al.*, 1991; Banchereau and Rousset, 1991). Stimulation of resting B cells in this system is an alternative to the EBV method to expand the B cell population before fusion.

CD40 is an integral membrane protein found on the surface of B lymphocytes, dendritic cells, follicular dendritic cells, hematopoetic progenitor cells, epithelial cells and carcinomas (Banchereau *et al.*, 1994). The ligand of CD40 (CD40L) is expressed

on activated T cells, mostly $CD4^+$ but also some $CD8^+$ as well as basophils and mast cells. Cross-linking of CD40 with anti-CD40 or CD40L induces strong proliferation of B cells and addition of IL-4 or IL-13 allows the generation of factor-dependent long-term normal human B cell lines and the secretion of antibody following isotype switching (Banchereau *et al.*, 1994).

Banchereau and colleagues (1991) reported that IL-4 and monoclonal antibodies to human CD40, when presented in a cross-linked fashion by Ltk^- cells (transfected mouse fibroblasts expressing FcγRII/CDw32) (Peltz *et al.*, 1988), permit establishment of factor-dependent long-term human B cell lines. Therefore, these factors can exert a direct proliferative effect on B cells when presented appropriately. The initial studies on this technique reported that within 5 weeks, the initial B cell population had expanded 150-400 times, and that 20-30% of the resting B cells stimulated by this procedure can generate B cell clones of 50 to a few hundred cells after 15 days of culture.

The advantages of using the CD40 system before fusion is that the system can enhance the number of actively dividing and differentiating B lymphocytes and induce class switching giving higher fusion efficiency. CD40 is thought to be advantageous over EBV transformation in that EBV cultures frequently grow poorly, producing unstable cell lines that secrete low levels of IgM antibodies. The CD40 system is thought to give higher fusion efficiency and induce class switching to IgG (Niebala and Stott, 1998). This system does however, suffer from the disadvantage that the rescued cells are the product of polyclonal activation and are therefore, unrepresentative of the activated B cell population *in vivo*, however, this is also the case for EBV transformed B cells.

As previously discussed in the general introduction of this thesis, there is little doubt that gingivitis and periodontitis are caused by plaque micro-organisms. The predominance of the B cells and plasma cells in periodontal lesions, and the often increased antibody titres seen in the serum directed against putative periodontal pathogens, underlines the importance of the humoral immune response in the pathogenesis of these diseases. However, little is known about the specificity of the

antibodies raised against the antigens, derived from the bacteria responsible for disease. The aim of the present study was to investigate in detail the target of the antibody response in patients with periodontal disease. In order to do this, the task was to produce human monoclonal antibodies from immortalised B cells isolated from the granulation tissue of diseased sites and the peripheral blood of patients with current periodontal disease. These antibodies were then screened using the ELISA technique against a series of oral pathogens. A number of techniques were employed to achieve this. Firstly, human peripheral blood B cells were expanded in the CD40 system and then fused with a heteromyeloma cell line by the method of PEG fusion. Secondly, B cells isolated from granulation tissue, taken from diseased sites in periodontitis patients were fused directly using PEG.

4.2 Methods

4.2.1 Subjects

The subjects used in this study were patients attending Glasgow University Dental Hospital. The criteria for inclusion in the study was that they were suffering from advanced chronic periodontitis. Twenty one millilitres of venous blood were collected from the anterior cubital region using butterfly needles and 3 x 7ml blue-capped citrate treated Vacutainer tubes.

The inclusion criteria for the subjects from which granulation tissue samples were taken, was that they were receiving surgery as part of their periodontal therapy. Granulation tissue was removed during surgery, which was carried out by an experienced periodontist, and immediately placed into sterile RPMI medium. The sample was stored at 4°C until use.

4.2.2 Cell culture

Culture of L-cells.

L cells were cultured in a 75cm^2 flask at 37°C, 5% CO_2 in complete medium. The medium was changed every 4 days.

Culture of CRL 1668 cells.

Cultures were maintained by the addition or replacement of fresh CRL 1668 culture medium every 2 to 3 days. The cells were cultured in a $75cm^2$ flask at 37°C, 5% CO_2.

4.2.3 The CD40 system

Irradiation of the L cells was carried out to arrest cell division. The cells were incubated at 37°C for 3-4 hours with 10μg/ml Mitomycin-C. The flask was then treated with 10ml of versene to remove adherent cells, and centrifugation was then carried out at 200 x g for 10 minutes. The cells were then washed 3 times in RPMI 1640.

The cells were then enumerated in white cell counting fluid using a Neubauer haemocytometer and resuspended at $5x10^4$cells/ml of complete medium. 3.5ml of cells suspension per well ($1.75x10^5$cells/well) was then placed into a 6 well plate, and the plate was incubated overnight at 37°C, 5% CO_2 to allow cell adherence to the bottom of the wells.

4.2.4 Lymphocyte preparation

The blood was collected from subjects using 3 x 7ml citrate treated vacutainers following treatment. 10-15ml of blood was then layered on top of 10ml of Histopaque-1077, in a sterile falcon tube, and centrifuged at 400 x g for 30 minutes. Following centrifugation half of the plasma layer was removed, aliquoted and stored at -80°C for future testing and the other half was discarded.

The mononuclear cell layer was then transferred using a Pasteur pipette into a clean sterile flacon tube and PBS was added to increase the volume to 50ml. Further centrifugation was then carried out at 200 x g for 20 minutes. The supernatant was then discarded and the pellet was again resuspended in 50ml of PBS, and again centrifugation was carried out at 200 x g for 10 minutes. Following this the supernatant was again discarded before the pellet was washed twice under the same

centrifugation conditions in RPMI 1640. The pellet was then resuspended in 1ml of RPMI and a cell count carried out in nigrosin in 7% acetic acid.

4.2.5 Preparation of 2-aminoethylisothiouronium bromide (AET) -treated sheep red blood cells (SRBC)

Sheep red blood cells (SRBC) were washed 5 times in sterile saline. This constituted centrifugation at 200 x g, for 10 minutes and resuspension of the pellet in 20ml of sterile saline 5 times. 0.143M AET solution was prepared and filtered, and then 1 volume of washed packed SRBC was mixed in a sterile falcon tube with 4 volumes of AET solution. This mixture was then incubated at 37°C for 15 minutes; however, mixing at every 5 minute interval was carried out.

The cells were then centrifuged at 200 x g for 10 minutes and washed 5 times in ice-cold saline, and once in RPMI 1640. The cells were then made up to a 10% solution in RPMI containing 10% FCS. This suspension can be stored for up to 5 days.

4.2.6 T cell depletion

Following cell counting the lymphocytes were prepared at $2x10^6$cells/ml of RPMI medium. The AET treated SRBC were prepared at a concentration of 0.5%. Equal volumes were then mixed together and incubated for 15 minutes at 37°C, with mixing at every 5 minute interval. The suspension was then centrifuged at 200 x g for 10 minutes and then incubated on ice for 30 minutes. The pellet was then resuspended by gentle shaking in the same supernatant. The suspension was then layered on to 15ml of Histopaque-1077 and centrifuged at 300 x g for 30 minutes. The interface of B cells was then removed and washed twice in RPMI.

The cells were counted in nigrosin in 7% acetic acid.

4.2.7 Expansion of the B cell numbers by the Banchereau method

B cells were added to the adhered L cells at a ratio of 10:1, therefore, $1.75x10^6$ B cells were added into each of the wells of the 6 well plate. As the volume of medium in each of the wells was required to be kept to a minimum and due to further additions 1ml of medium was removed from each well before the addition of the B cells. Since the majority of the L cells should have become adherent to the surface of the well, they were in little danger of being discarded.

Anti-human CD40 monoclonal antibody was then added at a concentration of 20ng/ml and human IL-4 at a concentration of 100u/ml. The 6 well plates were then incubated at 37°C, 5% CO2 for 5 days, before the cells were fed using complete medium.

After 11 days the cells were removed, centrifuged at 200 x g for 10 minutes and counted using nigrosin in 7% acetic acid.

4.2.8 Enzymatic dissociation of granulation tissue

Surgically removed granulation tissue was placed in a sterile 25ml universal. The tissue was then cut into 1-2mm^3 segments using sterile scissors. The tissue was then washed twice with RPMI 1640 medium, by centrifugation at 200 x g for 10 minutes. Cells were then dissociated from the tissue by use of Dispase.

Eight millilitres of Jockliks MEM containing dispase at 3mg/ml (pH 7.4) was added to the universal containing the tissue. The universal was then incubated at 37°C for 90 minutes with continuous stirring. The medium containing the cells was separated from the tissue by filtration using 41μm nylon net filters and mixed with excess RMPI 1640 medium.

The cells were harvested by centrifugation at 200 x g for 10 minutes before being resuspended in Iscoves medium and stored on ice. The cells were then placed on a mini gradient containing 1ml of Histopaque-1077. Centrifugation was then carried out at 400 x g for 20 minutes, and the interface of cells was collected, washed and

resuspended in Iscoves medium. The cells were then enumerated in white cell counting fluid using a Neubauer haemocytometer. T cell depletion was then carried out using SRBC as previously described for the peripheral blood lymphocytes.

4.2.9 PEG fusion

Twenty four hours prior to fusion the macrophage feeder layer required for cell survival was prepared. Macrophages were obtained by peritoneal lavage of a BALB/C mouse. The cells were counted using white cell counting fluid in RPMI 1640 medium (without phenol red) containing 10% FCS, penicillin, streptomycin and L-Glutamine at $4x10^5$cells/ml. The cells were then dispensed into 96 well flat bottomed plates at 50μl/well, ignoring the outside wells of the plate. These plates were then incubated overnight at 37°C, 5% CO_2.

The following day the fusion was carried out. The fusion partner cells, CRL-1668 cells were transferred to a 50ml centrifuge tube. The lymphocytes were also transferred into a 50ml centrifuge tube, and both tubes were centrifuged at 200 x g for 5 minutes. The supernatants were discarded. The cells were then resuspended in 1ml of serum free RPMI 1640 medium. Nine hundred microlitres of nigrosin was transferred into 2 separate microcaps and one was labelled partner and one lymphocyte. One hundred microlitres of each of the cell suspensions were then transferred into these containers, and each sample was counted using a Neubauer haemocytometer. The volume of each cell suspension was then adjusted with serum free RPMI 1640 medium to give a density of 10^6 cells/ml.

Equal volumes of each cell suspension was then transferred to a sterile 50ml centrifuge tube, and this was topped up with serum free RPMI 1640. Centrifugation was then carried out at 200 x g for 5 minutes and the supernatant discarded. The tube was then placed into a small water bath at 37°C. Using a 1ml pipette, 1ml of PEG was added to the cell pellet over a 1-minute time interval, whilst mixing the PEG into the cells with the pipette tip. The cell suspension was mixed for a further minute in the water. Again using a 1ml pipette, 1ml of serum free RPMI 1640 was added to the cell suspension, again over a minute.

Twenty millilitres of serum free RPMI 1640 was then added to the cell suspension over 5 minutes, starting slowly, dropwise at first then faster as the PEG was diluted out. The cells were then centrifuged at 200 x g for 5 minutes and the supernatant discarded. The cells were then resuspended in 30-50ml of HAT medium, and then cells dispensed into the 96 well flat-bottomed plates that contained the mouse peritoneal macrophages, that had been prepared the previous day. The wells were topped up with HAT medium.

The plates were then incubated at 37°C and 5% CO_2 for 6 days undisturbed. On day-6 the plates were examined under a microscope to note any wells that had hybrid growth. The next day, half of the supernatant from the wells was aspirated and the wells were topped up with HT medium.

The HT medium was then changed every 2-3 days for 7 days, and following this the cells were cultured in culture medium. When sufficient growth was apparent, the supernatant was screened using the ELISA technique to investigate the production of antibodies by the hybrids specific for *P. gingivalis*, *A. actinomycetemcomitans*, *B. forsythus*, *P. intermedia* and *T. denticola*, whole fixed bacteria.

Growth was correlated with antibody activity, and the cells were removed from any wells in which the surface was covered. Initially these cells were split among 2-4 wells in a fresh plate, then the growth was monitored and the cells were expanded into 8 wells of a 96 well plate, then 4 wells of a 24 well plate, then a 25cm^2 flask and then a 75cm^2 flask.

The cell supernatants were constantly checked to make sure that antibody secretion was maintained.

4.2.10 Growth and preparation of bacteria

P. gingivalis NCTC 11834, *P. intermedia* ATCC 25611, *B. forsythus* ATCC 43037 and *A. actinomycetemcomitans* ATCC 29523 (serotype A) were grown and fixed as

described in 3.2.2. *T. denticola* ATCC 35405 were cultured in medium OMIZ-pat + 1% human serum, and were a gift from Dr. C. Wyss, Institut fur Orale Mikrobiologie, Zurich, Switzerland (Wyss *et al.*, 1997).

4.2.11 Analysis of hybridoma supernatants by the Enzyme Linked Immunsorbent Assay (ELISA)

Analysis of the antibody production by the hybridomas was analysed by ELISA as described in 3.2.3. Instead of the addition of sera, the hybridoma supernatant was added to the wells at this step.

4.3 Results

4.3.1 L-cell culture

The growth of the L cells did not lead to any difficulties at all. The cells grew extremely quickly and were subcultured every 3-4 days. The cells were cultured in complete RPMI medium in 5% CO_2 at 37°C.

4.3.2 CRL-1668 cell culture

The heteromyeloma cell line CRL-1668 ATCC was much more difficult to culture. The cells were extremely sensitive to any fluctuations in temperature or CO_2 level. These cells were also very sensitive to cell density and had to be maintained very strictly between $4x10^5$ and 10^6 cells per ml of culture medium. This cell line was cultured in Iscoves medium containing FCS (20%), L-glutamine (4mM) and G418 (200μg/ml) to stabilise its human chromosome complement.

4.3.3 The CD40 system

In these studies we activated peripheral blood lymphocytes taken from patients with chronic periodontitis in the CD40 system. Stimulation of peripheral blood lymphocytes by the CD40 system dramatically increased the number of actively

dividing and differentiating B lymphocytes. The cells grew in tight clusters and the clusters were observed every 2 days. The numbers of cells increased constantly until days 10-11, when the cells were counted and then fused with the heteromyeloma cell line.

Figure 4.1 shows a culture of the L cells and figure 4.2 shows a culture of the CRL-1668 cells. Figure 4.3 and 4.4 show cultures of B cells in the CD40 system.

Figure 4.1

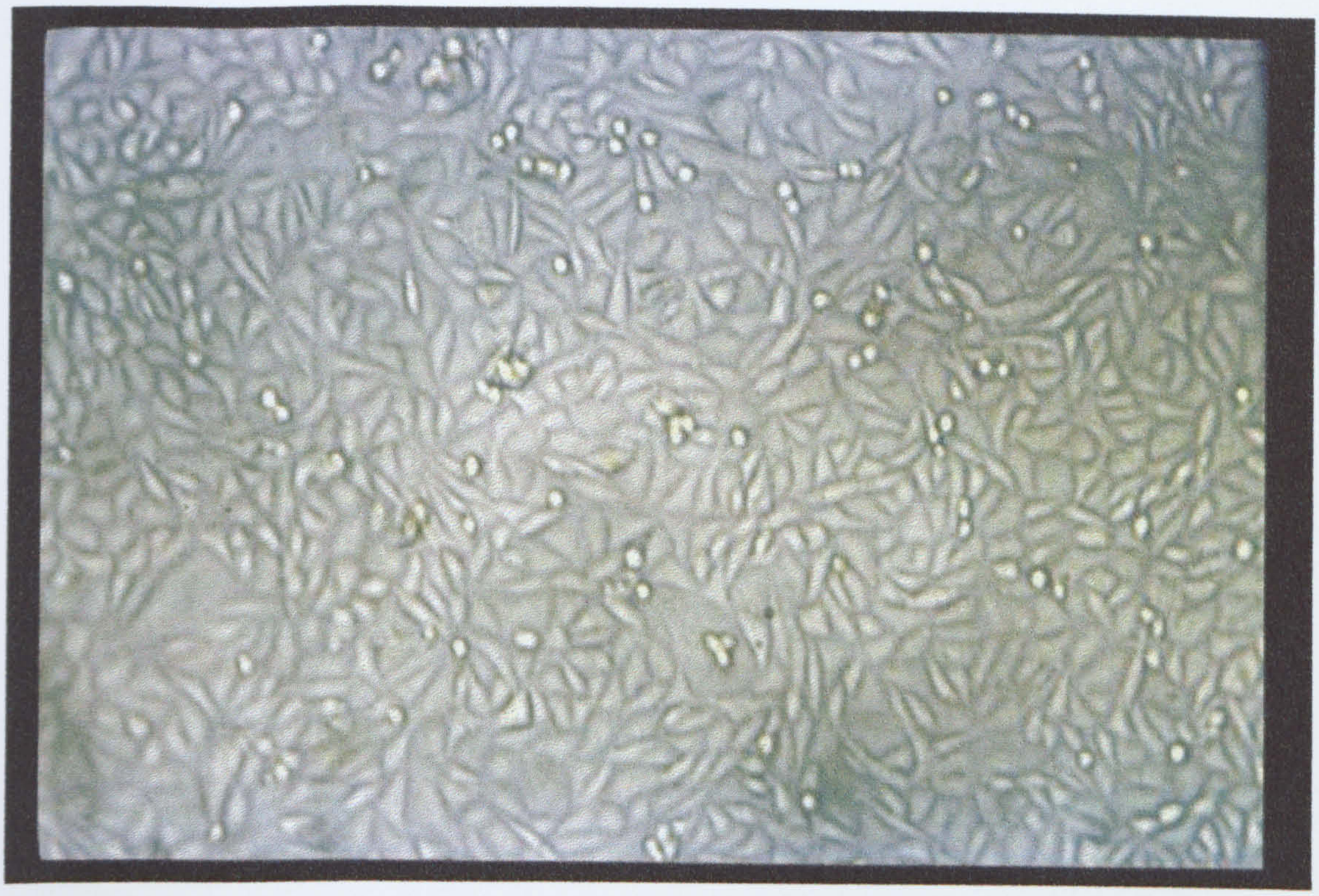


Figure 4.1 shows L cells in culture. (Magnification x400)

Figure 4.2

Figure 4.2 shows CRL-1668 heteromyeloma cells in culture. (Magnification x400)

Figure 4.3

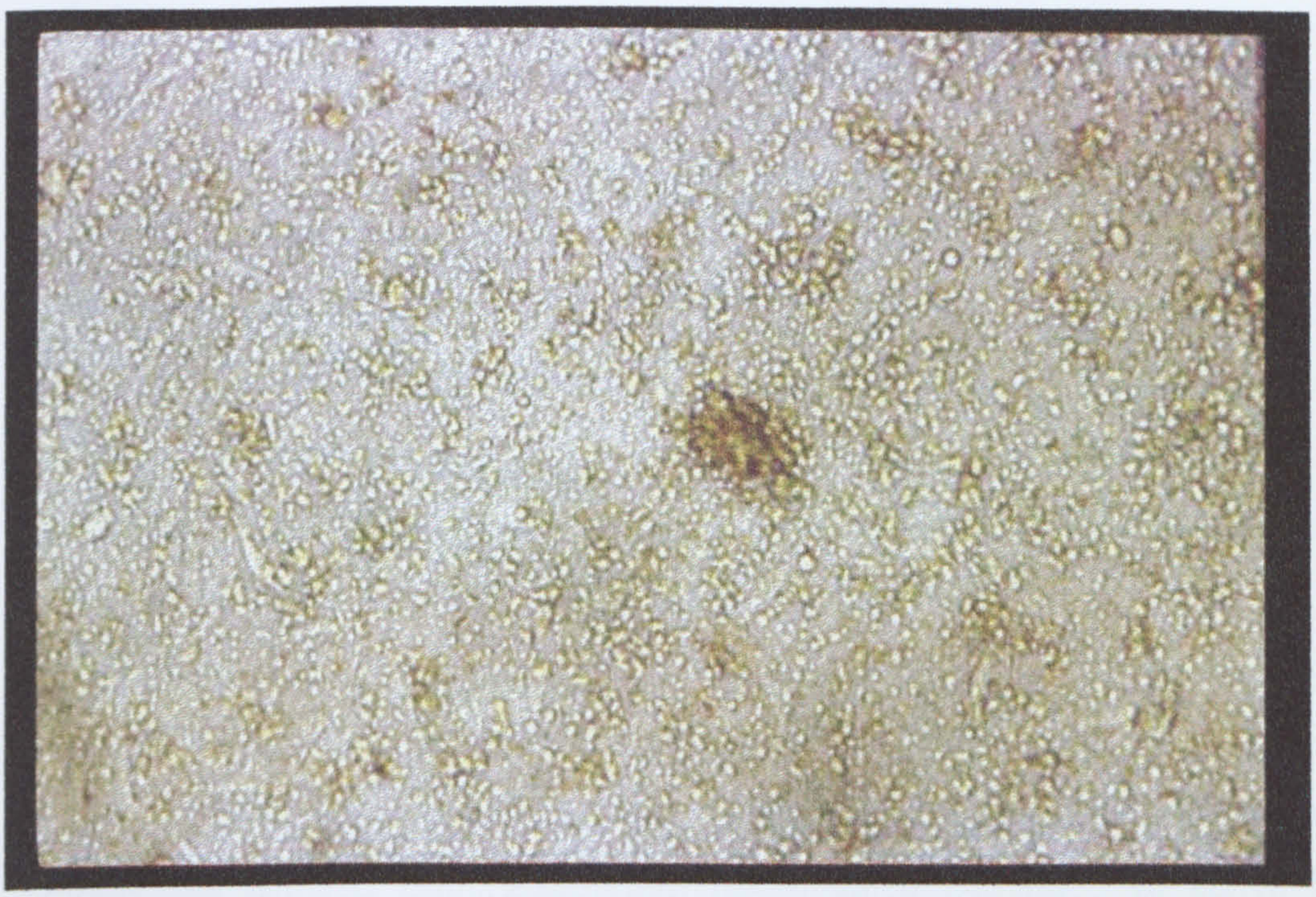


Figure 4.3 shows cells being cultured in the CD40 system. This photograph was taken on day 3 of culture. A cluster of B cells can be seen proliferating in the centre of the field. (Magnification x400)

Figure 4.4

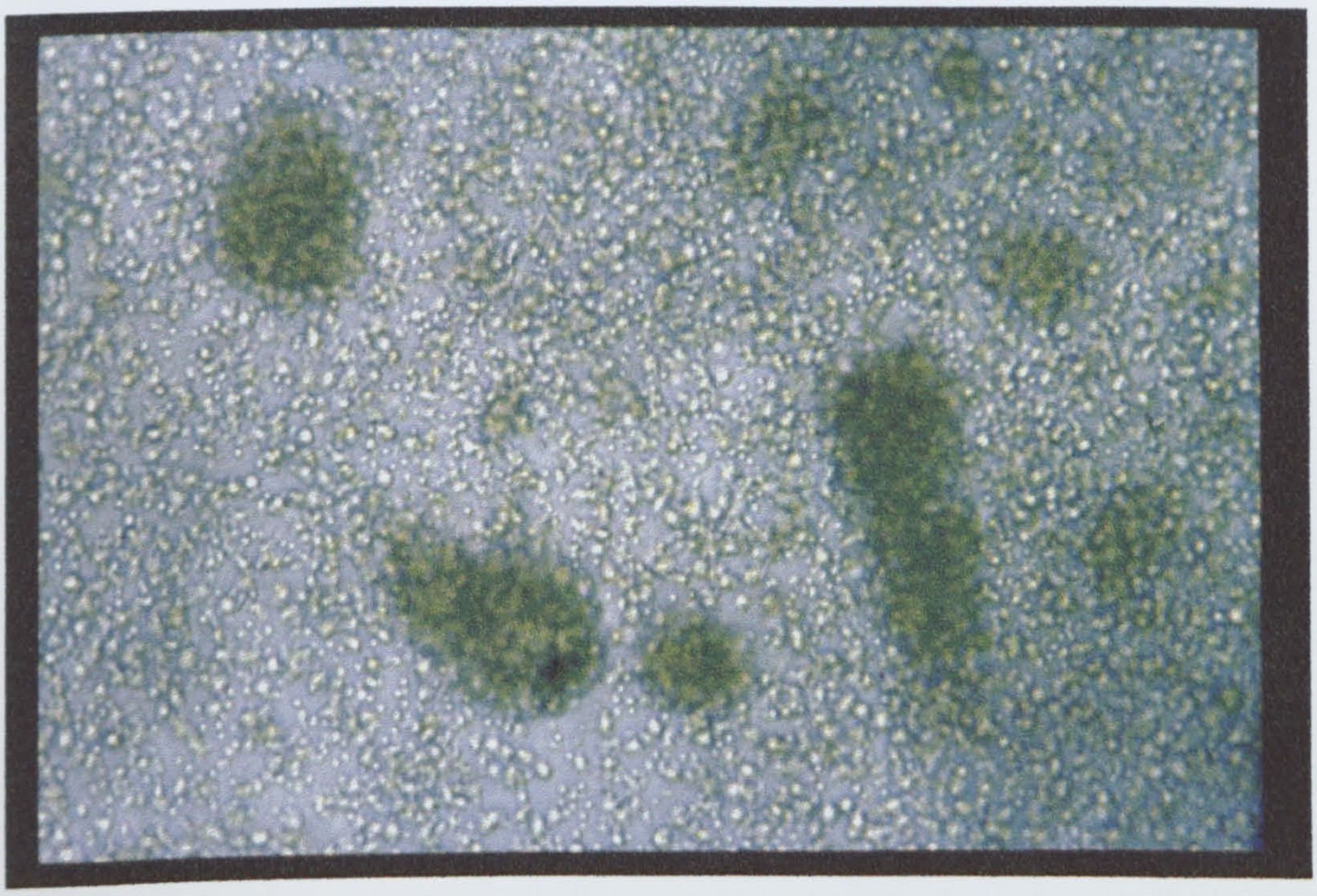


Figure 4.4 shows cells being cultured in the CD40 system. This photograph was taken on day 11. Numerous large clusters of B cells can be seen. (Magnification x400)

4.3.4 Cell fusion

After cell fusion, hybridoma formation was successful. The hybrids were visible by the eye as growing colonies by about day 10 following fusion. In wells that contained growing colonies, cloning was carried out by the limited dilution method. When the cloned cells had grown to visible colony size again the supernatant was removed from each well and screened for the presence of specific antibodies against periodontal pathogens. Antibodies specific to *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *B. forsythus* and *T. denticola* were screened for.

The screening stage was reached for peripheral blood lymphocytes of 5 patients expanded in the CD40 system and then fused using PEG, and for directly fused B lymphocytes isolated from granulation tissue for 6 patients. In all of the 11 experiments antibodies specific for the 5 pathogens were identified, and the clusters of cells were cloned. However, antibody production failed to continue and was not detected after 3-5 weeks of culture in all of the cases. Therefore, in all of the experiments, although fusion was achieved, and cloning was successful, the hybridomas stopped producing antibodies or had died after a period of a few weeks.

4.4 Discussion

The results obtained in this study are disappointing and unfortunately continuation of this project was not justified due to the lack of success following on from the laborious work that was carried out in this study. Although, no monoclonal antibodies were obtained, I feel that the rationale behind the project and the aims of the study were important to discuss and thus should be included in this thesis.

Two main techniques were employed and two different sources of lymphocytes were used. The CD40 system was used to expand the number of lymphocytes that were obtained from the peripheral blood of periodontitis patients. By following the methodology described by Niedbala and Stott (1998) the CD40 system was optimised for antibody production, culture conditions and the concentration of IL-4 and anti-CD40 antibody used. Cells that had been cultured with IL-4 and anti-CD40 presented

on Ltk$^-$ cells, were viable and grew in tight clusters. The cultures were kept for 10-11 days and provided regular enrichment of the culture medium. This resulted in a 3-5-fold increase of input B cells within the culture period of 10-11 days. Following expansion of the B lymphocyte numbers, cell fusion was carried out with the heteromyeloma cell line.

The second method used in this study involved direct fusion between B lymphocytes isolated from granulation tissue samples taken from patients with chronic periodontitis and the heteromyeloma cell line CRL 1668.

The first method involving amplification of peripheral blood lymphocytes in the CD40 system, followed by fusion, was carried out on 5 patients as previously mentioned. However, it was then decided to move the study towards direct fusion of B lymphocytes from diseased tissue. Although a small number of human heteromyelomas have been shown to be relatively stable, not all B cells fuse equally well. Schwaber *et al.* (1984) suggested that the differentiation state of the B cell seems to affect its ability to fuse. Glassy (1993) reported that small unstimulated memory cells are resistant to fusion, while larger activated B cells fuse more readily. Granulation tissue from individuals with periodontal disease is thought to contain large numbers of B lymphocytes and plasma cells which are thought to comprise a significant portion of the local cellular infiltrate (Seymour *et al.*, 1979). These B lymphocytes are thought to be large activated cells. In addition, since the frequency of antigen-specific B cells in human peripheral blood is very low, the number of activated B lymphocytes specific to periodontal pathogens present in the tissues would be expected to be higher.

Peripheral blood B lymphocytes are much more easily obtained from diseased patients and one blood sample can provide a large number. In addition, the number of B lymphocytes taken from the blood, can be amplified with use of the CD40 system, as in this system human resting B cells enter a state of sustained proliferation resulting in clonal expansion. Therefore, peripheral blood lymphocytes are the best source for practical and ethical reasons. However, peripheral blood lymphocytes do not make the best fusion partners. In the mouse it has been suggested that lymphocytes isolated

from tissues make better fusion partners than those from peripheral blood. Further to this it has been proposed that peripheral blood does not contain enough antigen reactive B cells in the appropriate state of differentiation and proliferation, and contains too many suppressor and cytotoxic cells and not enough antigen presenting and T helper cells (James and Bell, 1987).

A study carried out by Çelenligil and Ebersole (1997) to examine responses to *A. actinomycetemcomitans* in patients with chronic periodontitis compared to health, involved the examination of B cells that had been immortalised using EBV. The rationale for the study was to investigate whether immortalised B cells could potentially be used to study the host humoral immune response in this disease. The results of this study suggested B cells from both diseased patients and controls, were capable of being immortalised and that EBV transformation led to the proliferation and differentiation of B cells to give IgM, IgG and IgA secreting cell lines. An elevated frequency of IgM producing cells was reported and in addition, a higher proportion of immunoglobulin producing cells were found in the periodontitis patients compared with the normal controls. Thus Çelenligil and Ebersole suggested that either periodontitis patients demonstrate a higher proportion of immunoglobulin producing cells, or that the circulating B cell pool in periodontitis patients is enriched for the subset that are EBV transformable. The study went on to investigate the effects of polyclonal B cell activators, including LPS of *A. actinomycetemcomitans* on antibody production by EBV transformed cells.

This study is one of the first in the periodontal disease research area involving human cell transformation. EBV transformation is a useful tool for providing a resource of cells for studying human B lymphocytes in health and disease. The elevated frequency of IgM producing cells noted in this study is not surprising as many studies in this field have reported a bias in IgM secreting cells. EBV transformation can be used either alone, as in the study by Çelenligil and Ebersole (1997), or combined with cell fusion.

Plasma cells within the gingival tissues have been shown to produce antibody that is specific for micro-organisms in the subgingival plaque (Ebersole, 1990; Ebersole and

Taubman, 1994). Local humoral immune responses are implicated in the pathogenesis of periodontitis (Pulver *et al.*, 1978; Stern *et al.*, 1981; Torabinejad *et al.*, 1981; Matthews and Mason, 1983; Smith *et al.*, 1987) and B cells which are predominant in the lesions locally produce IgG, lower amounts of IgA and small quantities of IgM, as detected by immunofluorescence techniques (Pulver *et al.*, 1978; Stern *et al.*, 1981; Torabinejad *et al.*, 1981).

Studies have shown that infiltrating lymphocytes are more likely to arise through selective homing to the diseased tissues than by local cell division (Takahashi *et al.*, 1996). In addition, this study suggested that the plasma cells in the tissue samples observed were among the most active secretary cells in the gingiva. Therefore, it is thought that antibody-bearing cells specific for periodontal pathogens home to the site of infection, stay there if they meet relevant antigen or other stimuli, and once resident remain active, secreting antibodies in a response against the offending pathogenic bacteria.

Therefore, in theory to study the target of antibody-bearing cells that are present in the diseased tissue of individuals with periodontal disease, would appear to be a direct way of examining what has induced the immune response to begin with. The aim of the project was to fuse antibody-bearing cells from diseased periodontal tissue with a heteromyeloma fusion partner. A murine fusion partner was not chosen as the aim of the project was to produce human monoclonal antibodies that could be characterised fully. A few very satisfactory murine partner cell lines have been developed however, this is not the case for human partner lines. The fusion rates achieved when using mouse myelomas as partners are significantly higher than those achieved with human partners in comparative experiments (Cote *et al.*, 1983; 1984; 1986). In this study a heteromyeloma cell line was used as a partner for the fusions. This was chosen as it was more humanised than a mouse partner cell line, however, when using heteromyeloma cell lines there is always the chance that the heterohybrids may reject human chromosomes. Thompson *et al.* (1986) did report, however, that the loss of antibody secreting lines due to genetic instability is no worse in heterohybrids than in murine hybrids, hence it was decided that a heteromyeloma was a good choice of fusion partner.

Fusion is complex and extremely technique sensitive and reports have suggested that the success of the technique can depend on minor technical details such as a particular batch of PEG, the pH of the PEG when it is in contact with the cells and the length of time that the cells stay in contact with the fusion partner (Lane *et al.*, 1984; Davidson and Gerald, 1976). In addition, it is generally accepted that many technical problems still remain to be solved in terms of successfully fused cell lines that grow well for 1 to 2 months before their antibody titre suddenly declines (James and Bell, 1987). In the present study, it appeared that fusion was successful, however, the cell line did cease antibody production before cloning could be carried out.

In spite of the numerous difficulties encountered in monoclonal antibody production, this technology has huge potential. Various human monoclonal antibodies have been produced to date, many of which have been developed with prophylaxis and therapy in mind and are, therefore, directed against a wide range of bacterial, viral, erythrocyte and tumour antigens. The technique has great potential which will only expand and may eventually provide us with valuable tools for future diagnostics, therapy and research.

Chapter 5. Analysis of the Target of Antibody Secreting Cells From Diseased Tissue of Chronic Periodontitis Patients By ELISPOT

5.1 Introduction

Development of the ELISPOT assay was originally based upon the haemolytic plaque assay developed by Jerne and Nordin (1963). The development of the haemolytic plaque assay began a new era of detection that permitted antibody analyses to be undertaken at the cellular level. It allowed cellular immunoglobulin secretion to be assessed as well as analysis of the regulatory mechanisms that control the expansion of antibody-producing cell populations. The assay was originally developed to detect cells secreting complement-binding antibodies against erythrocytes. The use of haemolysis as a signal in this method necessitates the application of complement and red blood cells. Both of these components limit the selection of antigens and antibodies that can be used or assayed. Experimental failures and inconsistent results are generally related to the difficulties experienced when trying to efficiently couple antigens to red blood cells (Golub *et al.*, 1968; Pasanen and Mäkelä, 1969; Jerne *et al.*, 1974).

The ELISPOT technique was developed simultaneously by two different groups; Czerkinsky *et al.* (1983) first coined the term ELISPOT and immunohistochemically showed a spot-forming cell, and Sedgwick and Holt (1983) also reported development of the assay, but called it the ELISA-plaque. Since the development of the assay, other names have described the method, such as spot-ELISA and ELISA-spot, however, the generally accepted name is the ELISPOT. The development of the technique combined the principles of the ELISA and the plaque forming assay. However, unlike the ELISA technique, where the final quantification is done by a spectrophotometer at a defined wavelength, ELISPOT is usually counted manually using a stereo-microscope. The technique can be very time-consuming and laborious if there are large numbers of spots, and there is a certain amount of subjectiveness when counting. For these reasons computer-assisted methods have been developed to

aid counting (Cui and Chang., 1997; Herr *et al.*, 1997; Vaquerano *et al.*, 1998). The benefits that ELISPOT has over its precursor, is that adsorption of antigens to hydrophobic plastic surfaces is much simpler and easier to accomplish than trying to couple antigens to erythrocytes, which is the main limitation of the haemolytic plaque assay. Further to this, adsorption of antigens to plastic surfaces results in this step being stable for a much longer period of time.

Since the introduction of the ELISPOT assay it has been utilised to investigate a number of different cell products. It has become well established for studying cytokine-secreting cells (Herr *et al.*, 1997; Hagiwara *et al.*, 1996; Karttunen *et al.*, 1995; Merville, 1993) as well as antibody secreting cells in response to infections and vaccination. A lot of the work reported on ELISPOT has looked at antibody-secreting cells in infections that have entered the body via the mucosa such as Salmonella and Campylobacter (Kantele *et al.*, 1988), typhoid fever (Murphy, 1993) and infection with Shigella (Murphy, 1993; Orr *et al.*, 1992). The ELISPOT technique has also been utilised in studies examining urinary tract infections (Kantele *et al.*, 1994), otitis media (Nieminen *et al.*, 1996), gastritis (Sugiyama *et al.*, 1995) and some viral infections such as rotavirus gastro-enteritis (Kaila *et al.*, 1992), influenza (Mäkelä *et al.*, 1995) and HIV (Lee *et al.*, 1989; Nesheim *et al.*, 1992).

The ELISPOT technique has also been used to assess the efficiency of vaccines. This technique allows the response induced by the vaccine to be studied at the cellular level. Thus the results are not effected by any pre-existing antibodies that may be present in the serum of the vaccinated subjects. It has been suggested that vaccines given by a mucosal route may not increase serum antibody titres, whereas effects can be observed on a cellular level, by identifying antibody-bearing cells (Kantele and Mäkelä, 1991), therefore, the ELISPOT is a useful tool. The ELISPOT technique has been used in studies observing antibody secreting cells following many vaccine strategies including those against tetanus toxoid (Stevens *et al.*, 1979), influenza (Yarchoan *et al.*, 1981) and hepatitis B (Cupps *et al.*, 1984).

A complex inflammatory and immune response is involved in the progression of periodontitis. Tissue activity within the diseased periodontium comprises epithelial

and connective tissue turnover, and cellular activity that occurs continuously throughout the disease process which is associated with infiltrating inflammatory cells (Page and Ammons, 1974; Page and Schroeder, 1976). Previous work has suggested that B and T cells accumulate in large numbers in the periodontal tissues, and Takahashi *et al.* (1997) demonstrated that there are numerous plasma cells in periodontitis gingiva. They reported that IgG producing plasma cells predominate in periodontitis gingiva, with IgA and IgM producing cells being present, but in low numbers. It has been suggested that the humoral immune response is protective in the pathogenesis of periodontal disease, and if this is the case, the target or targets of the antibody secreting cells found in periodontal lesions are clearly of interest and may indicate the specific microbes involved in this disease.

Ogawa *et al.* (1989b) analysed the isotype and subclass of antibodies produced by single cells directed against fimbriae and LPS of *P. gingivalis* isolated from gingival tissues of patients with periodontal disease. The study was carried out using the ELISPOT technique. The periodontitis patients were divided into 3 groups: slight; moderate; and advanced chronic periodontitis. The total number of plasma cells increased in relation to the severity of disease, and the major antibody isotype detected in all subjects was IgG followed by IgA. The results of the study indicated that levels of antibody secreting cells, or spot forming cells, were higher to fimbriae than to LPS, which were found to be present in lower numbers. These results came as a surprise since it is generally assumed that LPS in gingival plaque induces polyclonal antigen-specific responses. The reasons for this is not clear. It is faesible that LPS of *P. gingivalis* is not very immunogenic, as it has been shown to differ chemically from LPS from other Gram negative bacteria (Mansheim *et al.*, 1978, Koga *et al.*, 1984). The overall results of this study, indicated that high levels of immunoglobulin, most notably of the IgG isotype and less, but significant, IgA subclasses are produced locally in diseased sites. The study also indicated that cells can be isolated from diseased gingival tissues, and used to investigate the target of the antibodies produced by these cells. This technique clearly permits us to study more closely the local immune response induced in this disease.

A further study was carried out by the same group (Ogawa *et al.*, 1991), to investigate anti-fimbriae responses in individuals with chronic periodontitis. The study compared mononuclear cells isolated from diseased and healthy sites of individuals with chronic periodontitis. Results were correlated with levels, isotypes and subclasses of anti-fimbriae antibodies found in the sera and those produced by peripheral blood mononuclear cells of the same subjects. Thus the study was a direct comparison of the local immune response versus the systemic. The results of this study suggested that serum antibodies to periodontal bacteria are derived from local stimulation of B cells in inflamed gingiva. Hence, indicating a need to examine the local immune response in this disease, as it may provide more details about the pathogens and antigens that are inducing the immune response, that begins locally and leads to systemic immunity.

Our understanding of the function of the immune response locally within the tissues is incomplete, for example we are unsure as to whether the plasma cells, found in large numbers in lesions, in diseased periodontal tissues are producing relevant or totally non-specific antibodies to periodontal bacteria. Work carried out on gingiva of periodontitis patients indicates that the infiltrating lymphocytes are more likely to arise through selective homing, than by local cell division (Takahashi *et al.*, 1996), hence indicating the plasma cells present in the lesion are there for a specific reason, probably producing specific antibody.

Many studies have indicated that antibodies directed against putative periodontopathogens are present in the serum and GCF of individuals with periodontitis. Some studies suggest that titres are elevated in these patients compared with healthy individuals. It is generally accepted that systemically antibodies are being produced against periodontal bacteria and play a role in the host protective system. Controversy lies, however, in how much the local immune response reflects the systemic specificity.

The present study is an investigation, utilising the ELISPOT technique, into the target of antibody secreting plasma cells isolated from granulation tissue taken from diseased patients. Moscow and Polson (1991) suggested that experiments carried out

on superficial gingival biopsies may not be a true reflection of the disease progression in the deeper granulation tissues. Therefore, the present study is an investigation of the targets of some of the plasma cells isolated from granulation tissue taken from patients with advanced chronic periodontitis. The specific aim of this study was to identify whether antibody-bearing cells removed from these lesions were secreting antibody specific for the putative periodontopathogens *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus*.

5.2 Methods

5.2.1 Patients and tissue samples

The subjects included in this study were 5 patients attending Glasgow University Dental Hospital. The criteria for inclusion was that they had advanced periodontal disease and were receiving surgery as part of their periodontal therapy. During surgery, which was carried out by an experienced periodontist, granulation tissue was removed and immediately placed into sterile RPMI medium. The tissue sample was then stored at 4°C until use.

5.2.2 Antigen preparation

P. gingivalis NCTC 11834, *P. intermedia* ATCC 25611, *B. forsythus* ATCC 43037 and *A. actinomycetemcomitans* ATCC 29523 (serotype A) were grown as described in 3.2.2.

Bacterial sonicate preparations were prepared as described in section 3.3.2.

The protein concentrations of the bacterial sonicate preparations were determined using the Pierce BCA protein assay kit.

5.2.3 Enzymatic dissociation of granulation tissue

Surgically removed granulation tissue from each subject was placed in a sterile 25ml universal. The tissue was then cut into 1-2mm^3 segments using sterile scissors, washed twice with RPMI 1640 medium, and then centrifuged at 200 x g for 10 minutes. Cells were then dissociated from the tissue.

8ml of Jockliks MEM containing dispase at 3mg/ml (pH 7.4) was added to the universal containing the tissue. The universal was then incubated at 37°C for 90 minutes with continuous stirring. The medium containing the cells was separated from the tissue by filtration using 41μm nylon net filters and mixed with excess RPMI 1640 medium.

The cells were harvested by centrifugation at 200 x g for 10 minutes before being resuspended in Iscoves medium and stored on ice. The cells were then placed on a mini gradient containing 1ml of Histopaque-1077. Centrifugation was then carried out at 400 x g for 20 minutes, and the interface of cells was collected, washed and resuspended in Iscoves medium. The cells were then enumerated in white cell counting fluid using a Neubauer haemocytometer.

5.2.4 Enumeration of specific immunoglobulin producing cells by ELISPOT

Multiscreen 96 well HA membrane-bottomed plates designed specifically for the ELISPOT technique were used in this experiment. The wells were coated with 100μl of phosphate buffered saline (PBS) containing sonicated bacterial antigens of *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus* at a concentration of 10μg/ml. Tetanus toxoid was diluted in PBS and added at a concentration of 20μg/ml. This was the control. The plates were then incubated overnight at 4°C. The following day the plates were washed with 200μl/well of PBS containing 0.1% Tween-20 (PBS-T) for 5 minutes. The plates were emptied by flicking and then they were washed twice with 200μl/well of PBS. The wells were blocked with 200μl/well of Iscoves modified Dulbecco's medium supplemented with

5% foetal calf serum (FCS), penicillin (10000IU) and streptomycin (1000μg/ml) at room temperature for 1 hour.

One hundred microlitres was then replaced with medium just prior to addition of the single cell suspension. The 100μl cell suspension obtained from the granulation tissue digest, described above, was then added to the wells. Two different concentrations of cells were used throughout the experiment; $1x10^8$ and $5x10^7$ cells/ml. The plates were then incubated at 37°C for 5 hours. Care was taken not to disturb the plates during this incubation period as it may cause "double image" spots to form.

After 5 hours the plates were emptied by flicking, the wells were then washed 3 times with 200μl/well with PBS and 3 times with 200μl/well with PBS-T. Biotinylated anti-human IgG was then diluted 1:1000 in PBS-T-FCS and 100 μl/well was added to the plates. The plates were incubated overnight at 4°C. Following the overnight incubation the plates were washed 4 times with 200μl/well PBS-T and then 100μl/well of HRP-conjugated avidin-D was added. This was diluted to a final concentration of 5μg/ml with PBS-T-FCS. The plates were then incubated for 1 hour at room temperature. The wells were then washed 3 times with 200μl/well PBS-T, followed by 3 times with 200μl/well PBS. 100ml per well of chromogen substrate was then added and left for 3 to 8 minutes or until red spots could be visualised. The reaction was stopped by washing the plate with running tap water. The plates were then blotted dry.

The nitrocellulose discs covering the bottom of the wells were then removed. They were then photographed and the spots enumerated using a stereo-microscope equipped with a vertical overhead white light. The results represent the number of spots counted per well.

5.2.5 Statistical methods

The data was statistically analysed by the Friedman's test using the Minitab statistical package. This test was chosen as it compares the number of cells detected against

different antigens, when observations have been repeated on the same subjects. In addition, this test makes no assumptions about the distribution of the data. It was not satisfactory just to reject the hypothesis and conclude that not all the ELISPOT results were not identical, therefore, a multiple comparison procedure suitable for use after the Friedman's test was carried out (Daniel, 1978).

The Wilcoxon Signed Rank test was used to compare the median paired differences between the results obtained when 10^8 and $5x10^7$ cells/ml were added. This test was chosen as it allowed the comparison of the differences (10^8 minus $1x10^7$ cells/ml) for each antigen separately whilst still taking into account that the data has been collected from the same subjects.

5.3 Results

Many different preliminary experiments were carried out in this area. Three of four different protocols for the ELISPOT technique were carried out and numerous problems were experienced. Experiments at the beginning were carried out to try and get the technique working correctly and these involved studies such as enumeration of the number of peripheral blood lymphocytes bearing antibody to tetanus toxoid in healthy individuals following a tetanus vaccination. The results presented in this chapter are the results obtained from the most successful protocol that was examined. Establishing the technique was extremely time consuming and laborious, however, the results presented in this study are the beginning of a project that should be continued to look at a greater number of chronic periodontitis patients.

Figures 5.1a - 5.4b Show examples of ELISPOT results obtained.

Figure 5.1a

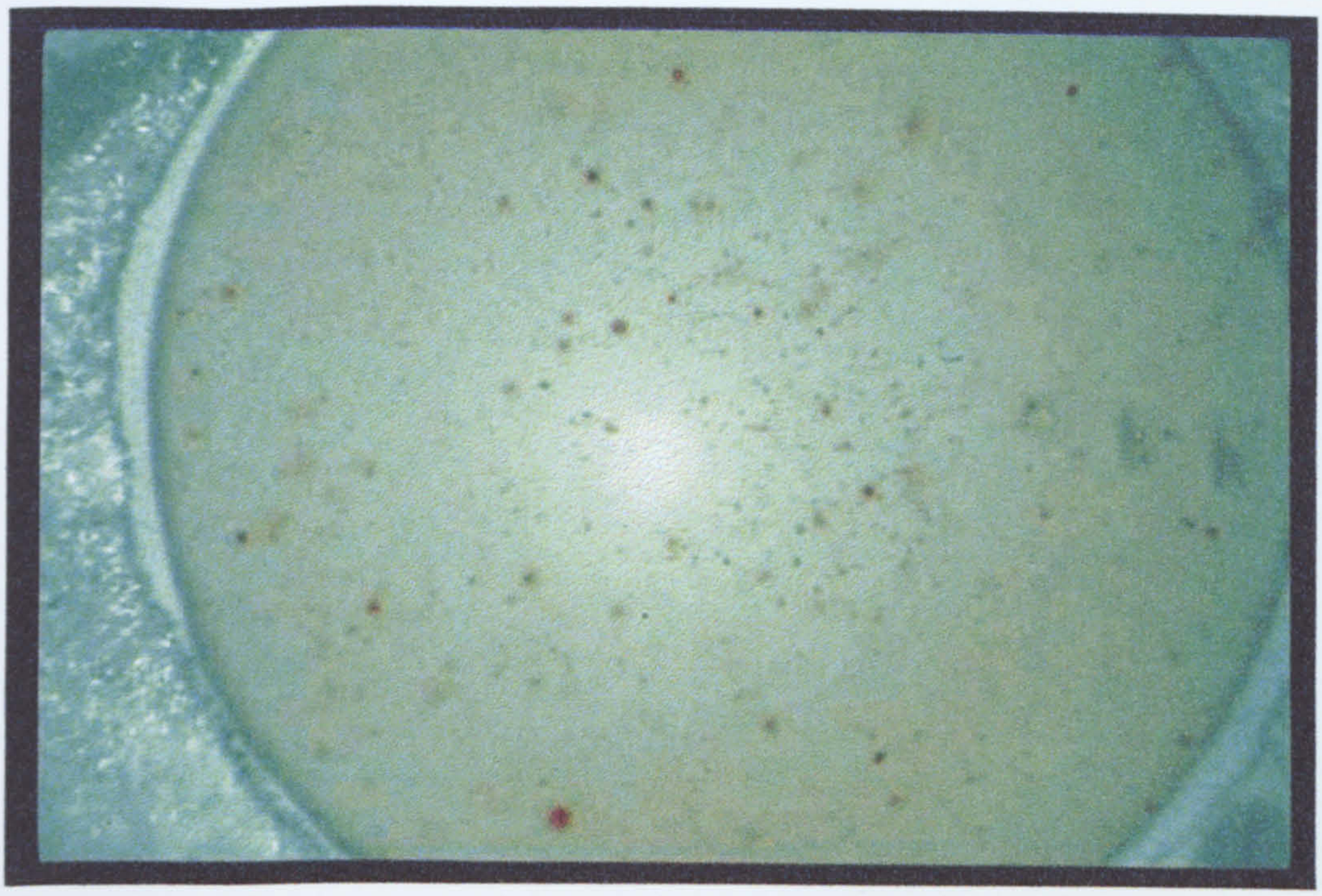


Figure 5.1a shows spots indicating plasma cells isolated from granulation tissue bearing antibodies on their surface specific for *P. gingivalis*. As measured by the ELISPOT technique. (Magnification x10)

Figure 5.1b

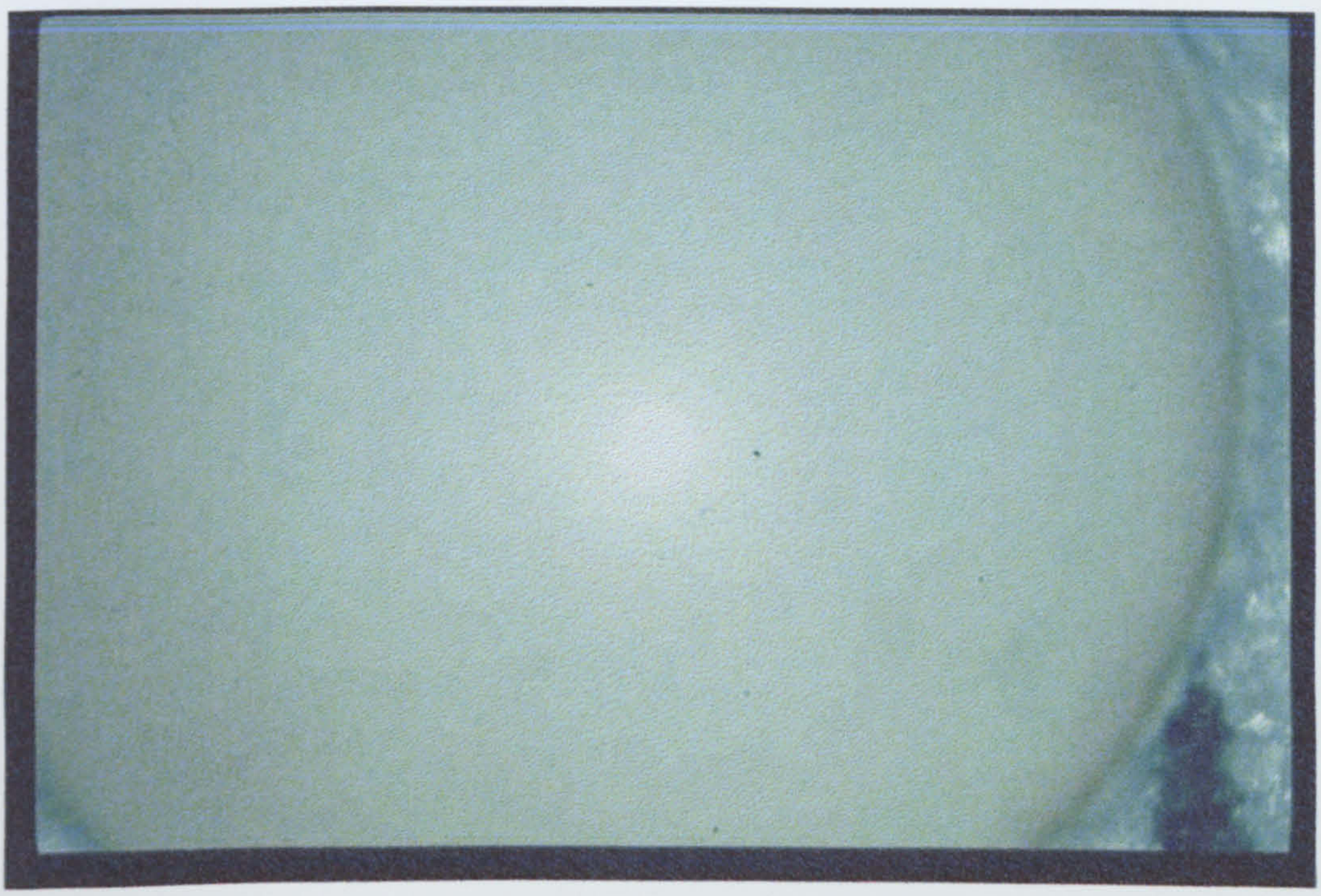


Figure 5.1b. Negative control for *P. gingivalis*. (Magnification x10)

Figure 5.2a

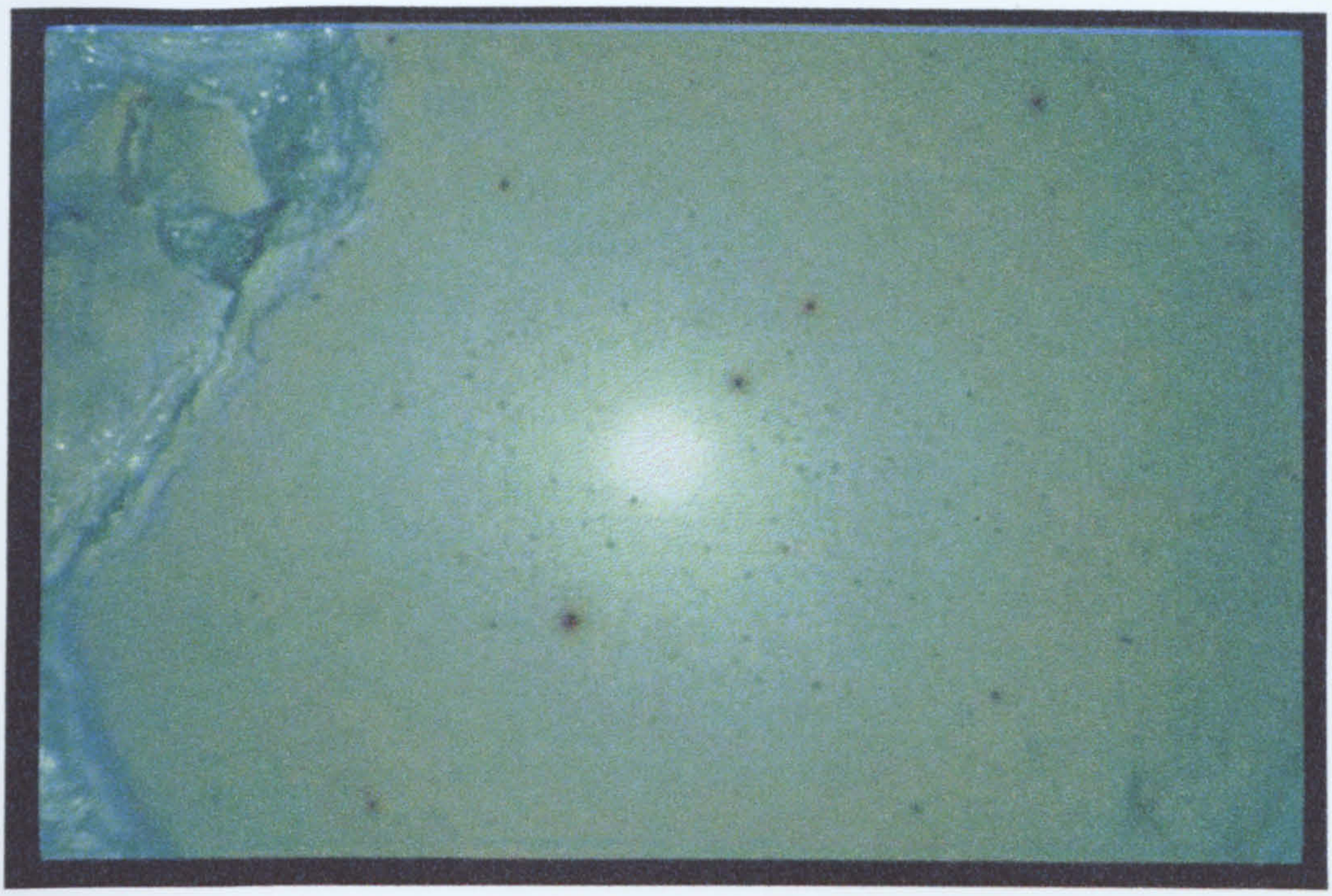


Figure 5.2a shows spots indicating plasma cells isolated from granulation tissue bearing antibodies on their surface specific for *A. actinomycetemcomitans*. As measured by the ELISPOT technique. (Magnification x10)

Figure 5.2b

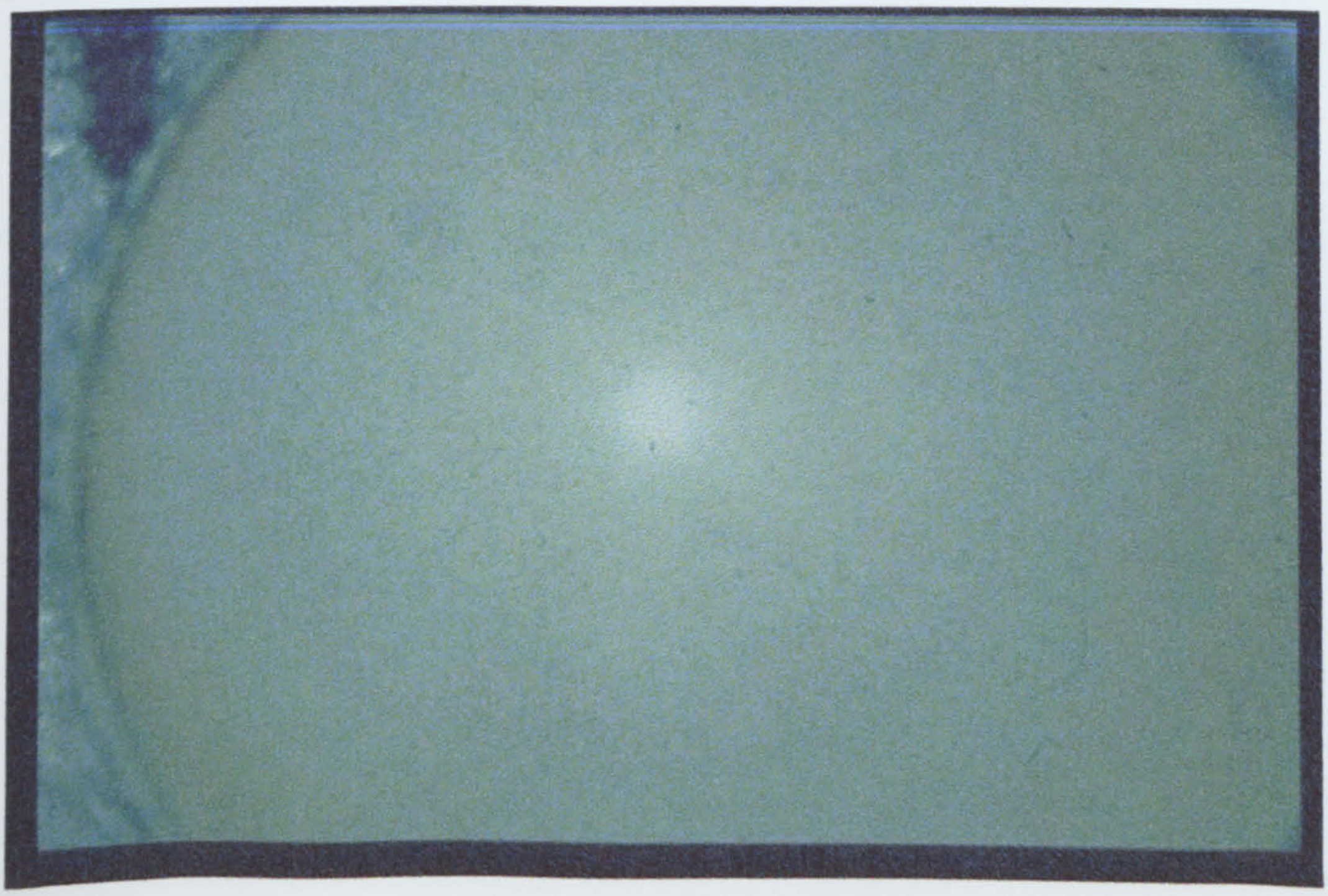


Figure 5.2b. Negative control for *A. actinomycetemcomitans*. (Magnification x10)

Figure 5.3a

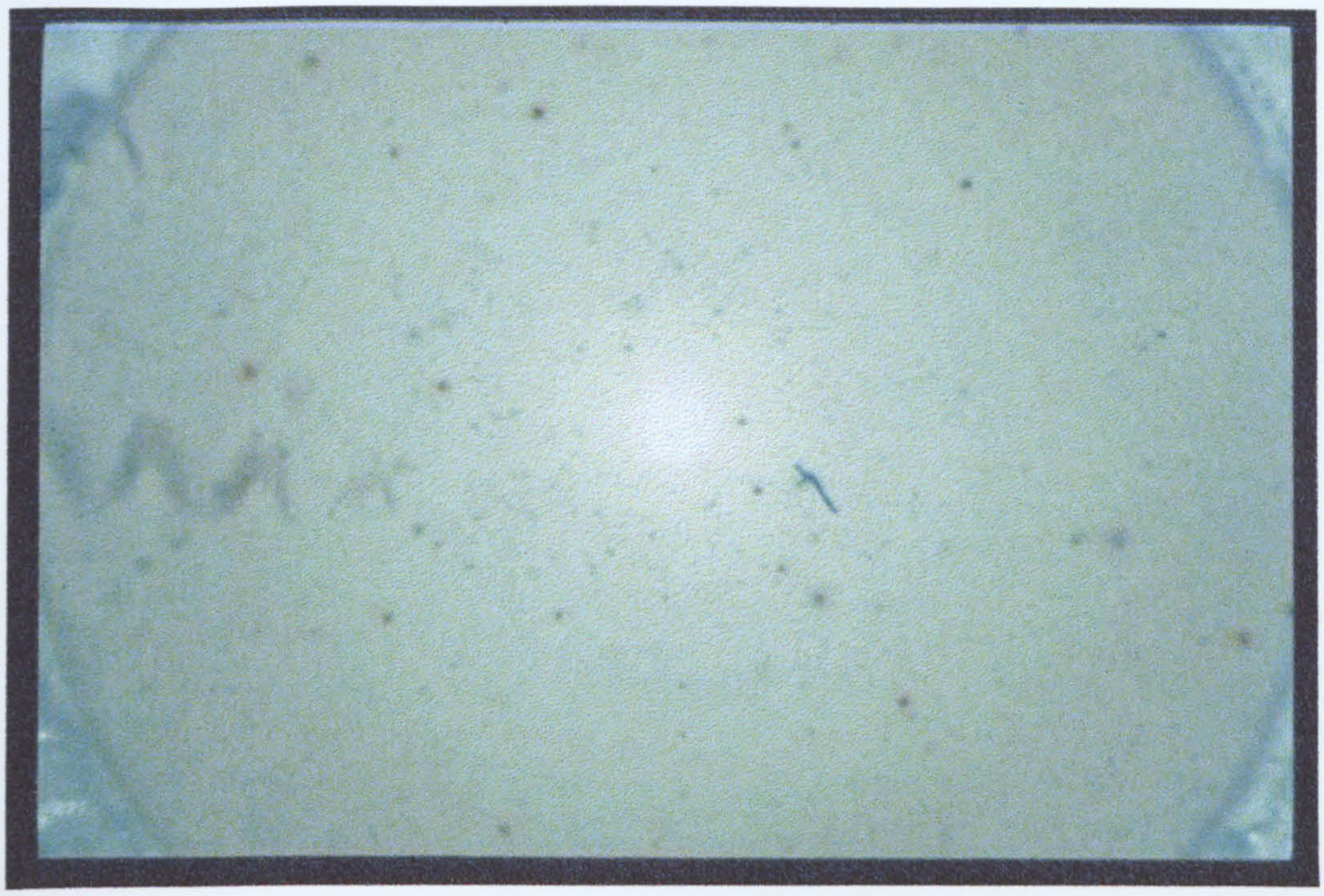


Figure 5.3a shows spots indicating plasma cells isolated from granulation tissue bearing antibodies on their surface specific for *P. intermedia*. As measured by the ELISPOT technique. (Magnification x10)

Figure 5.3b

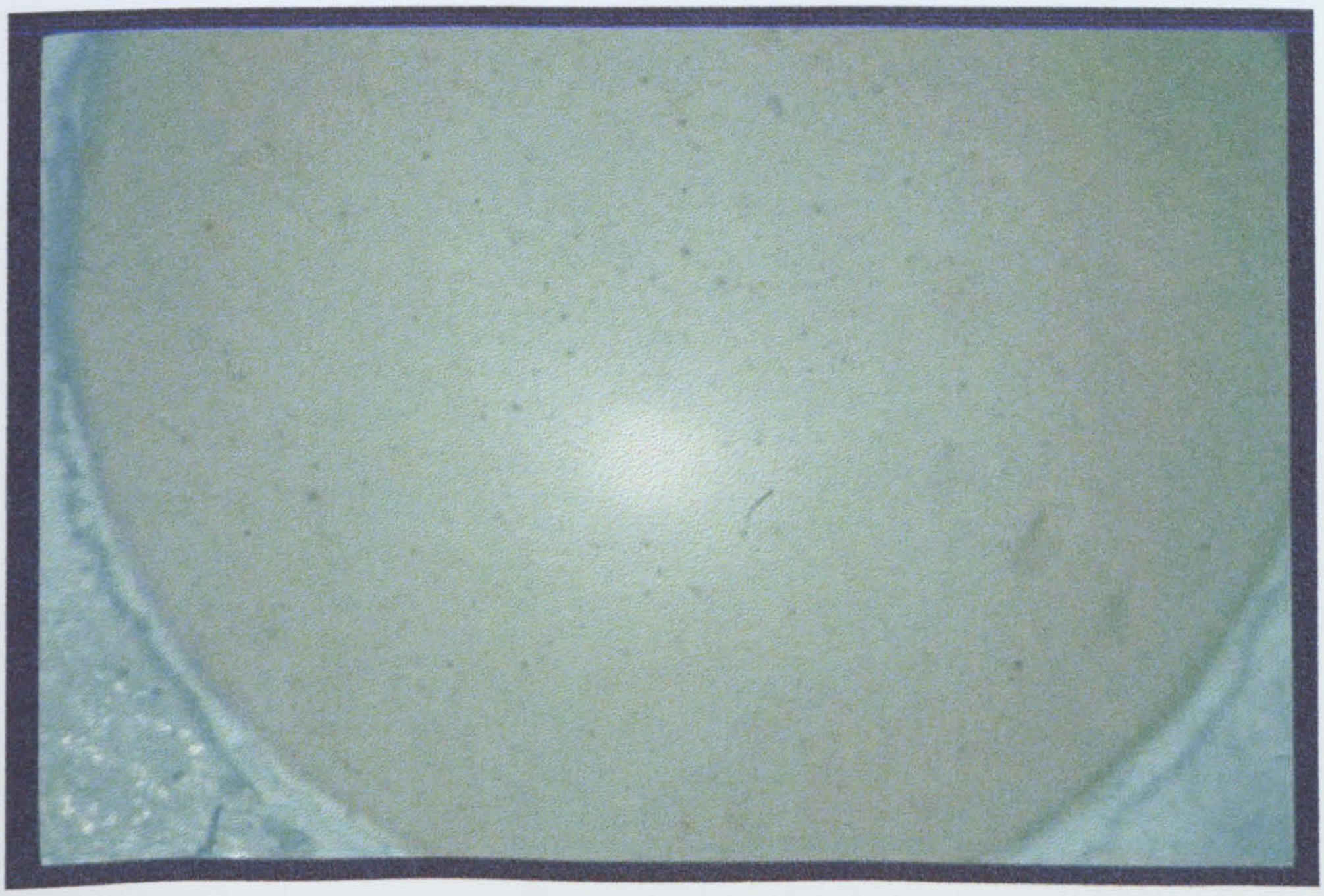


Figure 5.3b. Negative control for *P. intermedia*. (Magnification x10)

Figure 5.4a

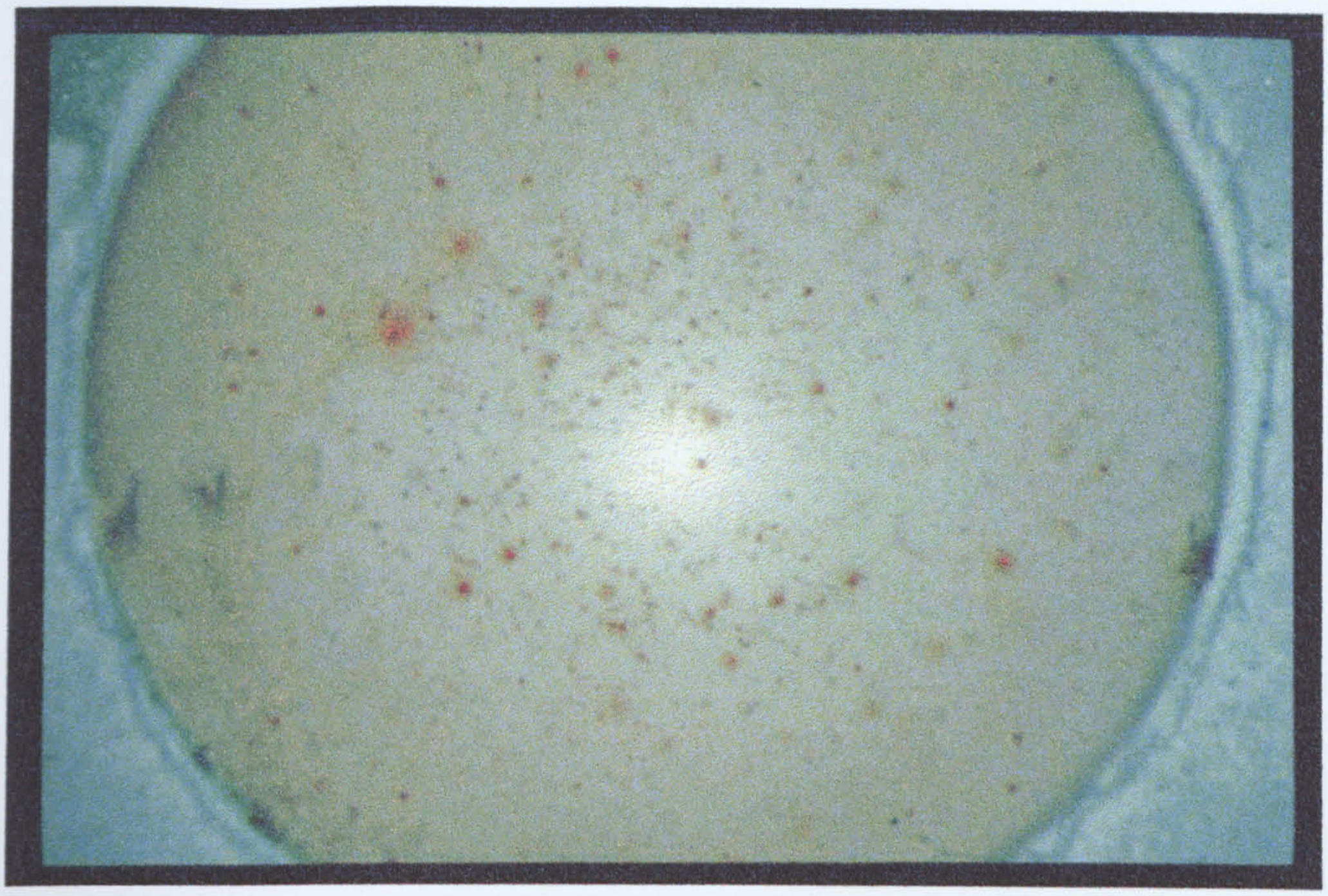


Figure 5.4a shows spots indicating plasma cells isolated from granulation tissue bearing antibodies on their surface specific for *B. forsythus*. As measured by the ELISPOT technique. (Magnification x10)

Figure 5.4b

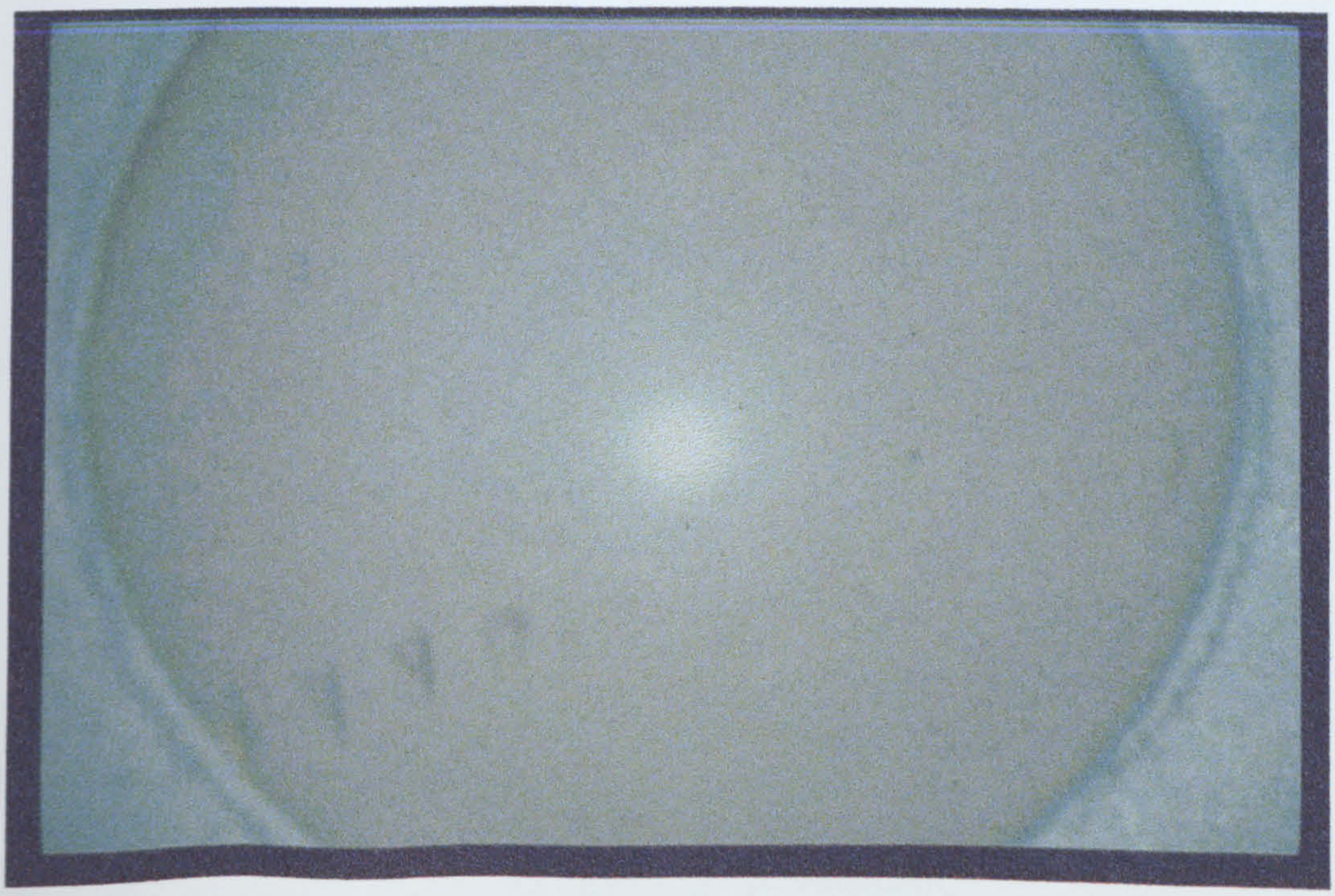
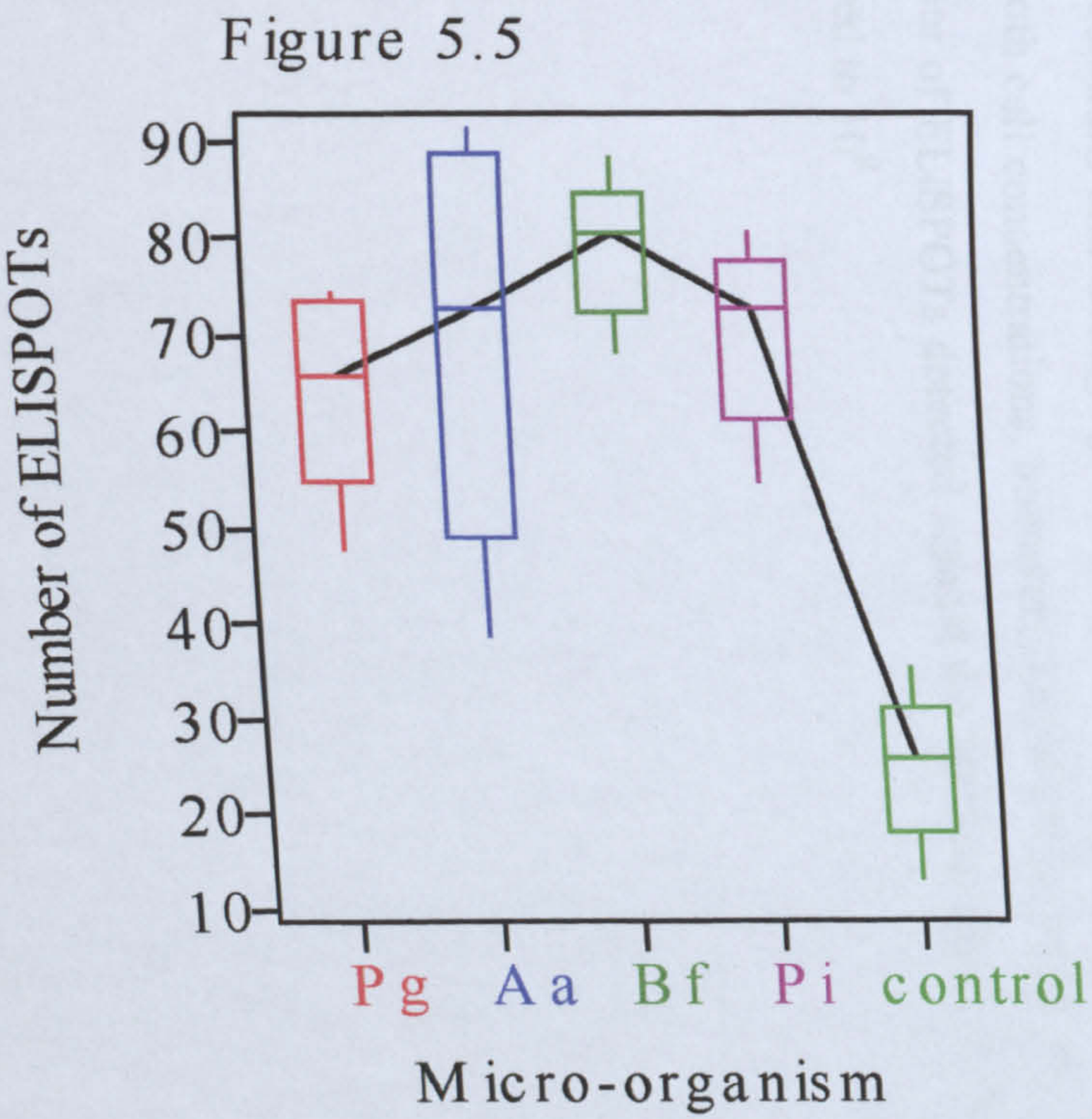
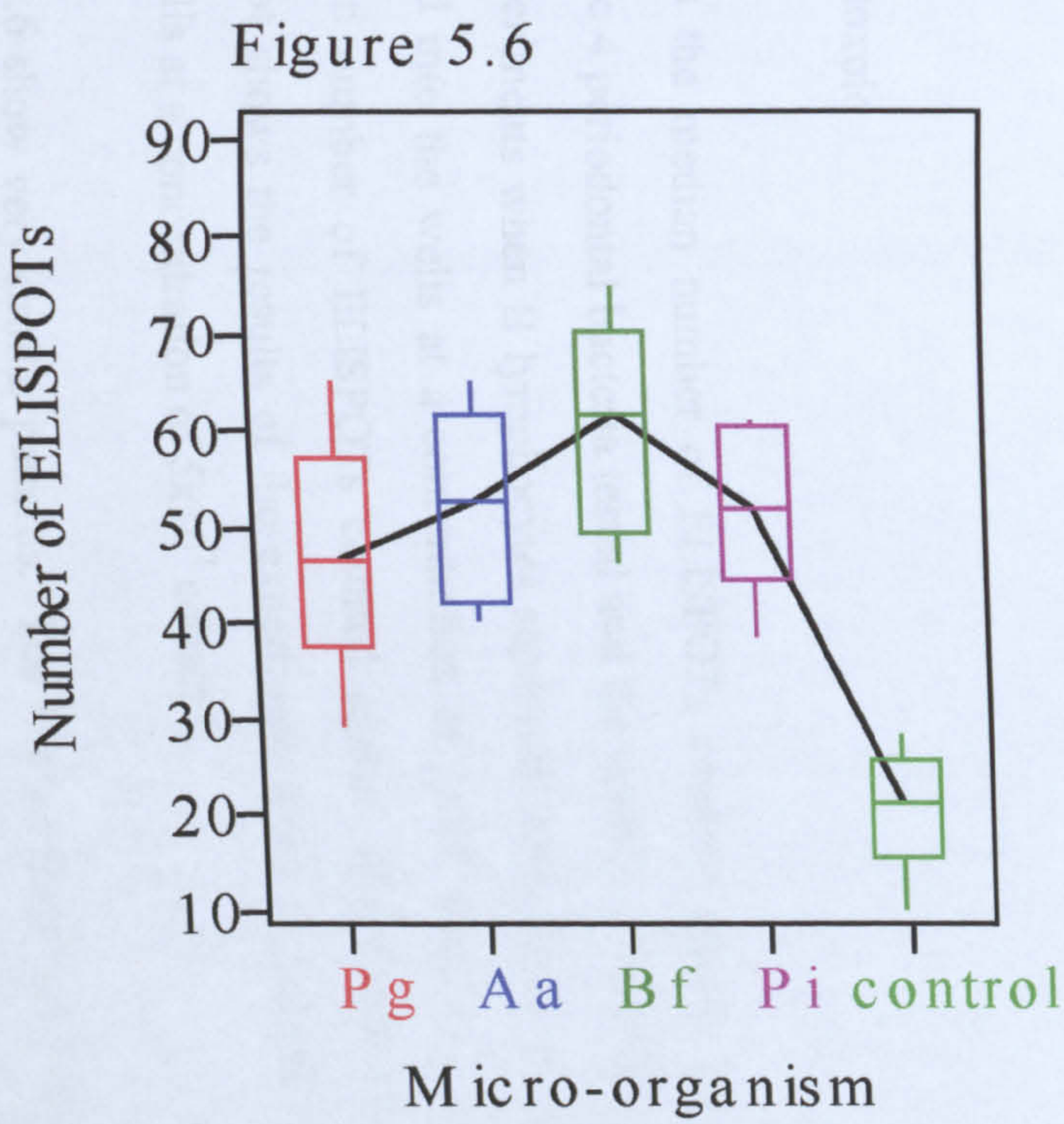


Figure 5.4b. Negative control for *B. forsythus*. (Magnification x10)

Figure 5.5 Repeated measures boxplot for the addition of 10^8 B lymphocytes.

Figure 5.6 Repeated measures boxplot for the addition of $5x10^7$ B lymphocytes





Pg - *P. gingivalis*

Aa - *A. actinomycetemcomitans*

Bf - *B. forsythus*

Pi - *P. intermedia*

control - Tetanus toxoid

Figure 5.5 shows the median number of ELISPOTs counted against the sonicate preparations of the 4 periodontal bacteria tested and the control. This plot shows the results of the experiments when B lymphocytes separated from diseased granulation tissue were added into the wells at a concentration of $1x10^8$ cells/ml. Figure 5.6 shows the median number of ELISPOTs counted against the 4 bacteria and the control. This plot shows the results of the experiment when B lymphocytes were added into the wells at a concentration of $5x10^7$ cells/ml.

Figures 5.5 and 5.6 show very similar patterns. The line on the graphs indicates the median values. There is a pattern that can be seen that indicates that the highest number of ELISPOTs is detected against *B. forsythus*, increasing from *P. gingivalis*, *A. actinomycetemcomitans* and falling again for *P. intermedia*. The patterns seen are very similar for both cell concentrations, however, the pattern is suggesting that there are a lower number of ELISPOTs detected against the antigens when $5x10^7$ cells/ml are added compared to 10^8.

Table 5.1 To show the median number of ELISPOTs enumerated against the periodontal bacteria tested

	Median (10^8)	Interquartile Range	Range	p-value of Friedman's test*
Pg	66.1	54.8 - 74.0	48.0 - 74.5	0.01
Aa	73.0	49.0 - 89.0	39.0 - 91.5	
Pi	73.0	61.5 - 78.0	55.0 - 81.0	
Bf	81.0	72.6 - 84.8	68.6 - 88.5	
Control	25.5	17.7 - 31.0	13.0 - 35.0	

	Median ($5x10^7$)	Interquartile Range	Range	p-value of Friedman's test*
Pg	46.50	37.5 - 57.3	29.5 - 65.0	0.007
Aa	53.00	42.0 - 61.8	40.5 - 65.0	
Pi	52.00	44.5 - 60.5	39.0 - 61.0	
Bf	61.70	49.4 - 70.5	46.8 - 75.0	
Control	21.00	15.2 - 25.8	10.0 - 28.0	

* p-value for hypothesis of test of no difference in the number of ELISPOTs detected against different antigens for each cell concentration independently.

Table 5.2 To show the median difference in the number of ELISPOTs enumerated for the two different cell concentrations

	Median Difference (10^8 - $5x10^7$ cells/ml)	Interquartile Range of Differences	p-value* for test of median difference equal to 0
Pg	16.6	12.3 - 23.3	0.059
Aa	20.0	7.0 - 27.3	0.106
Pi	21.0	9.5 - 25.0	0.059
Bf	10.5	6.5 - 35.4	0.059
Control	3.0	2.0 - 6.5	0.059

* p-value for hypothesis test of no difference in the number of ELISPOTs detected against an antigen for two different cell concentrations.

Table 5.1 shows the median values for the number of ELISPOTs enumerated against the four periodontal pathogens and the control for the two different cell counts.

Table 5.2 shows the median difference in the number of ELISPOTs counted for the two different cell concentrations added.

Statistical analysis of the results using the Friedman's test was carried out for the 2 different concentrations of cells separately. Both showed p-values of < 0.05. This indicates that there are differences in the numbers of ELISPOTs detected between at least 2 micro-organisms for both cell concentrations. On observation of the results in tabular form, it appears that the median number of cells detected for the control tetanus toxoid is lower than the median number of ELISPOTs detected against any of the periodontal pathogens, as expected. The graphical representation of the results suggests that the trend for the number of ELISPOTs detected against the micro-organisms when 10^8 cells/ml are added is very similar to the trend when $5x10^7$ cells/ml are added. By observing figures 5.5 and 5.6 it does seem that the number of cells detected when $5x10^7$ cells/ml are added is lower than when 10^8 cells/ml are added. This is suggested by the shift that is seen in the boxplots, i.e. the boxplot for $5x10^7$ cells/ml has shifted down compared with the one for 10^8 cells/ml.

Further analysis was carried out using a follow-up multiple comparisons procedure (Daniel, 1978). The results indicated that when 10^8 cells/ml were added, there is a significant difference between the number of ELISPOTs detected against *A. actinomycetemcomitans* and tetanus toxoid and between *B. forsythus* and tetanus toxoid. The analysis also suggested that when $5x10^7$ cells/ml were added there was a significant difference between the number of ELISPOTs detected against *A. actinomycetemcomitans* and tetanus toxoid and between *B. forsythus* and tetanus toxoid. These were the only significant differences found.

The results of the Wilcoxon Signed Rank test on the paired differences in the number of ELISPOTs detected between the 2 different concentrations of B lymphocytes used indicated that there was a borderline significant difference between the number of ELISPOTS detected for *P. gingivalis*, *B. forsythus*, *P. intermedia* and tetanus toxoid.

There was no significant difference in the number of ELISPOTs detected when 10^8 and $5x10^7$ cells/ml were added against *A. actinomycetemcomitans*.

5.4 Discussion

The ELISPOT technique is a cell-based immunoassay, which means that various inhibitors present in biological fluids, such as serum, are absent and hence cannot interfere. Further to this, the quantification of this method is based on cell numbers, and not arbitrary values, which are much more easily understood. Differences between high and low antibody secretors can also be disregarded with this method.

The ELISPOT was used to examine the number of antibody secreting cells specific to putative periodontal pathogens. The cells were isolated from granulation tissue taken from individuals with chronic periodontitis. The number of cells bearing antibody to *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus* and tetanus toxoid were enumerated.

Tetanus toxoid was used as a control, to ensure that binding was due to specific interactions between antibodies on the surface of cells and the antigens. Tetanus toxoid was chosen after preliminary studies were carried out to ensure that the technique could be undertaken with this particular antigen preparation. The antibody-bearing cells being utilised in this study were isolated from granulation tissue taken from patients with chronic periodontitis. Granulation tissue is highly vascularised tissue, full of inflammatory infiltrate. This area of tissue is very rich in immune cells. It has been reported that at least 50% of the cells in chronic periodontitis lesions are plasma cells (Kinane and Lindhe, 1997). One would suppose that the majority of the antibody-bearing cells found in the tissues are specific for periodontal micro-organisms associated with the disease. Therefore, the reason for choosing tetanus toxoid as the control is because there should be very few antibody-bearing cells specific for *Clostridium tetani* in a periodontitis lesion. Blood vessels are present in the tissues, and since most individuals have immunity to tetanus toxoid, it provides a useful control in discounting the fact that we may be merely measuring cells from the circulation and that the interactions are specific to the tissues.

The median number of spots against tetanus toxoid enumerated were 25.5 and 21.0 for 10^8 cells/ml and $5x10^7$ cells/ml respectively. Whether these spots are formed due to a small number of cells present in the periodontal tissues specific for or cross-reacting with tetanus toxoid, or that they represent non-specific background binding, is not clear. In addition, *Clostridium tetani* is a Gram negative bacillus, so the formation of spots due to cross-reactivity of antibodies cannot be disregarded.

The results suggest that there is no significant difference between the number of antibody-bearing cells in the granulation tissue against the 4 periodontal pathogens tested. In addition, the statistical analysis indicates that there is only a significant difference between the control and *A. actinomycetemcomitans* and *B. forsythus*. This is very difficult to accept because when the median values are observed, it does appear that there is a large difference between the number of ELISPOTs enumerated against the periodontal pathogens and tetanus toxoid. Further to this, one would certainly expect there to be a difference between the number of ELISPOTs detected against the periodontal pathogens and the number detected against the control. The reason for this is probably the small sample size. The multiple comparison analyses that were carried out, made 10 pairway comparisons based on 5 people. They also compared sum of ranks instead of median values. In addition, the data range is wide, which cannot be corrected for with a sample size of 5.

Therefore, although the statistical analysis does not indicate that there is a significant difference, it can be seen from table 5.1 that there does appear to be a difference between the numbers of ELISPOTs detected against the periodontal pathogens and the control tetanus toxoid. This suggests that a significant proportion of the ELISPOTs enumerated for the periodontal pathogens which are due to specific binding and are not non-specific or due to another phenomenon like those detected against tetanus toxoid.

The statistical analysis indicates that there is a borderline significant difference between the number of ELISPOTs detected when 10^8 and $5x10^7$ cells/ml were added. The results indicate that for *P. gingivalis*, *B. forsythus*, *P. intermedia* and tetanus

toxoid, there are significantly more ELISPOTs enumerated when 10^8 cells/ml are added compared to when $5x10^7$ cells/ml are added. It is expected that there would be a greater number of ELISPOTs detected when a greater number of cells per well are added. Further evidence for this, as already discussed, is seen by the shift in the boxplots in figures 5.5 and 5.6, where there is an obvious shift to a decrease in the number of ELISPOTs enumerated when $5x10^7$ cells/ml were added compared to 10^8 cells/ml.

Although statistical analysis of the results is important and various differences were significant. It must be stressed that in this study positive results were obtained. ELISPOTs were detected against the 4 periodontal pathogens tested, indicating that antibody-bearing plasma cells specific to *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus* are present in the granulation tissue of the patients in this study. This indicates that there is a local immune response directed against these periodontal pathogens occurring in the chronic periodontitis lesion of these patients. The importance of this phenomenon is that the ELISPOT technique could be utilised to observe antibody specificity in the local immune response of this disease. It could further be used for studies examining other diseases, where tissue can be removed ethically and the antibody-bearing cells isolated for investigation. Examining the target of the local immune response in periodontitis patients may provide us with knowledge of the specific immune response and its target. Answers to these kind of enigmas would lead to research into a new era, in terms of vaccine development and diagnostic tools.

This study utilises the ELISPOT technique to examine the bacterial target of antibody-bearing cells isolated from the granulation tissue of patients with chronic periodontitis. The main limitation experienced with the use of the technique was the need for a high number of cells. Much of the literature describes the use of the ELISPOT technique in enumeration of antibody-bearing cells following immunisation. Cox *et al.* (1994) reported a study that examined the number of antibody-bearing cells specific for the influenza virus over a time period directly following immunisation. Eight blood samples were taken during the period up to 22 days following immunisation. The aim was to examine the time course of appearance

of influenza antibody secreting cells in the circulation of individuals following vaccination. The mean number of specific antibody-bearing cells in the circulation peaked as high as 606 ± 283 cells (mean ± S.D.) per 500,000 cells assayed. In the present study, it was not expected to obtain a sample containing such a high number of antibody-bearing cells.

Blood samples would not have been useful in this study, as the length of time since the onset of chronic periodontitis in the patients was unclear, but certainly it was much greater than the initial primary immune response stage, which would be a matter of weeks. The number of cells in the circulation bearing antibody specific for periodontal pathogens would have been low and the chance of getting even one "hit" would have been extremely small. For this reason granulation tissue samples were chosen.

Very little information exists regarding the use of the ELISPOT technique in the periodontal literature. A study by Kusumoto *et al.* (1993) identified *P. gingivalis* fimbriae specific antibody secreting cells in the lymphoid tissues of mice. Hirsch *et al.* (1988) utilised the ELISPOT technique to enumerate antibody secreting cells specific for types I-VI collagen in periodontal tissues. Ogawa *et al.* (1989b) analysed the distribution of IgM, IgG and IgA secreting cells in gingival tissues from subjects with periodontal disease using the ELISPOT technique. In addition, Ogawa and co-workers, have also carried out studies utilising the ELISPOT technique to investigate the number of antibody-bearing cells specific to fimbriae and LPS of *P. gingivalis* (Ogawa *et al.*, 1989a; Ogawa *et al.*, 1991).

Although those latter studies were investigating the target of the human response, most of the studies in the literature utilise the ELISPOT technique for other purposes. The aim of the current study was to use the technique to investigate the target of the immune response, specifically to look at the number of antibody-bearing cells specific to 4 putative periodontopathogens. Many preliminary studies were carried out to optimise different factors, including experiments to optimise the concentration of bacteria used for coating and for the number of lymphocytes added. Problems were

encountered when whole fixed bacterial preparations were utilised, and background staining was very high.

Future studies are justified in this area, and would include a similar study with a much larger subject number. In addition, the technique could be used to investigate the presence of antibody-bearing cells specific for purified antigens and eventually for single antigenic epitopes.

Chapter 6. Detection of Antibody-Bearing Plasma Cells in Periodontitis Granulation and Gingival Biopsy Tissue

6.1 Introduction

The central theme of this thesis is the humoral immune response and its important role in periodontal disease. Numerous investigations have focused on the immunological parameters of body fluids such a peripheral blood or GCF. However, there is much less literature on the local immune response at the site of the lesion. Furthermore, studies on serum antibody levels or characterisation of peripheral blood lymphocytes in patients, may not reflect the activity of cells present in the periodontal lesion and gingival tissues.

Histological studies on inflammatory periodontal lesions were first published as early as the start of the last century (Zamensky, 1902; Hopewell-Smith, 1911). With the advent of improved microscope technology during the 1920's more detailed histopathological analysis of these tissues were reported (Box, 1924; James and Counsel, 1927; Gottlieb, 1927; Becks, 1927). Much work has been carried out since these early studies, and as laboratory techniques have been developed the knowledge of the disease histopathology has grown. It may be noted however, that the early structural observations are still considered accurate by many investigators.

The major pathological features of periodontal diseases are: (i) apical migration of epithelial attachment; (ii) an accumulation of inflammatory infiltrate within the periodontal pocket tissues; (iii) breakdown of connective tissue fibres anchoring the root to alveolar bone; and (iv) resorption of the marginal portion of the alveolar bone which eventually results in bone loss. The clinical and histopathological stages of health to periodontal disease were systematically defined originally by Page and Schroeder (1976), who developed a classification system to categorise the different stages of the disease process. However, much of this work was carried out on animal and adolescent biopsies, so for this reason the following discussion will use the more

recent system published by Kinane and Lindhe (1997), which is based on that of Page and Schroeder.

The system reported by Kinane and Lindhe (1997) classifies the disease progression from health to advanced periodontal disease. The classification system includes: the pristine gingiva (Figure 1.2), which is histological perfection; the normal healthy gingiva (Figure 1.3); early gingivitis (Figure 1.4); established gingivitis (Figure 1.5); and periodontitis (Figure 1.6).

The pathogenesis of the different stages are discussed elsewhere in this thesis. Of importance to this study is the histopathogenesis of periodontal disease. It is accepted that gingival tissues removed from individuals suffering from inflammatory periodontal disease, contain large numbers of lymphocytes and plasma cells, which are found in additional numbers with increasing severity of periodontal disease (Zachinsky, 1954; Brill 1960; Zachrisson, 1968; Payne *et al.*, 1975). Much work has been carried out to enumerate the plasma cells present in diseased tissues from patients with advanced periodontal disease, as compared with the number of lymphocytes present. The results of work carried out by different investigators are not in agreement, and do not present a clear picture.

Lindhe *et al.* (1973) reported that the cellular infiltrate of diseased tissues is predominantly made up of plasma cells. Wittwer *et al.* (1969) and Angelopoulas (1973) reported that the number of plasma cells was equivalent to or in some cases was exceeded by, the number of lymphocytes. Platt *et al.* (1970) reported that there are approximately 5 times more lymphocytes than plasma cells present. Mackler *et al.* (1977) carried out a study to try and define the relative distribution of lymphocytes and plasma cells by investigating 3 different types of tissues. The tissues were gingival biopsies taken from areas characterised as clinically normal, mild gingivitis and periodontitis. Mackler *et al.*, (1977) reported a difference in cellular profile between the mild gingivitis biopsies and those from periodontitis sites. The cellular profile of the gingivitis biopsies was characterised by non-immunoglobulin-bearing lymphocytes and very few plasma cells. Therefore, the non-immunoglobulin-bearing lymphocytes could either be T lymphocytes or immature B lymphocytes or a

combination of both (MacDermott *et al.*, 1975). In contrast to the cellular profile from the gingivitis biopsies, Mackler *et al.* (1977) reported that the cellular profile of the periodontal tissue biopsies was different, and mainly consisted of immunoglobulin-bearing lymphocytes and plasma cells. The number of immunoglobulin-bearing lymphocytes exceeded the number of plasma cells.

More recently Liljenberg *et al.* (1994), compared plasma cell densities in sites with active progressive periodontitis, and in sites with deep pockets and gingivitis but no significant attachment loss over a 2 year period. The density of plasma cells (51.3%) was significantly increased in active sites compared to inactive sites (31%), indicating that plasma cells are more dominant in the advanced lesion.

Although studies investigating the numbers of immunoglobulin-bearing lymphocytes and plasma cells in the tissues seem to be numerous, it is important to consider the function and target of these cells. The products of B cells and plasma cells in the tissues include antibodies which may be active against components and metabolites of periodontal pathogens (Brandtzaeg, 1966), which may result in immunopathology and tissue destruction (Carvel and Carr, 1982; Genco *et al.*, 1974). Further to this, examination of plasma cells and their targets in the diseased tissues, which are specific to the infection and have undergone affinity maturation and migration to the site, may lead us to a clearer idea of the more important pathogens and immune response inducing antigens.

Several studies using a variety of techniques to investigate local defence mechanisms and their specificity have been carried out over the last few decades.

Schneider *et al.* (1966) carried out a study to demonstrate that there is a local defence mechanism, present in gingival tissues, of adults with marginal gingivitis and periodontitis against bacteria present in the adjacent sulcus or pocket to the site of the tissue taken. Firstly, bacterial samples were taken from the sulci of inflamed gingiva and placed in acridine orange dye. Immediately following this, gingival biopsies were taken from the same area as the bacterial sample had been taken. Frozen sections of the biopsies were adhered to glass slides by the warmth of a finger. This avoided the

use of tissue adherent that could interfere with the analysis process. The first investigation carried out was of the reaction of one of the sections from each biopsy with fluorescein conjugated rabbit anti-human globulin globulin. This was to demonstrate specific staining of immunoglobulins in these diseased gingival tissues. Intense fluorescence was seen predominantly in the connective tissue. Staining was also seen within the cytoplasm of the perivascular plasma cells. The second part of the study utilised further serial sections from the biopsies. The acridine orange stained bacterial suspensions, prepared at the beginning of the study, were added to each section and incubated, before washing and the addition of rabbit anti-human globulin globulin conjugated to fluorescein isothiocyanate. Some of the sections were stained firstly with the fluorescein conjugated rabbit anti-human globulin globulin and then allowed to incubate with the acridine orange stained bacteria. The results showed that the bacteria reacted with the tissues in areas of high antibody concentration, indicating specific antibody-antigen reactions. The specificity was confirmed by the fact that there were also reactions seen between gingival tissues taken from one individual and the bacteria taken from another. Once the gingival tissues had been reacted with rabbit anti-human globulin globulin the bacterial reactions were inhibited as the antigen-antibody sites were blocked. This provided evidence that the binding of the bacteria to the tissues was specific. In most of the sections observed, individual plasma cells were also seen to be reacting with bacteria. The bacteria could be observed as single cells lined up around the periphery and overlying the cytoplasm of plasma cells. Although, this study showed that there are specific interactions between antibodies present in the tissues and antigens, the specificity of the bacteria and bacterial targets was not investigated. Further to this, the bacterial sample taken from the sulcus may not have contained any of the "relevant" or putative periodontal pathogens. Many antibodies could be present in the tissues that have migrated in with the exudative fluid during the inflammatory reaction. They may not be specific for the bacteria added for the experiment and may have bound to non-specific moieties present on the surface of the bacteria such as lectins. Such interactions are known to occur between some strains of *E. coli* and murine lymphocytes (Mayer *et al.*, 1978). The fact that bacterial samples taken from other individuals reacted, and that the staining in the serial sections always seemed to

be in exactly the same area, even though the bacterial samples were heterogeneous, appears to further indicate this.

Periodontal disease is a chronic disorder which is multifactorial, and there are a number of pathogens, with similar antigenic moieties that play a role in the induction of the immune response. The gingival tissues and periodontium are anatomically distinct and whether the immune response involved in periodontal lesions is mucosal, systemic or both is unclear.

Two years later Schonfeld and Kagan (1982) determined the percentage of plasma cells in initial and advanced lesions, from sections of gingival tissue biopsies taken from periodontitis patients, that were directed against *A. viscosus*, strain ATCC 27044 and T14-V, *P. gingivalis* and *A. actinomycetemcomitans*. The methodology used in this study was as theirs described previously (Schonfeld and Kagan, 1980). The results suggested an important role for *P. gingivalis* in the pathology of periodontal disease. In every tissue section studied, 10 initial lesions and 16 advanced periodontal lesions, at least 50 plasma cells were located. There was no significant difference in the binding of *A. viscosus* or *A. actinomycetemcomitans* between initial or advanced lesions, and the binding of these pathogens to specific plasma cells was quite low. However, the binding of *P. gingivalis* was seen in all of the advanced lesions, but only in 40% of the initial lesions, and a much higher number of plasma cells in the advanced lesions bound *P. gingivalis*. The low specificity towards the other putative pathogens tested remained unclear. It could be that the antigens associated with these particular bacteria are not powerful immunogens. The results obtained from this study seem to emphasise the importance of *P. gingivalis* in the pathogenesis of periodontal disease. Numerous other studies have indicated that patients with chronic periodontitis possess high titres of serum antibody to *P. gingivalis* (Slots and Listgarten, 1988; van Winkelhoff *et al.*, 1988a; Moore *et al.*, 1991; Mouton *et al.*, 1981), therefore, it seems that the specificity of the systemic immune response is reflected locally (or vice versa).

The aim of the present study was to determine the specificity and numbers of plasma cells for bacterial antigens present in gingival and granulation tissue sections taken

from individuals with chronic periodontitis. The number of specific plasma cells in gingival and granulation tissue sections were enumerated for *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *B. forsythus* and *T. denticola*. The number of plasma cells that bound *E. coli* were also enumerated as a control to investigate the phenomenon of natural or non-immunologically mediated binding. The experiments were further carried out with sonicate preparations of *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus*, and the purified antigens leukotoxin (of *A. actinomycetemcomitans*), HSP75 (of *P. gingivalis*) and the β segment of the RgpA protease (of *P. gingivalis*). The specificity of the interactions between the bacteria and the plasma cells was demonstrated by control studies where a blocking step was included which consisted of the addition of a vast excess of fab fragments of goat anti-human IgG.

The methodology required for this study was challenging to develop because of the need to counteract and discount any binding through the Fc portions of antibodies with any other cell types present, and ensure that all binding detected was specifically with the Fab portions of antibodies. It was novel and different from previous studies because of the need to use Fab fragments to blockade the specific interactions between labelled bacteria and the antibody secreting cells. The use of a biotin system, with its inherent amplification, means that there is no requirement for the use of fluorescence microscopy, making it a much easier, sensitive and quicker method.

6.2 Methods

6.2.1 Preparation of Fab fragments of goat anti-human IgG

$F(ab)_2$ fragments of goat anti-human IgG were obtained and digested with Papain. 0.5ml of 1mg/ml $F(ab')_2$ fragments were incubated with 5μg of Papain contained in 125μl of beaded agarose. The incubation was carried out for 30 minutes at 37°C with agitation. Centrifugation was then carried out at 6000 rpm for 5 minutes and the supernatant removed and transferred to a sterile tube, and stored at 4°C until use.

6.2.2 Subjects and samples

Granulation and gingival tissue specimens were obtained from 5 patients attending Glasgow Dental Hospital. The criteria for inclusion in the study was that they were to be suffering from advanced chronic periodontal disease and were receiving surgery as part of their periodontal therapy. During surgery, carried out by an experienced periodontist, granulation and gingival tissue was removed. The tissue was immediately fixed in 10% buffered formaldehyde at room temperature and subsequently processed and embedded in paraffin wax. Serial sections, 4μm thick were prepared on silane-coated glass slides using haematoxylin and eosin staining before immunohistochemistry was undertaken.

The following 3 sections, 6.2.3, 6.2.4 and 6.2.5 are a description of the 3 different methodologies that were used during the development of the technique. The beginning of the development process was method 1 and changes were made and the technique progressed through methods 1 to 2 and eventually to 3. The results were obtained by method 3, however, a description of the development method protocols are included for completeness and so that the reader can form a good understanding of the development of the technique.

6.2.3 Method 1

6.2.3.1 Bacteria preparation.

P. gingivalis NCTC 11834, *P. intermedia* ATCC 25611, *B. forsythus* ATCC 43037 and *A. actinomycetemcomitans* ATCC 29523 (serotype A) were grown and fixed as described in 3.2.2. *T. denticola* ATCC 35405 were cultured in medium OMIZ-pat + 1% human serum, and were a gift from Dr.C.Wyss, Institut fur Orale Mikrobiologie, Zurich, Switzerland (Wyss *et al.*, 1997).

6.2.3.2 Immunohistochemistry

The sections were firstly deparafinised by immersion in Xylene x2, then in 99% Ethanol x2 and in 95% Ethanol all for 5 minutes each. The sections were then washed in distilled H_2O, and PBS x2, again for 5 minutes each. The sections were then treated with 3% Hydrogen peroxide (H_2O_2) in Methanol for 15 minutes. The sections were then washed in distilled H_2O for 5 minutes twice. The sections were then microwaved in 1mM EDTA (pH 8) for 5 minutes x3, and then allowed to cool to room temperature before being washed for 5 minutes in PBS.

The slides were wiped dry and 200μl of blocking serum was then layered onto each section and incubated for 20 minutes at room temperature. The blocking serum was then tipped off the sections and 200μl of fixed bacteria at a concentration of $1.5x10^8$ bacterial cells/ml diluted in blocking serum was layered onto all but one section and incubated for 1 hour at room temperature. For counting purposes the bacteria were stained using nigrosin and counted in a Neubauer chamber under oil immersion. The sections were then washed twice in PBS-T for 5 minutes followed by a wash for 5 minutes in PBS. The sections were then wiped dry and 200μl of Fab fragments of goat anti-human IgG was added at a concentration of 0.05mg/ml, to block any endogenous IgG, to all but one section (a different section from the minus antigen section) and incubated for 1 hour at room temperature.

A sample of human serum taken from a patient with untreated chronic periodontal disease which was obtained previously and stored at -20°C, was diluted 1/200 in blocking serum. The sections were washed twice in PBS-T for 5 minutes and then once in PBS, again for 5 minutes. 200μl of the diluted human serum was then layered onto each section and they were incubated at room temperature for 30 minutes. The sections were then again washed x2 in PBS-T and x1 in PBS, for 5 minutes each. 200μl of Biotin labelled Fab fragments of goat anti-human IgG at a concentration of 0.05μg/ml in PBS was then added to all sections and incubated at room temperature for 30 minutes.

A biotin avidin HRP complex mixture was then prepared and allowed to stand at room temperature for 30 minutes. The sections were then washed x2 in PBS-T for 5 minutes and then x1 in PBS, again for 5 minutes. 200μl of the Biotin avidin HRP complex mixture was then placed onto each section, and the slides were incubated at room temperature for 30 minutes. The sections were the washed x2 in PBS-T for 5 minutes and with PBS for 5 minutes.

Developing solution was then prepared, and 300μl was placed onto each section. The sections were incubated at room temperature for 2-7 minutes. The reaction was checked during this period by viewing the sections at low power under the microscope. When the reaction was complete the sections were washed in tap water several times and were then counter-stained in Mayers haematoxylin for 10 minutes. The sections were then washed several times in tap water and mounted in hot glycergel with a 22mm x 22mm cover slip.

6.2.4 Method 2.

6.2.4.1 Antigen preparation.

The bacteria were cultured and fixed as described in 3.2.2. Human serum taken from a patient suffering from adult periodontitis was diluted 1/100 in PBS and then taken and heat treated in 10mM EDTA for 30 minutes at 56°C. The fixed bacteria were then incubated with the heat treated serum for 30 minutes at 37°C. The incubated preparation was then micro-centrifuged at 7000 rpm for 3 minutes and then washed in PBS x2. The preparation was then used at 1/50 dilution in goat serum.

6.2.4.2 Immunohistochemistry

The sections were deparafinised and prepared as described in section 6.2.3.2

The slides were wiped dry and 200μl of blocking serum was layered onto each section and incubated for 20 minutes at room temperature. The blocking serum was tipped off and 200μl per section of the antigen preparation added to all of the sections except

one, and incubated for 1 hour at room temperature. The sections were again washed x2 in PBS-T and x1 in PBS, for 5 minutes each. 200μl of Biotin labelled Fab fragments of goat anti-human IgG at a concentration of 0.05μg/ml in PBS was added to all sections and incubated at room temperature for 30 minutes.

A biotin avidin HRP complex mixture was prepared and allowed to stand at room temperature for 30 minutes. The sections were then washed x2 in PBS-T for 5 minutes and then x1 in PBS, again for 5 minutes. 200μl of the Biotin avidin HRP complex mixture was placed onto each section, and the slides were incubated at room temperature for 30 minutes. The sections were washed x2 in PBS-T for 5 minutes and with PBS for 5 minutes.

Developing solution was then prepared, and 300μl was placed onto each section. The sections were incubated at room temperature for 2-7 minutes, and the reaction was checked during this period by viewing the sections at low power under the microscope. When the reaction was complete the sections were washed in tap water several times and were then counter-stained in Mayers haematoxylin for 10 minutes. The sections were washed several times in tap water and mounted in hot glycergel with a 22mm x 22mm cover slip.

6.2.5 Method 3

6.2.5.1 Antigen preparation

(a) Fixed bacteria.

The bacteria were cultured and fixed as described in section 3.2.2.

(b) Sonicates.

P. gingivalis, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus* were grown as described as above in 3.2.2. Sonicated bacterial preparations of these were prepared as described in 3.3.2.

The protein concentrations were determined using the Pierce BCA protein assay kit.

(c) Purified antigens

Leukotoxin of *A. actinomycetemcomitans*

leukotoxin of *A. actinomycetemcomitans* was purified as described in section 3.2.2.

The leukotoxin was a kind gift from Dr.J.Korostoff, Leon Levy Research Center for Oral Biology, University of Pennsylvania, Philadelphia, USA.

The β segment of the RgpA protease of *P. gingivalis*

Domains of the RgpA protease of *P. gingivalis* was prepared as previously described in section 3.2.2.

The β segment of RgpA which was used in this study was a gift from Professor M. Curtis.

HSP75 of *P. gingivalis*

HSP75 (of *P. gingivalis*) was purified and prepared according to the method described by Hinode *et al.* (1996). This antigen was a kind gift from Dr.D.Grenier, Université Laval, Quebec, Canada.

6.2.5.2 Biotinylation of bacteria and bacterial sonicates

25μg Biotinamidocaproate N-Hydroxysuccimimide ester (Biotin) was diluted into 1ml DMF, which was then further diluted 1:5 with 100mM phosphate buffer. 5mg of sonicated bacteria and $3x10^9$ fixed bacterial cells were mixed with 200μl of the Biotin solution and incubated at room temperature for 3-4 hours with agitation. The bacterial suspension was then centrifuged at 13,000 rpm for 3 minutes, washed x3 with PBS and stored at 4°C until used.

6.2.5.3 Biotinylation of purified antigens

100μg Biotinamidocaproate N-Hydroxysuccimimide ester (Biotin) was added to 20μg of purified antigen and dissolved in a final volume of 1ml of sterile H_2O. Dialysis tubing was boiled in 10mM EDTA for 10 minutes. This was repeated 3 times. The dialysis tubing was thoroughly rinsed in distilled H_2O. 1ml of biotin and antigen solution was then placed inside a piece of dialysis tubing that had been tied at both ends. The tubing was then placed inside a large beaker containing PBS with 10mM EDTA with constant stirring. This was incubated at 4°C for 12 hours. Following this the dialysis tubing was then placed into a fresh beaker containing only PBS and incubated at 4°C for a further 12 hours with constant stirring. This step was repeated a further 4 times. The contents of the dialysis tubing was removed after all the necessary incubations had been carried out, and placed into a 1ml microcap tube. Microcentrifugation was then carried out at 13,000 rpm for 10 minutes. The supernatant was removed and stored at 4°C until use. This method was used for biotinylation of the antigens leukotoxin (of *A. actinomycetemcomitans*), HSP75 (of *P. gingivalis*) and the β segment of the protease RgpA (of *P. gingivalis*).

6.2.5.4 Immunohistochemistry

The sections were deparafinised and prepared as described in section 6.2.3.2.

200μl of blocking serum was then layered onto each section of half the slides and incubated for 20 minutes at room temperature. Blocking serum containing fab fragments of goat anti-human IgG at a concentration of 0.05mg/ml was added to the other half of the slides and these sections were then also incubated at room temperature for 20 minutes. These slides were to act as negative controls. The blocking serum was then tipped off and 200μl of biotin labelled antigen was added to each section. Whole bacteria were added at a concentration of $3x10^7$ cells/ml, sonicate preparations at 100μg/ml and purified antigens at a concentration of 1μg/ml. Incubation was carried out for 1 hour at room temperature. The sections were then again washed x2 in PBS-T and x1 in PBS, for 5 minutes each.

A biotin avidin HRP complex mixture was prepared and allowed to stand at room temperature for 30 minutes. The sections were washed x2 in PBS-T for 5 minutes and then x1 in PBS, again for 5 minutes. 200μl of the Biotin avidin HRP complex mixture was placed onto each section, and the slides were incubated at room temperature for 30 minutes. The sections were then washed x2 in PBS-T for 5 minutes and with PBS for 5 minutes.

Developing solution was prepared, and 300μl was placed onto each section. The sections were then incubated at room temperature for 2-7 minutes. The reaction was checked during this period by viewing the sections at low power under the microscope. When the reaction was complete the sections were washed in tap water several times and were counter-stained in Mayers haematoxylin for 10 minutes. The sections were then washed several times in tap water and then mounted in hot glycergel with a 22mm x 22mm cover slip.

Method 3 was chosen to be used as it was the most successful, and hence is the method that was used for further experiments which will be reported subsequently.

6.2.6 Data collection and analysis

Sections were examined under a light microscope, and the presence of positively stained cells was confirmed by the appearance of cell surface brown staining with the morphology of human leukocytes. Six microscopic fields at x200 magnification were chosen for counting in each of the 5 sections for each micro-organism, for purified antigens and for each of the tissue types. Serial sections were chosen as far as possible for each different antigen used, so comparable fields could be identified and counted. Once cell counts were complete median and standard deviation values of the population were calculated.

The data was statistically analysed by the Friedman's test using the Minitab statistical package. This test was chosen as it is used to compare the number of cells specific for the different antigens when observations have been repeated on the same subjects. In

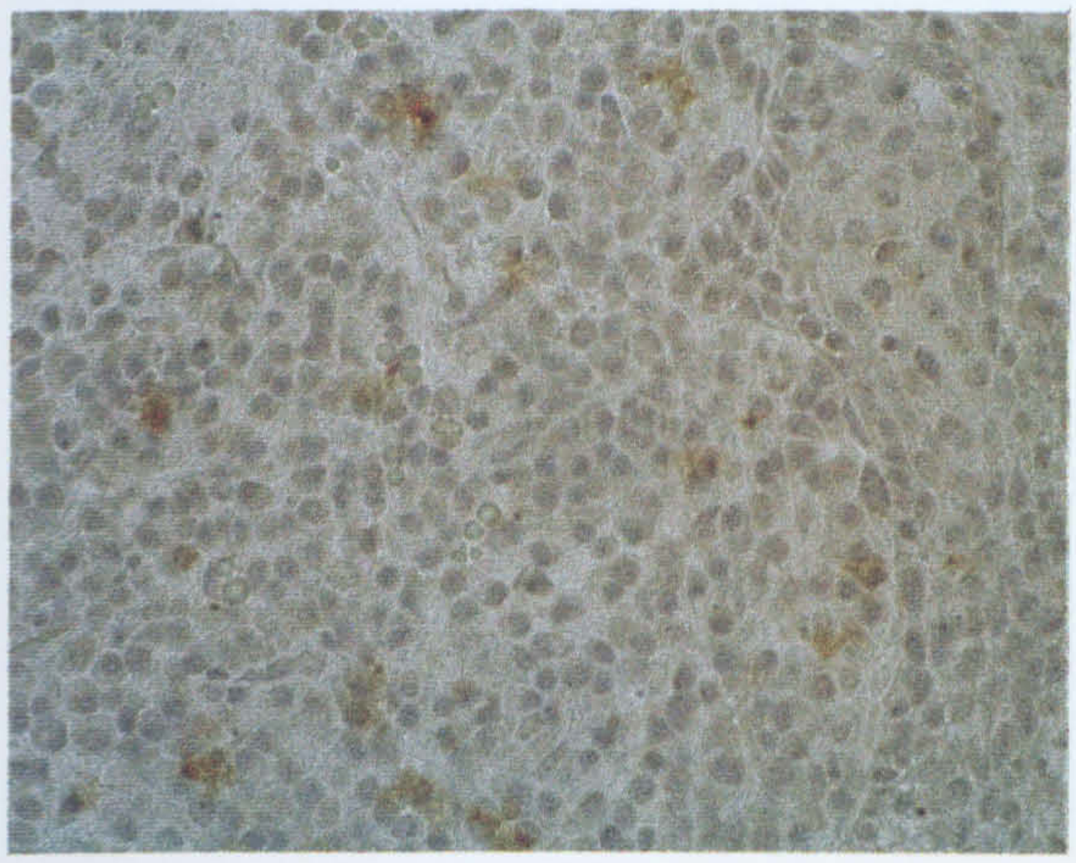
addition, this test makes no assumptions about the distribution of the data. It was not satisfactory just to reject the hypothesis and conclude that not all the cell counts were not identical, therefore, a multiple comparison procedure suitable for use after the Friedman's test was carried out (Daniel, 1978).

The Wilcoxon Signed Rank test was used to compare the median of the paired differences of the results for the granulation tissue samples minus the results from the gingival tissue samples. This test was chosen as it allowed the comparison of the differences (granulation tissue cell count for a particular antigen - gingival tissue cell count for the same antigen) for each antigen separately whilst still taking into account that the data has been collected from the same subjects.

6.3 Results

Figures 6.1-6.6 show examples obtained from experiments investigating the number of specific antibody-bearing cells against *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *B. forsythus*, *T. denticola* and *E. coli* whole fixed bacterial cells in granulation tissue sections. Figures 6.7-6.9 shows examples obtained from experiments investigating the number of specific antibody-bearing cells against HSP75 (of *P. gingivalis*), leukotoxin (of *A. actinomycetemcomitans*) and the β segment of RgpA (of *P. gingivalis*) in granulation tissue sections.

Figure 6.1a **Figure 6.1b**



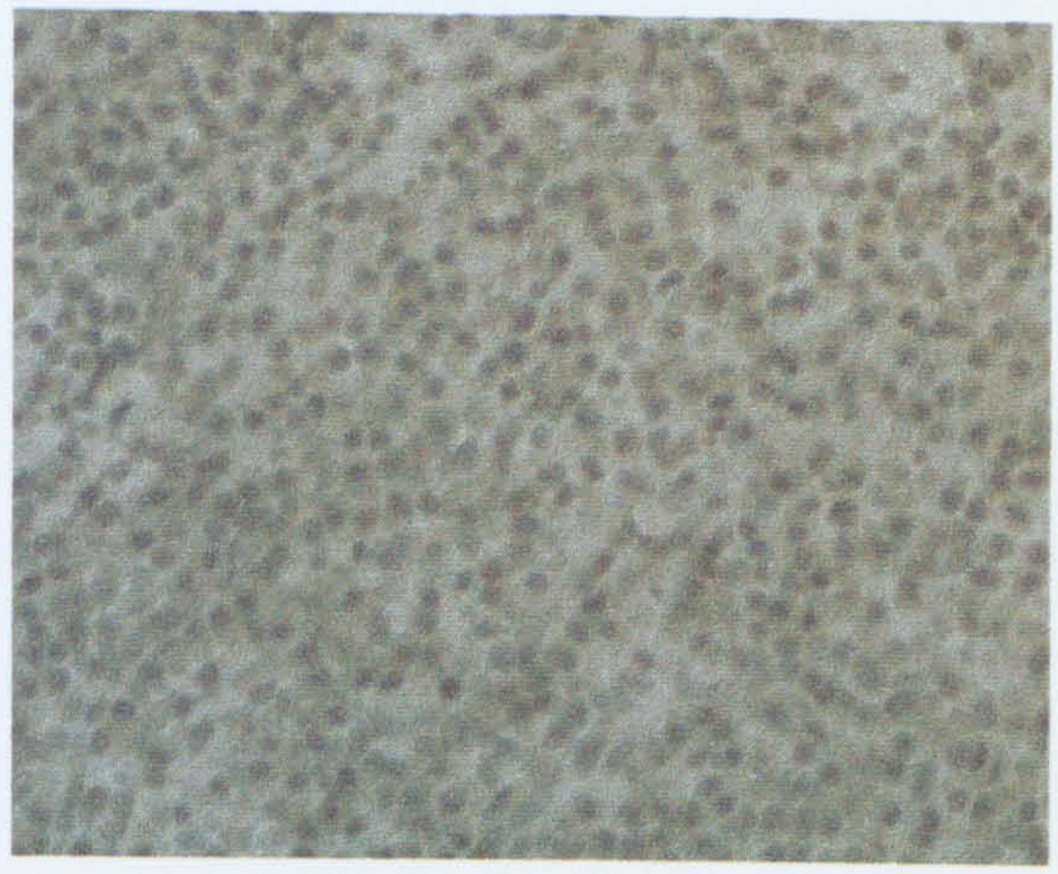
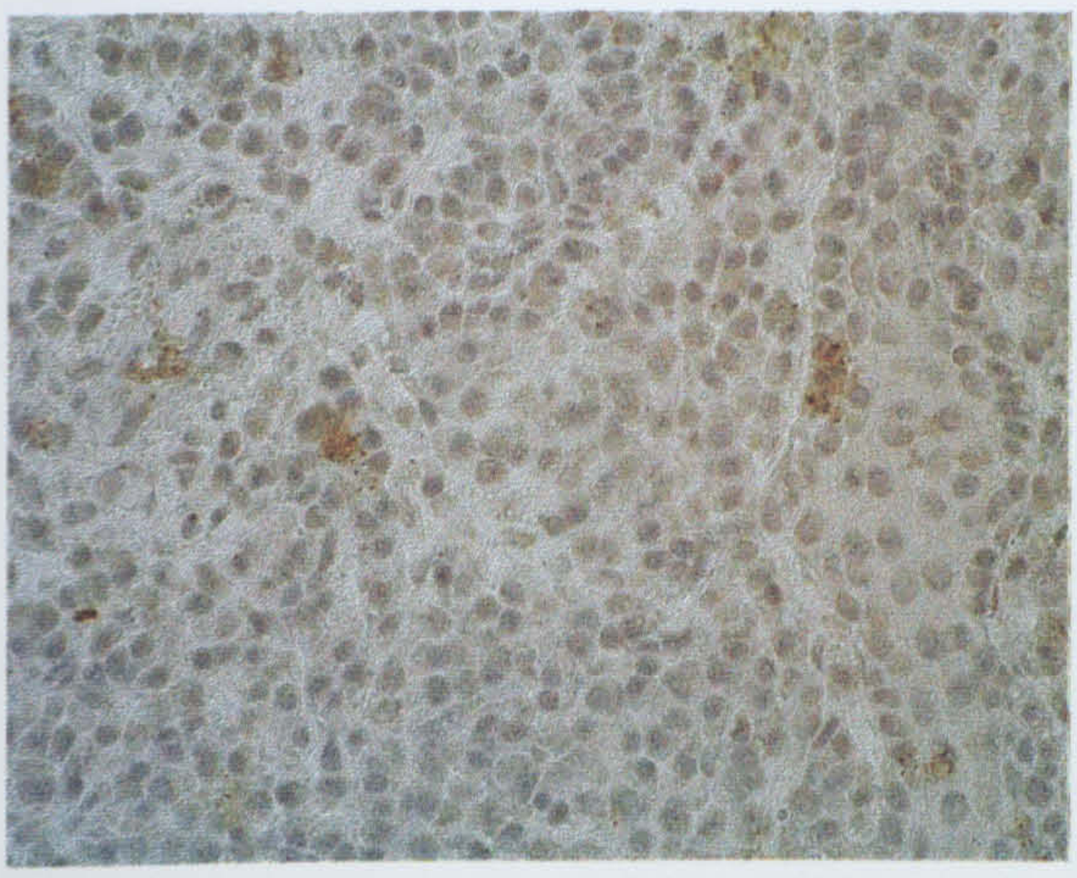


Figure 6.1a shows granulation tissue plasma cells bearing antibodies to *P. gingivalis*. Figure 6.1b shows the control, inhibition of specific *P. gingivalis* antibody interactions using Fab' fragments of goat anti-human immunoglobulin. (Magnification x200)

Figure 6.2a **Figure 6.2b**



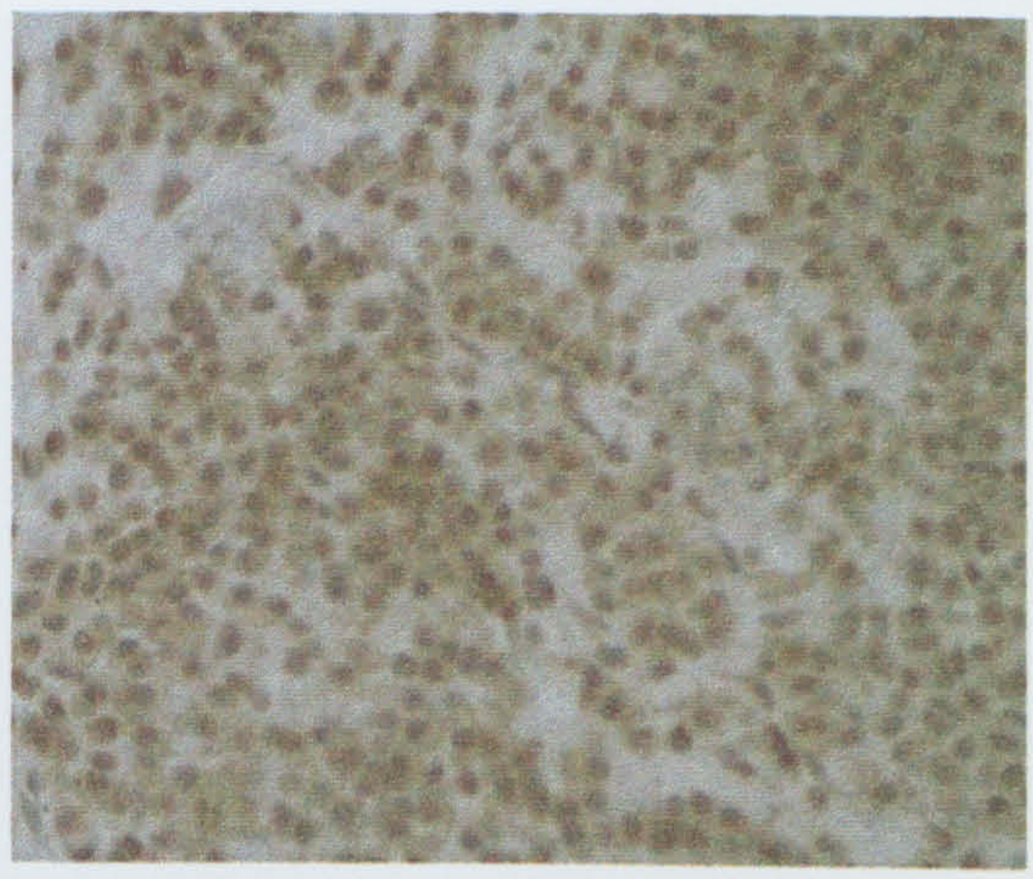


Figure 6.2a shows granulation tissue plasma cells bearing antibodies to *A. actinomycetemcomitans*. Figure 6.2b shows the control, inhibition of specific *A. actinomycetemcomitans* antibody interactions using Fab' fragments of goat anti-human immunoglobulin. (Magnification x200)

Figure 6.3a

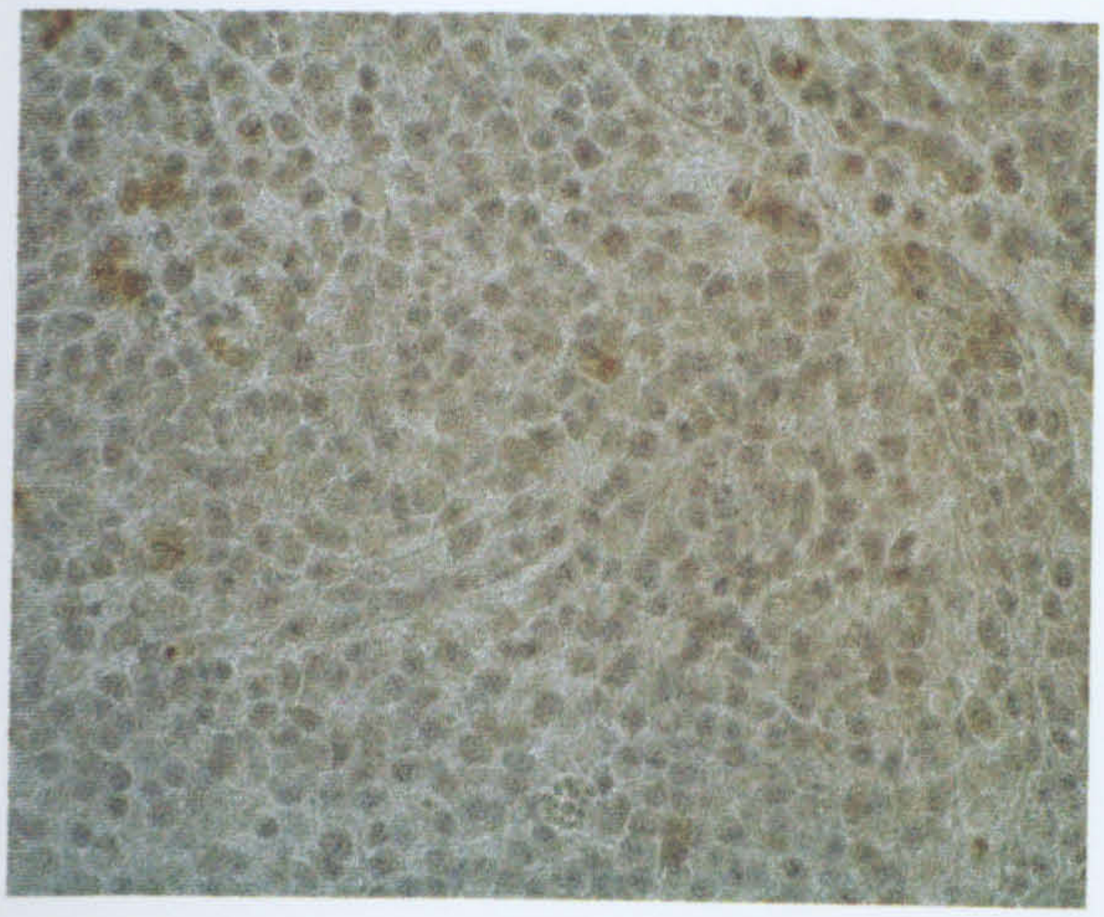


Figure 6.3b

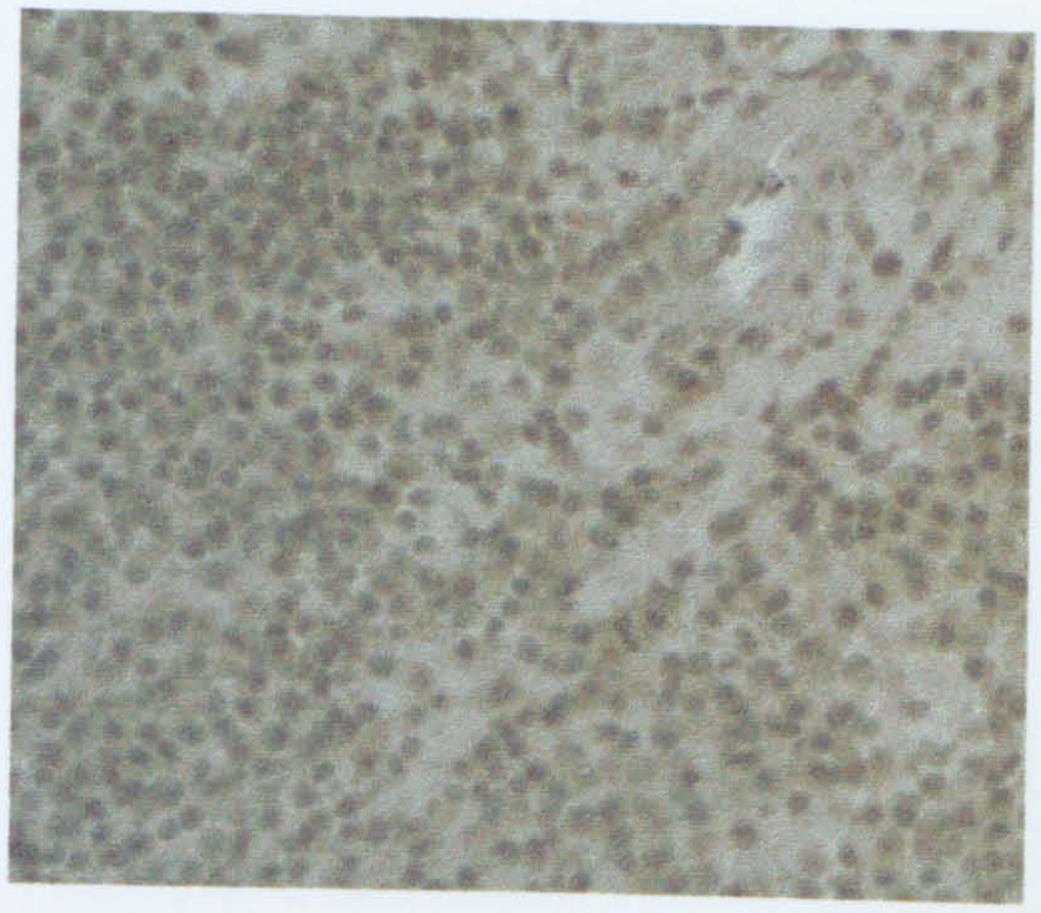


Figure 6.3a shows granulation tissue plasma cells bearing antibodies to *P. intermedia*. Figure 6.3b shows the control, inhibition of specific *P. intermedia* antibody interactions using Fab' fragments of goat anti-human immunoglobulin. (Magnification x200)

Figure 6.4a

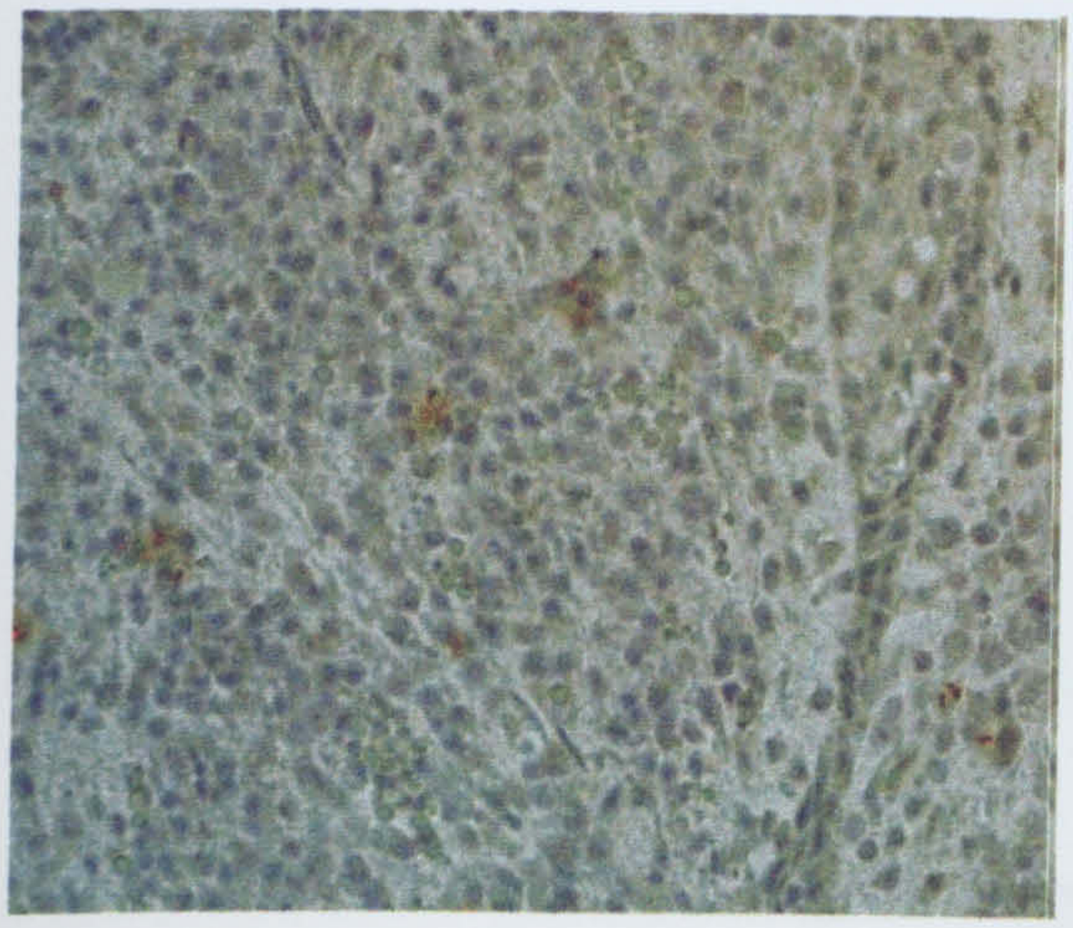


Figure 6.4b

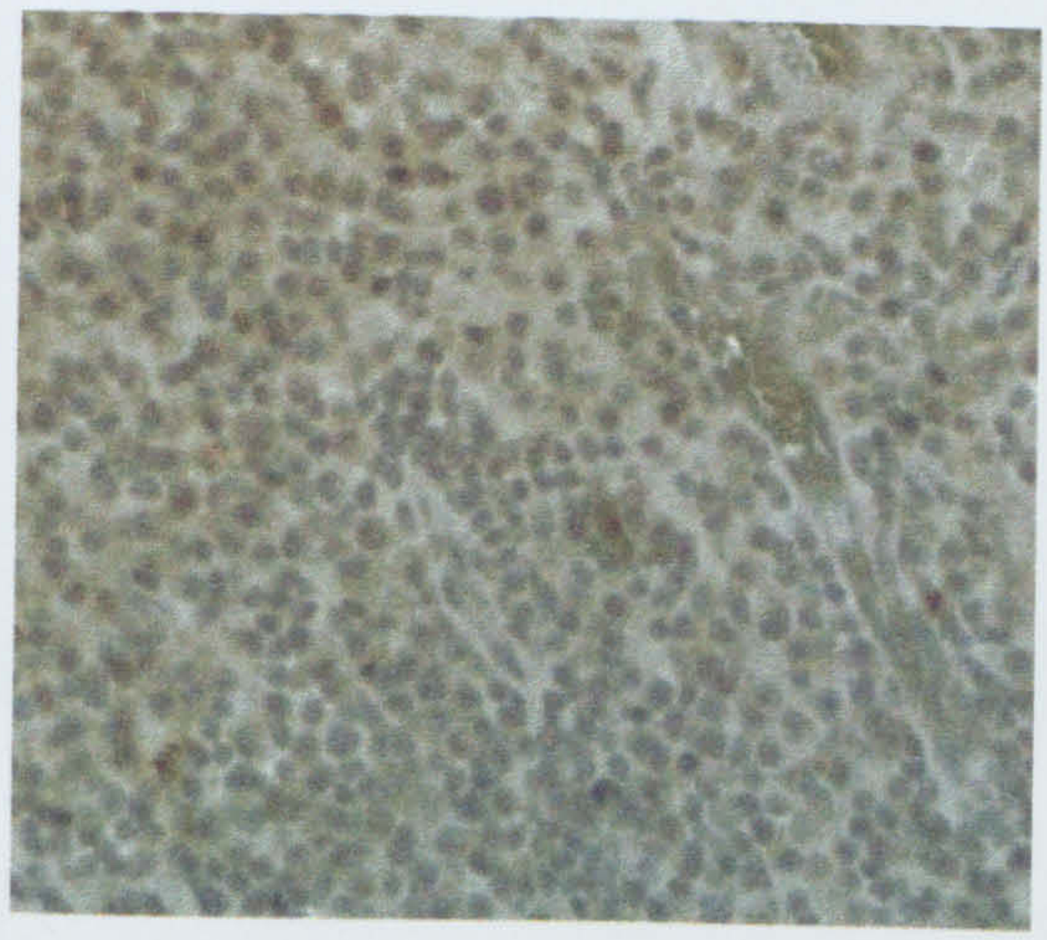


Figure 6.4a shows granulation tissue plasma cells bearing antibodies to *B. forsythus*. Figure 6.4b shows the control, inhibition of specific *B. forsythus* antibody interactions using Fab' fragments of goat anti-human immunoglobulin. (Magnification x200)

Figure 6.5a

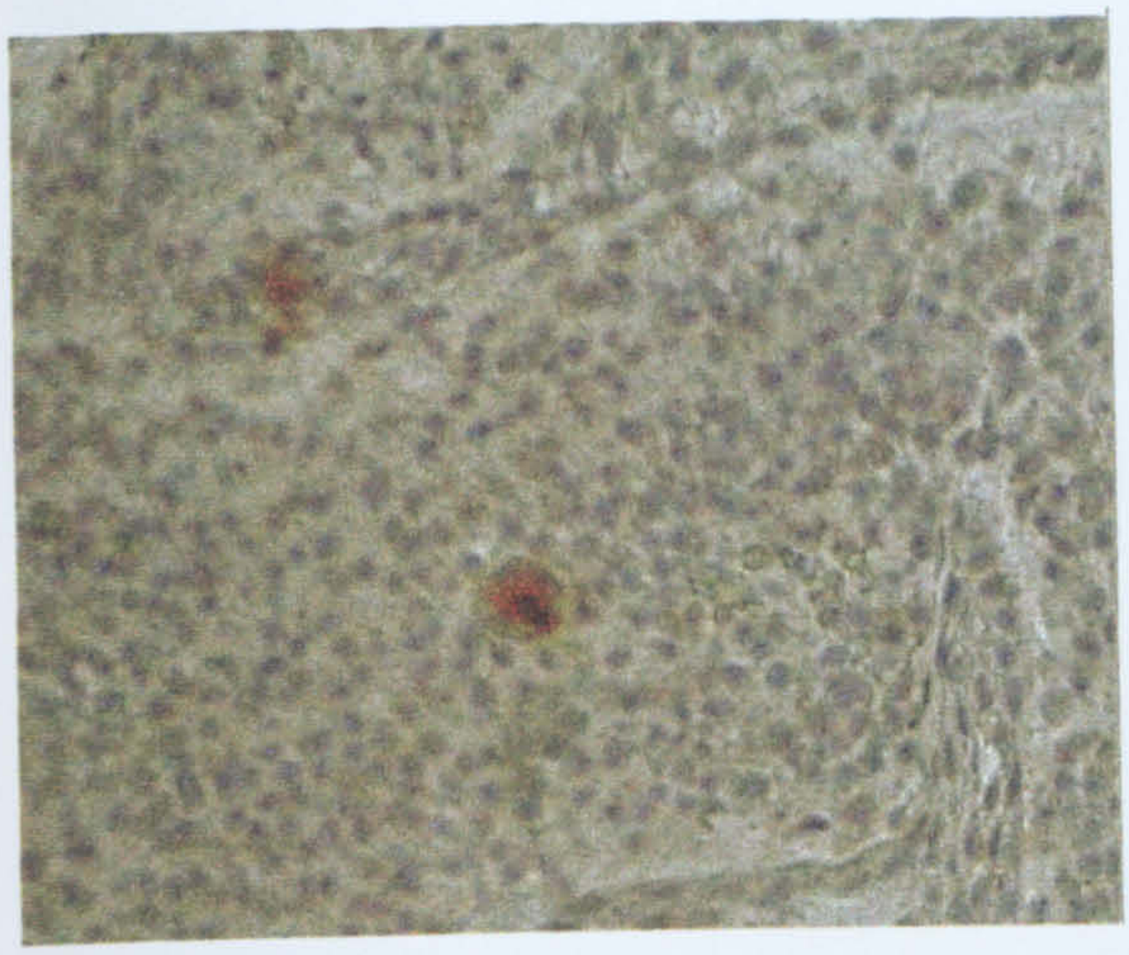


Figure 6.5b

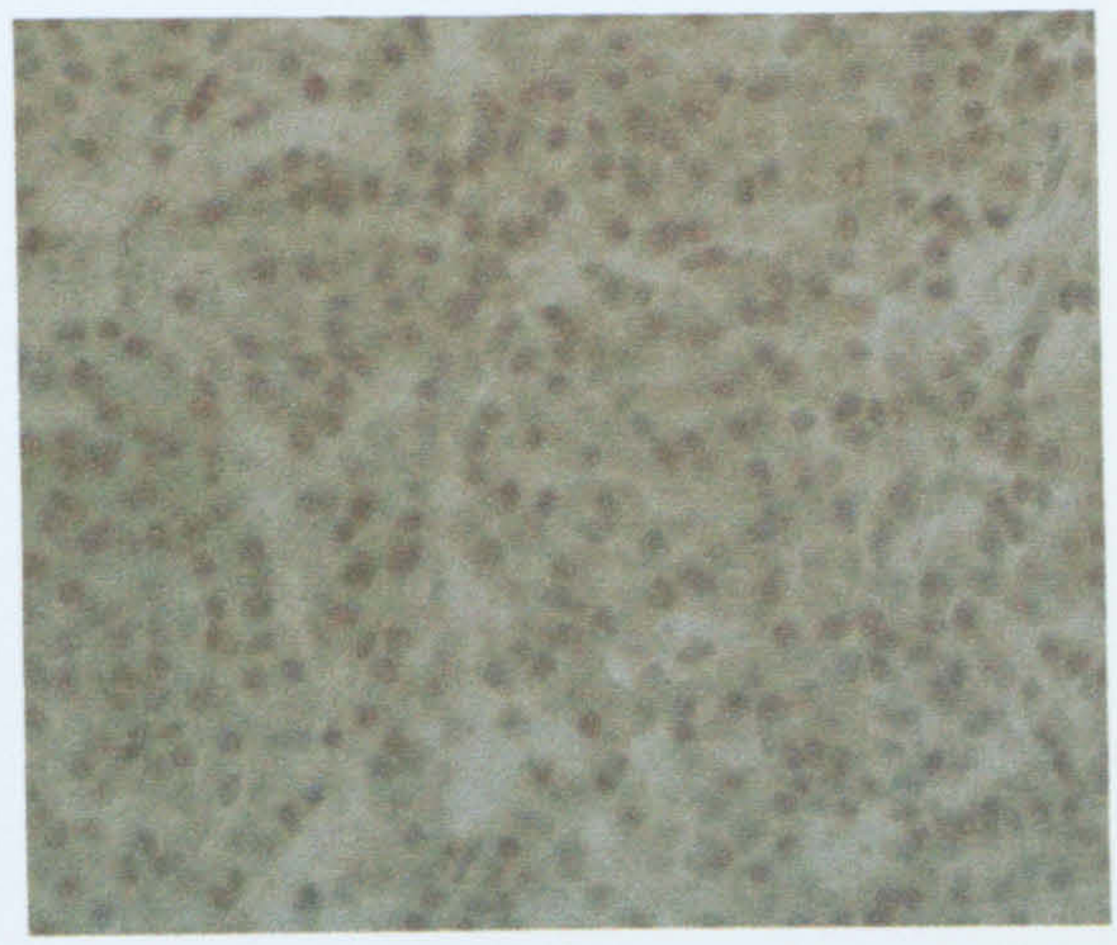


Figure 6.5a shows granulation tissue plasma cells bearing antibodies to *T. denticola*. Figure 6.5b shows the control, inhibition of specific *T. denticola* antibody interactions using Fab' fragments of goat anti-human immunoglobulin. (Magnification x200)

Figure 6.6a

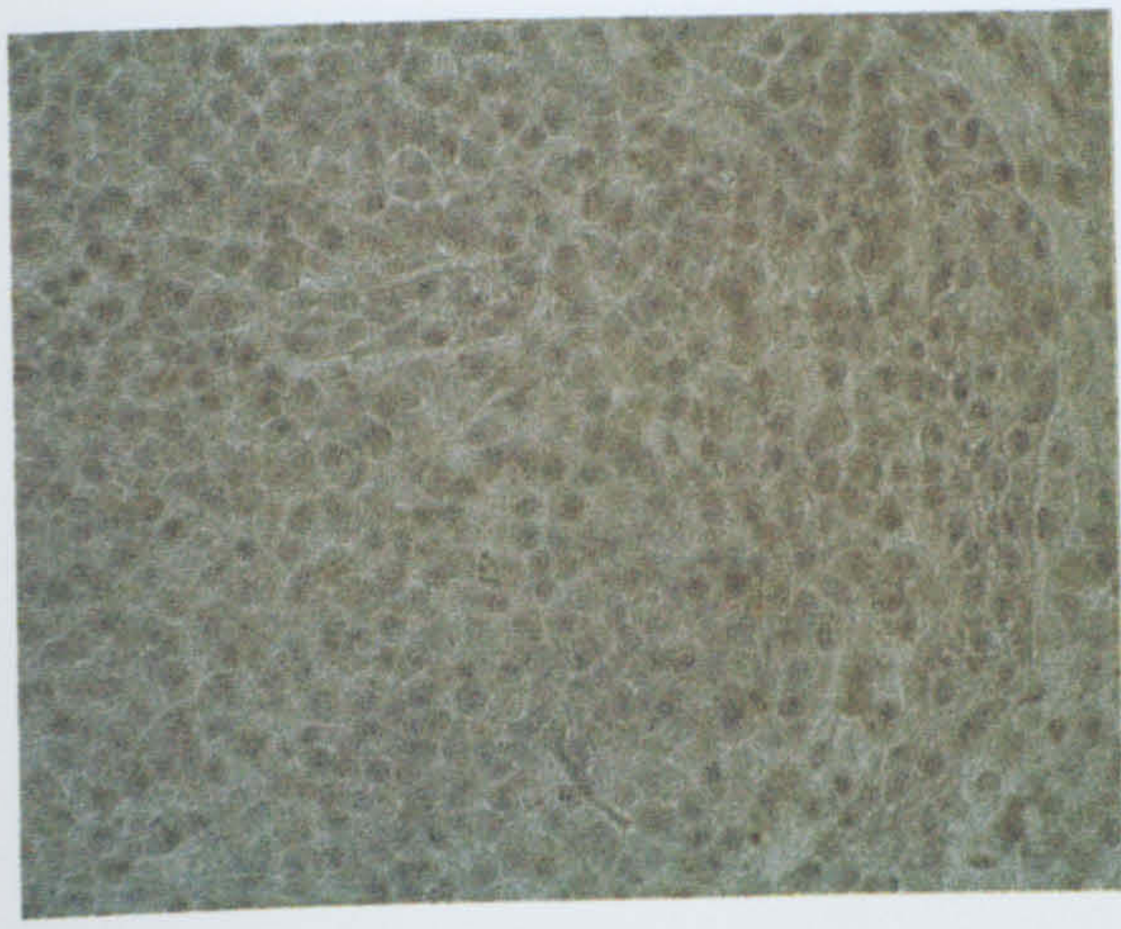


Figure 6.6b

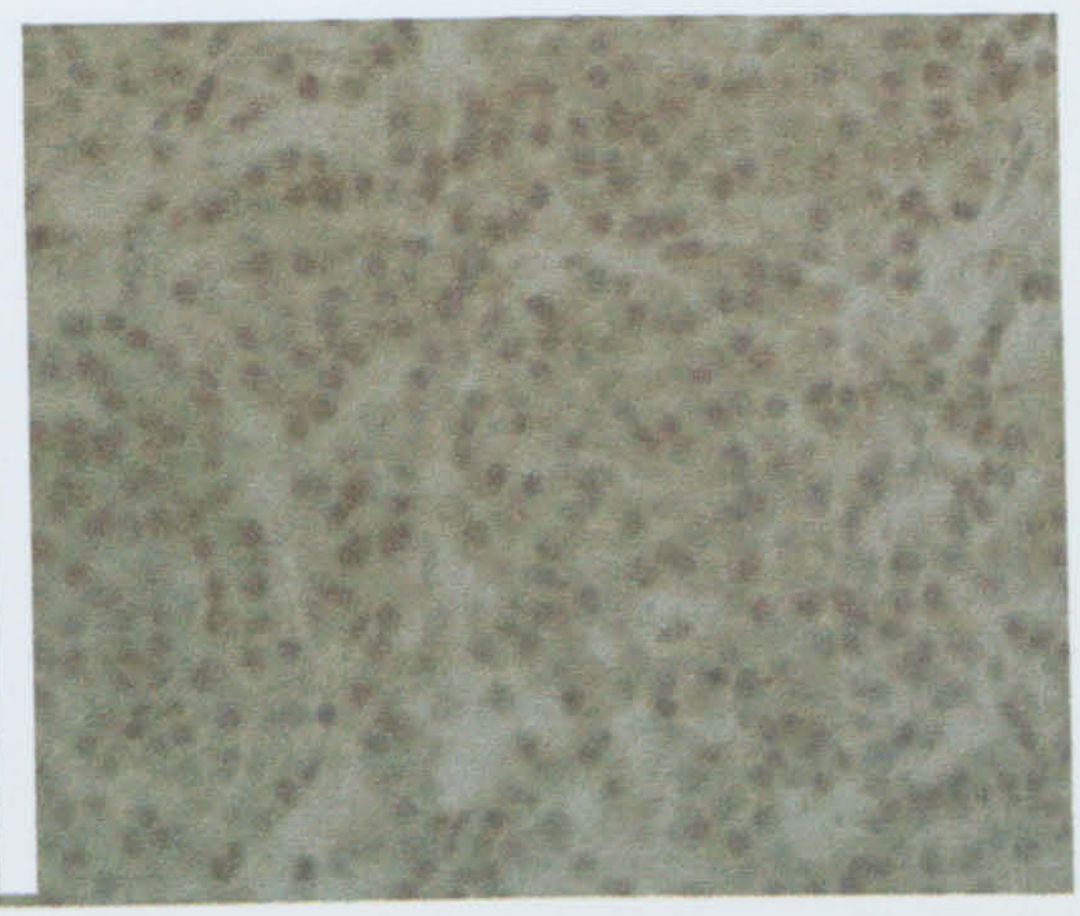


Figure 6.6a shows granulation tissue plasma cells bearing antibodies to *E. coli*. Figure 6.6b shows the control, inhibition of specific *E. coli* antibody interactions using Fab' fragments of goat anti-human immunoglobulin. (Magnification x200)

Figure 6.7a

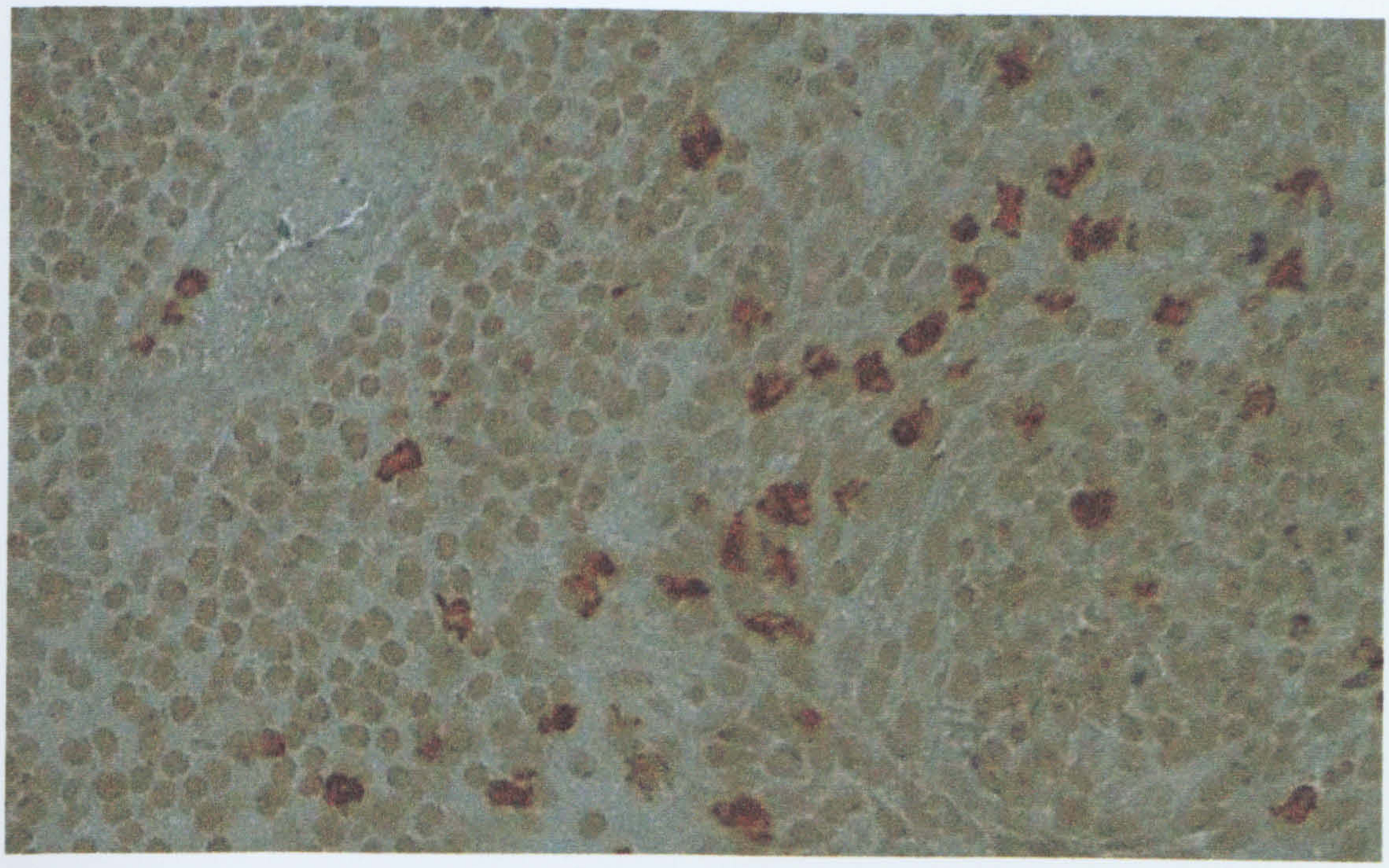


Figure 6.7a shows granulation tissue plasma cells bearing antibodies to HSP60 (of *P. gingivalis*). (Magnification x 200)

Figure 6.7b

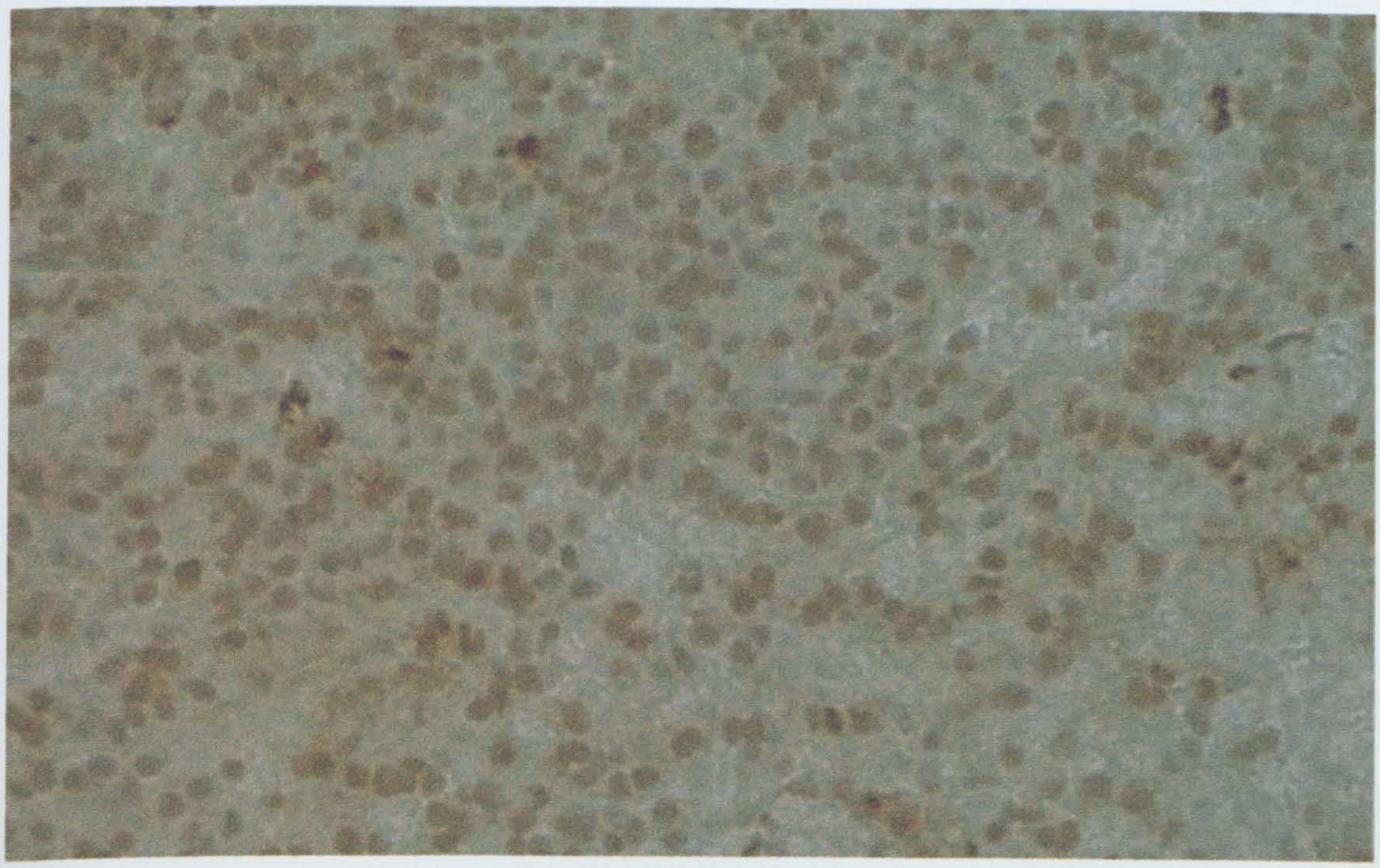


Figure 6.7b shows the control, inhibition of specific HSP60 (of *P. gingivalis*) antibody interactions using Fab' fragments of goat anti-human immunoglobulin. (Magnification x 200)

Figure 6.8a

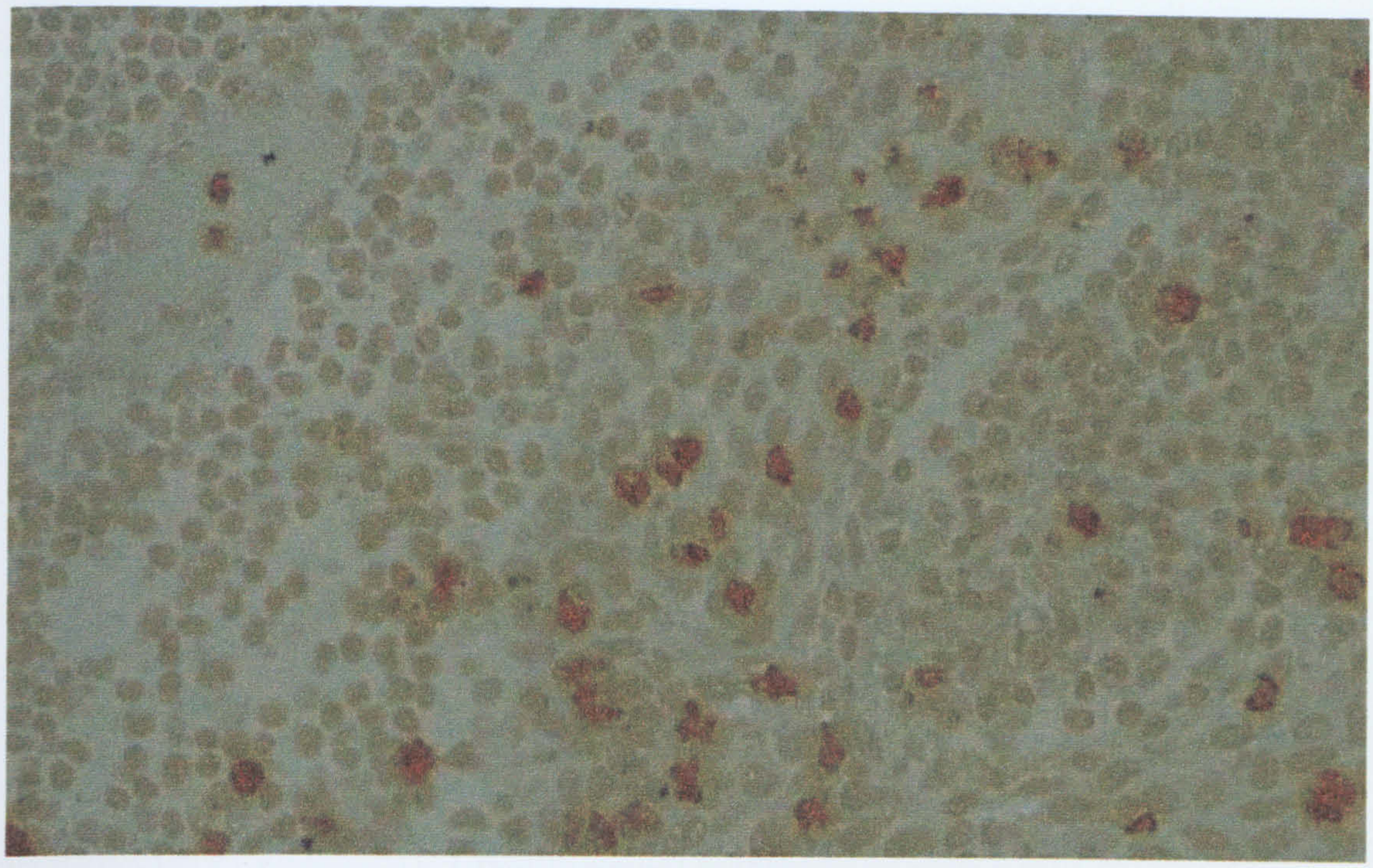


Figure 6.8a shows granulation tissue plasma cells bearing antibodies to leukotoxin (of *A. actinomycetemcomitans*). (Magnification x200)

Figure 6.8b

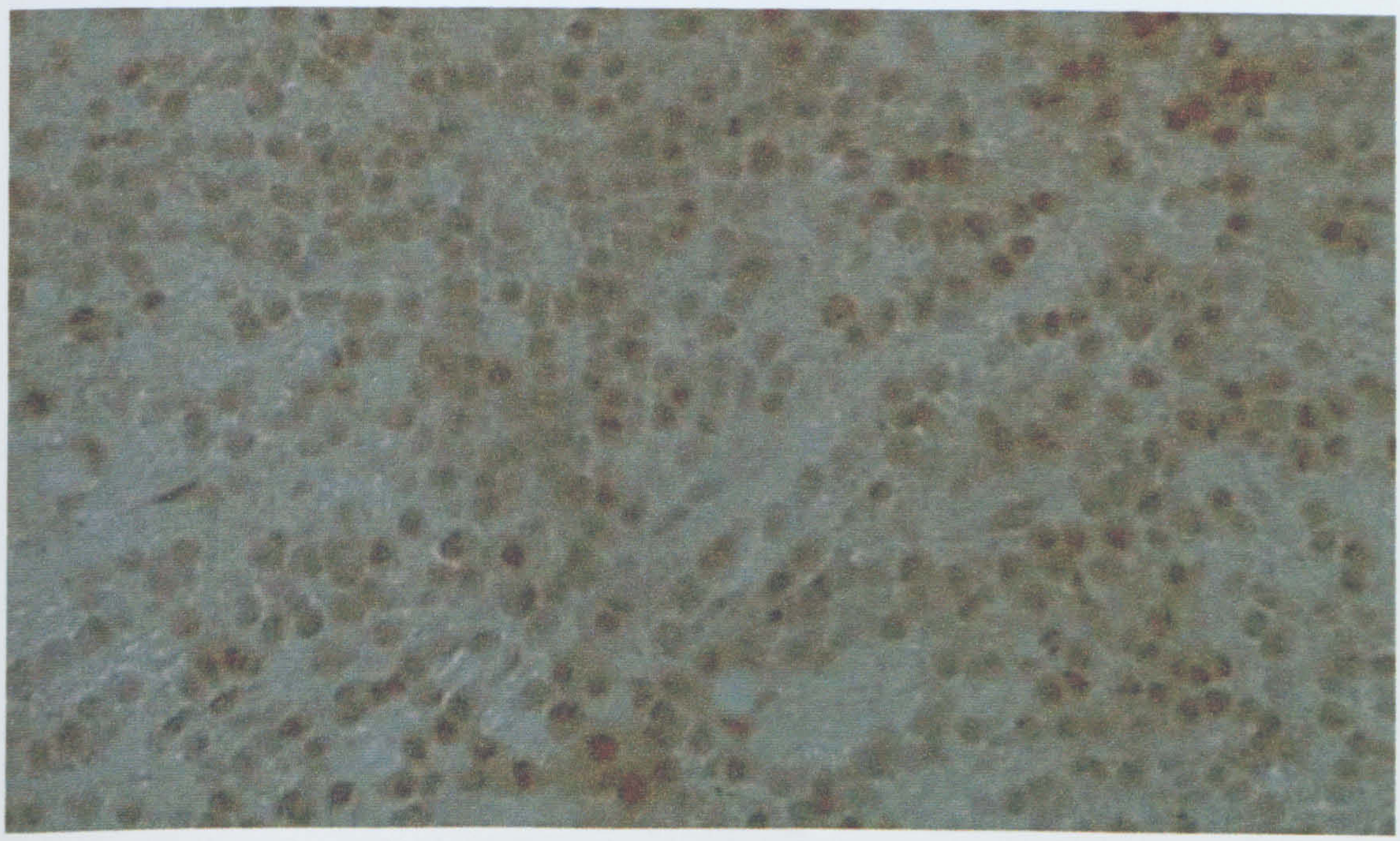


Figure 6.8b shows the control, inhibition of specific leukotoxin (of *A. actinomycetemcomitans*) antibody interactions using Fab' fragments of goat anti-human immunoglobulin. (Magnification x200)

Figure 6.9a

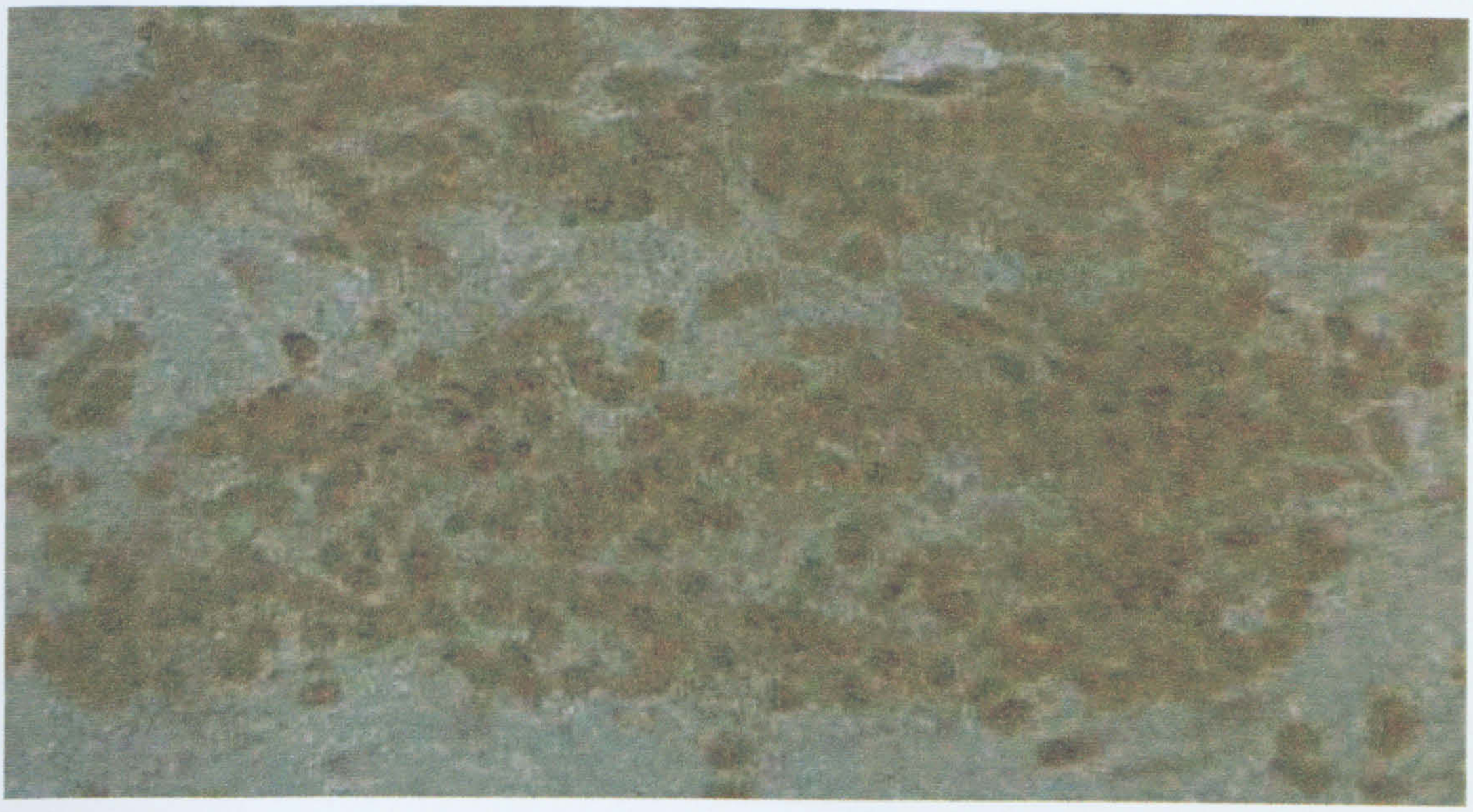


Figure 6.9a shows granulation tissue plasma cells bearing antibodies to the β segment of RgpA (of *P. gingivalis*). (Magnification x200)

Figure 6.9b

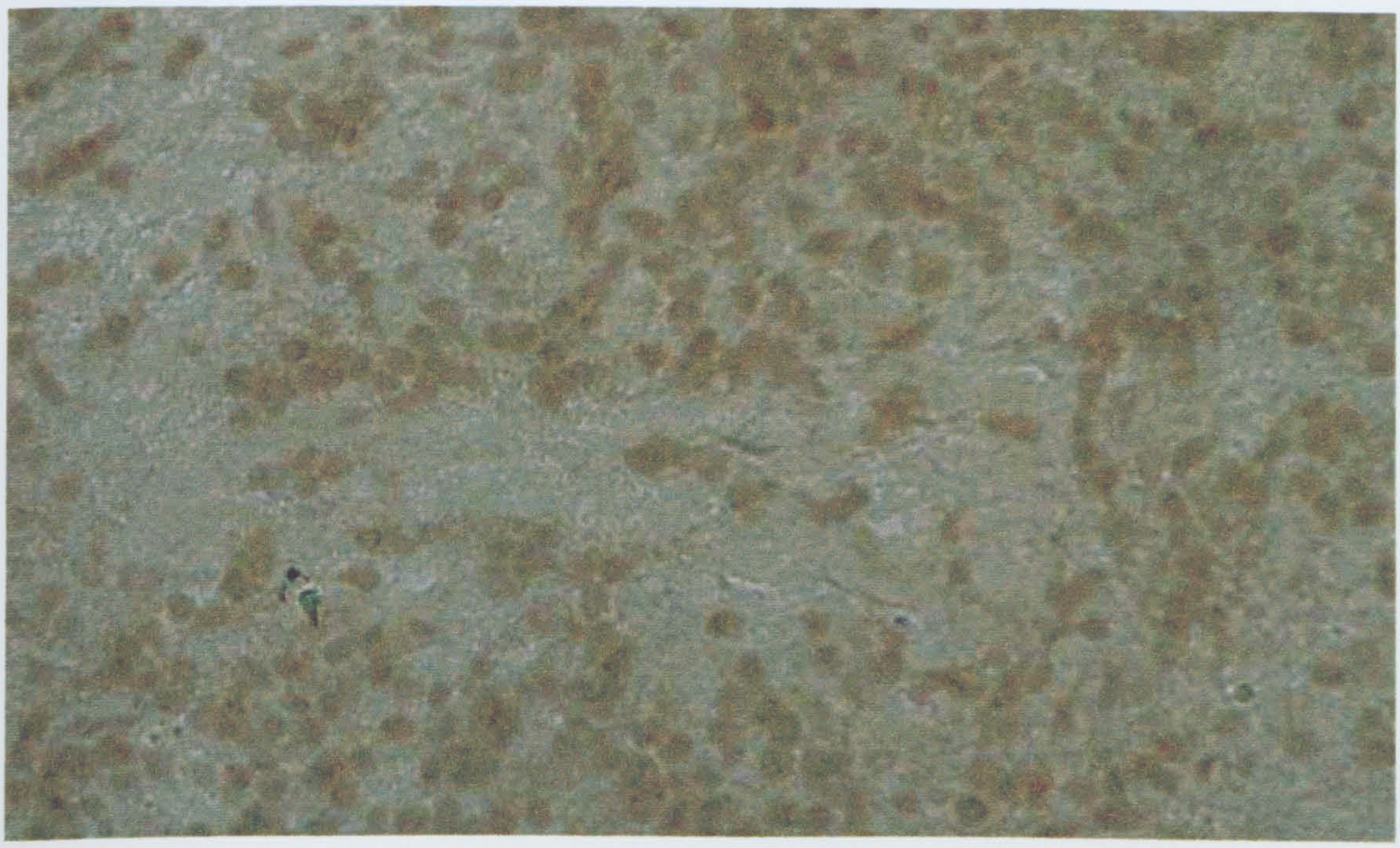
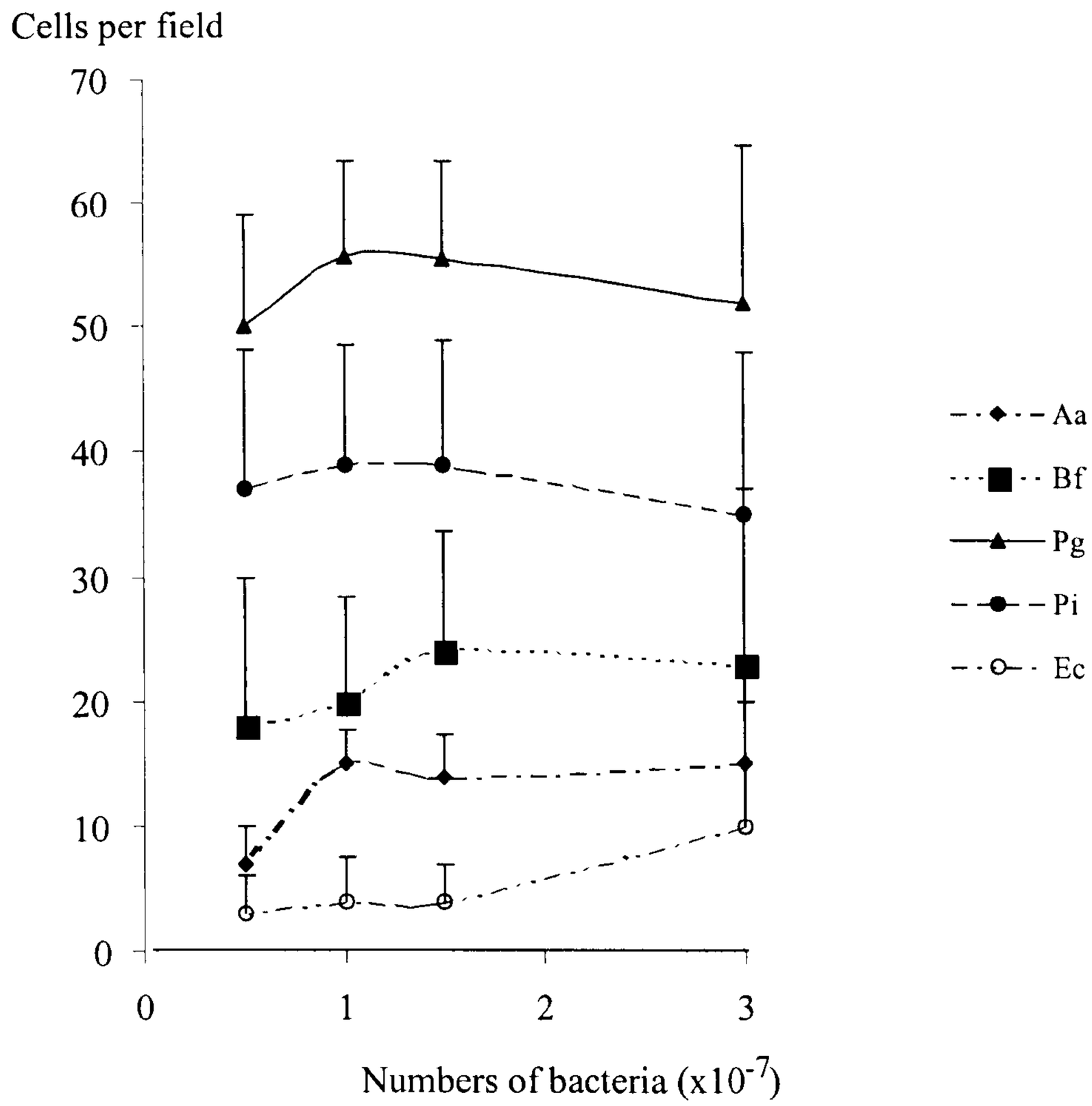


Figure 6.9b shows the control, inhibition of specific the β segment of RgpA protease (of *P. gingivalis*) antibody interactions using Fab' fragments of goat anti-human immunoglobulin. (Magnification x200)

Figure 6.10 Plot of number of specific antibody-bearing cells per field against different concentrations of whole fixed bacteria used. Magnification x200 for granulation tissue sections from patients with chronic periodontitis (n=1).



Pg - *P. gingivalis*

Aa - *A. actinomycetemcomitans*

Pi - *P. intermedia*

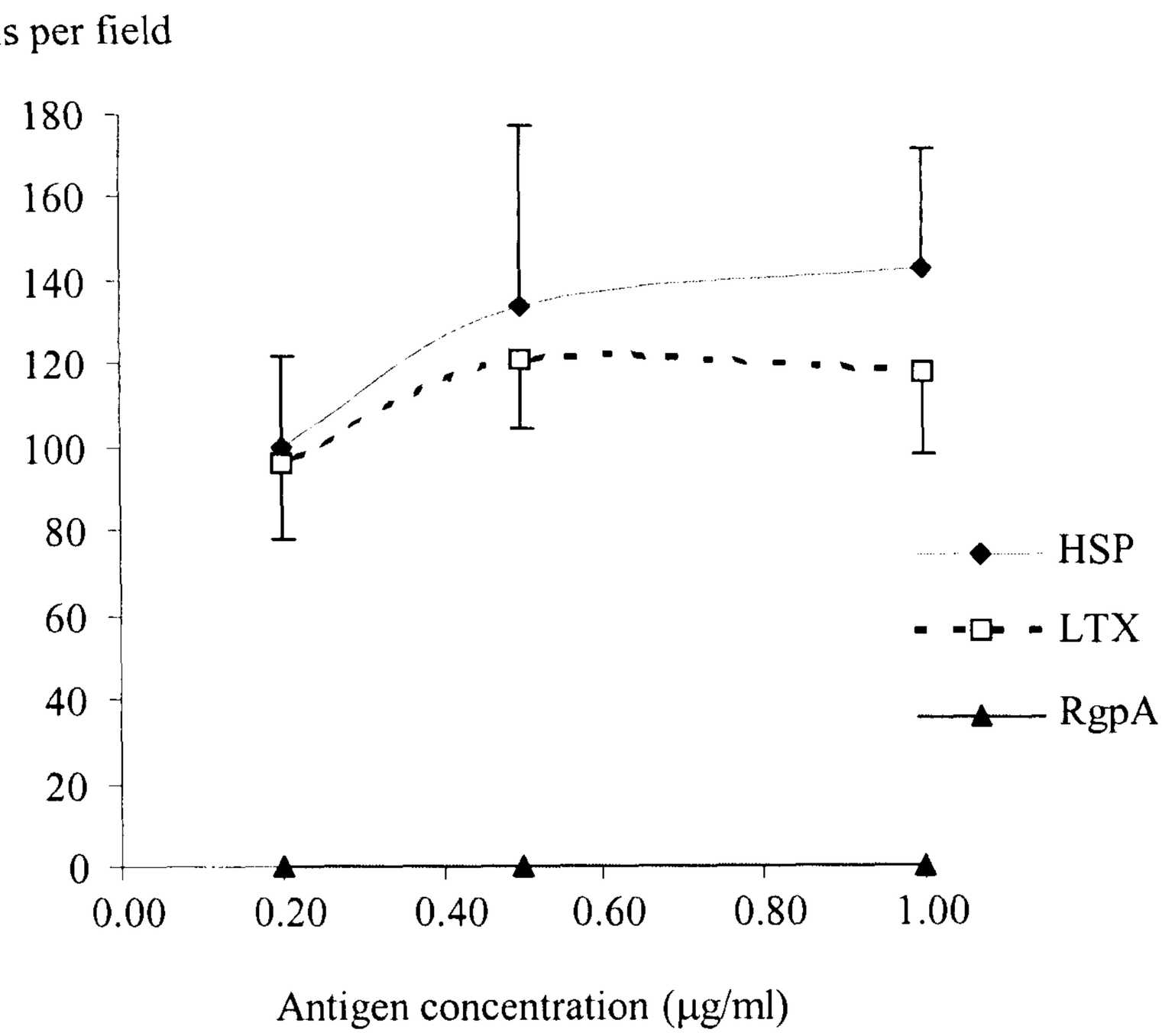
Bf - *B. forsythus*

Td - *T. denticola*

Ec - *E. coli*

Figure 6.10 shows the number of cells per field against the 3 different concentrations of whole fixed bacteria. There is an indication of a peak in the number of cells counted per field, when a concentration of $1x10^7$ bacterial cells/section ($1.5x10^8$ cells/ml) is used. As the concentration of whole fixed bacteria added is further increased, there is a decrease in the number of antibody-bearing cells detected. The experiment was therefore, carried out using $1x10^7$ bacterial cells/section. These are the results of a preliminary experiment that was carried out to identify the optimum concentration of the biotinylated bacterial cells to use in the study. The aim was to establish the optimum concentration for each bacteria to identify the concentration which gives a compromise between low background staining with adequate staining intensity of the appropriate cells. The experiment was performed in triplicate on one set of granulation tissue sections.

Figure 6.11 Plot of number of specific antibody-bearing cells per field against different concentrations of purified antigen. Magnification x200 for granulation tissue sections from patients with chronic periodontitis (n=1).



HSP - HSP75 (of *P. gingivalis*)

LTX - Leukotoxin (of *A. actinomycetemcomitans*)

RgpA - β segment of the protease RgpA (of *P. gingivalis*)

Figure 6.11 shows the number of cells per field against 3 different concentrations of purified antigens. These results were obtained from a preliminary experiment that was performed in triplicate on one set of granulation tissue sections. A positive correlation is evident between the antigen concentration and the number of cells counted per field. This indicates that the higher the concentration of antigen used, the greater the number of specific antibody-bearing cells are detected. The highest number of cells are detected when a concentration of 1μg/ml of antigen is used. The graph suggests that at this concentration a plateau is beginning to be established and hence this is the optimal concentration to use in the experiment. The aim of this preliminary study was to establish the optimum concentration for each antigen to ascertain the level which gives a compromise between low background staining with adequate staining intensity of the appropriate cells. The results suggest that the number of cells per field detected against *P. intermedia* is significantly higher than the number detected against *B. forsythus* in this individual patient. However, when the results from all of the patients are observed, there is no significant difference for the numbers of cells detected against *P. intermedia* and *B. forsythus*.

Table 6.1 Median figures for cells per field bearing antibody to the whole bacterial cells and purified antigens tested. Magnification x200 for granulation tissue sections taken from patients with chronic periodontitis (n=5).

	Median	Interquartile Range	Range	p-value of Friedman's test*
Pg	81.0	37.5 - 141.0	36.0 - 150.0	0.002
Aa	21.0	17.0 - 37.5	16.0 - 50.0	
Pi	93.0	30.0 - 121.5	26.0 - 130.0	
Bf	95.0	26.2 - 123.0	25.5 - 125.0	
Td	16.0	14.0 - 26.5	12.0 - 33.0	
Ec	9.0	5.5 - 15.0	3.0 - 17.0	
Pg HSP75	116.2	56.7 - 134.1	11.1 - 134.2	
Aa LTX	113.2	65.0 - 119.5	20.8 - 121.3	
Pg RgpA	0	-	-	-

Table 6.2 Median figures for cells per field bearing antibody to the whole bacterial cells and purified antigens tested. Magnification x200 for gingival tissue sections taken from patients with chronic periodontitis (n=5).

	Median	Interquartile Range	Range	p-value of Friedman's test*
Pg	36.0	18.5 - 37.7	15.0 - 39.3	0.006
Aa	16.0	10.9 - 17.0	9.3 - 18.0	
Pi	29.0	24.5 - 32.8	22.0 - 33.0	
Bf	26.0	20.4 - 37.7	15.7 - 49.0	
Td	12.0	11.0 - 14.0	11.0 - 15.0	
Ec	9.0	5.5 - 11.9	3.0 - 12.5	
Pg HSP75	48.2	18.4 - 67.5	9.8 - 78.0	
Aa LTX	17.0	11.9 - 43.0	10.0 - 54.8	
Pg RgpA	0	-	-	-

For all of the subjects, the number of cells detected against RgpA was zero, thus there was no variability in the number of cells detected against RgpA across subjects. For this reason it was decided that the RgpA data should be omitted from the statistical analysis to enable suitable comparisons to be made, using the Friedmans's analysis.

Table 6.1 shows the median number of specific antibody-bearing plasma cells detected in granulation tissue against the 6 whole fixed bacteria tested and the purified antigens HSP75 (of *P. gingivalis*), leukotoxin (of *A. actinomycetemcomitans*) and the β segment of RgpA (of *P. gingivalis*) in granulation tissue. The results represent the median of the results obtained from 5 patients. The results of the Friedmans analysis suggest that for at least one antigen there are a larger number of specific plasma cells identified than for at least one other antigen ($p = 0.002$). The number of cells detected by the addition of *E. coli* constituted the controls. A small median number (9.0) of cells was detected, suggesting that there is some non-specific binding or cross-reactivity occurring. Cross-reactivity is possible between antibodies induced against periodontal pathogens and specific for an epitope found on other Gram negative bacteria.

Table 6.2 shows the median number of specific antibody-bearing plasma cells detected in gingival tissue against the 6 whole fixed bacteria tested and the 3 purified antigens. The results of the Friedmans analysis suggest that for at least one antigen there are a larger number of specific plasma cells identified than for at least one other antigen ($p = 0.006$). The number of cells detected bearing antibody specific to *E. coli* was small (9.0), again suggesting that there is some non-specific binding or cross-reactivity occurring.

Experimental work was carried out on sonicated bacterial preparations of *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *B. forsythus*, *T. denticola* and *E. coli*. The results showed positively stained antibody-bearing cells being detected, however, the background staining was very high. It was very difficult to decipher if staining was due to specific interactions or non-specific binding. Observation of the stained sections suggested that the sonicate preparations had bound non-specifically to the tissue sections. The results of these experiments are not included in this chapter as

the technique is demonstrated much better with the results of the whole fixed micro-organisms and the purified antigens.

Further analysis was carried out using a suitable follow up multiple comparisons procedure (Daniel, 1978). The results of this test suggested that in the granulation tissue samples there was a significant difference in the number of cells bearing antibodies specific to *P. gingivalis* and *E. coli*. In addition there was also a significant difference (borderline) between the number of cells detected against *B. forsythus* and *E. coli*, *P. intermedia* and *E. coli*, leukotoxin (of *A. actinomycetemcomitans*) and *E. coli* and HSP75 (of *P. gingivalis*) and *E. coli*.

Multiple comparison analysis indicated that in the gingival tissue sections there was a significant difference (borderline) between the number of cells detected against *P. gingivalis* and *E. coli*, *P. intermedia* and *E. coli*, *B. forsythus* and *E.coli* and HSP75 (of *P. gingivalis*) and *E. coli*.

Table 6.3 Median differences in the number of cells per field for the micro-organisms and purified antigens tested in the granulation and gingival tissue samples. Magnification x200 for granulation and gingival tissue sections taken from patients with chronic periodontitis (n=5).

	Median Difference (Granulation - Gingival Tissue)	Interquartile Range of Differences	p-value for test of Median Difference equal to 0
Pg	**59.0**	**12.0 - 103.4**	**0.05**
Aa	**8.70**	**4.25 - 20.5**	**0.05**
Pi	**66.0**	**-0.8 - 94.0**	**0.05**
Bf	69.0	0.6 - 90.7	0.18
Td	5.0	0.0 - 15.0	0.14
Ec	4.0	-3.7 - 4.8	0.69
Pg HSP75	75.3	5.1 - 96.2	0.11
Aa LTX	99.1	26.2 - 101.9	0.11
Pg RgpA	-	-	-

Table 6.3 shows the median differences in the number of cells detected for the 2 different tissue types (granulation minus gingival). The Wilcoxon Signed Rank test was utilised to examine the paired differences in the number of cells detected against the micro-organisms and antigens tested in the 2 different tissue types. Table 6.3 shows the p-values which suggest that there is a significant difference in the number of cells detected against *P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia* between the granulation and gingival tissue samples. Therefore, a significantly higher number of cells specific for *P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia* were detected in the granulation tissue samples compared with the gingival tissue samples. There was no statistically significant difference in the number of cells detected in the granulation and gingival tissue samples against *B. forsythus*, *T. denticola*, *E. coli*, HSP75, leukotoxin and the β segment of RgpA.

Table 6.4 Mean figures for the percentage of the plasma cells per field specific for the antigens tested. Magnification x200 for granulation and gingival tissue sections taken from patients with chronic periodontitis (n=5).

	Granulation Tissue		Gingival Tissue	
	Mean	S.D.	Mean	S.D.
% of leukocytes which are plasma cells	52.3	6.6	23.3	3.1
% plasma cells bearing antibody specific to Pg	10.0	6.4	8.8	3.1
% plasma cells bearing antibody specific to Aa	3.0	1.9	4.7	1.8
% plasma cells bearing antibody specific to Pi	9.5	6.3	8.8	2.6
% plasma cells bearing antibody specific to Bf	9.3	6.3	8.3	3.7
% plasma cells bearing antibody specific to Td	2.2	1.2	3.9	1.4
% plasma cells bearing antibody specific to Ec	1.7	0.8	3.1	1.8
% plasma cells bearing antibody specific to HSP	11.1	6.1	14.5	10.3
% plasma cells bearing antibody specific to LTX	10.8	5.1	8.6	7.0
% plasma cells bearing antibody specific to RgpA	0	0	0	0

To express the results in a more relevant context the number of plasma cells were enumerated so that the percentage of leukocytes that were plasma cells could be calculated. Further to this the percentage of plasma cells that were being detected for each of the antigens were also determined, in both the granulation and gingival tissues. This enabled a comparison between the 2 tissue types of the percentage of cells for an antigen, adjusting for the size of inflammatory infiltrate. Table 6.4 shows the percentage of the plasma cells specific for each antigen used in the study. The values seen are the mean values. The counts were obtained by counting 6 fields of each section at x200 magnification.

As can be seen from the table of results 52.3 $\pm$ 6.6% (mean $\pm$ S.D.) of the infiltrate in the granulation tissue is made up of plasma cells, whereas in the gingival tissue the figure is only 23.3 $\pm$ 3.1% (mean $\pm$ S.D.). Therefore, the ratio of plasma cells to other leukocytes is much higher in the granulation tissue than the gingival tissue. The number of cells detected to be bearing antibody against *P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia* in the granulation tissue was found to be higher than the number of cells specific to these micro-organisms in the gingival tissue samples. However, when table 6.4 is observed it can be seen that when the counts are expressed as a percentage of the number of plasma cells per field, the percentage of the cells bearing antibodies to these micro-organisms do not appear to be significantly different in the 2 tissue types. The value for the percentage of plasma cells bearing antibodies to all of the micro-organisms and purified antigens tested shows no difference between the granulation and gingival tissue samples.

Table 6.5 Mean figures for the percentage of plasma cells per field specific for the whole bacterial cells and antigens tested for each of the patients individually. Magnification x200 for granulation and gingival tissue sections taken from patients with chronic periodontitis (n=5).

Mean figures for the percentage of the plasma cells per field bearing specific antibody to:								
GRANULATION TISSUE								
Patient	Pg	Aa	Pi	Bf	Td	Ec	HSP	LTX
1	12.7 (±5.6)	2.0 (±0.8)	11.2 (±7.2)	11.6 (±7.1)	1.1 (±1.2)	2.1 (±1.9)	12.9 (±5.2)	11.3 (±3.7)
2	19.0 (±6.7)	6.3 (±1.9)	12.4 (±7.3)	12.4 (±5.0)	2.6 (±1.6)	2.8 (±1.3)	16.7 (±4.9)	15.3 (±2.9)
3	4.2 (±1.9)	2.1 (±0.8)	3.2 (±1.3)	3.0 (±1.9)	1.9 (±0.7)	0.9 (±0.6)	1.3 (±0.8)	2.4 (±1.9)
4	3.7 (±2.3)	1.5 (±0.9)	3.1 (±2.2)	2.5 (±1.5)	1.4 (±1.2)	1.2 (±1.3)	9.6 (±2.3)	10.6 (±1.2)
5	10.7 (±4.1)	3.1 (±0.5)	17.6 (±8.3)	16.8 (±7.0)	4.1 (±1.3)	1.6 (±0.7)	15.0 (±3.6)	14.3 (±4.5)

GINGIVAL TISSUE								
Patient	Pg	Aa	Pi	Bf	Td	Ec	HSP	LTX
1	14.0 (±13)	6.4 (±6.8)	11.9 (±10)	14.5 (±6.2)	5.1 (±5.3)	5.0 (±5.3)	28.8 (±26)	20.0 (±18)
2	8.3 (±3.0)	4.4 (±1.8)	6.4 (±2.3)	6.2 (±3.4)	2.8 (±0.9)	2.0 (±1.1)	11.3 (±2.8)	4.2 (±2.0)
3	5.5 (±6.7)	3.0 (±3.1)	10.8 (±4.4)	8.9 (±5.9)	3.6 (±4.0)	1.5 (±2.9)	21.4 (±15)	10.4 (±4.3)
4	8.0 (±1.5)	3.0 (±2.0)	6.3 (±1.0)	6.0 (±2.1)	2.4 (±0.8)	1.9 (±0.6)	6.1 (±3.2)	3.2 (±1.4)
5	8.4 (±5.8)	6.7 (±5.9)	8.8 (±6.9)	5.8 (±3.3)	5.8 (±4.4)	5.0 (±5.5)	4.8 (±7.1)	5.3 (±8.2)

Figure 6.12a Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the granulation tissue sample of patient 1.

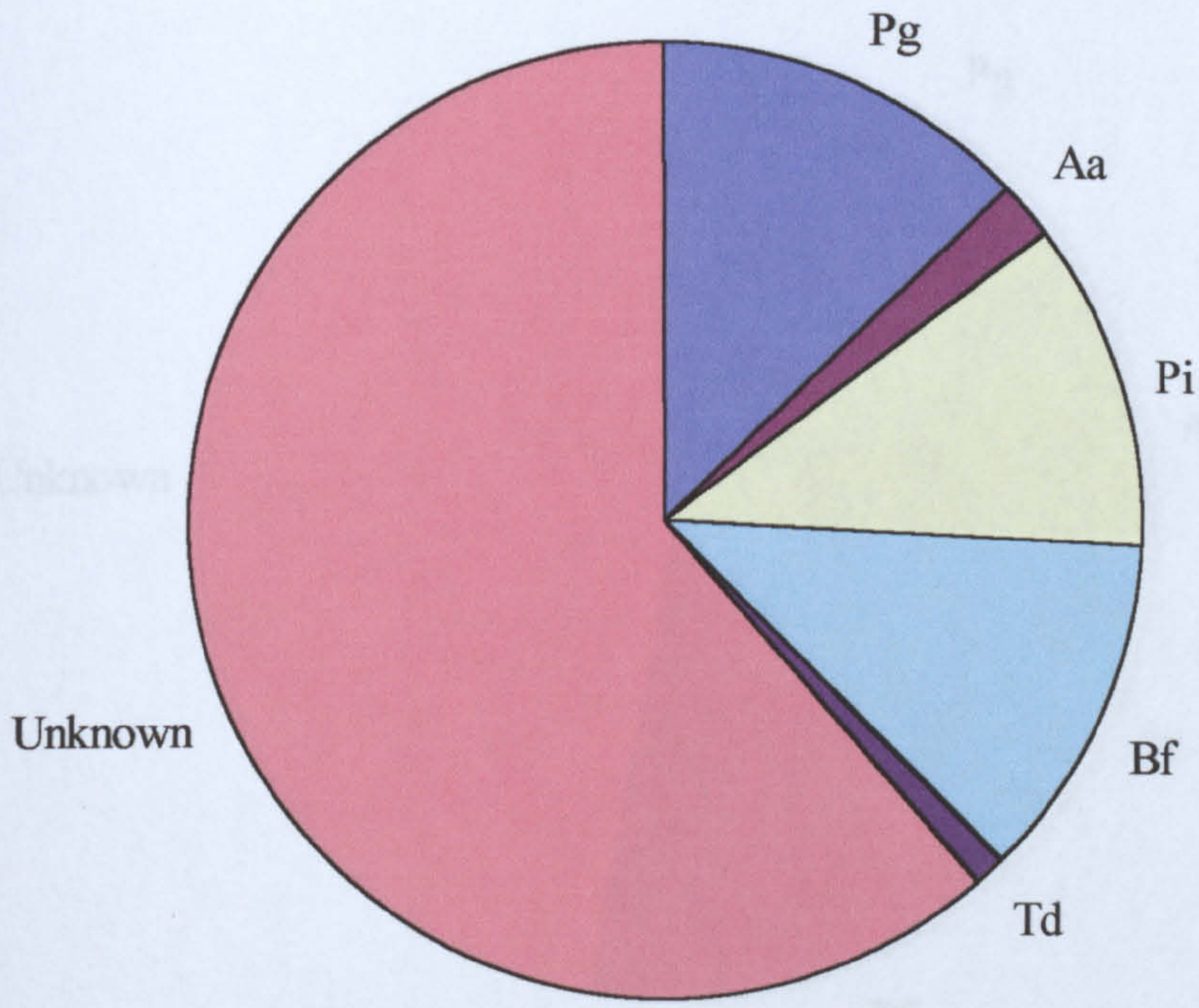


Figure 6.12b Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the gingival tissue sample of patient 1.

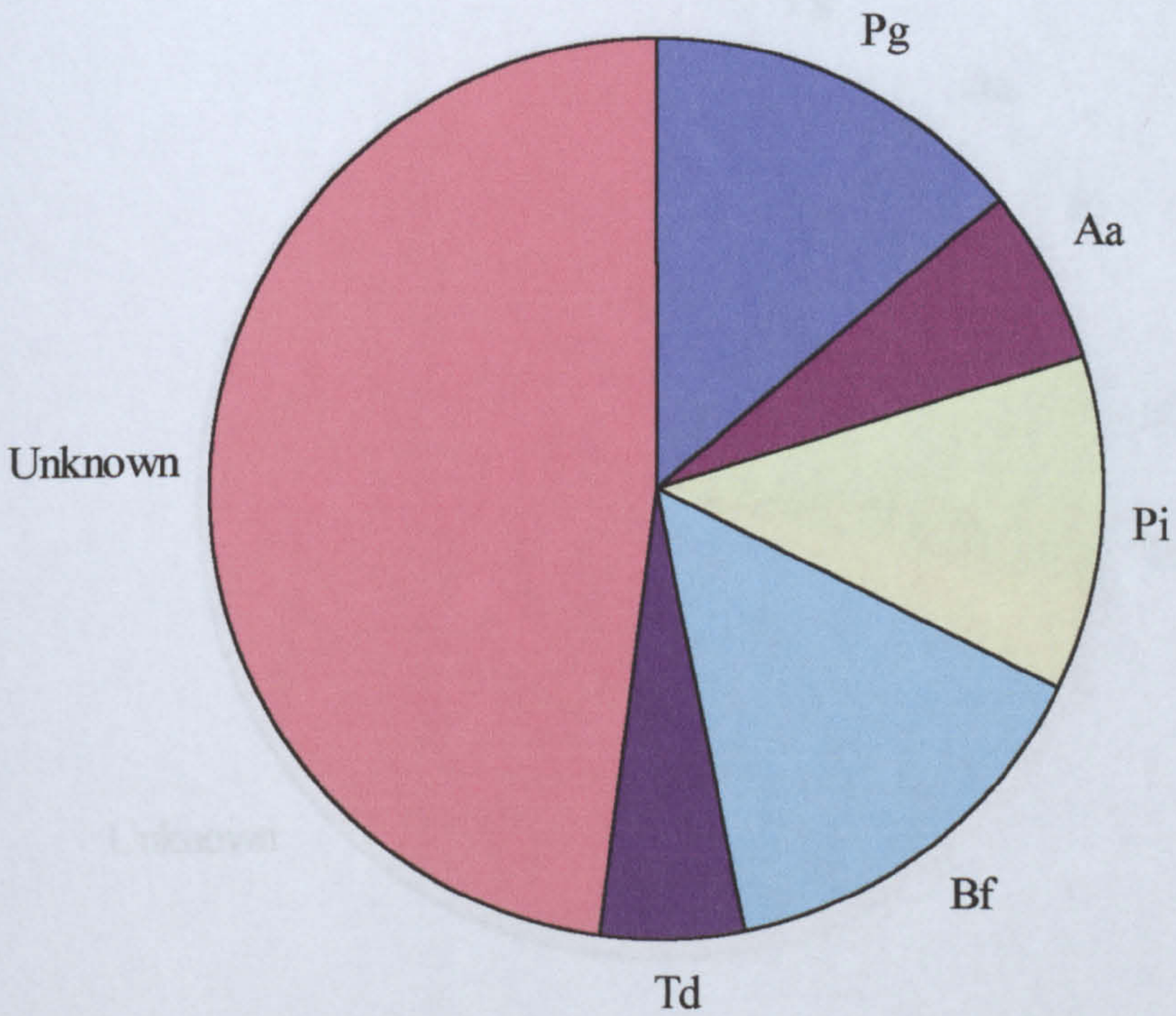


Figure 6.13a Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the granulation tissue sample of patient 2.

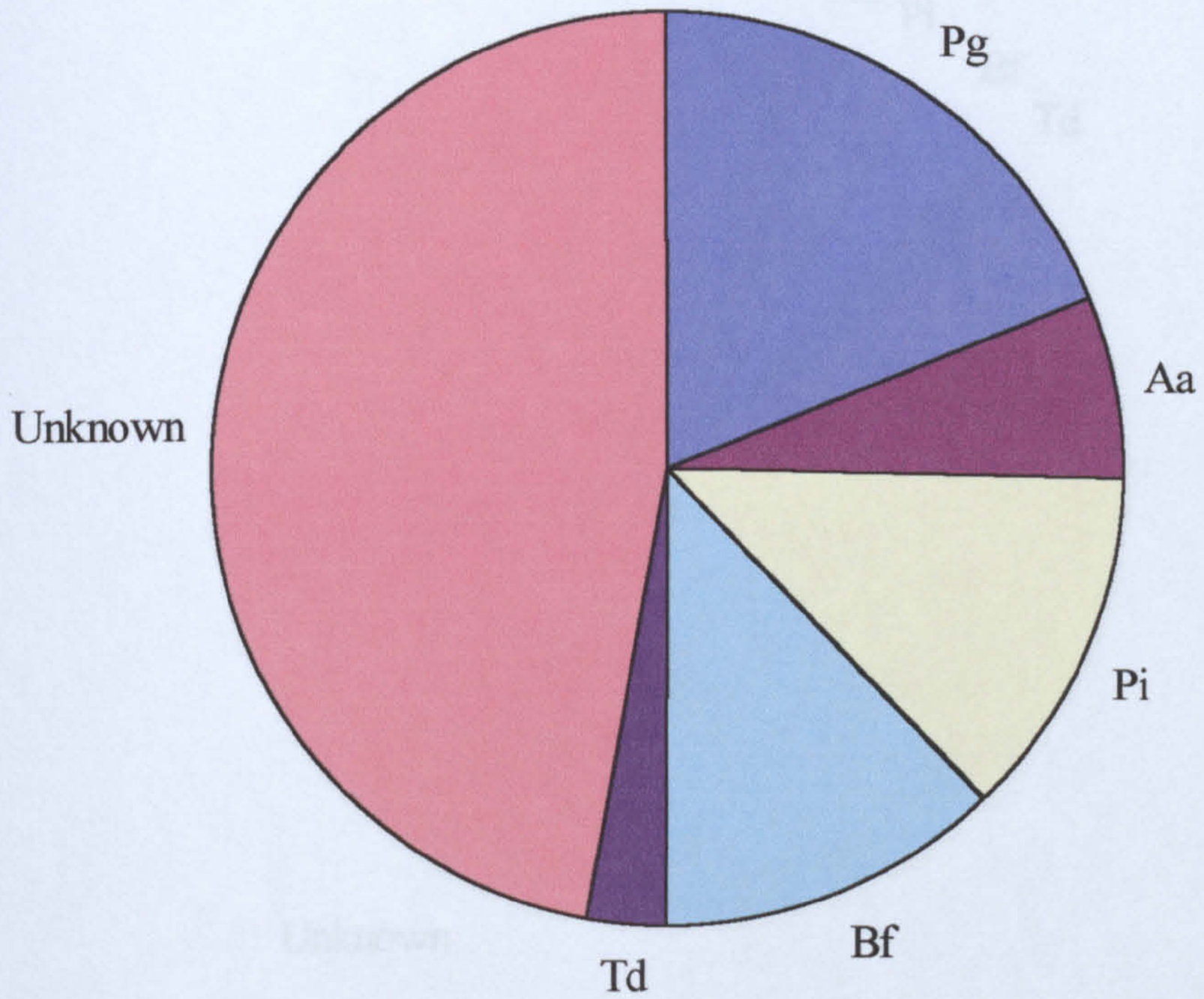


Figure 6.13b Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the gingival tissue sample of patient 2.

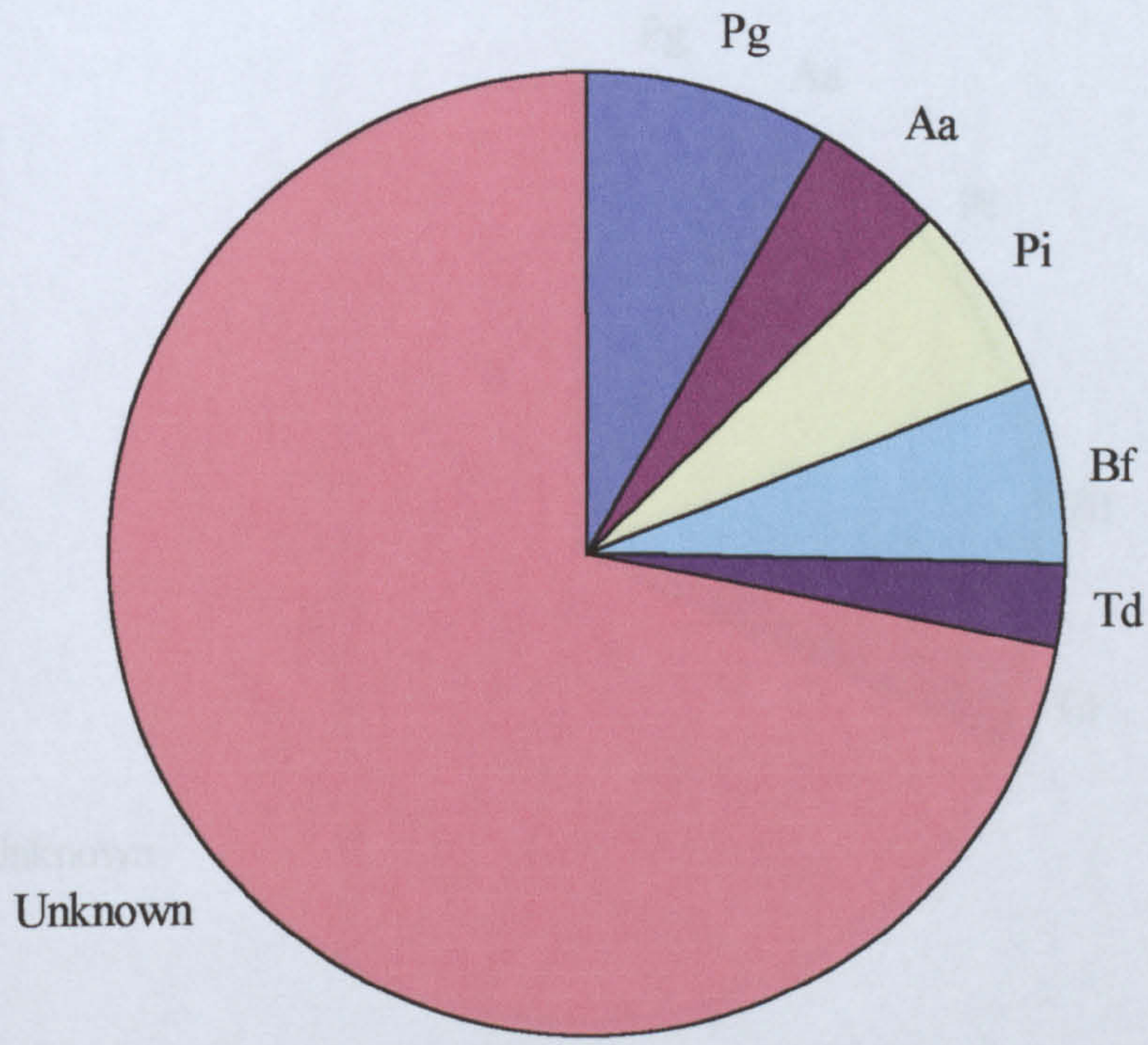


Figure 6.14a Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the granulation tissue sample of patient 3.

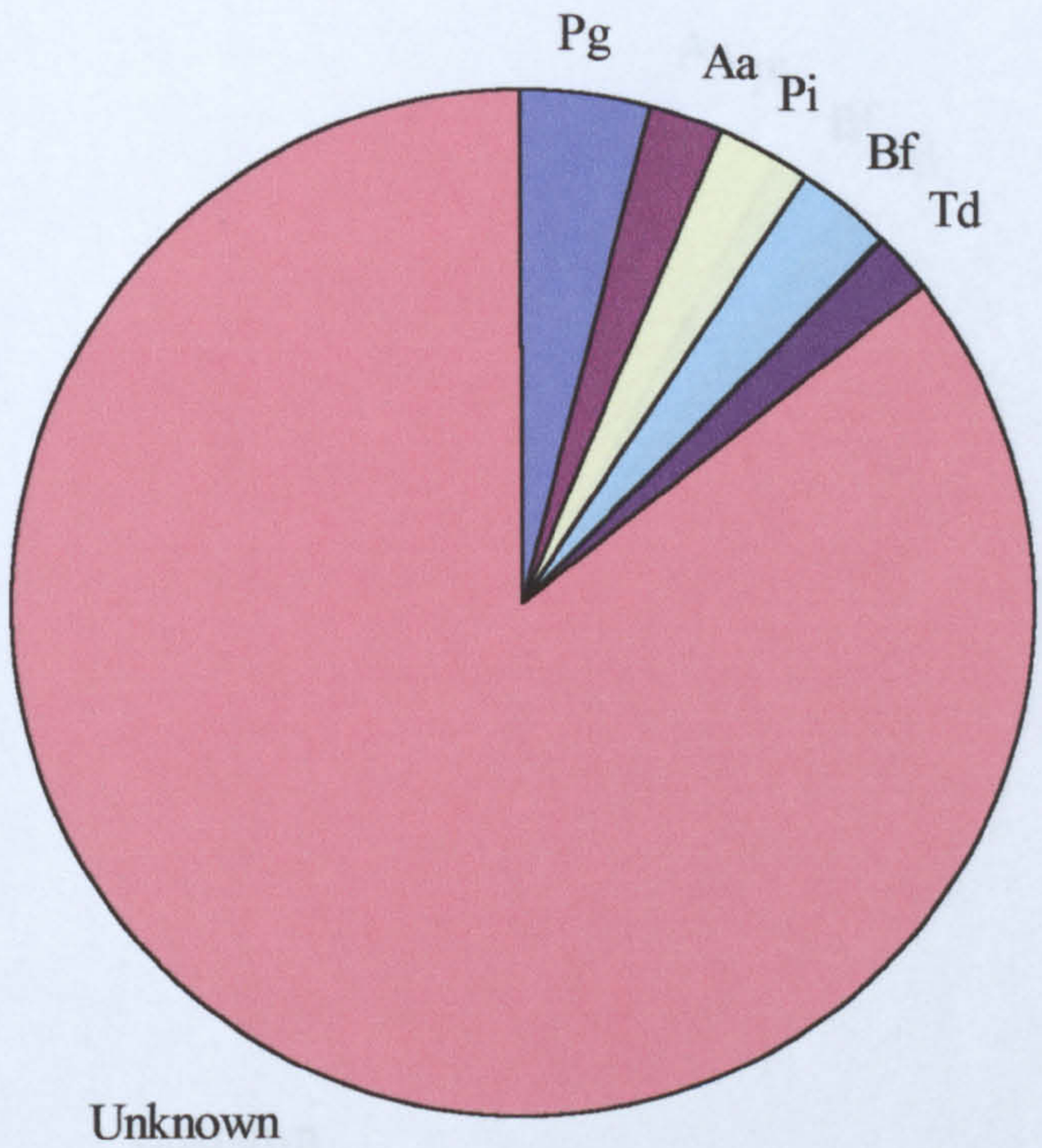


Figure 6.14b Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the gingival tissue sample of patient 3.

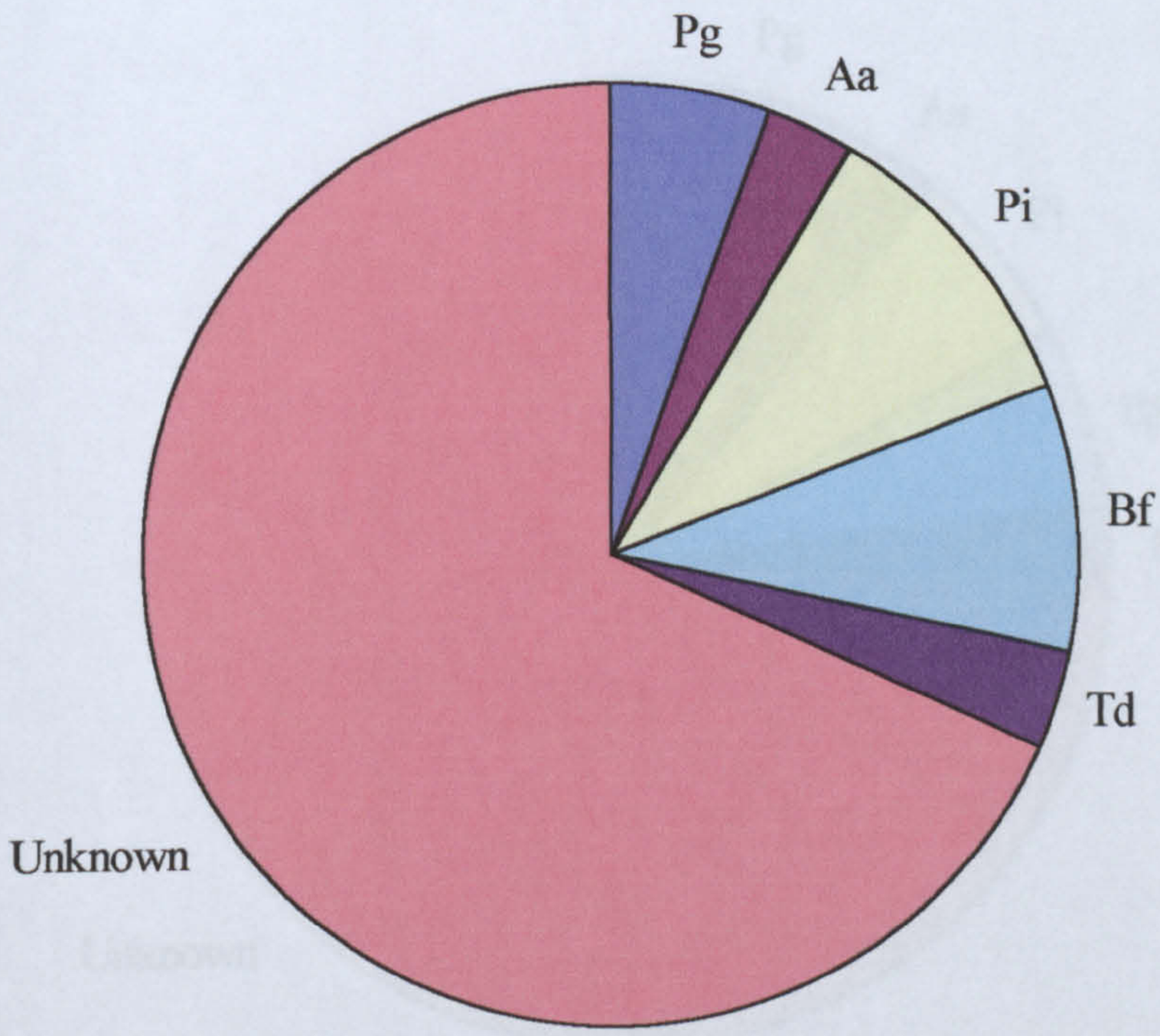


Figure 6.15a Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the granulation tissue sample of patient 4.

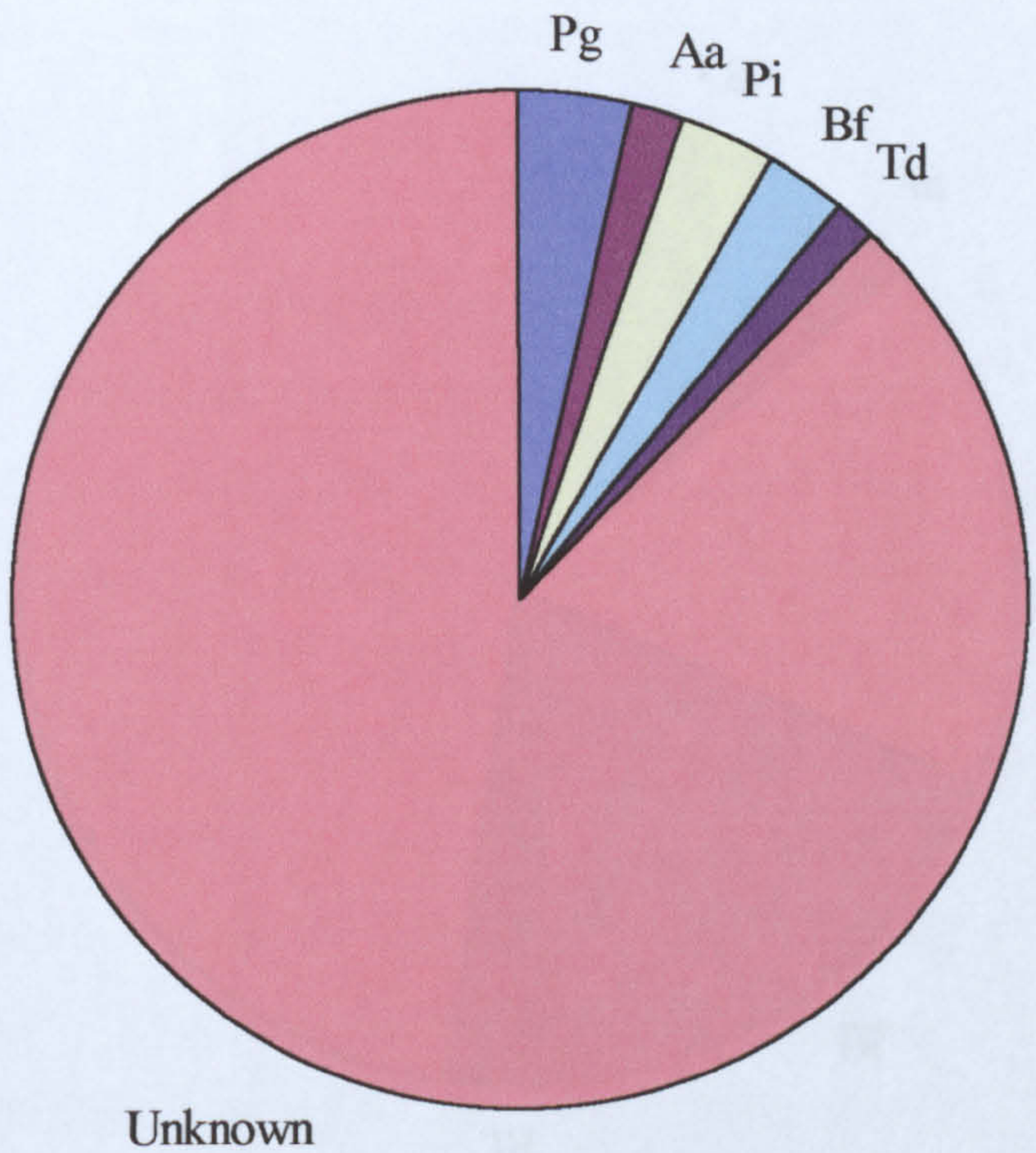


Figure 6.15b Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the gingival tissue sample of patient 4.

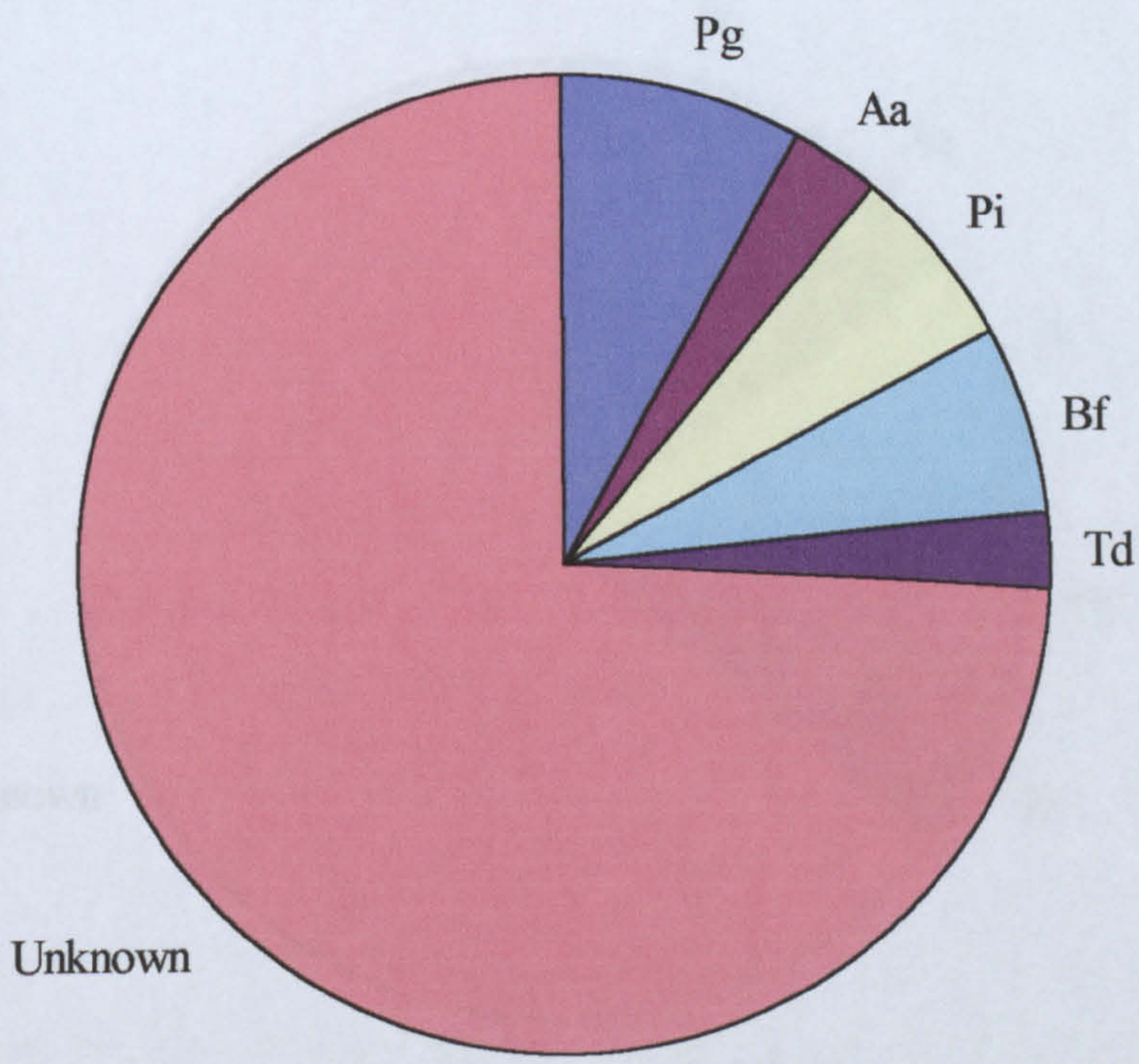


Figure 6.16a Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the granulation tissue sample of patient 5.

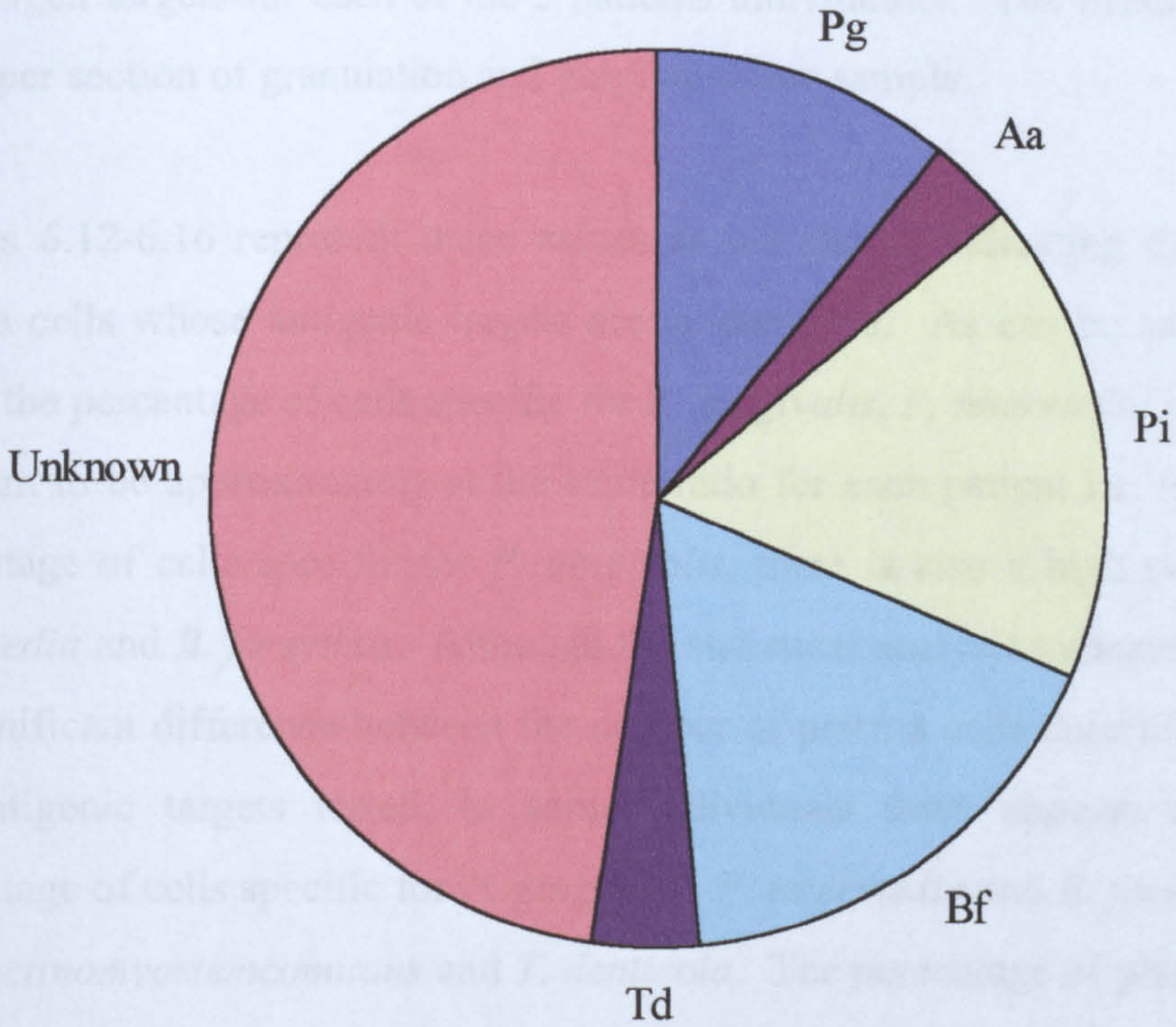


Figure 6.16b Pie chart to show the percentage of plasma cells specific for each antigenic target tested in the gingival tissue sample of patient 5.

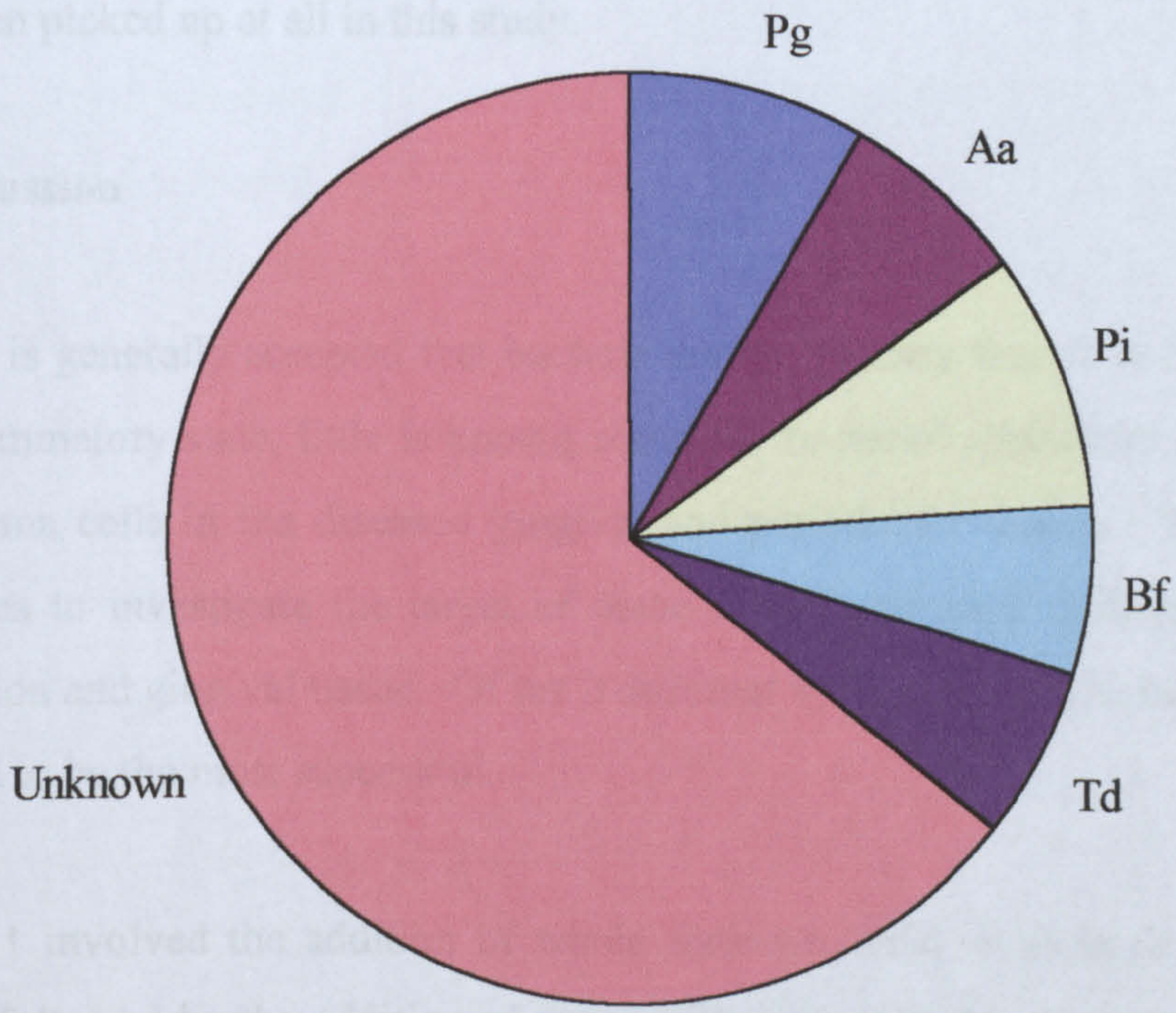


Table 6.5 shows the mean percentages (± S.D.) of the plasma cells specific for each of the antigen targets for each of the 5 patients individually. The mean values are of 6 fields per section of granulation and gingival tissue sample.

Figures 6.12-6.16 represent these values as pie charts, indicating the percentage of plasma cells whose antigenic targets are unidentified. As can be seen from the pie charts the percentage of cells specific for *P. gingivalis*, *P. intermedia* and *B. forsythus*, all seem to be approximately at the same ratio for each patient i.e. if there is a high percentage of cells specific for *P. gingivalis*, there is also a high percentage for *P. intermedia* and *B. forsythus*. Although the statistical analysis indicated that there was no significant difference between the number of plasma cells detected against any of the antigenic targets tested, in some individuals there appears to be a higher percentage of cells specific for *P. gingivalis*, *P. intermedia* and *B. forsythus* compared to *A. actinomycetemcomitans* and *T. denticola*. The percentage of plasma cells whose target is unidentified varies between patients, however, there does not appear to be a correlation between the percentage of cells unidentified and the tissue type. In 3 out of the 5 patients however, the main antigenic target or “”unknown” does not seem to have been picked up at all in this study.

6.4 Discussion

While it is generally accepted that bacteria are the primary factors in the induction of the inflammatory state, little is known about the bacterial specificity of lymphocytes and plasma cells in the diseased gingival and periodontal tissues. The aim of this study was to investigate the target of these cells found in 2 different tissue types; granulation and gingival tissue. Of the 3 different methods tested in this study method 3 proved to be the most successful.

Method 1 involved the addition of whole formalin fixed bacteria directly on to the section, followed by the addition of goat anti-human IgG Fab fragments to block any endogenous or unbound IgG. A sample of human serum taken from a patient with a an antibody titre previously determined by ELISA as “high” to the putative periodontal pathogens utilised in this study was then added to the sections. The

rationale being that antibodies in the serum would bind the bacteria that remained bound to antibodies on the surface of plasma cells in the section. Biotin labelled Fab fragments of goat anti-human IgG were then added, to detect bound serum antibodies, before amplification and detection of the signal by the addition of biotin avidin HRP complex and then developing solution. Biotin labelled Fab fragments were used instead of whole antibodies in the remote possibility that aggregated biotinylated antibodies would bind to Fc receptors. One potential problem with this method was that when the antigen bound antibodies on the surface of the plasma cell, epitopes of the antigen were engaged for binding to occur. Detection of this antigen-antibody complex was by the addition of human serum antibodies that also had to bind the antigen. Not all epitopes of the antigen were available for binding to the serum antibodies. If the serum antibodies that were added for detection purposes were specific for the epitopes that were not available, detection would not have occurred. Further to this, although blocking serum was added at the beginning of the experiment to hinder any non-specific binding sites, it cannot be guaranteed that all were blocked. Human serum antibodies could bind these unbound sites through Fc interactions, possibly leading to a false detection signal.

Method 2 was developed with the aim of reducing the number of steps in the protocol. In this method a sample of serum taken from a high antibody titre patient with periodontal disease was heat treated and then incubated with the formalin fixed bacteria. This allowed the addition of the antigen and the serum antibody to be added in one step. The problems experienced in the first method were still not solved by this method, as all of the epitopes on the antigen surface were still not available. Whereas before they were not all available for the human serum antibodies, in this method they were not all available to the antibodies on the surface of the plasma cells in the tissue sections. Again, there was no certainty that there were no interactions between the Fc portions of the human serum antibodies and other cells in the tissue with Fc receptors.

Method 3 involved direct biotinylation of the antigen, which was then added directly to the section. Detection was achieved through the addition of biotin avidin HRP complex mixture. This method was less laborious, time consuming and complicated, and more accurate than methods 1 and 2. Furthermore, all of the epitopes on the

surface of the antigen were available for binding to specific plasma cells and there was no addition of a further step that could lead to non-specific Fc interactions. The experiment allowed specific binding of antibody on the surface of plasma cells to antigen. The antigen had already been biotinylated, and therefore, amplification and detection was carried out in 2 simple steps. The one problem experienced was that although blocking serum was added at the beginning of the experiment, there was a certain amount of non-specific binding of the antigen to the section. More background staining, and hence non-specific binding was seen when sonicate preparations were used. The purified antigens showed very little non-specific binding.

Investigations carried out by Schneider *et al.* (1966) utilised fluorescein conjugated rabbit anti-human globulin globulin to demonstrate specific staining of immunoglobulins in diseased gingival tissue sections. Further to the detection of immunoglobulins, acridine orange stained bacterial suspensions were added on to the sections to observe if these bacterial preparations were bound by the detected immunoglobulins. The methods of Schonfeld and Kagan (1980) utilised bacteria that were labelled with rhodamine, which is a fluorochrome, and these were added onto gingival tissue sections taken from periodontitis patients.

Although method 3 is quite similar to the methods described by these workers there is a difference in the detection method. The methods used by Schneider *et al.*, (1966) and Schonfeld and Kagan (1980; 1982) utilised fluorescence to detect binding between antibodies on the surface of plasma cells and antigens, whereas in this study, biotin was used as the detection agent. In this study the Vectastain Elite ABC kit was used. The principles of this kit are based on avidin, a 68,000 molecular weight glycoprotein having an extremely high affinity ($10^{15}M^{-1}$) for biotin. As the affinity of this interaction is over 10^8 times higher than that of antibody for most antigens it is essentially irreversible. In addition the biotin/avidin system can be effectively exploited because avidin has 4 binding sites for biotin and most proteins, including antibodies and enzymes, can be conjugated with several molecules of biotin. The advantages of using biotin are that it provides a permanent record of the results, whereas fluorescence is known to fade after approximately 7 days. Also the biotin avidin HRP complex mixture provides an amplification step which can be magnified

further by the addition of avidin labelled anti-avidin. Fluorescence cannot be amplified, and therefore, provides a weaker signal.

A further difference between this work and those of Schonfeld and Kagan (1980, 1982) and Schneider *et al.* (1966) is that they used frozen tissue sections whereas paraffin embedded sections were used in this study. A preliminary experiment was carried out to examine the immunohistochemistry of a frozen specimen compared with a matched paraffin embedded section. One piece of granulation tissue was taken and divided into 2 pieces, one piece was frozen and the other paraffin embedded. This experiment was carried out in triplicate. The results showed that there was no significant difference in cell numbers detected in the 2 different types of section. Therefore, paraffin embedded sections were used in this study, as they have several advantages over frozen sections. They can be stored for many years and are easier to cut in thin sections (4μm) allowing the same cells to be apparent on adjacent cut sections. Thin sections hold an added advantage in that they require shorter incubation times for optimal staining. In addition, the morphology seen in paraffin embedded sections is much better than in frozen sections and they are much easier to obtain, as an archive of paraffin embedded sections can be retained. Paraffin sections are superior to frozen sections, as long as they are completely de-paraffinised before immunohistochemistry is performed. Ensuring that all of the antigenic epitopes are unmasked is important, however, this is not experienced with frozen sections. The preliminary study indicates that the epitopes of the paraffin embedded sections are being efficiently unmasked.

Preliminary experiments were carried out to establish the optimum concentration for each antigen. Various concentrations were attempted to ascertain a concentration which gives a compromise between low background staining with an adequate staining intensity of the appropriate cells. For the whole fixed bacteria 3 different concentrations were used $1x10^7$, $2x10^7$ and $3x10^7$ bacterial cells/section. Figure 6.10 shows that for *P. gingivalis*, *A. actinomycetemcomitans*, and *P. intermedia* there was a peak number of antibody-bearing cells detected when the antigen was used at a concentration of $1x10^7$ bacterial cells/section. It was therefore, decided that this concentration would be used for all of the experiments. Three concentrations; 0.2, 0.5

and 1.0μg/ml were tested for the purified antigens. The concentration of 1.0μg/ml was chosen to be used, as on average a higher number of antibody-bearing cells were detected.

The results suggest that the number of antibody-bearing cells detected per field specific for *P. gingivalis* were significantly higher than detected in the granulation tissue for *E. coli*. The number of cells detected against *B. forsythus*, *P. intermedia*, leukotoxin (of *A. actinomycetemcomitans*) and HSP75 (of *P. gingivalis*) were also found to be significantly higher (borderline) than the number of cells detected against *E. coli*. In addition, the results for the gingival tissue samples suggest that the number of cells specific for *P. gingivalis*, *P. intermedia*, *B. forsythus* and HSP75 (of *P. gingivalis*) are significantly higher (borderline) than detected for *E. coli*.

The number of cells per field found in the gingival tissue sections specific for *P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia* are significantly lower than those found in the granulation tissue section. The numbers of cells detected against the other periodontal pathogens appeared to be lower, however, this was not justified statistically. They are not significantly lower, even though on observation of the median values they appear to be the range values for these counts are large.

E. coli was used in the experiment as a control. *E. coli* is a Gram negative bacteria, but not a periodontal pathogen, so was utilised to address the issues of non-specific binding and cross-reactivity. There was a small number of cells detected against *E. coli* which could be due to non-specific binding, however, it seems more likely that it is due to the binding of *E. coli* to plasma cells bearing cross-reacting antibodies on their surface. As discussed in the general introduction of this thesis, many antigens e.g. LPS and HSP, have homology between species. Although the numbers of cells detected against *E. coli* were very small, because of the multiple comparison analysis which is comparing the cell numbers against 9 different antigenic targets for only 5 subjects, the results do not suggest a significant difference in the numbers of cells detected against *E. coli* and all of the periodontal pathogens and antigens, however, it was the case for a number of those tested. Although, statistically this therefore, does not suggest full confidence in the technique, when the numbers of cells detected

against *E. coli* are examined they are extremely low. In addition, most of the micro-organisms and antigens are found to have statistically more specific cells against them when compared to the number recorded against *E. coli*. Interestingly, the median number of cells detected against *E. coli* is almost the same in the granulation and gingival tissue sections, further suggesting that these cells are due to non-specific binding.

In this study, a number of antibody-bearing cells specific for the purified antigens HSP75 (of *P. gingivalis*), leukotoxin (of *A. actinomycetemcomitans*) and the β segment of the RgpA protease of (*P. gingivalis*) were examined. In the granulation and gingival tissue sections there was a high number of cells detected against HSP75 and leukotoxin. There was no significant difference between the numbers of cells detected for these 2 different purified antigens. There were no cells detected against the β segment of RgpA in either tissue types.

Table 6.4 shows the percentage of the plasma cells in a field that are specific to each micro-organism and antigen tested. The results suggest that a significantly higher percentage of the leukocytes in granulation tissue are plasma cells when compared with gingival tissue. The mean percentage of the infiltrate in granulation tissue that was made up of plasma cells was 52.3% ± 6.6% (mean ± S.D.) compared to 23.3% ± 3.1% (mean ± S.D.) for gingival tissue.

Granulation tissue is highly vascularised, full of inflammatory infiltrate and found adjacent to the bone. Gingival tissue is more superficial, associated with the periodontal pocket and often referred to as infiltrated connective tissue. Therefore, due to the nature of the 2 tissue types one would expect a greater percentage of antibody-bearing cells to be detected in the granulation tissue. This area of tissue is very rich in immune cells. In periodontitis, it has been reported that 50% of the cells in the established lesion are plasma cells. In addition several investigators have shown that the inflammatory cell infiltrate in human chronic periodontal lesions is predominated by lymphocytes and plasma cells (Lindhe *et al.*, 1980; Mackler *et al.*, 1977; Page and Schroeder, 1976). The results of this study are in agreement with these reports.

The percentage of the plasma cells specific for all of the whole micro-organisms and the purified antigens is the same for the granulation and gingival tissue samples. Thus, the percentage of the total number of plasma cells specific to *P. gingivalis* in the granulation tissue is not significantly different from the percentage of the total number of plasma cells in the gingival tissue samples. Therefore, although when looking at the mean number of plasma cells per field it appears that there is a significantly higher number of plasma cells specific to *P. gingivalis* in granulation tissue samples when compared to gingival tissue, the percentage of the infiltrate that is specific to *P. gingivalis* is not significantly different.

The results of the number of cells specific to HSP75 and leukotoxin suggested cell numbers, similar to those found against *P. gingivalis* whole bacteria, in granulation tissue samples. The high numbers of cells bearing antibodies to leukotoxin does not correlate with the results of the experiments with the whole fixed bacteria *A. actinomycetemcomitans*. There was a high number of cells detected against leukotoxin in the granulation tissue samples, and a moderate number in the gingival tissues. However, leukotoxin is an antigen taken from the surface of *A. actinomycetemcomitans*, and there were very few cells found to be bearing antibody specific for this micro-organism. One would expect there would be a correlation between the number of cells found to be bearing antibody against a purified antigen, and the whole bacteria that it were taken from. Examination of formalin-stable epitope recognition may provide different results to examination of "native" antigen preparations. Important epitopes may be destroyed or changed during the fixing process, and may no longer recognised by specific antibodies. In addition, leukotoxin is a much smaller molecule than that of the whole bacteria *A. actinomycetemcomitans*. The much higher number of cells detected against leukotoxin perhaps highlights a problem of steric hindrance that is experienced on addition of the whole bacterial cells. Addition of purified antigens possibly leads to a more sensitive detection technique. If this is true, the number of cells detected against the whole bacterial cells is possibly an under estimation, and there could be a higher percentage of plasma cells specific for the whole micro-organisms in general.

For all of the subjects tested there were zero cells detected against the β segment of the RgpA protease (of *P. gingivalis*). It has previously been reported that the β segment is the immunogenic portion of this protease (Curtis *et al.*, 1993). IgG antibodies specific for this portion of RgpA have been detected in the serum of patients with periodontal disease, e.g. in chapter 3 of this thesis. However, it has been reported that the β segment of RgpA has a primary sequence similar to that of adhesins and haemagglutinins of other molecules and micro-organisms (Curtis *et al.*, 1993). Therefore, it should be considered that perhaps the serum antibodies are cross-reacting with this segment of the *P. gingivalis* protease as there does not appear to be any evidence of a local immune response in the diseased tissues of periodontitis patients against this antigenic target. The lack of detection of antibody-bearing cells to this antigen could however, be due to there not being many in the tissues, either way this is an area requiring further research.

Figures 6.12-6.16 show the percentages of plasma cells specific for the whole bacterial cells *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *B. forsythus* and *T. denticola*. The percentages of cells detected against *E. coli*, HSP75 (of *P. gingivalis*) and leukotoxin (of *A. actinomycetemcomitans*) were not included in the pie charts. The percentages of cells detected against *E. coli* were not included as it is thought that the cells shown to be specific for this micro-organism are possibly cross-reacting antibody-bearing cells. The percentages of cells detected against leukotoxin and HSP75 were also not included as some of these cells could possibly have been counted as being specific for any of the other Gram negative bacteria tested in the case of HSP75, and against *A. actinomycetemcomitans* in the case of leukotoxin.

The results obtained from this study do not appear to agree with those reported by Kagan (1980). Kagan utilised the method developed by Schonfeld and Kagan (1980) to examine the percentage of plasma cells specific to *P. gingivalis* in initial/early lesions compared with the percentage in established/advanced lesions. The gingival tissue samples cannot really be compared with the early/initial lesion samples, as there is not enough information in the report to allow this. However, the results from the established/advanced lesion samples can be compared with the results from the

granulation tissue samples. Kagan reported that 31.6% of the plasma cells in the established/advanced lesions were specific to *P. gingivalis*, whereas this study reported a mean percentage of only 9.8%. Kagan suggested that a percentage of 31.6% was high, although this could have been due to the small sample size. The results indicated that established/advanced lesions were examined for only 3 subjects, which was small. Further to this, the number of plasma cells counted in total in the study carried out by Kagan (1980) was extremely low, much lower than the number reported in this study and also that reported in the study by Mackler *et al.* (1977).

This study was carried out using granulation and gingival tissue sections, to determine if the target of the antibody-bearing cells in these different tissues differed or exhibited any different patterns. Lappin *et al.* (1999) looked at the relative proportions of leukocytes in granulation tissues samples in patients with chronic and aggressive periodontitis, and compared these with numbers obtained from gingival tissues. The results of this study suggested that B cells in the gingiva and the granulation tissue are long lived cells, and the B cells from both tissue types behave in a similar fashion. Therefore, it was of interest to compare the targets of the antibody-bearing cells from both tissue types.

The results of the experiments that attempted to detect plasma cells bearing antibodies with sonicated bacterial preparations were not included in this study. The experiments utilising sonicated bacterial preparations showed much more background staining than seen when whole micro-organisms and purified antigens are used. These results suggested that some of the binding may have been non-specific and hence, it was difficult to differentiate between specific and non-specific binding to the sections. Sonicated bacterial preparations resulted in the exposure of many molecules that are not on the surface of an organism. Whether some of these are carbohydrate moeties that are "sticky" and can non-specifically adhere on to the tissue is unknown.

It is considered that the methodological approach to this study has achieved interesting results particularly as the study was largely developmental. This is not a standard technique and hence, many preliminary experiments were carried out in the developmental stages for each part of the technique, such as optimisation of antigen

concentrations, blocking techniques, use of frozen and fixed tissue and compete labelling of antigens. Unlike conventional immunohistochemical techniques where cells are detected by the use of antibodies, this approach is aimed at achieving the opposite. The technique is based on the rationale that by the addition of antigen, the detection of antibody-bearing cells will be accomplished. The resulting data at this point is difficult to utilise in a quantitative manner, as one will never be certain that all of the antibody-bearing cells are being detected. In fact, the number of cells detected against leukotoxin compared with the number detected against *A. actinomycetemcomitans* suggests that all of the cells are not being detected. Further to this, although the preliminary experiment was carried out to compare paraffin embedded with frozen sections, one can never be totally certain that all of the epitopes have been unmasked. Therefore, although the method described in this chapter has proved successful and is a great achievement in terms of a qualitative study, whether it can be used quantitatively is more debatable.

Method 3 used in this study, is a newly developed method that could prove useful for the examination of the target of antibody-bearing cells in diseased tissues from all areas of the body, and in certain infections this could play a role in diagnosis and choice of therapy. This is a little more difficult in the polymicrobic situation of periodontal disease, however, if a larger study were carried out, possibly on individuals in health, with gingivitis and periodontal disease, more patterns may emerge on the target of antibodies in the tissues at different stages of disease. Of course, there are always difficulties in such studies, to gain ethical approval for the removal of tissues from individuals in health and with gingivitis.

In the study, the results suggested that in both granulation and gingival tissue samples, a high proportion of the antibody-bearing cells were specific for the putative periodontal pathogen *P. gingivalis*. There is a higher load of *P. gingivalis* associated with active periodontal lesions, than inactive (Dzink *et al.*, 1988; Walker and Gordon, 1990), and following breakdown after treatment, large proportions of *P. gingivalis* have been found at sites, indicating an important role for the species in pathogenesis of recurrent disease. Therefore, from this study it seems that *P. gingivalis* is important in the induction of the humoral immune response at the site of infection. Antibody-

bearing cells specific for the other periodontal pathogens tested were also identified. There was no statistical significance difference found between the numbers of cells detected against *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *B. forsythus* and *T. denticola*. However, observation of the cell figures on an individual level indicates that the numbers of cells specific for *P. gingivalis*, *A. actinomycetemcomitans* and *T. Denticola* were markedly different. With a sample size of 5, and with large variations between subjects it is difficult to detect statistically significant differences which could possibly emerge in a study with a larger population.

Analysis of the results from this study proved interesting. It was felt to be an achievement to develop a technique such as this one, that has so much potential. It is a specific detection method, that could potentially provide results using purified antigens and even specific antigenic epitopes on serial sections of tissue. Molecular biological work investigating the antigenic target in individuals that have induced a humoral immune response in periodontal disease could potentially utilise this technique to test consecutive epitopes of a particular antigen. In addition, progress with the technique could lead to a potential diagnostic tool. Investigations utilising this technique to examine the percentage of the plasma cell infiltrate specific for a particular bacteria, and on a larger scale the percentage that is unidentified is exciting. The use of purified antigens which are smaller and possibly more sensitive could be the future for this technique.

Chapter 7. Methodological Considerations and General Conclusions

This chapter attempts to do two things, firstly, it deals with the methodological considerations and secondly it brings together the results and discussions from the previous four chapters to a final conclusion.

7.1 Methodological considerations

Sample size

The first study carried out to investigate the effect of periodontal treatment on antibody titres against a panel of microbial targets was based on a sample size of 25. This sample size was similar, and in some cases larger, than those of studies previously published (Tolo *et al.*, 1982; Sandholm and Tolo, 1986; Mouton *et al.*, 1987; Horibe *et al.*, 1995; Mooney *et al.*, 1995).

The second part of that study investigating the cross-reactivity between micro-organisms was based on a sample size of 9 people. In addition, the ELISPOT technique and immunohistochemical studies were carried out both on 5 subjects. The numbers of subjects involved in these studies are small. It was felt that these numbers were sufficient to pilot the techniques used, to get a general feel for the results and observe any trends. However, statistical analysis was difficult with such a small sample size, and although the most suitable analysis possible was undertaken, the power of the analysis was weak.

7.2 Conclusions

In chapter 3 the effect of treatment on the serum antibody titres of patients with chronic periodontitis was examined. The overall conclusion from this study was that there was no significant difference between the antibody titres measured before and after periodontal treatment. There could be several reasons for this. Firstly, it could

be that the microbial targets used in the study are not causing disease. The panel of micro-organisms and antigen preparations used were, however, extensive and made up of a number of the putative periodontal pathogens (Zambon, 1996). Secondly, it could be that the organisms were not eliminated by the treatment, however, this again is unlikely as there was significant clinical improvement seen in the patients following treatment. It could be that the numbers of organisms remained at a level sufficient to maintain antigenic stimulus but not enough to cause disease, or perhaps that they were present at sites in the mouth where treatment was not carried out. The reason for not detecting a change in antibody titre following treatment, could, however, be due to the time chosen for sampling, hence the length of treatment as discussed in chapter 3. A final reason which could explain the results obtained is that there could be cross-reactive antigens that have the ability to maintain levels of antibodies in the absence of the infecting organisms. The subject of cross-reactivity was investigated therefore, in the second part of chapter 3.

The second part of chapter 3 examined pre-treatment serum antibody titres in patients with chronic periodontitis. The study investigated cross-reactivity between antibodies specific for one periodontal pathogen against three other periodontal micro-organisms. The results of this experiment suggest that there is cross-reactivity between *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus*. The results indicate that when serum is adsorbed against one micro-organism e.g. *P. gingivalis*, approximately 60-80% of the antibodies against the other 3 periodontal pathogens (*A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus*) have also been adsorbed out, indicating a high level of cross-reactivity between the 4 periodontopathogens. Similar results were found for all of the pathogens except *B. forsythus* which seemed to have less cross-reactivity with the other micro-organisms.

Chapter 3 was an investigation into the systemic humoral immune response of patients with chronic periodontitis. The systemic immune response is often examined by observation of serum antibody titres. It is useful as it is thought to reflect the local immune response and because blood is obtained easily and gives a full patient immunological assessment as contrasted with GCF which is very much site specific. However, following the study carried out in chapter 3, the natural progression was to

begin to investigate the local immune response, to try and gain a clearer understanding of the target of the humoral immune response in periodontal disease. Therefore, the remaining studies described in this thesis concerned the local immune response, examined by observation of granulation and gingival tissue, removed from periodontally diseased patients during surgery. Histopathological techniques have indicated that diseased tissues taken from periodontitis patients are populated by a large number of B lymphocytes and plasma cells, thus suggesting local antibody production (Ranney, 1991; Sims *et al.*, 1991). Therefore, the rationale behind the studies carried out in chapters 4, 5 and 6 was that examination of the antibody bearing cells in the diseased tissues of periodontitis patients will lead to knowledge of the pathogenic target inducing their production, more efficiently than observation of antibodies found in the serum. Theories have been put forward (Kinane *et al.*, 1999a) suggesting possible ways of linking the local and systemic immune responses, however, they all stem from the induction of an immune response against an antigenic stimulus in the periodontal tissues.

The general conclusions that were made following the study entitled 'Immortalised B cells from periodontal patients' described in chapter 4 are really more a discussion of the technical complexities of this endeavour and the hypothesis driving this study. The aim of the study was to produce monoclonal antibodies from the peripheral blood and granulation tissue of patients with chronic periodontal disease. Expansion of numbers of peripheral blood B lymphocytes was successful using the CD40 system, and with both expanded peripheral blood lymphocytes and antibody-bearing cells isolated from granulation tissue, fusion with a heteromyeloma partner was also successful. Following fusion, antibody production was monitored and screened for 3-4 weeks but in all of the 11 experiments carried out, antibody production stopped at 3-4 weeks and did not return. This is a problem that is often experienced when utilising this technique as discussed in chapter 4.

The ELISPOT technique was utilised in chapter 5 to investigate the specificity of antibody bearing cells isolated from the granulation tissue of patients with chronic periodontal disease. The number of antibody bearing plasma cells specific for the putative periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans*, *P.*

intermedia and *B. forsythus* were enumerated. In addition, the number of cells that were detected against the control, tetanus toxoid, were also counted, to examine the specificity of the technique. Two different concentrations of cells were used in the study.

The results showed that the only statistically significant differences in cell number were between, *A. actinomycetemcomitans* and tetanus toxoid and *B. forsythus* and tetanus toxoid for both cell concentrations. Median values indicated that the number of cells detected against tetanus toxoid appeared to be much lower than detected against the periodontal pathogens, however, the differences were not all found to be statistically significant, probably due to the low sample size of 5.

This study indicated that, the ELISPOT is a technique that can be utilised to study the target of cells isolated from periodontal tissues and hence, the local immune response induced in individuals with periodontal disease. In addition, it has suggested that there is no significant difference between the numbers of cells bearing antibodies to the four periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *B. forsythus*. Chapter 3 indicated a high percentage of cross-reactivity between these pathogens. Therefore, we cannot confirm that when an antibody bearing cell is detected against one micro-organism, it has not been initially induced against another micro-organism.

For this reason the technique in chapter 6 was developed. The aim of this study was again the detection of antibody-bearing cells in the tissues of patients with periodontal disease, however, the technique developed held a number of advantages over that of the ELISPOT. Firstly, the specially designed 96 well ELISPOT plates were extremely variable with regard to the bacterial preparations and antigens that were able to bind to them. Fixed bacteria and the purified antigens tested did not bind successfully to the plates and the experiments were unsuccessful when these preparations were used. In addition, enumeration of the ELISPOTs was quite difficult. With regard to the problem discussed above, the further advantage of the immunohistochemical technique developed in chapter 6, was that when serial sections were used, it was

possible to detect whether antibodies on the surface of plasma cells were binding and hence cross-reacting with a number of micro-organisms.

The study described in chapter 6 examined paraffin embedded gingival and granulation tissue sections taken from chronic periodontitis patients. The results indicated that the number of cells bearing antibody to *E. coli* was significantly lower than to the majority of the putative periodontal micro-organisms and antigens tested, indicating that this technique was specific.

The results of this study firstly indicated that a novel technique had been developed that was able to detect antibody-bearing cells against whole fixed periodontopathogens and purified antigens from some of these. The technique was based on the biotin-avidin system making it inexpensive, easy to use and not labour intensive. The results indicated that there was no significant difference in the number of cells detected against any of the whole fixed micro-organisms tested and the purified antigens leukotoxin (of *A. actinomycetemcomitans*) and HSP75 (of *P. gingivalis*) in either of the tissue types. No cells were detected to be specific against the β segment of the RgpA protease (of *P. gingivalis*).

The results further indicated that there were significantly more cells detected against *P. gingivalis*, *A. actinomycetemcomitans* and *P. intermedia* in the granulation tissue compared with the gingival tissue. However, the numbers of cells specific for a particular antigenic target were then expressed as a percentage of the total plasma cell infiltrate and when the percentages were observed it became clear that there was no difference in the percentage of cells specific to a particular micro-organism in the different tissue types. The difference lay in the size of the infiltrate and, therefore, the number of plasma cells that had permeated into the tissues. These results were, therefore, consistent with the ELISPOT results which suggested that in general there is no significant difference between the number of cells bearing antibody to the different micro-organisms and antigens tested. The percentages of plasma cells specific for each of the targets were presented in the form of a pie chart for each subject individually. It was very interesting that the results could be expressed in this form, firstly because it gives the reader a clearer idea of the number of cells specific

for a particular target, and secondly because it allows the percentage of plasma cells whose target is unidentified to be highlighted.

7.3 Suggestions for future research

7.3.1 Examination of the effect of treatment on the humoral immune response

As discussed in chapter 3, it is very difficult to compare studies performed by different groups, as the length of treatment appears to vary so much. To truly measure the effects of treatment on the immune response, it would be interesting to analyse serum samples at much shorter time intervals following treatment. For example if a serum sample was taken every 2-4 weeks following treatment for a time period of 6 months. Examination of samples taken like this would allow the dynamics i.e. any increases or decreases in titre to be noted over time.

7.3.2 Further work on monoclonal antibody production

The aim of the monoclonal antibody production study was novel but proved too complex at this time. Obtaining monoclonal antibodies produced by the protocol described in chapter 4 could in future provide some interesting results. If monoclonal antibodies were produced and the antigen binding portion of these sequenced, it could lead to identification of the antigenic target that has induced the immune response, whether it be a known antigen or not.

7.3.3 Detection of antibody-bearing plasma cells in granulation and gingival tissue

The technique developed in this study has potential and should be utilised in studies of other chronic infectious diseases not only periodontitis. In terms of the study described in chapter 6, it would be interesting to compare tissue taken from patients with different disease forms (e.g. gingivitis, aggressive periodontitis, chronic periodontitis), and see if there are differences in the immune infiltrate between these different disease entities. In addition, analysis of immunoglobulin isotypes and examination of further antigens would be of interest. Cross-reactivity studies could

also be carried out by utilising serial sections. This technique could also be used to further observe the percentages of the plasma cell infiltrate specific to a particular antigenic target. Having the ability to be able to do this could prove valuable in the future as it puts the results into a more understandable context. The technique should be utilised to look at a larger panel of purified antigens. A study specifically investigating the phenomena of cross-reactivity is also very important and this technique would be an excellent tool to do this with.

7.4 Concluding remarks

The future identification of the target or targets of the humoral immune response in periodontal disease could have wide-ranging implications. They could potentially include the possibility of new diagnostics, prophylactics and therapeutics. Whether antibody responses to specific antigens are markers of disease susceptibility or activity is currently unclear. Progress is, however, being made in identifying marker antigens and species out of the 300-400 bacterial species identified in microbial plaque.

The studies presented in this thesis have provided an insight into how the antigenic targets of plasma cells in periodontitis may be elucidated. The results reported show the development of a new detection technique that can be used to identify plasma cells directly that are bearing antibodies to a particular antigenic target. The results also emphasise the uses of techniques such as the ELISPOT, another method that can be utilised to investigate the target of the immune response in periodontal disease. As well as highlighting aspects regarding technical issues, the studies reported in this thesis have addressed the issues of cross-reactivity between Gram negative bacteria, the target of antibodies being produced locally in diseased tissues of periodontitis patients and responses to antigens that have previously been described as immunodominant such as LPS, leukotoxin and HSP.

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List of Publications

Abstract presentations:

Podmore M, Lappin D, Mooney J, Kinane DF. Microbial targets in periodontal disease. J Dent Res 1998; 77 (Spec Iss):1029.

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Kinane DF, Podmore M, Ebersole JL. Etiopathogenesis of periodontitis in children and adolescents. Periodontology 2000 2001; 26 (in press).

Microbial immune targets in periodontal disease. M. PODMORE,* D.F.LAPPIN, J. MOONEY & D.F. KINANE. (University of Glasgow Dental School, UK)

The immune response against periodontal pathogens is considered important in periodontal disease. Knowledge of the microbial targets for serum and crevicular fluid antibodies is crucial in the development of diagnostic techniques and for immunisation strategies. The aim of the study was to examine the microbial targets of peripheral humoral immune cells in ten patients affected by severe chronic periodontitis (35 to 65 years of age). ELISPOT assays were used to measure the presence of antibody producing cells in patients with periodontal disease. This assay has provided useful preliminary data which will assist in the identification of immunodominant antigens against which the humoral immune response in this disease is directed. A second approach was to utilise cell culture supernatants from Epstein-Barr virus (EBV) transformed pre-B cells from patients with periodontal disease which were screened by ELISA for antibodies against *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia* and *Bacteroides forsythus*. After screening 500 EBV transformed wells for each patient we detected between 15 and 30 positive wells for each of the three putative periodontal pathogens tested. These EBV transformed clones were combined with a human myeloma cell line to form discrete hybridomas capable of producing monoclonal antibodies. Thus human monoclonal antibodies can be produced from pre-B cells of periodontitis patients and a proportion are directed against periodontal pathogens.

The effect of periodontal therapy on the humoral immune response in patients with chronic periodontal disease. M. PODMORE,* I.B. DARBY & D.F. KINANE. (University of Glasgow Dental School, UK)

Numerous bacteria and the host humoral immune response to their antigens have been implicated in the pathogenesis of periodontal disease. The aim of this study was to assess the effect of initial periodontal therapy on 25 untreated adult periodontitis patients, and specifically assess the patient's serum antibody titres to a range of bacteria and specific antigens. The antigenic targets investigated were the whole fixed bacteria of *Porphyromonas gingivalis* (P.g), *Actinobacillus actinomycetemcomitans* (A.a), *Prevotella intermedia* (P.i), *Treponema denticola* (T.d) and *Bacteroides forsythus* (B.f), crude outermembrane protein preparations of P.g, A.a, P.i and B.f and various purified antigens thought to play a role; Leukotoxin (A.a), Lipopolysaccharide (P.g), Heat shock protein 60 (P.g) and RIA protease (P.g). Antibody titres were assayed by ELISAs. Following the initial therapy the average reduction in pocket depth was 2.1 mm ($\pm$ 0.9). In addition, specific antibody titres increased after treatment for all whole bacteria and antigens tested, for example, the antibody titre to P.g whole cells before treatment was 0.510 ELISA units ($\pm$ 0.180) and after treatment was 0.927 ELISA units ($\pm$ 0.334). This increase was statistically significant ($P<0.05$). Interestingly, P.g outer membrane protein preparations had a 119% increase in titre whereas antibodies to Lipopolysaccharide from P.g increased by only 25.1%.

<u>In conclusion, periodontal therapy influenced the magnitude of the humoral immune response to all of the putative periodontal pathogens and specific antigens tested. Initial periodontal treatment does not increase antibody titres to certain periodontal pathogens and their antigens to the same extent and this may indicate the relevance of certain candidate antigens in the disease process.</u>

TEXT BOUND INTO

THE SPINE

IMMUNODOMINANT ANTIGENS IN PERIODONTAL DISEASE: A REAL OR ILLUSIVE CONCEPT?

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ABSTRACT: The humoral arm of the immune system provides protection from many medically significant pathogens. The antigenic epitopes of the pathogens which induce these responses, and the subsequent characteristics of the host response, have been extensively documented in the medical literature, and in many cases have resulted in the development and implementation of effective vaccines or diagnostic tests. There is a substantial body of literature on the humoral immune response in periodontal disease, which is targeted at micro-organisms present within periodontal pockets. However, the significance and specificity of the immune response in periodontal disease have proved difficult to elucidate, due to the large number of potential pathogens in the plaque biofilm and the apparent commensal nature of many of these opportunistic pathogens. This review addresses our current knowledge of the approaches and strategies which have been used to elucidate and examine the concept of immunodominant antigens in medical infections and, more recently, periodontal disease. An identification/understanding of the immunodominant antigens would be informative with respect to: (i) the relative importance of the implicated pathogens, (ii) new approaches to immunological diagnosis, (iii) specific bacterial virulence determinants, (iv) natural protective responses, and (v) the selection of potential vaccine candidate antigens. We conclude that immunodominance of antigens in periodontal disease may be relevant to our understanding of periodontal disease pathogenesis, but due to the complexity and diversity of the 'pathogenic microbial ecology', it is currently an enigmatic topic requiring a multidisciplinary approach linking clinical, microbiological, and immunological investigations. We also conclude, after assessing the literature available on the topic of immunodominance, that it is a term that, if used, must be clearly defined and understood, since it is often used loosely, leading to a general misinterpretation by readers of oral and medical literature.

Key words. Immunodominance, periodontitis, oral pathogens, antigens, detectable antibody response.

(I) *Definition of the Term "Immunodominant Antigen"*

In dealing with this topic, we must first define the term 'Immunodominance'. This concept is often misused by investigators who go on to provide data and an interpretation that specific antigens or epitopes of a micro-organism or macromolecule are 'immunodominant', with little explanation as to the basis of the finding and, importantly, its significance. The term 'immunodominance or immune dominance' was initially coined by Eli Sercarz (1989) to provide a framework for describing the immune response gene control of antibody responses to hen egg lysozyme (HEL). HEL is a globular protein with a known three-dimensional structure, which enabled these investigators to define antigenic epitopes using peptides derived from cyanogen bromide cleavage of this protein. Specifically, immune responses of H-2^b mice to HEL were restricted to a domain at the amino terminus of the molecule—in fact, to 3 amino terminal residues. Wicker *et al.* (1984) reported that if these 3 residues were cleaved, 50% of the primary response antibody molecules bound poorly if at all to the HEL, and therefore, the immunogenicity of the protein was drastically affected. The term 'immunodominance' was used to describe the predominant immune recognition of these 3 amino terminal residues. Sercarz (1989) thus suggested that, for efficient and regulated activity of the immune response to a particular antigen, there should be a small number of immunodominant epitopes on an antigen.

The primary, secondary, and tertiary structures of a protein antigen present a vast array of potential epitopes; however, the number recognized by the immune system appears to be considerably smaller than the full, potentially antigenic repertoire of the immunogen (Laver *et al.*, 1990). Some of these antigenic epitopes will dominate the resulting specificity of the immune response, which can vary from species to species and even among individuals within a species, and will not only induce a more pronounced immune response, but may also suppress the primary immune response to other antigenic determinants. Recent studies with a similar experimental system have demonstrated the broad heterogeneity of proliferative responses to the immunodominant determinants within HEL. The heterogeneity of response was suggested to relate to the competitive, as well as regulatory, nature of the interaction among various factors, including different Major Histocompatibility Complex (MHC) molecules, determinant capture by antigen-presenting mechanisms, and the available T-cell repertoire. The authors suggested that these results, while directed toward focused studies of an isolated protein, may have important implications for studies of autoimmunity, infection, and vaccine design in human populations, where heterozygosity is the characteristic of the population (Moudgil *et al.*, 1998). The basis for the existence of immunodominant epitopes is less well-understood. Recent findings have

wn that peptide competition for MHC binding may not repre- the most important event in processes leading to immuno- minance (Lo-Man *et al.*, 1998). As has been noted with the ulting antibody to a particular protein antigen, T-lymphocyte ponses to a protein antigen are restricted to a limited number determinants and not to all peptides capable of binding to HC class II molecules. This focusing of the T-cell immune ponse is also defined as immunodominance and has been served with numerous antigens. Recent investigations by pin *et al.* (1998) have suggested that dendritic cells play a major le in the focusing of the immune response against a few anti- nic determinants, while B-lymphocytes may diversify the T-cell ponse by presenting a more heterogeneous set of peptide- HC complexes. Moreover, this T-cell focusing has been extend- to cellular immune responses, which are directed against a row set of immunodominant peptides derived from complex igens. The existence of epitopes that are hidden or infrequent gets of immune responses (cryptic epitopes) has also been ntified. Although the identification of immunodominant epi- pes is important for vaccine development, an understanding of munological reactivity to these cryptic epitopes may be impor- t in the development of autoimmunity. Blum *et al.* (1997) have ggested that targeting exogenous antigens into specialized ocessing compartments within antigen-presenting cells pears to be important in epitope selection and immunodomi- nce. The final outcome of the immune response may be an munochemical phenomenon that is related to the protein ucture or the host's immunoregulatory mechanisms; however, also appears to be effectively used by various pathogens as a st evasion tactic. This occurs when the microbial immuno- minant epitopes are sterically close to antigens which have the pacity to demonstrate antigenic diversity or are capable of anti- nic variation. Thus, they help to conceal the micro-organism m the host-protective responses (Nara *et al.*, 1998). **(AQ)**

In the field of periodontal research, the term 'immunodomi- nce' has been used imprecisely, and is poorly defined and ten misrepresented. There are multiple reports in the literature urportedly characterizing immunodominant antigens of oral cro-organisms, yet no clear definition of what is meant by munodominance is included, and the term is used to describe e detection of a high antibody titer in an ELISA, or substantial tibody reactivity to an antigenic molecule that has been visu- lized on a Western immunoblot. Studies by Califano et al (1992) nd Boutsi et al (1996) provide examples of investigations mis- sing the term 'Immunodominant antigens' in their reports of udies on *Actinobacillus actinomycetemcomitans* and *Porphyromonas ingivalis* antibody levels in patients. Do these antigens truly xpress an epitope that is the focus of the primary immune sponse, and which is essential for the initiation of the response the antigen and micro-organism? (Wicker *et al.*, 1984) The obvi- us answer is that currently we do not know. Due to the chronic ature of periodontal infections, these objective measures may nly be defining the gravimetric quantity of a particular antigen, ather than describing a function of its unique antigenicity. Thus, he original definition of immunodominance has been distorted describe a microbial antigen to which a predominance of anti- ody is detected when compared with the myriad other antigens resented by the micro-organism and recognized by the host. For he purposes of this review, we utilize the term 'detectable anti- ody response' to overcome these difficulties and to avoid con- usion when addressing the periodontal humoral immune esponse reports.

(II) The Complexities of the Periodontitis Humoral Immune Response

Periodontal disease is a multifactorial disease with a complex polymicrobial etiology. This not only varies between various forms of clinical disease, but may also vary at the individual patient level. For example, a subject may exhibit a disease process associated with a different species or consortia of bacteria at different disease sites, or recurrent disease exacerbations may occur in the context of infection and/or emergence of different pathogenic bacterial species/consortia temporally in the site. In this regard, periodontal disease has been difficult to study, due to the large number of species comprising the microbial biofilm at sites of health and disease, proposed genetic predispositions, behavioral variables such as adoption of oral hygiene, and environmental factors (*i.e.*, smoking, and hormonal and socio-economic factors). In addition to these factors, the nature of the humoral immune response itself is also controversial. Whether the antibody response in this disease is protective or in fact a facet of the pathology is unknown. One would think that disease activity would be abrogated if the antibody response was effective at eliminating or controlling the growth of the micro-organisms present. That is of course not to say that all pathology seen in this disease is directly due to the harmful effects of micro-organisms. Production of high levels of pro-inflammatory cytokines (IL-6, IL-8, TNF) has been reported (Tsai *et al.*, 1995; Yavuzyilmaz *et al.*, 1995), which could result in considerable tissue destruction. In this review, however, we are dealing with the humoral antibody response in periodontal disease and hence will not discuss this issue further. A further consideration in periodontal disease is that critical clinical features of the disease are often identified a substantial period after the biologic processes have initiated the disease course.

(III) Immune Responses in Polymicrobial Infections

This review addresses our current knowledge of the antigens to which a detectable immune response in periodontal disease may be directed. However, in addressing this complex issue, one must consider that within a complex microbial biofilm, such as the subgingival plaque, it appears that selected micro-organisms elicit greater antibody levels (*i.e.*, dominate the immune response) than others (Zambon, 1996). This characteristic of the host response does not appear to depend entirely on the absolute numbers of an individual micro-organism, but may be more related to their structural and antigenic components or their pathogenic significance (virulence), as well as their distribution within the biofilm and/or host tissues. An understanding of the antigens, which elicit a detectable antibody response, should contribute to a better understanding of specific bacterial virulence determinants, natural protective responses, and even possible strategies for vaccine development, or new approaches to immunological screening and diagnosis of the disease.

(IV) Immunodominance in Microbial Diseases

A survey of the scientific literature on immunodominant antigens in medical infectious diseases provides an indication of the significance of this concept for identifying potential virulence, diagnostic, or vaccine candidates. In the following discussion, we have reviewed carefully selected studies to provide examples of the variation in the use of the terminology, as well as the potential limitations if one equates detectable antibody response with immunodominant antigen and protective immune responses.

Examples of studies identifying immunodominant and there-e candidate diagnostic antigens in parasitic infections include dies on *Neospora canium* (Howe *et al.*, 1998), *Theilera parva* tende *et al.*, 1998), *Trypanosoma cruzi* (Pereira *et al.*, 1998), and *istosoma mansoni* infection (Chen and Boros, 1998). It is clear m the literature that often an antigenic relationship to similar igens from other parasites, and prevalence of the response ring infection, is used as evidence to speculate on the impor-ce and function for potentially "important" antigens during ection. Also, identification of important antigens from these es of studies can lead to a higher degree of sensitivity and ecificity in diagnostic documentation. Some studies have even covered information on the disease pathology and progres-n. For example, Chen and Boros (1998) identified that the mmunodominant T-cell epitope, P38, of *Schistosoma mansoni* elicit-the pulmonary granuloma formation, which is a disease-asso-ted hypersensitivity lesion of this infection.

There have also been several similar studies which focused dominance in bacterial or fungal infections. The mortality e for systemic candidosis remains high. However, the diagno-s is somewhat difficult, and there has been considerable inter-t in developing a reliable serodiagnostic test. Mathews *et al.* 988) identified the 47-kDa component of *Candida albicans* as an mmunodominant antigen' in the serology of systemic candido-s. These investigators suggested that the 47-kDa antigen ems to be immunodominant, and 92% of patients with candi-al antibodies produced a response to an antigen of 47 kDa Mathews *et al.*, 1984, 1987). Therefore, it was proposed that this tigen is conserved in structure between strains, and an assay tecting this antigen would be the basis for a sensitive and ecific diagnostic test.

Further interesting and important knowledge has been ained from studies involving immunodominant antigens and e quest to identify them. The *Mycobacterium avium* complex AC) consists predominantly of *Mycobacterium avium* and M. *intra-llulare* (Inderlied *et al.*, 1993). MAC members are ubiquitous envi-nmental micro-organisms, and infection in healthy individuals rare. However, MAC infection is a major cause of bacterial mor-dity and mortality in AIDS patients. A recent study by Triccas *et* (1998) reported, after a molecular and immunological analy-es, that a 35-kDa protein of M. *avium* is a homologue of the 35-Da immunodominant antigen of M. *leprae* (Triccas *et al.*, 1996). e 35-kDa protein of MAC was confirmed to contain T-cell and B-ll epitopes recognized by human immune responses following ycobacterial infection, but it was discovered that, immunologi-ally, there is an inability to distinguish between MAC and M. *uberculosis*, even though the 35-kDa protein is not recognized by uberculosis patients. Although this is an example of a study here an antigen eliciting detectable antibody responses is iden-fied, it does not lead us to a potential vaccine candidate or any ther markedly positive conclusion. For both tuberculosis and ycobacterial infections, early diagnosis is crucial for the prog-osis of the patients, and therefore emphasizes a situation where e use of a detectable antibody response for differential diagno-s is critical.

Studies investigating dominant antigens, even if they are not rimarily designed to do this, can answer crucial questions. Otitis edia is a common problem associated with high morbidity, in hich a subset of children experience repeated episodes caused y non-typeable *Hemophilus influenzae* (NTHI). Murphy and Yungcheol (1997) have shown that the principal antibody esponse in animals to an NTHI strain was directed at an immunodominant epitope of the P2 molecule, which is the major outer membrane protein of NTHI. However, what this group hypothesized is that the otitis-prone children develop an anti-body response to an immunodominant region of the P2 mole-cule, but that this region is strain-specific. Therefore, the child's protective response is directed exclusively at the infecting strain, and hence, he/she will experience recurrent episodes of otitis media, since he/she has no protection against a new strain. The discovery of the dominant antigen associated with this infection has further important implications, in that it contributes to our understanding of the recurrent nature of otitis media, and pro-vides useful data on a potential vaccine design for this disease.

As mentioned in the preceding section, the importance of T-cell immunodominant epitopes is clear and must be distin-guished from those eliciting a dominant B-cell response. *Listeria monocytogenes* secretes proteins associated with its virulence in the infected cells, which are degraded by host cell proteasomes,**(AQ)** processed into peptides, and bound to MHC class I molecules. These antigen epitopes are effectively presented to cytolytic T-cells (CTL). Interestingly, results of Pamer *et al.* (1997) indicated that immunodominant T-cell responses cannot be predicted by the prevalence of antigens or epitopes. In fact, one of the least prevalent epitopes primes for the immunodominant CTL response in mice.

These are all examples of the purported significance of iden-tification of immunodominant antigens and the potential bene-fits in medically important infections. Utilization of the antigen(s) to create diagnostic tools and develop vaccines attaches greater value and relevance to the search for these types of antigens.

(V) *Periodontal Pathogens*

Concerns about identifying periodontal pathogens as true etio-logical agents in the disease process still exist. Many of these candidate pathogens may be merely opportunistic and depend on the biofilm "food web" to create the conditions or niche with-in which they can colonize, obtain nutrients, and replicate. Thus, these micro-organisms would not classically be considered as the true pathogens to which host defense mechanisms should be directed. In spite of the potential microbial complexity of this disease, several candidate species have emerged which are thought to play a role in the pathogenesis of periodontal disease. These micro-organisms generally fulfill the modified Koch's pos-tulates as described by Socransky and Haffajee (1992).

Examination of numerous proposed periodontal pathogens has resulted in the determination that these micro-organisms possess a variety of potential virulence factors and structures that may be crucial to their pathogenicity. In the quest for the identi-fication of the more important bacterial antigens in periodontal disease, different research groups report, sometimes with con-flicting results, different potential candidate antigens.

(A) Outer Membrane Proteins

Much of the periodontal literature applicable to this review topic covers studies on the Outer Membrane Proteins (OMPs) of perio-dontal pathogens. OMPs of Gram-negative bacteria may reside on the inner or outer layer, or can span the entire outer mem-brane. Gram-negative bacteria possess multiple OMPs which pro-vide important biological functions, such as phage receptors or receptors for uptake of nutritional substrates. In this regard, vari-ous specific microbial antigens have been identified from the outer membranes of oral pathogens, and have been suggested to play a role in periodontal disease. However, many studies have

ified outer membrane antigens of the same micro-orga-s, which remain uncharacterized and may be of importance otection afforded by the adaptive host immune response anabe *et al.*, 1989; Califano *et al.*, 1989, 1992; Wilson *et al.*, **(AQ)** Page *et al.*, 1992). These studies have tended to focus ther sonicate antigens or Outer Membrane Proteins which be sheared from the surfaces of the bacteria (Nakagawa *et al.*, 5). Since these two preparation methods differ in the fact that produces just surface antigens and one consists of the rity of the antigens of the bacteria, both internal and exter-one could estimate both antibodies to intracellular antigens, ell as to the surface antigens. Similar results, however, might xpected. It seems unlikely that patients would produce an y of antibodies to "inaccessible" intracellular proteins, and e likely that antigens eliciting a detectable antibody response ld be expressed on the microbial surface or as secreted pro-s. The majority of the outer membrane components of puta-periodontopathogens remain uncharacterized with respect eir metabolic or structural functions or their activity as viru-e determinants. In general, patients with strong responses to e individual antigens often tend to be those who have a ng response to the whole bacterial cell.

Nakagawa *et al.* (1995) reported detection of antibodies to arent 'immunodominant antigens' of P. *gingivalis* in the sera of lt periodontitis patients, which were specific for antigens of 75 31 kDa molecular weight from outer membrane preparations, a 46-kDa antigen from sonicated extracts of the micro-orga-m. Further studies identified 75-, 55-, and 43-kDa bands in le-cell sonicates of P. *gingivalis* to be 'immunodominant anti-s' in rapidly progressive periodontitis patients (Chen *et al.*, 5). A similar approach was utilized by Ebersole and Steffen 4). They documented 'immunodominant antigens' in outer mbrane preparations from various strains of P. *gingivalis*. The lts identified some similarities in antibody recognition of gen bands across strains; however, a striking variability was ced between the strains. Additionally, variations in response erns were noted to be associated with high- and low-response ents, regardless of the clinical disease classification.

Ebersole *et al.* (1995) and others (Watanabe *et al.*, 1989; stad *et al.*, 1990; Sims *et al.*, 1991) have used Western munoblot approaches to detect antibody responses specific antigens in the outer membranes from A. *actinomycetemcomi-*. Interestingly, these studies revealed a striking variability in ponses among individual patients, thereby posing a potential gma regarding the identification and/or importance of an anti-n(s) that dominates throughout a patient population. rnatively, Flemmig *et al.* (1996) demonstrated IgG/IgA anti-dy activity in Early Onset Periodontitis (EOP) (65%/70%) and lt Periodontitis (AP) (45%/55%) patients reactive with a 110-a OMP of A. *actinomycetemcomitans*. In contrast, control subjects d no IgG, and only 5% had any IgA antibody activity. This proach exemplifies a strategy whereby a high frequency and/or el of antibody is detected to a particular antigen in patients *vs.* trols, which is then suggested to be an immunodominant gen. Each of these studies aimed to identify immunodomi-t antigens as macromolecules which may play an important e in the pathogenesis of periodontal diseases, and to correlate response with disease resistance. In fairness to the authors, ny of these studies probably identified the molecular weight antigens which elicited a detectable antibody response in this ease, but none of these studies obtained sufficient evidence dicative of the presence of an immunodominant antigen.

Importantly, the OMPs are uniformly immunogenic, and many demonstrate antigenic diversity (heterogeneity) (Holt and Bramanti, 1991; Holt and Ebersole, 1991; Mitchel, 1991).**(AQ)** Moreover, environmental changes altering the growth of numerous micro-organisms, such as *Bordetella pertussis*, have been shown to result in antigenic modulation of outer membrane proteins (Robinson *et al.*, 1986). Genco and colleagues (1991) presented the concept of the potential importance of antigenic heterogeneity as a virulence strategy for oral micro-organisms. Recent studies by Ebersole and colleagues (1995) with A. *actinomycetemcomitans*, Chen *et al.* (1995) with P. *gingivalis*, and Sims *et al.* (1998) with B. *forsythus* have supported antigenic diversity of these pathogens within infected patients, but there remains minimal information of its potential role in virulence within the periodontal microbiota.

The previously mentioned studies are representative of strategies which have been used and provide examples of some potentially interesting targets. However, they only "scratch the surface" in providing an understanding of the antigenic epitopes which activate the regulatory T-cells and effectively stimulate regional B-lymphocytes which could provide a functional host immunity in periodontal disease. Current genome sequencing of these pathogens will allow us to visualize the organization of these protein genes, as well as enhancing studies of genetic and antigenic heterogeneity within the population. Sequencing, purification, and functional studies of these antigens will encourage evaluation of their significance in well-characterized patient and control populations, as well as in *in vitro* and *in vivo* models of disease. More importantly, from the perspective of this review, it is not clear that the concept of a 'dominant antigen' is critical to the ultimate goals of periodontal research. As mentioned previously, high antibody levels or frequency to an antigen does not necessarily define immunodominance of the antigen, but rather delineates a dominant antibody response. If this is directed to a 'flak antigen', an antigen capable of significant diversity or variation, or having little contribution to the pathogenicity of the micro-organism, the detection of a dominant antibody response does not inherently describe an immunodominant antigen or provide insight into immune-protective responses.

(B) Fimbriae

The available literature supports that fimbriae of P. *gingivalis* are a target of the humoral immune response in periodontal disease patients, although a lack of antibodies reactive with fimbriae in patients has not been defined as increasing disease risk. This has been delineated in various disease populations, with a general finding of increased levels and frequency of serum antibody to the fimbriae or fimbrillin subunits (Okuda *et al.*, 1988;**(AQ)** Ogawa *et al.*, 1989; Genco *et al.*, 1991). Recently, Condorelli *et al.* (1998) reported the detection of IgA antibodies in the gingival crevicular fluid (GCF) of patients with acute recurrent periodontitis. The antibodies were specific for the purified 43-kDa fimbrial antigen. Thus, it appears clear that both a local and a systemic humoral immune response are generated to this antigen in many periodontitis patients. Evidence that fimbriae are immunodominant antigens in periodontal disease, or in fact in other infectious diseases, is generally lacking.

(C) Lipopolysaccharide

Numerous studies have demonstrated that the serotype determinants of A. *actinomycetemcomitans* are associated with a high-molecular-weight LPS-associated antigen (Califano *et al.*, 1989) or carbohydrate smear antigen (Califano *et al.*, 1989; Sims *et al.*,

). Interestingly, in Early Onset Periodontitis (EOP) patients the highest serum antibody levels to A. *actinomycetemcomitans* the predominant antibody specificity is to the LPS (Okuda *et al.*, 1986; Wilson, 1991; Califano *et al.*, 1992; Wilson and Hamilton, 1995). Moreover, while LPS has generally been thought to exhibit immune-stimulating characteristics which are T-cell-independent, and thus induce principally an IgM response, it is clear that EOP patients produce substantial levels of IgG antibody to this antigen from A. *actinomycetemcomitans* (Lu *et al.*, 1993). This antibody also demonstrates apparent subclass specificity, in that it is primarily/exclusively of the IgG2 subclass (Lu *et al.*, 1993). The level and characteristics of this dominant antibody response suggest that an anti-LPS response is a significant marker of A. *actinomycetemcomitans* infecting periodontitis. However, whether the LPS represents a virulence determinant, a true immunodominant antigen, or an antigen to which the adaptive immune response can provide protective immunity remains to be delineated.

The literature is replete with studies reporting a significant prevalence of periodontal disease patients with high titers of serum IgG antibodies to P. *gingivalis*, although the temporal acquisition of the antibody response relative to colonization and/or the infectious processes associated with this micro-organism have not been clearly delineated. Thus, strategies involving animal models have been developed to examine the acquisition and specificity of potential protective immunity. A recent study by Hasel *et al.* (1996) reported that immunization of monkeys with a vaccine containing a monkey isolate of P. *gingivalis* induced protection against alveolar bone destruction.

Consequently, the validity of targeting LPS from oral pathogens as an immunodominant antigen from the perspective of identifying naturally protective responses for the purpose of prevention or intervention through vaccine implementation is still fraught with critical unknowns. Nevertheless, it appears that, at least with certain periodontopathogens, the detectable antibody response to these types of antigens may provide some useful diagnostic information.

(D) Heat-shock proteins

Studies supporting the importance of Heat-shock Proteins have been reported in the periodontal literature for some time. Immunoblot analyses with purified P. *gingivalis* GroEL revealed that it was recognized by antibody in 8/10 sera from patients tested and in only 3/9 healthy controls (Maeda *et al.*, 1994). Evidence of this nature, however, does not warrant a further discussion of this antigen in a review looking at immunodominance.

(E) Leukotoxin

Periodontitis patients with A. *actinomycetemcomitans* infections generally demonstrated extreme elevations in antibody to leukotoxin (Califano *et al.*, 1989; Ebersole *et al.*, 1991; Gunsolley *et al.*, 1991), suggesting that it is a strong immunogen. This has been confirmed in both non-human primate (unpublished observations) and rodent models (Shenker *et al.*, 1993). Details of the human response consistently showed elevated leukotoxin antibody in localized early-onset periodontitis (LEOP) patients (McArthur *et al.*, 1981; Tsai *et al.*, 1981; Ebersole *et al.*, 1983). Relative to the focus of this review, a crucial point is that the antibody to the leukotoxin was not a dominant antibody response in these patients. As mentioned previously, the magnitude of response was clearly to the LPS and serotype determinants from A. *actinomycetemcomitans*. The resulting interpretations suggested that LPS was an immunodominant antigen, and thus the response to this molecule should be the focus of immunological efforts to interfere with its pathogenicity. Although the biologic relevance of the leukotoxin as a virulence factor has not been clearly demonstrated *in vivo*, recent genotypic studies support the concept that A. *actinomycetemcomitans* isolates which have an altered promoter region, resulting in enhanced leukotoxin production, are most frequently associated with disease sites in selected populations (DiRenzo *et al.*, 1994;**(AQ)** Haubek *et al.*, 1995). Additionally, analysis of recent data suggests that leukotoxin antibody levels may be more discriminatory than antibody to the whole bacteria (Califano *et al.*, 1997). Furthermore, limited evidence from animal models has suggested that challenge by leukotoxin, of a human immune system reconstituted into mice, led to the induction of antibodies which correlated with some protection from lesion formation (Shenker *et al.*, 1993). Therefore, while the reactivity of humans to the leukotoxin cannot be considered a 'dominant' response, there appear to be 'dominant epitopes' on this antigen which can consistently elicit antibody in infected subjects that appears**(AQ)** to ameliorate the effects of this toxin. Consequently, the use of antibody to the leukotoxin as a biomarker for this disease, and as an immunological target, seems worthy of continued investigation.

(VI) *Concluding Remarks*

As mentioned previously, the examples in this review are to provide an overview of the approaches and strategies which have been used in medical infections and are currently being used in periodontal infections to examine the concept of immunodominant antigens.**(AQ)** Where does the future lie for the immunodominance concept in periodontal disease research? 'Immunodominance' in periodontal disease may be a relevant entity. However, with the complexity of this polymicrobial infection and the intertwining of the relationships within the microbial ecology, the plethora of host and microbial variables may make any successful exploitation of any intervention strategy based on the immunodominance concept difficult to implement in this disease. This concern is exemplified by the breadth of studies supplying often-disparate findings regarding the antigenic specificity of dominant host responses to the group of putative pathogens.

One important consideration emphasized in this review is that future studies and published reports must be more careful in their use of the term 'immunodominance'. Authors should be clear what they mean by this term and must define it for their readers. 'Immunodominance' is a term too readily used in an unqualified manner, leading to much confusion and misinterpretation.

If the identification of immunodominant antigens is relevant and additional supportive knowledge is gained, we would suggest that utilization of these critical antigens for screening and diagnosis of the disease, as well as using them as targets for vaccine development, will be not only feasible but also desirable. This is a rich area for future periodontal disease research, and we anticipate that additional data will more accurately identify whether critical antigens are immunodominant or are just the target of a detectable antibody response. Importantly, this area of research will require multidisciplinary expertise from "chairside to lab bench" in linking clinical, microbial, and immunological knowledge and techniques to elucidate this as-yet-elusive target.

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Etiopathogenesis of periodontitis in children and adolescents

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This chapter provides an account of knowledge on the etiopathogenesis of the various periodontal diseases that affect children and adolescents. This is currently an area of active research, and many challenging questions remain. In many cases we have insufficient knowledge to be definitive about the processes involved. This chapter describes the processes pertinent to the periodontal diseases, with particular emphasis on the inflammatory and immune responses occurring in the periodontal region during gingivitis and periodontitis.

This chapter will be structured in the following way: firstly the basic elements of the host response processes relevant to periodontal disease will be related; then follows a description of how the processes interact in the pathogenesis of the diseases; and lastly the specifics of the host response which may be relevant to the causation and diagnosis of the various forms of periodontal disease will be considered.

As described in the first chapter in this volume, the periodontal diseases affecting children and adolescents are numerous. These diseases can be conveniently grouped into the following entities: gingivitis; early-onset forms of periodontitis; necrotizing gingivitis and periodontitis; incipient adult periodontitis; and periodontitis associated with systemic diseases. Further categories exist within these broad definitions, in particular, gingivitis has many forms, but in children the puberty-related form is common and most significant. Similarly, early-onset periodontitis has several categories namely: prepubertal, which can be localised or generalized; early-onset periodontitis, which again can be localised or generalized; and incidental early-onset periodontitis (not extensive enough to fit any other category). All of the early-onset periodontal diseases are initiated by dental plaque and result in destructive disease progression in susceptible individuals. The susceptibility criteria for these diseases remain elusive; the immune causation could be due to variations or defects in the host response to the plaque, however, the immune and inflammatory responses during the development and progression of periodontitis are highly complex, making inferences more difficult to draw. For example, reduced neutrophil chemotaxis has been reported in localized early-onset periodontitis and generalized early-onset periodontitis (93, 94, 319, 320, 323–325) but is not a consistent finding across all populations (152, 153). If the abnormalities actually exist, they do not appear to predispose the patient to other diseases, suggesting that these are either mild abnormalities or only relevant in the periodontal environment (152, 153).

In the many cases where periodontal disease is predisposed to by systemic disease, identifiable components of the immune or inflammatory process may be impaired or absent. In leukocyte adhesion deficiency or neutropenia, the systemic defect is obvious. The effect of the absence of this component in the host response can be deduced from the description of the normal immune and inflammatory responses in periodontal disease. The pathogenesis of the periodontal destruction in the forms related to systemic diseases is covered in the chapter by Gonzalez & Meyle (203) in this volume.

A pertinent aspect when considering periodontal disease in children is the hormonal influences on the tissues and the host response, which are particularly relevant in pubertal gingivitis. In addition, the known variations in the host response in early-onset periodontitis are described, but the genetics of this and other periodontal diseases are dealt with fully in a further chapter in this volume. Briefly, early-onset periodontitis is clearly a familial disease with an autosomal dominant mode of inheritance. Genes coding for several aspects of the host immune response have been suggested as candidate markers to be investigated in early-onset periodontitis. These have included allelic variations in the Fc receptor for immunoglobulin G_2 (264, 343). The interleukin-1 polymorphisms associated with adult periodontitis

have not, however, been found in the early-onset periodontitis patients studied and further may suggest intrinsic differences between these disease entities.

The early-onset forms of periodontal disease most probably share a common pathogenic pathway, which may, in fact, be similar to that of adult periodontitis, although the causal pathways may differ considerably. The predisposing aspects responsible for the much earlier onset and more rapid destruction in early-onset forms of periodontitis are probably genetically related but are still not fully understood. Thus, the pathogenesis of adult periodontal disease is covered in some detail and points relevant to children and adolescents are highlighted. The etiopathogenesis of necrotizing forms of periodontitis are quite unique and are also discussed.

The host defenses in periodontal diseases

The host defense system comprises a collection of tissues, cells and molecules whose function is to protect the host against infectious agents (275). The immune response may be subdivided into two broad divisions, the innate (nonspecific) and the adaptive (specific) responses. Innate reactions include the inflammatory response and do not involve immune mechanisms. Adaptive or immune responses tend to be more effective, as the host response is a specific immune response to the offending pathogens.

Innate immunity represents an important first line of defence against infectious agents. This type of immunity is present from birth, is not enhanced by prior exposure and lacks memory. The innate response has the advantage of speed, but lacks specificity and may cause host tissue damage. Innate immunity entails a number of elements, both cellular and noncellular. Physical barriers such as the skin and mucous membranes represent a component that infectious agents must breach to gain access to the host. The washing action of fluids such as tears, saliva, urine and gingival crevicular fluid keeps mucosal surfaces clear of invading organisms and also contain bactericidal agents.

The intact epithelial barrier of the gingiva, sulcular and junctional epithelium normally prevents bacterial invasion of the periodontal tissues. It is normally an effective physical barrier against bacterial products and components. The epithelial cell wall, secreted proteins and fatty acids are toxic to many microbes. Salivary secretions provide a continuous flushing of the oral cavity as well as providing a continuing supply of agglutinins and specific antibodies. Furthermore, the gingival crevicular fluid flushes the gingival sulcus and delivers all the components of serum, including complement and specific antibodies.

The microbial biofilm that colonizes the tooth surface releases large quantities of metabolites that may diffuse through the junctional epithelium. These metabolites include fatty acids, peptides and lipopolysaccharides of gram-negative bacteria. By releasing proteolytic and noxious waste products, plaque microorganisms may damage cellular and structural components of the periodontium. As well as releasing these waste products, microorganisms could also invade the soft tissues.

Microorganisms produce a large variety of soluble enzymes in order to digest extracellularly host proteins and other molecules, thereby producing nutrients for growth. They also release numerous metabolic products, such as ammonia, indole, hydrogen sulfide and butyric acid. Among the enzymes released by bacteria are proteases capable of digesting collagen, elastin, fibronectin, fibrin, and other components of the intercellular matrix of epithelial and connective tissues. The released waste products stimulate junctional epithelial cells to release various inflammatory mediators including interleukin-1, prostaglandin E_2 and matrix metalloproteinases, all of which can transverse the junctional epithelium and enter the crevicular fluid (1).

The normal flora of the body can also act as an effective buffer against infection, by inhibiting the growth of pathogenic organisms by competition for nutrients or production of inhibitors. Phagocytic cells in the blood stream and tissues can destroy invading agents. These include polymorphonuclear leukocytes (or neutrophils), monocyte/macrophages, and natural killer cells. Finally, there are the soluble components. These consist of a wide range of molecules that are normally protective, but, however, can also damage microbial cell walls, aid phagocytosis and cell recruitment or prevent cellular infection. These soluble components include lysozymes, antimicrobial peptides, cytokines, acute-phase proteins, complement components and interferons.

The persistence of an infection in spite of the actions of the innate immune response leads to the induction of an adaptive immune response. Adaptive immune responses are characterized by 1) specificity

for the offending antigen(s), 2) memory, which allows a more rapid and heightened response upon reinfection by the same or closely related antigen, 3) diversity, the ability to respond to a wide range of different antigens, and 4) self versus non-self recognition. The adaptive immune response can be subdivided into humoral and cell-mediated immunity. Humoral immunity is mediated by antibodies, whereas cell-mediated immunity involves the direct action of immune cells.

The lipopolysaccharides of gram-negative microorganisms are capable of invoking both the inflammatory and immune responses as it interacts with host cells. The immune response will result in further release of cytokines and proinflammatory mediators, which in turn will increase the inflammation and thus be more harmful to the host. The quality of the host inflammatory response is critical to the disease process. Although its purpose is protection and prevention of bacterial invasion into the tissues, it is also detrimental, as it can be an ineffective, chronic and frustrated response that actually causes much of the tissue damage that occurs in periodontal disease. Inflammation, acute and chronic, nonspecific and immune-mediated, all play a role in periodontal disease. The components of these responses are now considered.

Inflammatory mediators

Inflammatory mediators play a major role in acute and chronic inflammation, and there is a strong evidence for participation of these mediators in periodontitis. These comprise a range of interacting molecules that include the cytokine system, proteinases, proteinase activators, thrombin, histamine, prostaglandins, leukotrienes, tissue and blood factors such as Hageman's factor, complement and clotting factors. The purpose of these and many other mediators is to initiate and perpetuate and eventually terminate an inflammatory response to insult. In the periodontium they are produced by activated resident gingival cells and infiltrating leukocytes. In the blood plasma they are produced by the complement cascade and kinin system. Monocytes from individuals susceptible to or suffering from severe periodontitis produce elevated amounts of mediators (87). Mediators are present in inflamed gingiva and gingival crevicular fluid from diseased sites in high concentrations (221). Concentrations of inflammatory mediators typically decrease following successful periodontal therapy. There now follows a discussion of the various components capable of promoting, sustaining and terminating inflammatory and immune reactions.

Cytokines

The term "cytokine" is derived from the Greek words *kytos* meaning cell, and kinesis, meaning movement. Cytokines are low-molecular-weight polypeptides of (5–70 kDa) (17). They function as soluble mediators produced by cells, to regulate or modify the activity of other cells. The cytokines can be broadly split into two groups, those involved in inflammatory and immune reactions and those involved in tissue growth and repair (growth- and colony-stimulating factors).

The first group is further subdivided into:

- the interleukins transmit information between leucocytes;
- the chemokines (chemotactic cytokines) involved in cell recruitment; and
- the interferons that influence lymphocyte activity.

Various cells secrete cytokines in response to injury or stimulation. These proteins are biologically active in even femtomolar concentrations. Most cytokines are multifunctional molecules that act locally and have a variety of target or effector cells (341). Cytokines act by binding to specific receptors on the surface of effector cells, which then stimulate intracellular activation of the cell. In certain situations receptors become saturated and cytokines such as tumor necrosis factor, interleukin-1 and interleukin-6 may spill over into the systemic circulation and stimulate distant tissues and organs (216).

Collectively, the cytokines and other related molecules form a complex network that controls the inflammatory and immune responses (356) and subsequent tissue healing. Many cytokines have overlapping functions, and some may inhibit the action of others (156). A cytokine may have different forms of receptors that may produce opposing actions, and paradoxically, elevated levels of proinflammatory cytokines may be evident during periods of healing as well as during destructive phases. These are important considerations when interpreting periodontal studies of single or even combinations of cytokines. These studies may not reflect the true situation in the living organism (356).

Cytokine regulation is achieved through several mechanisms. Control of cytokines can take place at

the gene activation level, during secretion and circulation and at the cell receptor level (17). Cytokine production is usually short-lived and self-limiting (137). In addition, many cytokine receptors exist in soluble forms, which may be cleaved from the target cells, allowing them to bind and neutralize cytokines extracellularly (17). Thus, the local tissue environment influences cytokine and cytokine receptor activity. Proinflammatory cytokines are also regulated by high-affinity autoantibodies and anti-inflammatory cytokines such as interleukin-1 receptor antagonist, interleukin-4 and interleukin-10 (17).

Interleukin-1

In humans, two distinct interleukin-1 gene products have been cloned interleukin-1α and interleukin-1β. Sources of interleukin-1 production includes mononuclear phagocytes, keratinocytes (186), fibroblasts (126), endothelial cells (204) and osteoblasts (120). Interleukin-1 is a major mediator in periodontitis (235, 289). Production is induced by lipopolysaccharide and other bacterial components and by interleukin-1 which is autostimulatory (216). Interleukin-1 is mainly secreted by monocytes or macrophages but can also be secreted by most nucleated cells (144, 197).

All three members of the interleukin-1 group (interleukin-1α, interleukin-1β and interleukin-1 receptor antagonist) bind to interleukin-1 receptors I and interleukin-1 receptors II. Corticosteroids induce interleukin-1 receptors II messenger RNA synthesis and the release of secreted interleukin-1 receptors II, mostly from neutrophils, which may partly explain their anti-inflammatory and immunosuppressive actions (17).

Tumor necrosis factor

Tumor necrosis factor-α is a multipotential cytokine, produced mainly by macrophages, with a wide variety of biological effects similar to interleukin-1 (43, 170). Tumor necrosis factor-α and interleukin-1 both act on endothelial cells to increase recruitment of polymorphonuclear leukocytes and monocytes to sites of inflammation (20). Tumor necrosis factor-α and interleukin-1 are key mediators of chronic inflammatory diseases and have the potential to initiate tissue destruction and bone loss in periodontal disease (22). Tumor necrosis factor also mediates connective tissue destruction through its action on the matrix metalloproteinase system (51, 200). Tumor necrosis factor receptors exist in membrane-bound and soluble forms similar to the interleukin-1 receptors (17). These receptors (secreted tumor necrosis factor receptors I and secreted tumor necrosis factor receptors II) regulate the activity of both tumor necrosis factor-α and the less potent form, tumor necrosis factor-β.

In addition to interleukin-1 and tumor necrosis factor-α, we must consider additional proinflammatory cytokines such as interleukin-6 and related molecules such as the prostaglandins and leukotrienes and the inhibitors of inflammatory cytokines such as interleukin-10.

Interleukin-6

Interleukin-6 is produced by macrophages, fibroblasts, lymphocytes and endothelial cells. Production is induced by interleukin-1 and lipopolysaccharide and suppressed by estrogen and progesterone. It may be through interleukin-6 that these hormones exert their effects on gingiva. Interleukin-6 causes fusion of monocytes to form multinuclear cells that resorb bone.

Interleukin-8

Interleukin-8 is produced by a wide variety of cell types, including polymorphonuclear leukocytes, monocytes, fibroblasts and keratinocytes in response to microorganisms, mitogens and endogenous mediators such as interleukin-1 and tumor necrosis factor. One of the major functions of interleukin-8 is its ability to induce the directional migration of cells, including polymorphonuclear leukocytes, monocytes and T cells (230), thus playing a key role in the accumulation of leukocytes at sites of inflammation.

Interleukin-10

Interleukin-10 plays a major role in suppressing immune and inflammatory responses. It is produced by T cells, including human Th0, Th1 and Th2 cells, B cells and monocytes and macrophages after activation (55). Interleukin-10 inhibits the antigen-presenting capacity of macrophages by downregulating class II major histocompatability complex expression. It has also been reported to inhibit antigen presentation by Langerhans cells (77). Human interleukin-10 reduces significantly the proliferation and production of cytokines by both Th1 and Th2 clones exposed to specific antigen and phytohemagglutin (55). It also has direct inhibitory effects on inter-

feron-γ production (54, 56). Interleukin-10 has been found to act as a specific chemotactic factor towards $CD8^+$ T cells while suppressing the ability of $CD4^+$ T cells to migrate in response to interleukin-8 (145). Interleukin-10 is a potent growth and differentiation factor for activated human B cells and thus plays an important role in amplifying the humoral immune response (253). Interleukin-10 inhibits the synthesis of interleukin-1α, interleukin-6 and interleukin-8 but enhances the production of the interleukin-1 receptor antagonist, so that this cytokine dampens immune proliferation and the inflammatory response (54, 89). Interleukin-10 abrogates antigen presentation by macrophages and indirectly inhibits T-cell activation (17).

The lymphocyte cytokines

The lymphocyte-signaling cytokines interleukin-2, interleukin-3, interleukin-4 and interleukin-5 are all involved in lymphocyte clonal expansion, differentiation of B cells into antibody-producing plasma cells and immunoglobulin isotype switching. Interleukin-2 is produced by both $CD4^+$ (helper) and $CD8^+$ (suppresser) (142), activated T lymphocytes and induces expression of clones of specific T cells and secretion of interleukin-3 and interleukin-4. Interleukin-4 regulates immunoglobulin production and suppresses activated macrophages and causes their apoptosis.

Transforming growth factor-β

Transforming growth factor-β is produced by activated T cells. It is a chemoattractant for monocytes and it suppresses their activation. It also induces production of immunoglobulin A and immunoglobulin G2b. Transforming growth factor-α is produced by macrophages and serves as a mitogen for fibroblasts, epithelial and endothelial cells. Interferon-γ is produced by $CD8^+$ T cells and is responsible for recruiting and activating macrophages and also upregulates the major histocompatibility complex on virally infected cells and targets them for killing.

In summary the cytokine network has a crucial role in immune homeostasis. Complex summative, overlapping, synergistic and inhibitory interactions exist between the various members of this family of molecules. This allows for redundancy within the system and a high level of fine control which prevents devastating effects to the host when dysfunction or dysregulation occurs.

Role of matrix metalloproteinases in periodontal disease

Loss of attachment, as occurs in periodontal disease, is associated with extensive breakdown of collagen fibers in the periodontal tissues. Since the major structural protein of the periodontium is collagen, the assessment of matrix metalloproteinase levels during inflammation will reflect the association of matrix metalloproteinases in collagen destruction in periodontitis (Fig. 1). The involvement of matrix metalloproteinases in pathological tissue destruction is well documented, and the evidence for their role in periodontal destruction has increased over the years. In earlier studies, collagenases has been identified in explanted gingival tissues and their culture fluids (22, 83, 249) and in homogenates of gingival biopsies (316). Collagenase activity has also been demonstrated in gingival crevicular fluid, and this activity increased with the severity of inflammation (101). Further evidence that matrix metalloproteinases are involved in tissue destruction in human periodontal disease were summarized by Birkedal-Hansen (22), which includes:

- cells isolated from normal and inflamed gingiva express matrix metalloproteinases in culture;
- several metalloproteinases can be detected in human gingiva cells *in vivo*;
- polymorphonuclear leukocytes collagenase and gelatinases can be detected in gingival crevicular fluid from gingivitis and periodontitis patients.

Each of the major cell types of human periodontal tissues is capable of expressing a unique complement of matrix metalloproteinase when properly stimulated. The cells include polymorphonuclear

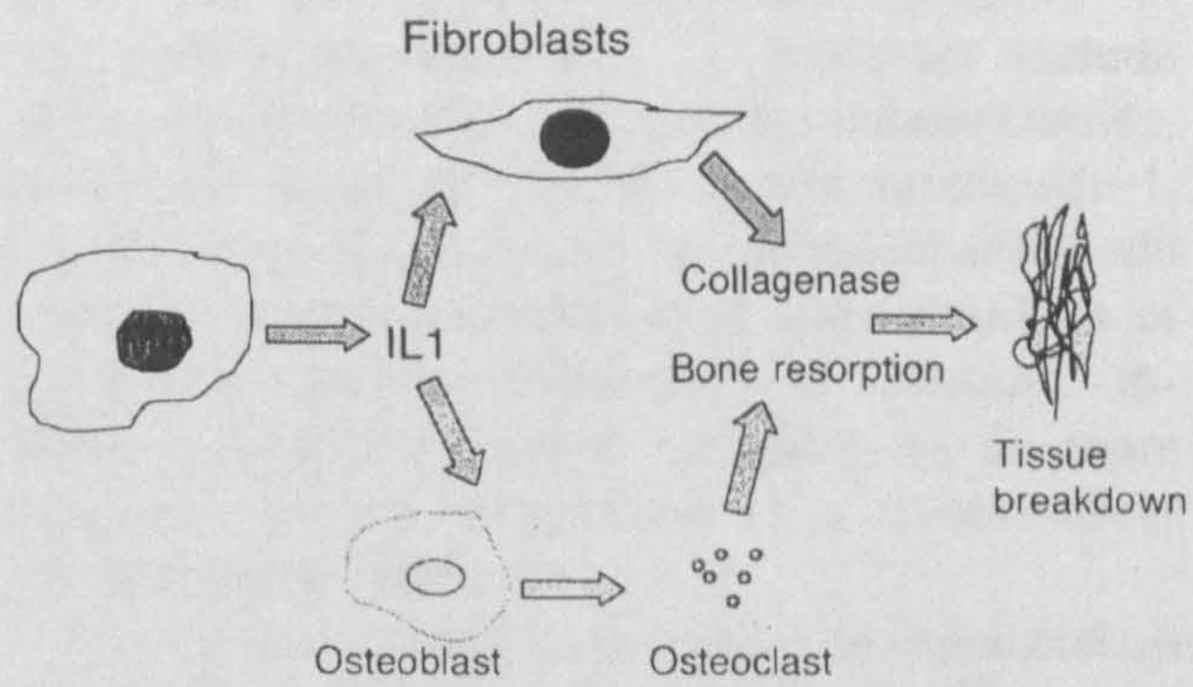

Fig. 1. Macrophages produced cytokines (such as interleukin-1) induce fibroblasts and osteoblasts to produce proteases which result in bone and tissue breakdown.

leukocyte, fibroblast, keratinocyte, macrophage and endothelial cells.

Collagenases in tissues may existed in different forms, that is, in active form, in inactive form that is an enzyme inhibitor complex, or in latent form (proenzyme). Total collagenase activity has been shown to be high in gingival crevicular fluid of inflamed dogs gingiva (161), in gingival crevicular fluid of localized early-onset periodontitis and chronic adult periodontitis patients compared with gingivitis and control patients (329, 330), in gingival crevicular fluid of untreated localized early-onset periodontitis patients and also that the activity decreased after treatment (169). Gingival crevicular fluid collagenase activity has been noted to increase with disease severity, that is, adult periodontitis and localized early-onset periodontitis > gingivitis > healthy gingiva (329, 330). Ingman et al. (139) demonstrated that saliva samples did not reflect periodontal tissue destruction clearly as they found only low amount of total collagenase activity in saliva of untreated localized early-onset periodontitis patient and the level was comparable to normal patients. Ingman et al. (140) also demonstrated that in saliva no difference in collagenolytic activity could be seen in adult periodontitis, localized early-onset periodontitis and control patients.

Latent collagenase levels were found to be higher in gingival crevicular fluid of gingivitis sites in humans and dogs compared to healthy sites (161, 162, 171), and also in normal sites (161). It was also detected in the explant of clinically normal tissue (125). Higher latent collagenase levels seems to reflect either the normal healthy state or the gingivitis state. Little or no active collagenase activity was demonstrated in the gingival crevicular fluid of healthy and gingivitis sites in dogs (167), but higher levels were found in gingival crevicular fluid of localized early-onset periodontitis compared with normal patients (169). Higher levels were also detected in gingival crevicular fluid of progressive periodontitis patients compared with stable and gingivitis patients (171). In whole saliva, high active collagenase activity was demonstrated in untreated adult periodontitis (85, 139) and localized early-onset periodontitis, but as treatment progressed more collagenase is present in the latent form (85). Higher levels of active collagenase correlates with active destructive phases of periodontal disease (171).

Although there is no direct evidence for a causal relationship between matrix metalloproteinases and periodontal tissue destruction, the evidence for the involvement of collagenase in collagen breakdown during chronic inflammatory periodontal disease is strong. Matrix metalloproteinase-8 is detected at high levels in gingival crevicular fluid and saliva during gingivitis or periodontitis, whereas it is undetectable in healthy individuals (139, 285). In extracts or homogenates of diseased periodontal tissues, matrix metalloproteinase-1 is abundantly present in contrast to healthy specimens and, as with matrix metalloproteinase-8, a positive correlation was found between its presence and the severity of inflammation (231). In addition, matrix metalloproteinase-1 has been immunolocalized in inflamed but not in healthy periodontal tissue (348). These data may suggest that, following production during inflammation, most matrix metalloproteinase-1 remains in the gingival tissue, whereas most released matrix metalloproteinase-8 finds its way to the pocket.

Adhesion molecules

It has been recognized that blood vessels play a central role in orchestrating the inflammatory and immune processes. Blood vessels are lined by endothelial cells that are activated by cytokines and bacterial lipopolysaccharide to become more adhesive to circulating polymorphonuclear leukocytes, monocytes, eosinophils, basophils and T and B lymphocytes. Thus, the endothelial lining is transformed from being anti-coagulant to becoming pro-coagulant. The changes in the vessels contribute to the inflammatory changes in periodontal disease, but the most important features is the migration of leukocytes from the circulation. When the endothelial cells become activated they express adhesion molecules, such as the intercellular adhesion molecule-1, that enable specific subpopulations of leukocytes to bind and subsequently migrate into the tissues under the influence of chemotactic gradients (Fig. 2). Activators include the proinflammatory cytokines, lipopolysaccharide, interleukin-4 and interferons. *In vivo*, interleukin-1, tumor necrosis factor and lipopolysaccharide will result in rapid accumulation of large numbers of polymorphonuclear leukocytes. In contrast, interleukin-4 and interferon are likely to be more important in the recruitment of a mononuclear cell dominated infiltrate.

Intercellular adhesion molecule-1 is expressed on endothelial cells and shows an increase in expression after stimulation with interleukin-1, tumor necrosis factor and interferon (Fig. 2, 3). It plays an im-

portant part in the adhesion of all leukocytes to endothelial cells and migration. Evidence for this is leukocyte adhesion deficiency, a congenital disease with low levels of adhesion molecules. Patients subsequently suffer from recurrent infections due to inability of their leukocytes to extravasate into damaged or injured tissue. A constant and characteristic feature of these individuals is a very rapid severe generalized prepubertal periodontitis affecting both the primary and permanent dentition (335).

The endothelial cell leukocyte adhesion molecule-1 has been found to play an important role in polymorphonuclear leukocytes binding to activated endothelial cells. It is rapidly induced by interleukin-1, tumor necrosis factor or lipopolysaccharide.

In clinically healthy gingiva, endothelial cell leukocyte adhesion molecule-1 and intercellular adhesion molecule-1 are found in postcapillary venules in the vicinity of the gingival crevice, with more distant vessels being negative. A further finding in the study of Moughal et al. (211) was the observation that junctional epithelium expresses intercellular adhesion molecule-1. This specialized epithelium may be adapted to express intercellular adhesion molecule-1 constitutively even in the absence of interferon-γ stimulation. This may reflect the fact that junctional epithelium is the site of extensive polymorphonuclear leukocytes migration both in health and in disease (12, 266, 269).

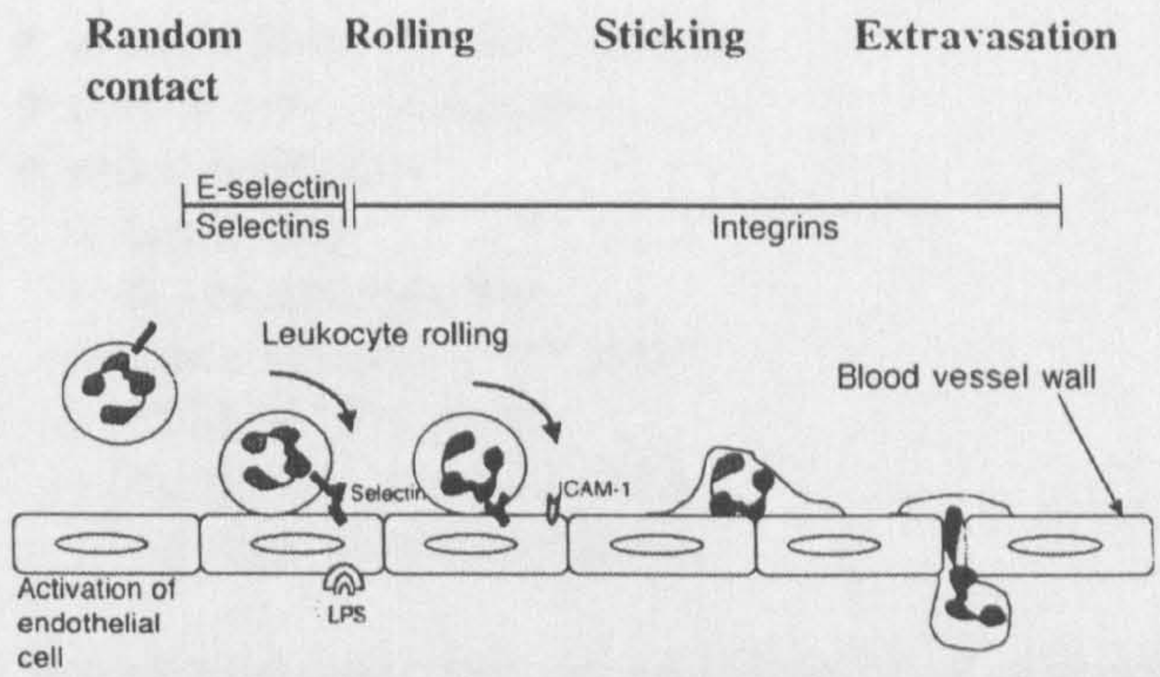

Fig. 2. The leucocyte contacts, rolls, sticks and eventually extravasates out of the blood vessel prior to beginning its journey to the site of inflammation.

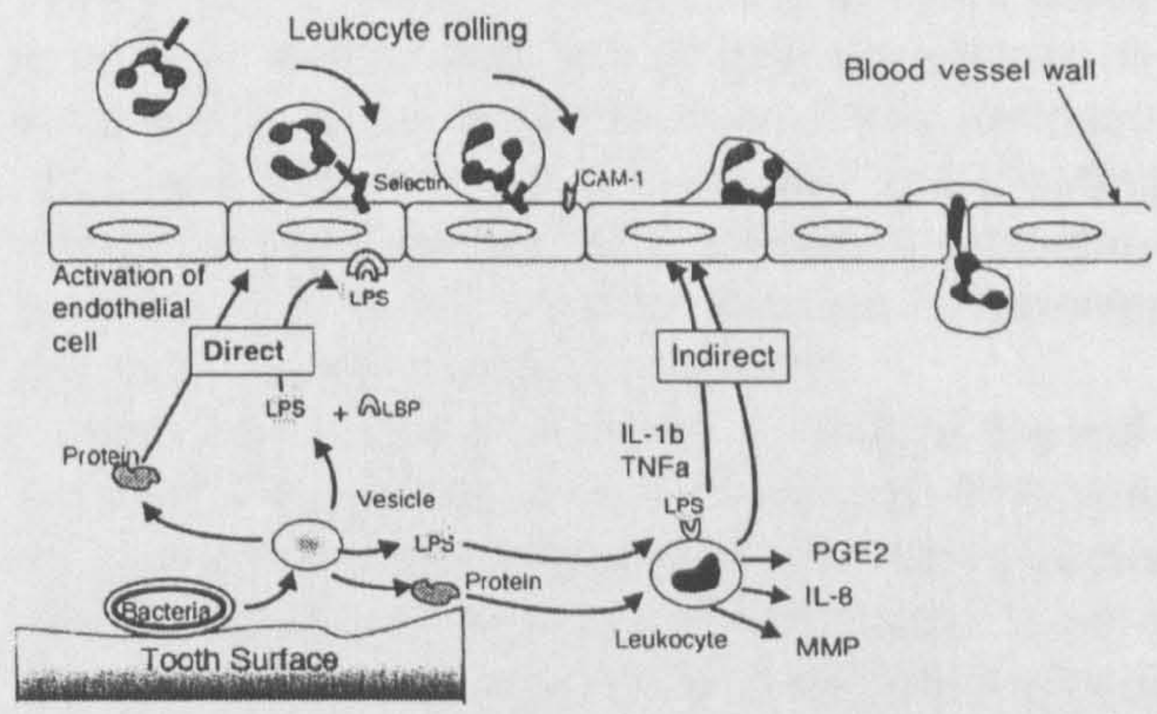

Fig. 3. Molecules, cells and process influencing the increased adherence of leucocytes to bllod vessels so that they can extravasate and begin to chemotact towards the microbes.

Polymorphonuclear leukocytes

The polymorphonuclear leukocyte is a phagocytic cell and is the predominant leukocyte in blood and in the gingival crevice (12). It forms about 62% of peripheral white blood cells and about 91% of gingival crevicular cells recovered by aspiration (278). Polymorphonuclear leukocytes are found at sites of both gingival health and disease (12). The polymorphonuclear leukocytes is an important cell because, along with the integrity of the physical surface barrier, it forms the first-line defense against invading pathogens. Evidence for its crucial role in the defense of the periodontal tissues comes from experiments of nature where neutrophils are depleted or malfunctioning, such as in neutropenia or Chèdiak-Higashi syndrome, and there is severe and rapid loss of alveolar bone and teeth.

Polymorphonuclear leukocytes develop from pluripotential bone marrow stem cells and the cells go through several stages prior to maturity. The lifespan of a polymorphonuclear leukocyte outside the bone marrow is short, on average of 7 to 58 hours. As polymorphonuclear leukocytes mature they become more deformable, alter their surface charge and acquire new cell surface receptors (337). Following these changes mature polymorphonuclear leukocytes can cross endothelial barriers in the bone marrow and enter the bloodstream.

In the circulation polymorphonuclear leukocytes form two pools; circulating and marginated. Marginated polymorphonuclear leukocytes are stored in the small vessels, particularly of the lung and spleen. They can be mobilized at short notice in times of acute need. The average range of polymorphonuclear leukocyte numbers in the circulation is 2.5 to 7.5×10^9 cells/liter. This number can increase 10 fold within 28 hours in times of acute inflammation. This rapid and substantial increase in polymorphonuclear leukocyte number occurs as a result of several processes; transfer of cells from the marginated to the circulating pool, increased

release of mature cells from the bone marrow and an increase in the rate of maturation of cells within the bone marrow.

Numerous surface receptors are present on polymorphonuclear leukocytes (251) of which there four major classes:

- receptors for inflammatory mediators and bacterial products;
- receptors for lymphokines and monokines;
- receptors for opsonization (Fc); and
- receptors for endothelium and proteins of the tissue matrix.

These receptors are used for all stages of polymorphonuclear leukocyte emigration from blood vessels, chemotactic movement, recognition of foreign materials, its binding and phagocytosis and for the control of these processes via lymphokines and monokines.

The polymorphonuclear leukocyte has a characteristic light microscopic appearance with a multilobed nucleus and numerous cytoplasmic granules. The granular component of the cell consists of many cytoplasmic granules with an outer membrane enclosing the densely packed protein in a mucopolysaccharide matrix.

Three types of granules exist:

- primary or azurophil granules;
- secondary or specific granules; and
- tertiary or C particles.

Primary granules are identified by their peroxidase content. Positive staining for peroxidase is seen in the granules, at all stages, and in the secretory apparatus of polymorphonuclear leukocytes during their formation. The contents of the primary granules are largely responsible for the non-oxidative killing mechanisms of the neutrophil (205), and these granules contain:

- antimicrobial agents or defensins, which include myeloperoxidase, lysozyme and cationic proteins; and
- proteinases, which include elastase and cathepsin G.

Secondary granules are peroxidase negative, of low density and are twice as numerous as primary granules in the mature polymorphonuclear leukocyte. Their size is variable and they are typically spheres or rods containing:

- antimicrobial agents – lysozyme
- proteinases – collagenase
- other molecules
  - lactoferrin
  - B_{12} binding protein
  - laminin receptor (67 kDa)
  - CD11b/CD18 (C3bi)
  - fibronectin receptor alpha
  - vitronectin receptor alpha.

The release characteristics and kinetics of primary and secondary granules are different. Secondary granules are released chiefly extracellularly, with the granules being found close to the advancing edge of cells responding to a chemotactic stimulus (349). Evidence of secondary granule release extracellurly is more prominent than that of primary granule release and occurs at an earlier stage. These phenomenon have been interpreted in the past as indicating a secondary and extracellular function of secondary granules and an intracellular function of primary granules related to phagocytosis (349).

The process of degranulation is calcium dependent and is not related to cell death (301, 349) with lysozymal enzymes released without a rise in lactate dehydrogenase, an indicator of cell death. *In vitro*, granule release can be stimulated by dental plaque (301), binding of immune complexes and complement, binding to nonphagocytosable surfaces and passage through membranes (349).

Polymorphonuclear leukocytes in inflammation

When functioning efficiently against infectious agents, polymorphonuclear leukocytes leave blood vessels and move across tissues towards a chemotactic source where they attempt to kill the organism. Adhesion molecules relevant to the movement of polymorphonuclear leukocytes from vessels are expressed on endothelial cells and polymorphonuclear leukocytes (251). During inflammation the molecules appear before those for other leukocytes, thus polymorphonuclear leukocytes are the first cells to cross the vessel wall. The immediate adhesion of polymorphonuclear leukocytes is mediated via upregulation of P-selectin on endothelial cells by thrombin or histamine. Stimulation of post-capillary venules by inflammatory mediators triggers the upregulation of expression of the endothelial cell adhesion molecules (291). This functions over 24

hours, increasing the interaction of leukocytes with the cells of the vessel walls. Various endothelial markers including intercellular adhesion molecule-1, intercellular adhesion molecule-2 and vascular cell adhesion molecule-1 bind to the LeuCAM family of integrins, which are expressed on polymorphonuclear leukocytes. Leukocyte function–associated antigen-1 binds specifically to intercellular adhesion molecule-1 and intercellular adhesion molecule-2.

In response to the expression of surface markers, the cells change shape and roll along the blood vessel wall (Fig. 1). They finally stop moving, flatten and their membranes develope a ruffled border. They then migrate through the endothelial wall. Endothelial cell leukocyte adhesion molecule-1, which is necessary for this migration, is expressed in response to interleukin-1 and tumor necrosis factor. Interleukin-1 and tumor necrosis factor-α thus have a crucial role in the control of inflammatory mechanisms following the initial stimulus.

Polymorphonuclear leukocytes are capable of responding to many different chemoattractants including *N*-formyl peptides, complement-derived C5a, leukotriene B_4, interleukin-8 and platelet-activating factor (124). Chemoattractant receptors span the cell membrane and are GTPase-coupled receptors. Once outside the vessel, cells migrate through the connective tissue towards the chemotactic stimulus (Fig. 4). Directed movement of polymorphonuclear leukocytes comes about by the interaction of specific chemoattractants with surface receptors on its plasma membrane. This binding activates a cascade of secondary intercellular events that lead to either chemotaxis or the respiratory burst, dependent on the chemotactic agent (199). Neutrophils stimulated by chemoattractants undergo morphological changes, becoming elongated cells with a ruffled border. During movement, the anterior edge extends forwards and the posterior edge retracts towards the body of the cell. Adherence of the cells to a substratum occurs via the glycoproteins and proteoglycans of the extracellular matrix. The concentration of extracellular matrix glycoproteins and proteoglycans changes at sites of gingival inflammation (243), and these molecules can become chemotactic for inflammatory cells. Actual cell movement occurs by the polymerization of globular actin to fibrillar actin, and current models propose that the fibrillar actin is involved in the generation of the force required for cell movement (229).

Chemoattractant receptors exist in two interconvertible forms: high- and low-affinity states (279). Chemotaxis is initiated at receptors by concentrations of chemoattractants that are much lower than those required to produce the respiratory burst. Snyderman & Pike (284) proposed that binding of chemoattractant to high-affinity sites results in chemotaxis, whereas binding to low-affinity sites results in degranulation. Chemoattractants appear to be most effective in producing chemotaxis in the following order of potency: interleukin-8, leukotriene B_4, C5a, *N*-formyl peptides and platelet-activating factor (199).

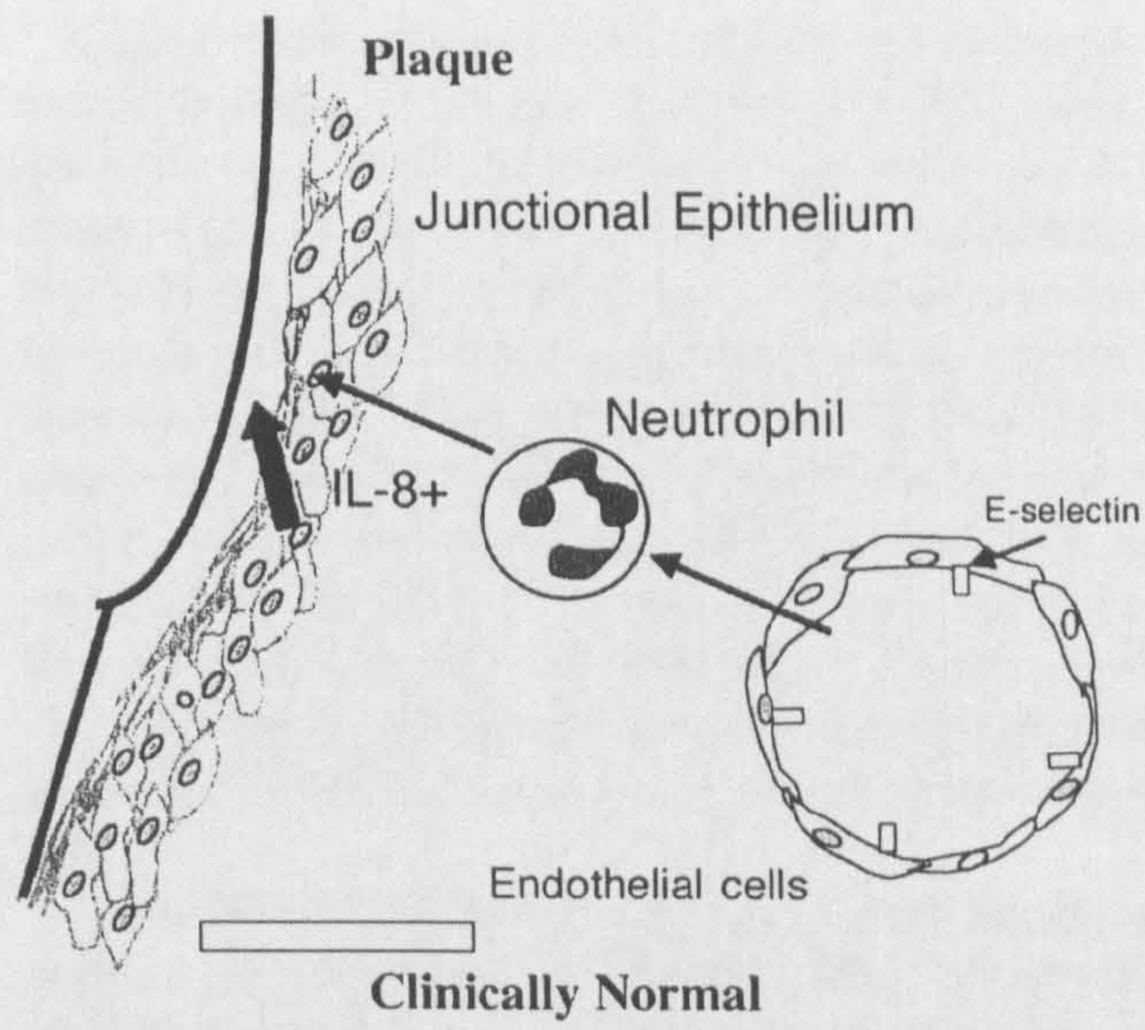

Fig. 4. Interleukin-8 is one of the major host produced chemotactic agents to attract neutrophils to the gingival crevice.

Once at the site of the chemotactic stimulus and prior to attachment of the polymorphonuclear leukocyte to the invading organism, opsonization takes place either in the presence or absence of complement and antibody. In the presence of complement and antibody, activated C3 and immunoglobulin G bind their respective polymorphonuclear leukocyte receptors, CR1 and CR3 for complement and Fcγ receptor II and Fcγ receptor III for antibody. Capsular organisms are able to evade this opsonization and phagocytosis due to the masking of the antigenic portion of their cell wall by the capsule and by avoiding the deposition of complement on their surface (280).

If antibody or complement are not available, then alternative systems exist for the opsonization of bacteria. Generally, cell wall antigens determine the ease with which an organism is phagocytosed. Lipopolysaccharide-binding protein is an acute-phase reactant which binds lipopolysaccharide, the cell wall component of gram-negative bacteria. The binding

of lipopolysaccharide-binding protein to bacteria enables binding to the CD14 receptor on polymorphonuclear leukocytes and macrophages, thus enhancing phagocytosis (350). Since gram-negative bacteria are abundant in the gingival crevice and complement and antibody can be locally destroyed (92), it is likely that this system is in operation.

Following opsonization, phagocytosis can occur. Phagocytosis is the process by which the polymorphonuclear leukocyte takes up a particle. It involves attachment of the phagocyte to the particle and its subsequent engulfment. It starts with the receptor-ligand binding between polymorphonuclear leukocyte and the microbe. This interaction activates the ingestion phases which involve actin, myosin and actin-binding proteins. The membrane surrounds the particle, pseudopodia are produced and a phagocytic vacuole is formed. Simultaneously cytoplasmic granules fuse with the phagosome membrane to form a phagolysosome. This final fusion of the membranes and release of granule contents generally results in destruction of the engulfed particle (328).

Once phagocytosed and engulfed by the polymorphonuclear leukocyte, two mechanisms exist to kill the organism; oxygen-dependent and oxygen-independent mechanisms (323). Oxygen-dependent killing is activated when the polymorphonuclear leukocyte undergoes a respiratory burst coincident with phagocytosis. NADPH in the phagolysosome membrane is activated and reduces O_2 to superoxide (340). Following the generation of hydrogen peroxide and OH in this reaction, a second enzyme system involving myeloperoxidase, a primary granule component, becomes active. Myeloperoxidase is released in large quantities but on its own has little effect. In combination with H_2O_2, myeloperoxidase is capable of catalyzing the oxidation of plasma halides, the most important of which is chloride because of its concentration. It is at this stage that the interaction between chlorinated oxidants, protease inhibitors and proteases can occur such that the proteases can act efficiently on their substrate (Fig. 5).

OH is considered the most toxic of all the free radicals. If this reaction occurs *in vivo* then the rate-limiting step is the availability of iron. Little free iron is available due to its binding to transferrin and lactoferrin extracellularly and apoferritin intracellularly (259). In the phagocytic vacuole the pH is reduced and some iron may be lost from transferrin; however, lactoferrin retains its ability to bind iron at low pH, thus protecting the cell and limiting the production of OH (118).

Oxygen-independent killing is based on the polymorphonuclear leukocyte granule network. Antimicrobial agents that are independent of O_2 are released into the phagolysosome during phagocytosis (311). These granule components, including bactericidal and permeability-increasing protein, chymotrypsin-like cationic protein and defensins (328, 340) and those outlined above, are strongly bacteriocidal and in most cases effectively kill bacteria (205). In addition to killing bacteria, the proteolytic enzymes, including elastase and the metalloproteinases, have the potential to damage extracellular tissue and have been suggested to operate in periodontal tissues (22).

It has been demonstrated that sera from localized early-onset periodontitis patients with decreased neutrophil function can modify the activity of healthy neutrophils (2). The serum factors responsible for these effects have been identified as being inflammatory mediators such as prostaglandins and cytokines. Small quantities of bacterial lipopolysaccharide (in the picogram range) can induce the secretion of tumor necrosis factor-α, interleukin-1 and prostaglandin E_2 from monocytes. The production of interleukin-1–tumor necrosis factor-α and tumor necrosis factor-α–prostaglandin E_2 are strongly correlated (208). Very small increases in serum cytokine levels can alter neutrophil function. Prostaglandin E_2 and cytokines also stimulate fibroblasts to release matrix metalloproteinases, which break down the extracellular matrix. In addition, interleukin-1, tumor necrosis factor-α and prostaglandin E_2 are potent stimulators of bone resorption. Adherent mononuclear cells from localized early-onset periodontitis patients have been found to secrete higher levels of prostaglandin E_2 and tumor necrosis factor-α than generalized early-onset periodontitis patients or healthy controls (274). More recently, insulin-dependent diabetic patients with periodontitis have been reported to manifest excessive levels of inflammatory mediators (256–258). It has been suggested that these observations indicate an underlying immune defect of monocytes in certain diagnostic groups (216).

The concept of excessive inflammatory mediator production by monocytes has been proposed as a possible causative pathway for periodontitis, and indeed one of the mechanisms whereby periodontitis can influence other systemic conditions such as cardiovascular disease (216). Early investigations of genetic polymorphisms of cytokines have attempted to identify a possible link between different periodontal categories and specific alleles related to an

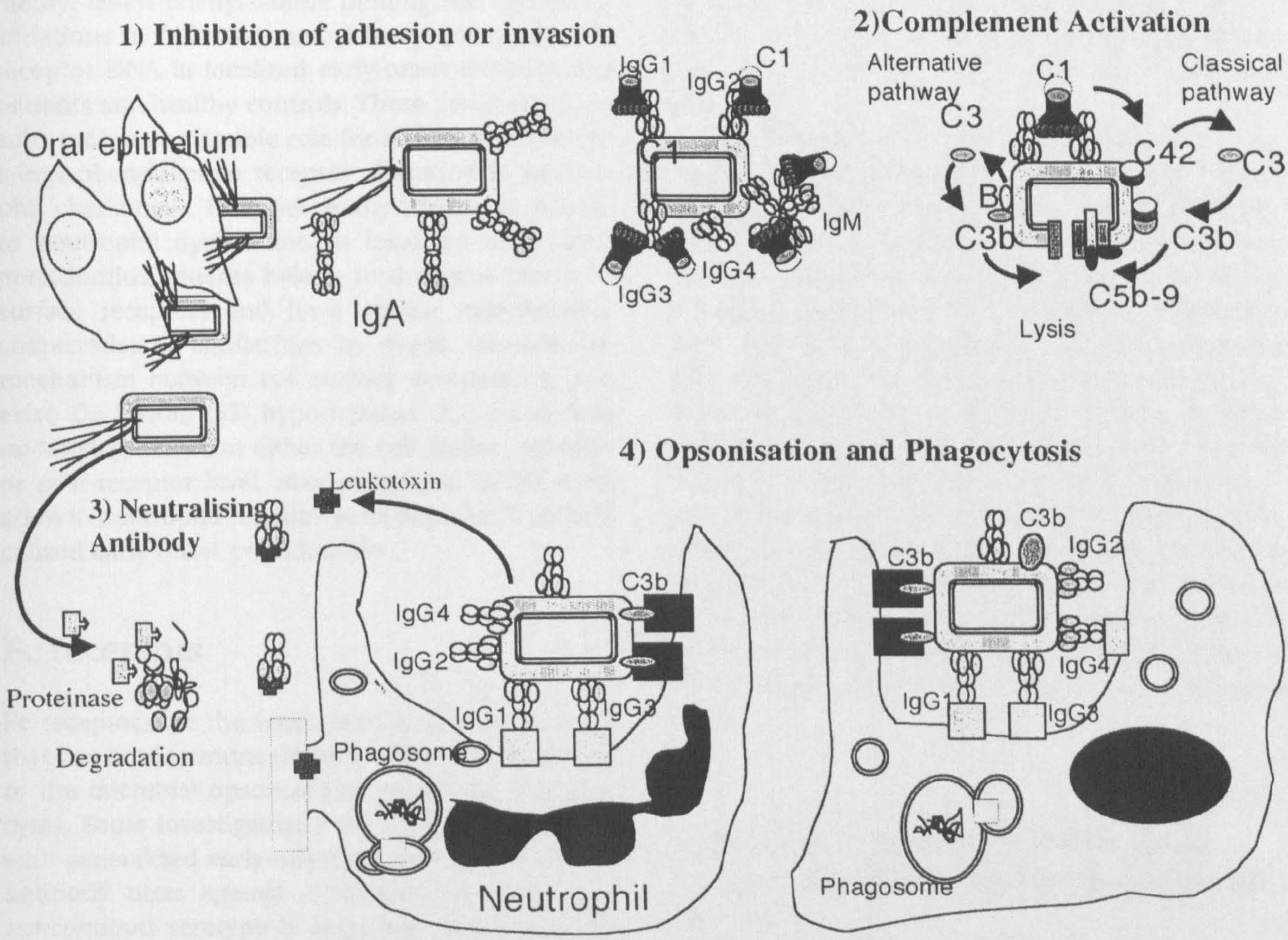

Fig. 5. The four main processes whereby antibodies can detrimentally affect bacteria: 1) inhibition of adhesion or invasion; 2) complement mediated killing; 3) neutralisation of microbial toxins (cf leukotoxin); and 4) opsonisation of microbes prior to phagocytosis and enhancement of phagocytosis.

ability to overproduce cytokines (57, 84, 102, 159). In addition, a family linkage study of early-onset periodontitis suggested that the COX-1 enzyme system, which acts as a catalyst for the breakdown of arachidonic acid, one of the products of which is prostaglandin E_2, may be associated with susceptibility to early-onset periodontitis (89).

A further host defense defect that may increase susceptibility to early-onset periodontitis is neutrophil chemotactic defects. A high percentage of members of families with a background of localized early-onset periodontitis have been reported to show abnormal neutrophil chemotaxis (323). Analysis of 22 families with localized early-onset periodontitis demonstrated that, in 19 of these families, the presenting localized early-onset periodontitis patient and their family members all demonstrated neutrophil chemotaxis disorders. In these 22 families the chemotactic defect was found in almost 50% of the siblings, indicating a dominant mode of inheritance (323). Whether the trait is due to an intrinsic defect of neutrophils or is caused by extrinsic factors in the sera and thus is environmentally influenced remains controversial (2). The neutrophil chemotactic defect is not noted in all populations exhibiting localized early-onset periodontitis, and a significant number of patients and families with localized early-onset periodontitis do not show evidence of the defect (152, 153, 232).

Decreased neutrophil chemotaxis in localized early-onset periodontitis has been linked to a reduced receptors density for chemoattractants such as *N*-formyl-methyl-leucyl-phenylalanine, complement fragment C5a, leukotriene B_4 and interleukin-8 (49, 52). A cell surface glycoprotein (gp110), which is thought to be involved in neutrophil movement and secretion, is reduced in localized early-onset periodontitis patients (324). In addition, several other post-receptor defects have been identified (49). De Nardin (53) found that decreased *N*-formyl-

methyl-leucyl-phenylalanine binding was related to variations in *N*-formyl-methyl-leucyl-phenylalanine receptor DNA in localized early-onset periodontitis patients and healthy controls. These preliminary results indicate a possible role for a *N*-formyl-methyl-leucyl-phenylalanine receptor alteration in neutrophil chemotaxis. However, many molecules related to neutrophil dysfunction in localized early-onset periodontitis patients belong to the same family of surface receptors and have similar morphogenic characteristics. Similarities in signal transduction mechanism between cell surface components also exist. De Nardin (53) hypothesized that a common underlying defect, at either the cell surface receptor or post-receptor level, may contribute to the alteration in neutrophil function seen in patients with localized early-onset periodontitis.

Fc receptors

Fc receptors are the receptors found on leukocytes that can bind immunoglobulin and are thus relevant to the microbial opsonization properties of phagocytes. Some investigators have shown that patients with generalized early-onset periodontitis have high antibody titers against *Actinobacillus actinomycetemcomitans* serotype b outer membrane antigens (107, 310, 342). It has also been suggested that the subclass IgG2 is a poor opsonin because it fixes complement less effectively than IgG3 and IgG1 and binds weakly to Fc receptors on the surface of phagocytes (250), which tends to complicate theories of protective effects afforded by high anti-*A. actinomycetemcomitans* titers of IgG2 in black localized early-onset periodontitis patients.

However, a further aspect of the immune response may yet explain this phenomenon. Fc receptors are the crucial link between the humoral and inflammatory components of the host defense system. Fcγ receptors on phagocytes recognize the Fc region of IgG and facilitate opsonization of microbes. It has been shown that neutrophils express low-affinity variants of Fcγ receptors (Fcγ receptors II and Fcγ receptors III) under normal conditions. Fcγ receptors II binds all subclasses of IgG, but Fcγ receptors III only binds IgG3 and IgG1. Many more Fcγ receptors III than Fcγ receptors II are expressed on the surface of neutrophils. However, while Fcγ receptors III can evoke lysosomal degranulation, they cannot promote the necessary respiratory burst activity and phagocytosis needed for microbial killing of opsonised bacteria. Fcγ receptors II, on the other hand, can precipitate all three activities. It is possible that Fcγ receptors III may prepare polymorphonuclear leukocytes for phagocytosis mediated by Fcγ receptors II (343).

It has been reported that the numbers of Fcγ receptors II and Fcγ receptors III on peripheral blood neutrophils of localized early-onset periodontitis patients are within the normal range (172). Miyazaki et al. (205) found that both Fcγ receptors II and Fcγ receptors III expression and neutrophil phagocytosis were significantly depressed in gingival crevicular fluid compared with peripheral blood in adult periodontitis patients. The reduction in Fcγ receptors was significantly correlated with phagocytic activity. In addition, the messenger RNA level of Fcγ receptors III was also significantly lower in gingival crevicular fluid neutrophils than in peripheral blood neutrophils but not the messenger RNA level of Fcγ receptors II. These findings indicate local suppression of Fcγ receptor expression and/or the production of Fcγ-binding proteins by periodontal pathogens (130).

Adaptive aspects of the immune response in early-onset periodontal disease

In contrast to the innate immune mechanisms discussed previously, adaptive immune responses are not initiated at the site where a pathogen first establishes a focus of infection.

The humoral immune response

The main function of the humoral immune response is the destruction of extracellular microorganisms and prevention of the spread of intracellular infections. This is achieved by antibodies secreted by B lymphocytes. Antibodies induced have many functions ranging from neutralization, to leading to opsonization. The activation of B cells and their differentiation into antibody-secreting cells is triggered by antigen and usually requires helper T cells.

Many studies over the years have demonstrated that antibody responses to various periodontal pathogens are increased in patients with periodontal disease compared with subjects without the disease. Therefore, antibody titers, isotypes and the responses of the humoral immune system have been investigated over the years.

Mouton et al. (212) established that antibody to

Porphyromonas gingivalis is found in a significant proportion of healthy adults as well as being detectable in children as young as 6 months. In both adults and children there was a positive correlation between antibody levels and age. Nevertheless, serum IgG antibody increased by 500–800% in adult periodontitis and generalized early-onset periodontitis patients. Multiple subsequent studies confirmed these findings and showed a significant increases in levels and frequency of antibody to *P. gingivalis* in adult periodontitis and a subset of generalized early-onset periodontitis patients (10, 11). Chen et al. (39) noted that only some generalized early-onset periodontitis patients displayed an elevated humoral immune response to *P. gingivalis*, albeit these antibodies were of low avidity and increased following periodontal therapy. Çelénligil & Ebersole (32) demonstrated a significantly higher level of antibody to the *P. gingivalis* strains (that is, strains generally derived from United States populations) in United States patients compared with similar disease groups derived from a Turkish population. However, the Turkish early-onset periodontitis patients exhibited a significantly higher frequency of elevated antibody to the *P. gingivalis* compared with a geographically matched normal group. The four concepts originally put forth by Mouton et al. (212) regarding this pathogen were: (i) based on the humoral immune response, *P. gingivalis* is probably an causative agent in periodontal disease; (ii) the humoral immune response is probably protective; (iii) diseased and healthy individuals can be distinguished in terms of their antibody response to this organism; and (iv) differences in the antibody response characteristics may denote different periodontal disease classifications. To these can now be added that the humoral immune response to *P. gingivalis* may contribute a better understanding of patient susceptibility, diagnostic categories, treatment effects and immune protection and that this pathogen may have the capacity to express antigenic diversity between subjects in a population and across patient populations.

A wealth of literature supports the existence of local specific antibody production by plasma cells present in inflamed tissues of the periodontal pocket (68, 72, 167, 283, 295). Likewise, a proportion of gingival crevicular fluid samples within a given subject have local antibody levels significantly greater than can be accounted for by a serum contribution (68, 71, 146). In addition to these inflammatory mediators, evidence has been provided that indicates that both C3 and C4 components of the complement system are cleaved in gingival crevicular fluid from early-onset periodontitis (237, 265), indicating the potential of antigen-antibody complexes to contribute to local immune modulation. Cross-sectional studies have suggested that those gingival crevicular fluid samples with elevated antibody frequently harbor the homologous bacteria and suggest that a combination of the antigen and the host-response is frequently associated with progressing disease (72); these local antibody levels decreased with pocket depth, attachment level stabilized and inflammation resolved following therapy (72).

Lu et al. (185) have shown that, in certain populations, serum IgG_2 levels are increased in localized early-onset periodontitis patients, but not generalized early-onset periodontitis, adult periodontitis or periodontally healthy individuals. IgG_2 is the predominant immunoglobulin subclass that reacts with carbohydrate antigens preferentially and is particularly reactive with *A. actinomycetemcomitans* serotype b outer membrane antigens. *A. actinomycetemcomitans* is a microbe commonly associated with early-onset periodontitis (see Darby & Curtis in this volume). Serum IgG_2 levels increase with age and a rise can be detected at puberty (185). An interesting association is that black subjects in general have higher levels of IgG_2 than whites, and the incidence of localized early-onset periodontitis among black individuals has been shown to be up to 15 times greater than among whites (182, 201, 263). Furthermore, the increase in serum IgG_2 levels in localized early-onset periodontitis patients was due to high anti–*A. actinomycetemcomitans* serotype b levels, which actually only accounted for around 10% of the IgG_2 excess in localized early-onset periodontitis patients. It thus appears that localized early-onset periodontitis patients, particularly black individuals, have a tendency to produce increased levels of IgG_2 (310).

A family study of early-onset periodontitis indicated the possibility of genetic transmission of IgG_2 levels (193). Tew et al. (310) suggest a protective role for high levels of IgG_2 in patients with localiazed early-onset periodontitis. Smoking has been found to suppress the IgG_2 response in adult periodontitis and generalized early-onset periodontitis patients and is associated with more severe destruction, but this does not appear to occur in localized early-onset periodontitis patients (310). Furthermore, the paradoxically lower IgG_2 titers seen in black generalized early-onset periodontitis smokers may be specific to *A. actinomycetemcomitans*, and the IgG_2 response against antigens of other bacteria may not be lower in generalized early-onset periodontitis subjects who smoke (306).

The cell-mediated immune response

Through nonspecific interactions of the T lymphocyte with other cells, controlled by a varying array of adhesion molecules, the naive T-cell migrates through lymph nodes, makes initial interactions with antigen-presenting cells and, after specific activation, eventually moves to peripheral tissues, where it can interact with target cells. This is thought to be the case during the maturation of T cells in periodontal disease, culminating in the homing of specific T cells to the gingival tissues where they can effect their protective function.

Effector T cells fall into 3 functional classes that detect antigens derived from different types of pathogens, presented by the 2 different classes of major histocompatibility complex molecule. Antigens derived from pathogens that multiply in the cytosol are carried to the cell surface by major histocompatibility complex class I molecules and presented to $CD8^+$ cytotoxic T cells, and those derived from extracellular bacteria and toxins are carried to the cell surface by major histocompatibility complex class II molecules and presented to $CD4^+$ T cells. These cells, often referred to as helper cells, can differentiate into two types of effector T-cell; Th1 and Th2 cells. Th1 cells secrete both interleukin-2 and interferon-γ when activated by certain types of T-dependent antigens so that they can enhance cell-mediated responses. The Th2 subset of T helper cells produce interleukin-4, interleukin-5, interleukin-10 and interleukin-13 and thereby promote the humoral immune response.

Although much of the literature discusses the importance of the humoral aspect of the immune response in periodontal disease, models used in earlier years suggested that the initial periodontal lesion is composed mainly of T lymphocytes, with B cells and plasma cells predominating at a later stage (188, 272, 273). Studies have been carried out reporting differences in CD4 : CD8 ratios in lesions and peripheral blood of periodontitis patients (226, 308) compared with healthy controls, further indicating that the cell-mediated immune system is potentially as important in a discussion of this disease as that of the humoral arm.

It appears that, in terms of periodontal disease, the antigen is picked up by an antigen-presenting cell, such as a Langerhans cell, at the site of infection, whereupon it is carried to a primary lymphoid tissue where the presentation to a circulating naive T cell takes place. This leads to antigen-specific clonal activation, where some activated cells become effector cells and others remain in the circulation as memory cells, naive T cells expressing CD45RA and memory cells CD45RO.

CD45 is a transmembrane tyrosine phosphatase with three variable exons that encode part of its external domain. In naive T cells, high-molecular-weight isoforms CD45RA are found. In memory T cells, the variable exons are removed by alternative splicing of CD45 RNA and this isoform is known as CD45RO. The general dogma indicates that memory T cells ($CD45RO^+$) greatly outnumber naive ($CD45RA^+$) T cells in periodontitis lesions (88, 155, 168, 354). However, it has also been shown that a proportion of these cells are $CD45RA^+$, which suggests reactivation of the memory cell population by specific but as yet identified antigens in the tissues. It has been reported that memory T cells adhere to vascular endothelial cells and augment their permeability to macromolecules (48). This functional ability of memory T cells to control endothelial permeability and to adhere to endothelial cells may be a dominant factor that contributes to the preferential migration of memory T cells into sites of chronic inflammation (240), such as the diseased gingival tissues.

The realization that T lymphocytes are present in large numbers in the diseased tissues of early-onset periodontitis patients and not in health has led to investigations of the numbers, ratios and functionality of T cells in both the peripheral blood and diseased tissues of patients with periodontal disease. Studies of peripheral blood T-lymphocyte subsets in early-onset forms of periodontal disease have revealed a wide variety of contradictory results with either depressed $CD4^+ : CD8^+$ ratios (154) or mixed (148), or even not correlated to the disease (76). A number of studies have also looked at the distribution of the T-cell subsets in the tissues. One study reports that the $CD4^+ : CD8^+$ ratio in the tissues of early-onset periodontitis patients and even their families tend to be increased (300). A study by Stoufi et al. (295) and one by Celenligil et al. (36), however, clearly states that the ratio is decreased in patients with this disease. What is obvious and further indicated by a study looking at juvenile periodontitis (202) is that the ratios are highly variable between different patients with the same disease, making generalizations and conclusions extremely difficult. What the above study did suggest however, was that the high $CD4^+ : CD8^+$ ratios were seen in those patients that had a greater degree of inflammatory cell infiltration, while the low $CD4^+ : CD8^+$ ratio tissues were less inflamed.

When diseased gingival tissue is removed and the cells extracted, both $CD4^+$ and $CD8^+$ lymphocytes

are present in large numbers (296, 308). Many studies have indicated raised levels of $CD8^+$ cells in diseased tissues; however, $CD4^+$ cells, although prominent, were found at lower levels than in the peripheral blood. Overall ratios of $CD4^+$: $CD8^+$ were not found to be altered in early-onset periodontitis and chronic periodontitis patients (196); however, these ratios were found to be depressed in patients with localized early-onset periodontitis and generalized early-onset periodontitis (154).

T-cell functions
The role of $CD4^+$ T cells in periodontal disease

T-cell functions in periodontal granulation and gingival tissues can be elucidated by their cytokine synthesis profile (353). There are two main subsets of T-helper cells, Th1 and Th2, which are usually determined by the cytokine profile that is characteristic of them (see Table 1 for characteristics of cytokines that play an important role in the progression of periodontal disease). ThO cells have also been identified, and these cells are thought to secrete interleukin-4 and interferon-γ. Their actual role is yet to be confirmed; however, it is thought that they may play a role as a precursor cell to T-helper cells yet to have differentiated to become either Th1 or Th2 cells (207). Although the cytokine profile is a good tool for T-cell subset investigations, the results reported are often conflicting, causing confusion. Often the results cannot assess the relative importance of the Th1 and Th2 subsets.

Fujihashi et al. (81) investigated Th1 and Th2 cytokine messenger RNA expression by $CD4^+$ T cells from diseased gingival tissues. They detected interferon-γ, a Th1 cytokine, but failed to detect the Th2 cytokine interleukin-4, thus indicating a more dominant role for Th1 cells. Other groups, however, indicate a more dominant role for Th2 cells after detection of interleukin-4, interleukin-5, interleukin-6 (10, 192, 355) and interleukin-10 (90). One of the current theories on the Th1/Th2 paradigm is that both cells have an important but different role. Gemmell et al. (89), have suggested that Th1 cells remain tightly localized at sites undergoing an active disease process, whereas Th2 cells are more widely distributed throughout the tissue and typify a more quiescent stage of the disease; that is, the immune response in periodontal disease is predominantly Th2 driven, with active phases of the disease leading to a foci of Th1 activity.

The role of these cell types is that of help for the immune response. Th1 cells, characterized often by their production of interferon-γ, are important in microbial killing and are known to augment cytotoxic T-cell functions through their production of interleukin-2 and interferon-γ. Th2 cells, however, inhibit much of the work done by Th1 cells. The production of interleukin-4 and interleukin-10 by these cells inhibits the actions of interferon-γ, hence has anti-inflammatory characteristics. The production of

Table 1. The role of cytokines in the pathogenesis of periodontal disease

Cytokine	Produced by	Role
Interleukin-1	Large quantities produced by macrophages	Proinflammatory properties Mediator of tissue destruction in peridontal disease (6)
Interleukin-4	Activated T cells	Inhibits production of interleukin-1, tumor necrosis factor and interleukin-6 An absence of interleukin-4 in the periodontal tissues has been suggested to trigger disease progression (80, 132)
Interleukin-6	Lymphoid and non-lymphoid cell types Production is triggered by interleukin-1, tumor necrosis factor and interferon-γ	Mediates inflammatory tissue destruction Thought to be an important cytokine in B-cell differentiation and hence to play a role in the induction of the elevated B-cell response in patients with periodontal disease (80)
Interleukin-8	Mononuclear monocytes and many of the tissue cells	Attracts and activates neutrophils into the tissues of the periodontium, particularly as it is produced by gingival fibroblasts (304)
Interleukin-12	Monocytes, macrophages, B-cells and accessory cells	Pleiotrophic effects on natural killer cells and T cells in the tissues Induces interferon-γ production and is necessary for Th1 induction
Tumor necrosis factor α and β	Macrophages and lymphocytes respectively	Similar effects to interleukin-1 and interleukin-6
Interferon γ	Activated T cells	Potent inhibitor of interleukin-1, tumor necrosis factor-α and tumor necrosis factor-β

interleukin-4, interleukin-5 and interleukin-6 elicits and helps maintain a humoral immune response.

Natural killer cells

Natural killer cells are also said to constitute a large part of the cell-mediated immune system, although they are classed as a component of the innate immune system. These cells are large granular lymphocytes that are often detected by the marker CD16, thus making the actual numbers detected very difficult to report, since this marker is also found on other peripheral blood lymphocytes and monocytes. In contrast to $CD8^+$ cytotoxic T cells and killer cells, which kill infected targets cells in an antigen-specific manner, natural killer cells kill infected target cells or tumor cells in an antigen-nonspecific manner (196).

The role of natural killer cells in periodontal disease are present is unknown. However, there are reports of an increase in the numbers of natural killer cells in the peripheral blood of patients with early-onset periodontitis (35, 158, 339).

Other studies have reported no increase in the numbers of natural killer cells found in the peripheral blood of diseased patients compared with health (358). In the tissues of diseased patients an increase of natural killer cells is seen, which is actually not difficult or surprising considering the fact that, in health, very few natural killer cells are present in the gingival tissues. The numbers of natural killer cells in the tissues are seen to increase from health to gingivitis to periodontitis (44, 82, 351), although the proportion of these cells relative to total lymphocyte numbers actually decreases (44).

The activity of natural killer cells is highly increased by soluble mediators such as interleukin-2, interferon-α, interferon-β and interferon-γ. Interferons α and β are antiviral proteins synthesized and released by leukocytes, fibroblasts and virally infected cells; interleukin-2 and interferon-γ are released by activated T cells (18). Surface lipopolysaccharide from gram-negative bacteria, however, appear to provide the major activation signal for natural killer cell–mediated cytotoxicity in periodontal disease (174), which leads to natural killer cell cytotoxic activity against host cells.

Cytokines in periodontal disease

Interleukin-1

In the periodontal tissues, interleukin-1β predominates over interleukin-1α and is present at higher levels in active diseased sites compared with healthy or stable diseased sites (144, 289). The variation in interleukin-1 concentrations between sites in the same individual, and the lack of variation in serum interleukin-1β levels between adult periodontitis patients and healthy controls, suggests local rather than systemic production of interleukin-1 (38). Activated keratinocytes and Langerhans cells produce interleukin-1α and interleukin-1β (131). Macrophages, polymorphonuclear leukocytes, B cells and fibroblasts secrete interleukin-1β (90, 144, 302). Not surprisingly, interleukin-1 receptor antagonist has been detected in periodontal macrophages and found in the periodontal crevicular fluid of patients with periodontitis (137, 147).

Numerous periodontal bacteria can stimulate host cells to produce interleukin-1 (88, 90). Levels of interleukin-1β correlate with the periodontal status (242). Interleukin-1 stimulates fibroblasts, endothelial cells, monocytes and granulocytes resident in the gingival connective tissue to express adhesion molecules (303). These adhesion molecules aid the passage of immune response cells from the capillaries into the inflamed tissues.

Interleukin-1 can induce the gingival fibroblast production of plasminogen activator, interleukin-6 and prostaglandin E_2 as well as the matrix metalloproteinases, which are involved in tissue destruction, turnover and remodeling (246). Both interleukin-1 and tumor necrosis factor-α strongly induce matrix metalloproteinase expression in resident and immigrant cells, but interleukin-1 is more potent than tumor necrosis factor-α (22, 318). Interleukin-1 stimulates osteoclasts to resorb bone (136) while inhibiting the formation of bone (288). More specifically, interleukin-1-mediated resorption of bone can be induced by periodontal pathogens (141).

Tumor necrosis factor

The proinflammatory cytokines tumor necrosis factor-α, interleukin-1α and interleukin-1β have been identified in periodontitis lesions from patients with adult periodontitis at higher levels than in healthy sites (197, 290). However, cells containing interleukin-1β were found in much greater numbers than those producing tumor necrosis factor-α and interleukin-1α (290). In contrast to interleukin-1, only very low concentrations of tumor necrosis factor-α have been demonstrated in gingival crevicular fluid from both adult and early-onset periodontitis patients (252, 357). However, high levels of tumor necrosis factor-α in periodontal lesions appear to rep-

resent an established inflammatory and immune response. Salvi et al. (256) examined gingival crevicular fluid interleukin-1β, tumor necrosis factor-α and prostaglandin E_2 levels in insulin-dependent diabetes mellitus patients with periodontal disease. They found that diabetics had significantly increased levels of prostaglandin E_2 and interleukin-1β but not tumor necrosis factor-α, compared with non-diabetic controls with similar periodontal status. In addition, diabetics with moderate to severe disease had almost two-fold higher levels of prostaglandin E_2 and interleukin-1β compared with diabetics with gingivitis or mild periodontitis.

Prostaglandin E_2 causes increased vascular dilation and permeability. It also stimulates macrophages to secrete matrix metalloproteinases and has been shown to trigger bone resorption *in vitro* (22), thus acting synergistically with interleukin-1 and tumor necrosis factor-α. Furthermore, it upregulates receptors for complement and immunoglobulin on monocytes and neutrophils (216). The major source of prostaglandins and leukotrienes in inflamed periodontal tissue is the activated macrophages, although they can also be produced by fibroblasts. Prostaglandins, especially prostaglandin E_2, comprise the primary pathway of alveolar bone destruction in periodontitis. Leukotrienes, especially leukotriene B_4, are potent chemoattractants for polymorphonuclear leukocytes.

Interleukin-10

Interleukin-10 may have a role in the progression of adult periodontitis. This was suggested by Gemmell et al. (90), who found that T-cell lines from both the peripheral blood of these patients and their gingival tissue secreted interleukin-10, but clones derived from the peripheral blood of an individual with gingivitis did not. It has also been suggested by Stein et al. (292, 293) that interleukin-10 may contribute to autoimmune reactions against gingival tissues. Interleukin-10 is able to favor the development of a particular clone of B cells (CD5), which can secrete high levels of autoantibody and is found in increased numbers in inflamed gingival sections (297). It was found that a subset of type I diabetics may be more susceptible to developing periodontitis through an autoimmune type of reaction directed against gingival connective tissue when exposed to periodontal pathogens (292, 293).

Gemmell & Seymour (90) have demonstrated a significantly reduced number of interleukin-10^+ $CD8^+$ cells from periodontitis lesions (adult periodontitis) than from healthy or gingivitis sections. They suggested that in gingivitis interleukin-10 might suppress inflammation by decreasing macrophage activity, thereby preventing progression to periodontitis. No differences were found in the percentages of interleukin-10^+ $CD4^+$ cells between the two disease categories. However, some individuals were found to have higher numbers of interleukin-10^+ T cells regardless of disease category. It appears that the overall variation between the two diseases entities in interleukin-10^+ $CD8^+$ cells may have been due to individual variation in interleukin-10 secretion patterns rather than differences in pathogenesis between the two disease categories. Further studies are needed to elucidate the role of interleukin-10 in periodontal disease.

In summary, interleukin-1, interleukin-6 and tumor necrosis factor are proinflammatory cytokines which are detected and may be modulated within the periodontium. They are important and ubiquitous aspects of the host defense system, operating in the periodontal tissues during health, destruction and healing phases. Similar claims may in future be made for other members of the cytokine network, in particular interleukin-10 and various soluble and cellbound receptors and inhibitors of cytokine function. These molecules are inter-related and are part of the immune, inflammatory, breakdown and repair homeostasis ongoing within the periodontium.

The pathology of the periodontal diseases affecting children and adolescents

Gingivitis

Various hypotheses have been made over the years linking the severity of gingivitis in childhood with various factors including puberty.

An increase in the sex steroid hormones during the period of puberty is believed to have a transient effect on the inflammatory status of the gingiva (194). Enlargement of the gingivae occurs in both males and females in areas of local irritation. The inflammation has been described as marginal in distribution, characterized by prominent bulbous proximal papillae. Enlargement is often only found on the facial gingiva, whereas the lingual surfaces remain unaltered (98). Although this view is widely accepted, the evidence obtained from cross-sectional and longitudinal studies for this hypothesis is mainly circumstantial and often fragmented, although still of importance.

Sutcliffe (299) reported a 6-year longitudinal study that had been carried out to observe the changes in gingivitis in 127 children 11–17 years old, and the relationship of these changes with oral hygiene. He reported an increase in gingivitis associated with puberty. It was noted that girls tended to experience their maximum gingivitis before boys, girls at a mean age of 12 years and 10 months and boys at 13 years and 7 months. Hence, he noted that the distributions of age were consistent with the hypothesis that there is an increase in gingivitis associated with puberty.

The problems with this and further studies is that they only provide circumstantial evidence linking puberty and gingivitis. Thus, the association between the increase in sex hormone levels and gingivitis needs confirmation. Chronological age is a poor indicator of puberty, the measurement of the maximum gingivitis experience is extremely subjective and, further, none of the studies reported have measured the hormone levels of the subjects. An alternative explanation for increased gingivitis during puberty is that this is a period of mixed dentition where erupting and exfoliating teeth present many sites for plaque retention. The fact that gingivitis decreases after puberty may reflect the general improvement seen in children as they improve their dexterity and become more aware of oral hygiene.

Other clinical indications of a hormonal effect include fluctuations in gingivitis with phases of the menstrual cycle (133, 176), increased incidence and severity of gingivitis during pregnancy (27, 138), gingival enlargement induced by oral contraceptives (149, 187, 287) and circumstantial evidence suggesting that there is an increased number of desquamative gingival lesions in menopausal females (216). The mechanisms by which the hormones may influence the gingiva have so far not been fully elucidated.

Steroid hormone receptors are located in hormone-sensitive tissues, where the hormones tend to be retained and allowed to accumulate. In a number of species accumulation of androgens, estrogens and progestins have been observed in the periodontium (194), and particularly pertinent are autoradiographic studies that have demonstrated nuclear localization of estradiol in human gingival epithelium and gingival fibroblast cells (332–334). The actual accumulation and effect of sex steroid hormone on the gingiva is unknown; however, a number of theories have arisen.

Microbial effects of hormones

Microbial plaque and its presence is a very important factor on the onset, progression and severity of gingivitis. It has been reported in other studies of a hormonal nature that microorganisms are effected. Kornman & Loesche (138, 149) reported higher levels of *P. intermedia* in pregnant females with elevated levels of sex hormones and also in women taking oral contraceptives. Therefore, it seems logical that if levels of sex hormones can effect the microflora at one time they must be affected during other periods of hormonal fluctuation. Many studies have been carried out often leading to reports of speculative results. Several reports of a transient increase of black pigmented gram-negative anaerobic rods in children during puberty have arisen (46, 47, 347). However, none of these studies have used hormone levels as a measure of puberty, and therefore it is difficult to draw inference in this situation.

Vasculature effects of hormones

Investigations in animals suggest that the sex hormones, particularly progesterone, may produce increased inflammation by generating changes in the gingival vasculature (138, 149). Local or systemic administration of progesterone produces an increase in vascularity in ear chamber wounds in rabbits (178). Following systemic administration of progesterone, certain observations suggested that the hormone might induce alterations in the plasma-endothelial lining of the post-capillary venules (178), resulting in an increased leakage of plasma proteins and leucocytes. Gingival exudate also increased following administration of sex hormones to dogs (177), and progesterone enhanced acute inflammation during wound healing in rabbits (220).

Immune effects of hormones

The importance of sex hormones is emphasized by the findings related to gender-related disease susceptibility. For example, a number of diseases can be modulated by hormones, mainly estrogens, such as rheumatoid arthritis (128) and Graves disease (8), which are both affected during pregnancy. Therefore, some immune responses and reactions are affected in the gingiva leading to a possible role for sex hormones in this disease. The degree of influence of this is unknown, however.

Cellular effects of hormones

Sex hormones are known to effect the cells of the body. Histological studies have shown that estrogens increase epithelial keratinization, stimulate prolifer-

ation (247, 248, 362) and increase the downgrowth of epithelial attachment (219). Androgens have also been shown to have effects.

Human gingiva is capable of metabolising sex hormones (334), and receptors for estrogen have been found in gingival tissues (16). Furthermore, inflamed gingiva has been found to metabolize progesterone more rapidly, and these metabolites differ from those produced by healthy gingiva (121). Vittek et al. (334) have shown a positive correlation between progesterone and gingival inflammation. It is known that the gingival levels of progesterone and its metabolites increase during pregnancy and that these metabolites increase the inflammation in preexisting gingivitis (225). These effects, produced by progesterone alone or in combination with estrogen, could account for many of the changes seen in puberty and other conditions in which there are increased circulating sex hormones. In the treatment of children undergoing puberty with gingivitis, the increased susceptibility to plaque-induced inflammatory changes should be reduced by the use of optimal levels of plaque control.

The pathogenesis and host responses of gingivitis and periodontitis

The normal healthy gingiva is characterized by its pink color and its firm consistency. Interdentally, the healthy gingival tissues are firm and do not bleed on gentle probing and fill the space below the contact areas between the teeth. Healthy gingiva often exhibits a stippled appearance and there is a knife-edge margin between the soft tissue and the tooth. In theory, normal gingiva are free from histological evidence of inflammation, but this ideal condition is very rarely seen due to the ever present microbial plaque. Even in the very healthy state, the gingiva has a leukocyte infiltrate that is predominantly comprised of neutrophils or polymorphonuclear leukocytes. The primary purpose of these phagocytes is to kill bacteria, which they do by emigrating through and out of the tissues into the gingival crevicular area.

Clinical changes in the early stages of inflammation of the gingival tissues following plaque accumulation are subtle, but quite rapidly the gingiva appears red, swollen and the soft tissue has an increased tendency to bleed on gentle probing. Histologically, this stage is described by increased vascular dilation and permeability, which leads to an influx of exudative fluid, proteins and inflammatory cells into the tissues giving them their swollen appearance. At this stage there is intense recruitment of polymorphonuclear leukocytes, macrophages and their progenitor cells monocytes into the tissues. These leukocytes migrate up a chemoattractant gradient to the crevice and are aided by adhesion molecules such as intercellular adhesion molecule-1 and endothelial cell leukocyte adhesion moelcule-1, which assist leukocyte binding to the post-capillary venules and help cells to leave the vessels (151). In addition, as the microbes damage the epithelial cells, the epithelial cells release cytokines, which further encourage recruitment of leukocytes (predominantly neutrophils) into the crevice. The neutrophils within the crevice can phagocytose and digest bacteria and so remove these bacteria from the pocket. In addition to this, if the neutrophil is overloaded with bacteria, it degranulates or explodes and, by doing so, causes tissue damage due to the toxic enzymes it releases. The neutrophil can thus be viewed as being both helpful and potentially harmful. This neutrophil defense may in some instances operate well and reduce the bacterial load and can be considered important in preventing the gingivitis lesion from being established. If, however, there is an overload of microbial plaque, then the neutrophils and the barrier that is comprised of epithelial cells will not be sufficient.

After approximately 7 days of plaque accumulation, the initial lesion is said to progress to the early lesion (26, 238, 267, 268). Lymphocytes and macrophages predominate, and the infiltrate occupies 10–15% of the gingival connective tissue. Both clinically and histologically the changes are very visible. The established lesion as defined by Page & Schroeder (233) is one dominated by plasma cells. Plasma cells have been shown to be predominantly IgG-producing cells, with a lower proportion being IgA (188, 189, 223, 336). In early-onset periodontitis, local IgG4-producing cells in particular seem to be elevated. Clinically in the established lesion, collagen loss continues both laterally and apically and the dentogingival epithelium continues to proliferate. Two types of established lesion, however, appear to exist. One remains stable not progressing for months or years (175, 234), and the second becomes more active and converts to progressive destructive lesions. The very nature of early-onset periodontitis and the knowledge that it is a rapidly progressing destructive disease make it clear that the established lesion here will progress speedily to the destructive advanced lesion. The advanced lesion is characterized by bone loss, periodontal ligament loss, apical migration of the junctional epithelium to form a true

pocket and histopathologically by an increase to over 50% of plasma cells in the infiltrated connective tissues (151).

Gingivitis is primarily a response to the bacteria in plaque. It includes a vascular response with increased fluid accumulation and inflammatory cell infiltration. Serum IgG antibody levels to *Actinomyces* spp. were found to be higher in gingivitis (73), and a similar study showed that antibodies to at least three bacteria (*Actinomyces* spp., *Bacterionema matruchotti* and *Leptotrichia buccalis*) were detected in gingivitis patients with a wide variation in levels (73). Multiple reports have suggested that gingivitis patients can present with antibody to suspected oral pathogens (50, 228), including *P. gingivalis* and *A. actinomycetemcomitans*. Bimstein & Ebersole (21) noted that IgM antibody levels were significantly different to many bacteria when comparing children versus adults with gingivitis. In contrast, the IgG levels were unrelated to disease in the adult groups but did help to distinguish between the children with and without gingivitis. Thus, generally, the gingivitis patients exhibit antibody to a wide array of these bacteria; however, the levels to suspected periodontal pathogens are uniformly significantly lower than those seen in periodontitis patients (62, 63). Finally, we observed lower IgA levels in gingivitis sites compared to healthy sites of normal subjects (65), and Grbic et al. (103) demonstrated that IgA levels were significantly increased in gingival crevicular fluid from gingivitis sites compared with periodontitis sites, suggesting the potential for IgA to function as a protective factor in this local environment. Generally low levels of antibody to bacteria associated with periodontitis are found in gingival crevicular fluid from gingivitis sites.

The progression from gingivitis to periodontitis

Brecx et al. (25) suggests that more than 6 months may be needed in the normal situation for gingivitis to change to a periodontitis lesion. Furthermore, this move from gingivitis to periodontitis probably only occurs in 10% to 15% of the population. The reason why some patients develop periodontitis more readily than others is highly elusive and thought to be multifactorial. It should be borne in mind that the changes during gingivitis, even in prolonged established gingivitis lesion, are to a large extent reversible, whereas the changes during periodontitis, that is, the bone loss and apical migration of the epithelial attachment, are irreversible. Periodontitis is a cumulative condition whereby once there is bone loss, it is almost impossible to have it re-established and therefore, patients lose bone incrementally over the years. The worst effects of periodontitis are therefore, commonly seen in older patients rather than younger patients, who have still to develop these deep pockets or this extensive bone loss or the associated gingival recession that occurs in periodontitis. The early-onset forms of periodontitis are, however, situations where the normal slow destructive process is greatly accelerated.

Differences between chronic gingivitis and periodontitis

The similarities in the inflammatory infiltrate between the stable established chronic gingivitis lesion and advanced periodontitis lesions have encouraged many investigators to look for qualitative and quantitative differences that may be involved in determining the progression of gingivitis to destructive periodontitis.

Seymour et al. (271) hypothesized that a change from T-cell to B-cell dominance causes periodontitis. However, Page (236) has disagreed with this view, as have Gillett et al. (97), who showed a predominantly B-cell infiltrate to be associated with stable, non-progressive lesions in childhood gingivitis. Liljenberg et al. (173) compared plasma cell densities in sites with active progressive periodontitis and in sites with deep pockets and gingivitis but no significant attachment loss over a 2-year period. The density of plasma cells (51.3%) was very much increased in active sites as compared with inactive sites (31.0%). It is now generally accepted that plasma cells are the dominant cell type in the advanced lesion (86).

Necrotizing ulcerative gingivitis and periodontitis

Necrotizing ulcerative gingivitis is an acute inflammatory condition associated with a fusospirochaetal microbial complex. Necrotizing ulcerative gingivitis begins as linear erythema of the marginal gingiva and extends to form ulcerated, punched-out, painful and necrotic papillae and later may extend to ulceration of the gingival margins (134). The ulcers are covered by a grayish slough, which if removed causes bleeding from the exposed ulcers. The condition is painful and is associated with marked halitosis often termed foetor oris. As the necrosis progresses, deep bone cratering appears in the interproximal regions in the areas where the papillae have been lost. If left

untreated, necrotizing ulcerative gingivitis will progress to necrotising ulcerative periodontitis, which involves destruction of the interproximal alveolar bone and the periodontal ligament (134, 198). Marked gingival recession and sequestra formation is a sequel of the rapid necrosis. In industrialized countries, adolescents and young adults are most predisposed to this condition, and a prevalence of approximately 0.5% has been reported (135).

In a classic electron microscopic study by Listgarten in 1965 (179) four zones were identified. These zones are the: 1) bacteria-rich zone or plaque zone, 2) neutrophil-rich zone in which neutrophils predominate; 3) necrotic zone, which has dead cell, spirochetes and other bacteria; and 4) the spirochetal infiltration zone, in which tissue structures is preserved but many invading spirochetes are noted.

Necrotizing ulcerative gingivitis has been characterized by the emergence of selected members of the oral microbiota: *Treponema* spp. and *P. intermedia*, which develop as a response to altered resistance to infection of the host tissue (immunosuppression). These oral spirochetes have been shown to be capable of invading the infected host tissue (79, 181, 255). IgG antibody to oral spirochetes (*Treponema denticola* and *Treponema vincentii*) has been reported; the data have shown below normal to selected elevations in disease subjects (14, 41, 143, 166, 213, 270, 346). Therefore, while treponemal species are frequent in subgingival plaque and comprise a major proportion of some plaques in periodontitis and necrotizing ulcerative gingivitis (281, 282), there does not appear to be an elevation in systemic antibody responses, indicative of an active host response, to the cultured treponemal species. Serum antibody to *Actinomyces viscosus*, *Actinomyces israelii*, *Prevotella melaninogenica* and *P. gingivalis* were within normal limits in necrotizing ulcerative gingivitis during acute and convalescence; however, antibody to *Prevotella intermedia* was significantly elevated in convalescent sera (73), suggesting that acute disease may exhibit a specific pathogenic microbiota to which the host responds. The contribution of this response to resolution of the disease remains unknown.

Cancrum oris or noma is a devastating orofacial gangrenous disease that is considered a very severe form of necrotizing ulcerative periodontitis and is commonly reported in underprivileged African children (78). The children often suffer from chronic malnutrition, numerous endemic communicable diseases, including viral diseases, and severe adverse physical conditions. These severe debilitating factors may deplete the subject's immune and inflammatory system. Measles is the most common infection preceding the development of noma in Nigerian children (78). Acquired immunodeficiency in combination with the impaired endocrine balance in malnourished children results in widespread infection with the measles virus. Anergy resulting from the combination of malnutrition and measles virus infection may promote overgrowth and invasion of anaerobic organisms, including gram-negative baccilli and spirochetes. Due to the severe debilitation of the malnourished child, the infection is not confined locally as necrotizing ulcerative gingivitis but spreads rapidly across normal anatomical barriers. Severe necrosis and possible sequestration as exemplified by noma are then seen. Minimal data are available on host responses in this disease.

Murray et al. (214) also demonstrated that the microbiota of periodontitis in human immunodeficiency virus (HIV) patients was similar to that seen in systemically healthy periodontitis individuals; however, there were significant increases in the numbers of *Campylobacter* spp. and *Treponema* spp. Serum IgG antibody to *P. intermedia*, *P. gingivalis* and *Fusobacterium nucleatum* have been found to be elevated in HIV-positive homosexuals compared with control normals and seronegative homosexuals (105). Both necrotizing ulcerative gingivitis and necrotizing ulcerative periodontitis (74) occur in a proportion of HIV-infected patients. Rams et al. (244) compared the distribution of several bacterial morphotypes in necrotizing ulcerative periodontitis and clinically normal periodontitis subjects and found that the morphotypes were very similar from areas of necrotizing ulcerative gingivitis–like tissue necrotic lesions and necrotizing ulcerative periodontitis sites. Importantly, all the patients with the necrotizing ulcerative gingivitis–like lesions had significantly high levels of spirochetes in their pockets. However, the levels of serum antibody to oral microorganisms in HIV subjects has had minimal investigation. Grieve et al. (104) suggested that HIV periodontitis (necrotizing ulcerative periodontitis) patients exhibited elevations in serum antibody to *P. gingivalis*, *P. intermedia*, *F. nucleatum*, *A. actinomycetemcomitans* and *Eikenella corrodens*. We recently examined serum IgG antibody to a battery of oral pathogens, including *T. denticola*, in HIV patients. We found that, while some of the patients with necrotizing ulcerative periodontitis exhibited elevated antibody to suspected periodontal pathogens, the antibody level to *T. denticola* isolates was low, and selected HIV patients had minimal antibody to all of the oral micro-

organisms. Additional studies will be required to delineate the capability of the host response in managing periodontal infections in these patients.

Pathogenic features of adult and early-onset periodontitis

Experimental short-term clinical studies have shown that microorganisms quickly colonize clean tooth surfaces when an individual stops toothbrushing. Within a few days, microscopical and clinical signs of gingivitis are then apparent. These inflammatory changes can be resolved when adequate tooth-cleaning methods are resumed (183, 317). Thus, microorganisms that form dental plaque and cause gingivitis probably do so by the release of components from the bacterium that induce inflammation in the tissues. Short- and long-term clinical trials have underlined the importance of supragingival plaque and subgingival microbial plaque in both the treatment of gingivitis and periodontitis. Furthermore, animal experiments have indicated that gingivitis only develops in animals that accumulate bacterial plaque deposits. Gingivitis is necessary prior to the tissue developing periodontitis, and this implies that prevention of gingivitis is also a primary preventive measure for periodontitis.

The pathogenic processes are largely the response to microbe-induced destructive processes. These processes are initiated by the microbes but are undertaken by the host cells, and thus it is the host tissue itself that results in the destruction seen. The host produces enzymes that break down host tissue. This is a necessary process that the host initiates and controls in order to allow the tissues to retreat from the microbial destructive lesions.

Microbial plaque accumulation occurs on the surface of the teeth adjacent to gingival tissues, bringing the oral sulcular and junctional epithelium into contact with colonizing bacteria and their waste products such as proteases, enzymes, lipopolysaccharide and toxins. As these products build up, the irritation to the tissues increases, until production of chemical mediators of inflammation commences and a host inflammatory response is induced.

Elements of the host response that could differentiate adult and early-onset forms of periodontitis

The various aspects of the host response that may contribute to peridontal disease have been covered generally, but several aspects supported within the literature may be specifically relevant to early-onset periodontitis. These include aspects of the innate, inflammatory and immune defense systems.

The innate defense system includes epithelial, connective tissue elements and soluble products that are antimicrobial and in the normal healthy state may protect against periodontal disease. There are, however, defects reported in epithelial, connective tissue (fibroblasts and cementum), body fluids (saliva) and enzymes (alkaline phosphatase) that may be relevant to early-onset periodontitis (5, 9, 15, 45). Structural defects such as the absence of endothelial adressins for leukocyte adherence and subsequent migration from the bloodstream into lesions (seen in leukocyte adhesion deficiency with marked periodontal destruction) and defects of epithelium and connective tissue (as seen in Papillon-Lefèvre syndrome) as well as more obvious periodontal defects such as that of hypophosphatasia (where defective cementum results in early tooth loss) can all have major effects on the periodontal tissues and lead to early loss of teeth.

Pathogens

The microbes involved in adult periodontitis are largely gram-negative anaerobic bacilli with some anaerobic cocci and a large quantity of anaerobic spirochaetes. The main organisms linked with deep destructive periodontal lesions are *P. gingivalis*, *P. intermedia*, *Bacteroides forsythus*, *A. actinomycetemcomitans* and *T. denticola* (360).

P. gingivalis is more frequently detected in severe adult periodontitis, in destructive forms of disease and in active lesions than in health or gingivitis or edentulous subjects. *P. gingivalis* is reduced in numbers in successfully treated sites but is seen in sites with recurrence of disease after therapy (16, 112, 326). It has been shown that *P. gingivalis* induces elevated systemic and local antibody responses in subjects with various forms of periodontitis (61). *P. intermedia* levels have been shown to be elevated in certain forms of periodontitis (9). Elevated serum antibodies to this species have been observed in some but not all subjects with refractory periodontitis (31). *B. forsythus* has been found in higher numbers in sites exhibiting destructive periodontal disease than in gingivitis or healthy sites (47). In addition, *B. forsythus* was detected more frequently in active periodontal lesions (13). Although spirochetes such as *T. denticola* are notoriously difficult to culture in the laboratory, these strict anaerobes may comprise more than 30% of the periodontal subgin-

gival microflora. The above mentioned putative pathogens are always part of a large and varied microflora found in the subgingival plaque. Some are not detected in certain sites with periodontitis and can even be completely absent in cultures from multiple sites within an untreated periodontitis patient.

The dominant microorganisms involved in early-onset periodontitis include *A. actinomycetemcomitans*, *Capnocytophaga* spp., *Eikenella* spp., *P. intermedia* and anaerobic rods such as *Campylobacter rectus* (312). Much of the literature regarding the bacterial effects in early-onset periodontitis have centred around studies on *A. actinomycetemcomitans*. *A. actinomycetemcomitans* is a short, facultatively anaerobic gram-negative rod that is nonmotile and regarded as a key microorganism in early-onset periodontitis. Studies of association have shown increased levels of *A. actinomycetemcomitans* and increased frequency of antibody to *A. actinomycetemcomitans* in localized early-onset periodontitis (24). A later study by these researchers (25) showed a significantly increased level of IgG antibody to *A. actinomycetemcomitans* serotype b in 90% of localized early-onset periodontitis patients, 40% of generalized early-onset periodontitis and only 25% of adult periodontitis patients. Furthermore, clinical studies carried out to observe the effect of treatment on *A. actinomycetemcomitans* load have related poor therapeutic outcome with an inability to reduce the subgingival load of *A. actinomycetemcomitans* (113, 114, 214).

In localized early-onset periodontitis when present, *A. actinomycetemcomitans*, is predominant. Genco et al. (91) indicated that organisms of the normal flora play a key role in gingivitis, while exogenous organisms or more unusual anaerobic organisms seem to be implicated in periodontitis. Immunological studies involving patients with localised early-onset periodontitis indicate significantly elevated titers of serum antibodies to *A. actinomycetemcomitans* in localized early-onset periodontitis patients (67, 69, 73, 95, 180, 191, 260, 315, 331). These patients have also been seen to produce antibodies locally to *A. actinomycetemcomitans* (260).

Accompanying the documentation of specific infections in periodontitis patients has been the definition of active host systemic immune responses to these bacteria (73). In particular, it appears clear that, in most populations, patients with localized juvenile periodontitis exhibit elevated serum IgG antibody to *A. actinomycetemcomitans* (30, 73). These results have been derived from cross-sectional studies and demonstrated some correlation with the ability to identify the homologous microorganism in the subgingival plaque (66, 309). Accumulating evidence has supported the concept that the predominant serotype of *A. actinomycetemcomitans* appears to differ throughout the world (11, 40, 60, 75, 99, 100, 109, 210, 224, 262, 314, 352, 359) and may represent some more general genotypic, phenotypic, and pathogenetic heterogeneity in this species. Ebersole et al. (30) reported a study that examined the frequency of oral disease in an adolescent population and assessed the relationship to *A. actinomycetemcomitans*. Heavy accumulations of plaque and calculus were frequently observed and were associated with gingival inflammation and, significantly, 25.7% of the students exhibited early-onset periodontitis with 1.7% diagnosed as localized early-onset periodontitis. The findings supported the hypothesis that *A. actinomycetemcomitans* may represent a pathogen in periodontitis and, while oral health care may be poor, contact with the microorganism appears to be required to initiate disease in this population (30). Ebersole et al. have reported humoral responses in periodontitis patients in various Turkish populations (32). All localized early-onset periodontitis patients from Turkey exhibited elevated antibody levels to *A. actinomycetemcomitans*, particularly serotypes c and a, while antibody levels to *A. actinomycetemcomitans* serotype b were highest in United States localized early-onset periodontitis patients. Fifty percent of the Turkish generalized early-onset periodontitis patients also showed elevated anti-*A. actinomycetemcomitans* antibody. These data suggested that considerable variation exists in the systemic antibody levels to periodontal pathogens and support potential differences in subgingival colonization or antigenic composition of these pathogens between patient populations from different geographical regions. Additional studies by this group have also noted elevated serum antibody responses to *A. actinomycetemcomitans* and selected other oral periodontopathogens in Sjögren's syndrome patients with periodontitis (34) and in patients with Behçet's disease and periodontitis (33). Thus, numerous investigations of many ethnic and geographically disparate groups tend to confirm that *A. actinomycetemcomitans* is frequently associated with localized early-onset periodontitis, and probably an causative agent in the vast majority of cases and in a substantial proportion of other early-onset periodontitis patients.

Our findings and those of others (23, 28, 235, 277, 338, 344, 345) suggest that human serum antibody

reactivities to *A. actinomycetemcomitans* are observed to a wide variety of antigens derived from the outer membrane of this pathogen. The results indicated a response to certain antigens that were distinctive in active disease patients and may: (i) indicate changes in antigen expression by *A. actinomycetemcomitans* (115); (ii) reflect the overall increase in serum antibody levels noted in active disease patients; or (iii) relate to the increase in the *A. actinomycetemcomitans* burden, resulting in greater antigenic load, (iv) reflect the extent of existing disease (59) or (v) be associated with more severe disease.

The previously mentioned studies concentrated their efforts on analysis of serum IgG antibody to *A. actinomycetemcomitans* or antigens isolated from the bacteria. The IgG isotype of immunoglobulin consists of four subclasses, IgG1–4, which have been shown to be elicited selectively by certain types of antigens and to have diverse functions. Thus, the isotype and subclass of antibodies in the patient serum specific to *A. actinomycetemcomitans* may reflect restrictions on the capacity of host antibody to bind to particular antigens (61, 63, 64, 96, 157). The repertoire of immunoglobulin isotypes and IgG subclass responses to particular antigens may account for variations in host resistance to infection and disease.

A wealth of literature supports the existence of local specific antibody production by plasma cells present in inflamed tissues of the periodontal pocket (68, 72, 167, 283, 294). Likewise, a proportion of gingival crevicular fluid samples within a given subject have local antibody levels significantly greater than can be accounted for by a serum contribution (68, 71, 72, 146). In addition to these inflammatory mediators, evidence has been provided that indicates that both C3 and C4 components of the complement system are cleaved in gingival crevicular fluid from early-onset periodontitis (237, 265), indicating the potential of antigen-antibody complexes to contribute to local immune modulation. Cross-sectional studies have suggested that the gingival crevicular fluid samples with elevated antibody frequently harbor the homologous bacteria and suggest that a combination of the antigen and the host-response is frequently associated with progressing disease (72). These local antibody levels decreased with pocket depth, attachment level stabilized and inflammation resolved following therapy (72).

Studies on early-onset periodontitis patients demonstrating infection with *A. actinomycetemcomitans* have demonstrated elevated gingival crevicular fluid antibodies to *A. actinomycetemcomitans* in the majority of the patients (116, 117, 260). The presence of all subclasses of IgG have been identified in gingival crevicular fluid, with IgG1, and/or IgG4 levels in gingival crevicular fluid were elevated relative to serum concentrations (241, 245). The results support a unique local response in individual sites within certain patients and are consistent with a progression of subclass responses at sites of infection and disease. An intriguing facet of host responses in periodontitis has been the relationship between local and systemic antibodies. The general paradigm exists that gingival crevicular fluid is comprised primarily of a serum transudate at the site of inflammation (37, 42). However, other findings are consistent with systemic antibody being a manifestation of the local antibody responses in the gingival tissues and that a portion of serum antibody may be derived from local gingival responses to this infection. The studies described above document some characteristics of the local immune response and their relationship to infection and clinical presentation in early-onset periodontitis patients, particularly within *A. actinomycetemcomitans* infected periodontitis patients. The findings are consistent with the potential to utilize antibodies or local mediators in gingival crevicular fluid as adjuncts in the diagnosis of periodontitis and in defining the mechanisms of disease.

A. actinomycetemcomitans is not always found in localized early-onset periodontitis patients (108, 119, 150, 184, 227, 327). Furthermore, *A. actinomycetemcomitans* has been found in patients with no clinical attachment loss (47, 75, 209). Leukotoxin production, a factor considered crucial in the virulence attributes of *A. actinomycetemcomitans*, varies among strains and has been found in *A. actinomycetemcomitans* isolated from localized early-onset periodontitis rather than adult periodontitis or healthy patients (361). A study in Denmark, however, found no occurrence of the high leukotoxin-producing strains of *A. actinomycetemcomitans* in their population of localized early-onset periodontitis patients (123). In summary *A. actinomycetemcomitans* does not seem to be found in all cases of localized early-onset periodontitis. Nevertheless, clinical studies carried out observing effect of treatment with *A. actinomycetemcomitans* load have linked failure of treatment with inability to reduce the subgingival load of *A. actinomycetemcomitans* (114, 214).

Inflammatory mediators

It has been demonstrated that sera from localized early-onset periodontitis patients with decreased

neutrophil function can modify the activity of healthy neutrophils (2). The serum factors responsible for these effects have been identified as being inflammatory mediators such as prostaglandins and cytokines, which have correlated with periodontal disease (208). Adherent mononuclear cells from localized early-onset periodontitis patients have been found to secrete higher levels of prostaglandin E_2 and tumor necrosis factor-α than generalized early-onset periodontitis patients or healthy controls (274). More recently, insulin-dependent diabetic patients with periodontitis have been reported to manifest excessive levels of inflammatory mediators (256–258). It has been suggested that these observations indicate an underlying immune defect of monocytes in certain diagnostic groups (222).

Early investigations of genetic polymorphisms of cytokines have attempted to identify a possible link between different periodontal categories and specific alleles related to an ability to overproduce cytokines (57, 159). In addition, a family linkage study of early-onset periodontitis suggested that the COX-1 enzyme system, which acts as a catalyst for the breakdown of arachidonic acid, one of the products of which is prostaglandin E_2, may be associated with susceptibility to early-onset periodontitis (89).

Neutrophil chemotactic defects

A further host defense defect that may increase susceptibility to early-onset periodontitis is neutrophil chemotactic defects. A high percentage of members of families with a background of localized early-onset periodontitis have been reported to show abnormal neutrophil chemotaxis (323). Whether the trait is due to an intrinsic defect of neutrophils or is caused by extrinsic factors in the sera and thus is environmentally influenced remains controversial (2). The neutrophil chemotactic defect is not noted in all populations exhibiting localized early-onset periodontitis, and many families with localized early-onset periodontitis do not show evidence of the defect (152, 153). Decreased neutrophil chemotaxis in localized early-onset periodontitis has been linked to a reduced receptors density for chemoattractants such as *N*-formyl-methyl-leucyl-phenylalanine, complement fragment C5a, leukotriene B_4 and interleukin-8 (49, 52). De Nardin (53) found that decreased *N*-formyl-methyl-leucyl-phenylalanine binding which was related to variations in *N*-formyl-methyl-leucyl-phenylalanine receptor DNA in localized early-onset periodontitis patients and healthy controls. These preliminary results indicate a possible role for a *N*-formyl-methyl-leucyl-phenylalanine receptor alteration in neutrophil chemotaxis. However, many molecules related to neutrophil dysfunction in localized early-onset periodontitis patients belong to the same family of surface receptors and have similar morphogenic characteristics. De Nardin (53) hypothesized that a common underlying defect, at either the cell surface receptor or post-receptor level may contribute to the alteration in neutrophil function seen in patients with localized early-onset periodontitis.

Humoral immune aspects

Lu et al. (185) have shown that in certain populations serum IgG2 levels are increased in localized early-onset periodontitis patients, but not generalized early-onset periodontitis, adult periodontitis or periodontally healthy individuals. An interesting association is that black subjects in general have higher levels of IgG2 than whites and the incidence of localized early-onset periodontitis among black individuals has been shown to be up to 15 times greater than among whites (182). It thus appears that localized early-onset periodontitis patients, particularly black individuals, have a tendency to produce increased levels of IgG2 (310).

A family study of early-onset periodontitis indicated the possibility of genetic transmission of IgG2 levels (193). Smoking has been found to suppress the IgG2 response in adult periodontitis and generalized early-onset periodontitis patients and is associated with more severe destruction but this does not appear to occur in localized early-onset periodontitis patients (310). Furthermore, the paradoxically lower IgG2 titers seen in black generalized early-onset periodontitis smokers may be specific to *A. actinomycetemcomitans* and the IgG2 response against antigens of other bacteria may not be lower in generalized early-onset periodontitis subjects who smoke (306).

Fc receptors

It has been suggested that the subclass IgG2 is a poor opsonin because it fixes complement less effectively than IgG3 and IgG1 and binds weakly to Fc receptors on the surface of phagocytes, which tends to complicate theories of protective effects afforded by high anti-*A. actinomycetemcomtians* titers of IgG2 in black localized early-onset periodontitis patients (108, 310). However, a further aspect of the immune response may yet explain this phenomenon. Fc re-

ceptors are the crucial link between the humoral and inflammatory components of the host defense system. It is possible that Fcγ receptors III may prepare polymorphonuclear leukocytes for phagocytosis mediated by Fcγ receptors II (343).

It has been reported that the numbers of Fcγ receptors II and Fcγ receptors III on peripheral blood neutrophils of localized early-onset periodontitis patients are within the normal range (172). There may be local suppression of Fcγ receptor expression and/or the increased production of Fcγ-binding proteins by periodontal pathogens (130).

Conclusion

The various aspects of the host response that may contribute to peridontal disease have been covered in this chapter as have several aspects that may be specifically relevant to early-onset periodontitis, including aspects of the innate, inflammatory and immune defense systems. In summary, the differences in the causation and pathogenesis of adult and early-onset forms of periodontitis are not yet sufficiently elucidated (322). However, multiple specific host defense features are emerging in the early-onset forms of periodontitis that may have a genetic basis and that may serve to differentiate the different forms of periodontitis and may have utility in terms of screening, diagnosis and therapy.

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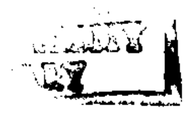
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