CELL MEDIATED IMMUNITY IN PIGEON BREEDERS' DISEASE

by

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ABBREVIATIONS USED IN THE TEXT

EAA	extrinsic allergic alveolitis
MHC	major histocompatibility complex
BALT	bronchus associated lymphoid tissue
B cell	bone marrow derived lymphocyte
T cell	thymus derived lymphocyte
FAE	follicle associated epithelium
BAL	bronchoalveolar lavage
Ig	immunoglobulin
LT	leukotriene
PG	prostaglandin
IL	interleukin
IFN	interferon
GM-CSF	granulocyte/macrophage - colony stimulating factor
NK	natural killer
TCR	T cell receptor
DTH	delayed type hypersensitivity
ADCC	antibody dependent cellular cytotoxicity
LAR	late asthmatic reaction
PBD	pigeon breeders' disease
IL-2R	IL-2 receptor
MIF	macrophage migration inhibition factor
LIF	leucocyte migration inhibitory factor
LMF	leucocyte mitogenic factor
CMI	cell mediated immunity
CD	cluster designation
WBCC	white blood cell count
ELISA	enzyme linked immunosorbent assay
Ab	antibody

SEM	standard error of the mean
SI	stimulation index
r	ratio
PS	pigeon serum
PBS	phosphate buffered saline
PMSF	phenyl methyl sulfonyl fluoride
Tris	tris hydroxymethyl aminomethane
SDS	sodium dodecyl sulphate
FITC	fluorescein isothiocyanate
PE	phycoerythrin
MAb	monoclonal antibody
PWM	pokeweed mitogen
PBMC	peripheral blood mononuclear cell
DNA	deoxyribose nucleic acid
SR	spontaneous release
cpm	counts per minute
P	probability
vs	versus
TNF	tumour necrosis factor
Ab.no	absolute number
PDE	pigeon dropping extract
^{3}H thymidine	tritiated thymidine
LPS	lipopolysaccharide
HPS	hypersensitivity
PFT	pulmonary function test
T_H	helper T lymphocyte
cmm	cubic milimetre

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Declaration

These studies represent original work carried out by the author, and have not been submitted in any form to any other University. Where use has been made of material provided by others, due acknowledgement has been made.

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To mum and dad, without whose help and encouragement this would not have been possible.

SUMMARY

This thesis explored the role of cell mediated immunity in the pathogenesis of extrinsic allergic alveolitis (EAA), using pigeon breeders disease (PBD) as a model. The first part of the thesis looked at the total and differential white blood cell count (WBCC) and lymphocyte subsets in the peripheral blood of pigeon breeders, grouped into well defined categories according to symptoms, presence of serum antibody to pigeon antigen and whether or not they were acutely symptomatic at the time of blood sampling. The aim was to determine whether there were any changes in peripheral blood leucocyte phenotypes and if these changes reflected the symptomatic status of the subjects.

The initial analysis revealed that symptomatic, antibody positive, pigeon breeders, who were acutely symptomatic at the time of blood sampling, had a leucocytosis, comprising neutrophils and T lymphocytes of the $CD4^+$ and $CD8^+$ subsets. The number of $CD56^+$ cells was also increased, but B cell numbers were unchanged. Pigeon breeders who were normally symptomatic and antibody positive, but who were not acutely symptomatic at the time of blood sampling, had normal peripheral blood cell profiles. These results suggest that acute exacerbations of PBD lead to a transient leucocytosis.

An increased proportion of asymptomatic, antibody positive subjects had high CD4:CD8 ratios compared with controls, due to increased numbers of $CD4^+$ cells. The increase in the numbers of $CD4^+$ cells may reflect the presence of an active humoral immune response, in these subjects.

Asymptomatic, antibody positive and asymptomatic, antibody negative pigeon breeders had normal peripheral blood cell profiles.

A proportion of pigeon breeders (41%) with severe longstanding PBD, had reversed CD4:CD8 ratios in their peripheral blood compared with controls. This was due predominantly to increased numbers of $CD8^+$ cells, which were both $CD3^+$ T cells and $CD3^-$ cells, possibly $CD56^+$ NK cells. Further phenotypic analysis of these cells revealed increased percentages of $CD3^-CD16^+$ NK cells, which were predominantly $CD8^-$, but this was not associated with increased cytotoxicity of these cells, as measured (against K562 target cells) in a chromium release assay. It is possible that these cells may be involved in an antibody dependent cellular cytotoxicity reaction in the lung. They might also act as inflammatory mediators. These pigeon breeders also had significantly reduced percentages of $\gamma\delta$ T cells, predominantly due to a decrease in $BB3^+$ cells, the predominant subpopulation of $\gamma\delta$ T cells in the lung and the peripheral blood. Both $CD8^+$ and $CD8^-$ $\gamma\delta$ T cells were reduced. It is possible that these cells are being recruited to the lung. Although there was no change in the levels of "naive" ($CD45RA^+$) $CD4^+$ and "memory" ($CDw29^+$) $CD4^+$ cells in the peripheral blood of these pigeon breeders, total levels of $CD45RA^+$ cells were increased, predominantly due to $CD4^-CD45RA^+$ cells. These cells were not identified. The changes recorded, in the levels of different lymphocyte subpopulations in the peripheral blood of these pigeon breeders, with severe

longstanding PBD, did not correlate with each other. This suggests that the changes occurring, in each lymphocyte subpopulation, are independent and may be associated with different pigeon breeders, who are perhaps at a different stage of disease.

The remaining part of the thesis attempted to determine the stimulus for the changes in the lymphocyte subpopulations, within the group of pigeon breeders with severe longstanding PBD. This was done by assessing whether antigens extracted from pigeon material could provoke cell mediated immune responses by lymphocytes in vitro. The initial studies used pigeon serum as an antigen source. This was found to stimulate lymphocytes from both pigeon breeders and controls. To determine whether this mitogenicity was masking an underlying antigenic response, pigeon serum was fractionated into a "globulin" and "albumin rich" fraction. The "globulin" fraction failed to induce a proliferative response in either pigeon breeders or controls. In contrast, the "albumin rich" fraction was found to possess significant mitogenicity, although with reduced activity, compared with whole pigeon serum. This mitogenicity was not masking a specific antigenic response to pigeon albumin, since a commercial source of pure pigeon albumin failed to induce lymphocyte proliferation. Therefore these studies did not identify any specific response of lymphocytes from pigeon breeders to pigeon serum antigens.

The nature of the mitogenic agent in pigeon serum was explored by testing the hypothesis that it was endotoxin,

which could be detected in pigeon serum at a concentration of 5pg/ml. Equivalent doses of LPS, similar to those that would be present in a mitogenic dose of pigeon serum, were used to try to mimic the effect of pigeon serum. Although no proliferative response of lymphocytes from either pigeon breeders or controls was induced, the results were inconclusive, since recommended doses of LPS also failed to stimulate lymphocytes to proliferate. As an alternative approach, the endotoxin inhibitor, Polymyxin B was included in the cultures stimulated with pigeon serum. However, this did not inhibit the proliferative response induced by pigeon serum.

Pigeon droppings were used in in vitro lymphocyte proliferation assays, to determine whether the mitogenic agent in pigeon serum was excreted in the droppings and therefore likely to be inhaled. Although these studies did not find any mitogenic agent in pigeon droppings, they do not rule out the possibility that the mitogenic agent may be present in another source of pigeon antigen, such as bloom. A role for a non specific agent, as an initiator of an acute inflammatory reaction in the lung, prior to the induction of EAA, has been previously suggested.

The overall conclusions from this thesis are that there are changes in the phenotypes of peripheral blood lymphocytes in pigeon breeders. The cellular changes vary depending on the stage of disease and the symptomatic state at the time of blood sampling. The reduced levels of $\gamma\delta$ T cells and the lack of specific T cell responses to pigeon antigen in pigeon breeders with severe longstanding

PBD suggest that lymphocytes may be recruited to the lung. Analysis of cells from BAL fluid and peripheral blood in parallel after a controlled laboratory challenge or in the natural loft environment would allow a more accurate definition of the course of disease. However, it is important to stress that while sampling the airspaces of the lung for the local cell profile is likely to be more relevant, it is also invasive enough to be ethically doubtful as an experimental protocol and even if it was, repeated sampling during the course of an acute symptomatic episode would not be permitted. Under these circumstances repeated blood sampling would be the only way to identify the cellular changes.

CHAPTER 1
INTRODUCTION

The lung is a complex organ that comes into contact with large volumes of air, to accomplish its primary task of gas exchange. As a result, the respiratory epithelial surfaces are in virtually constant contact with airborne organisms and particles. To protect itself against injury, infection and antigenic stimulation, the lung has evolved a highly efficient system of pulmonary defence which includes mechanical, phagocytic and immune defence mechanisms.

1.1 ANATOMY OF THE LUNG

At the end of a deep breath, about 80 percent of the lung volume is air, 10 percent is blood and only the remaining 10 percent is tissue (Weibel & Taylor, 1988).

The port through which blood vessels and airways enter the lungs is the hilus. The airways reach the two hili by the main bronchi, one leading to each lung. Inside the lungs the bronchi course downwards and divide sequentially to form a mass of very fine bronchioles. The bronchial tree terminates in air passages called respiratory bronchioles, which are about 0.5nm in diameter. These then branch into many short tubes of equal diameter called alveolar ducts which end in tiny hollow bags called alveolar sacs. The alveoli are the gas exchanging surface of a mammal.

Throughout this thesis, the upper respiratory tract will be considered to include the terminal bronchioles and the airways proximal to them, whereas the lower respiratory tract will be considered as the respiratory bronchioles and the distal alveolar spaces (Kaltreider, 1976a).

The whole outer surface of each alveolus is covered by a dense network of capillary blood vessels. The epithelial lining of the lung in the alveoli is very thin, being usually only one cell thick and the basement membrane of the alveoli and capillary endothelium are fused, which aids gaseous exchange.

1.2 MECHANICAL AND PHAGOCYTIC DEFENSE MECHANISMS

Since the vast majority of airborne antigenic material is non-pathogenic, the immune system of the lung has devised a series of mechanical and phagocytic exclusion barriers to limit contact between inhaled antigens and the local immune cell populations situated beneath the respiratory epithelium. This normally prevents unnecessary immune responses to non invasive antigens (pollens, animal danders, insect excretions etc), and limits active immune responses to those inhaled antigens, which are capable of breaching these barriers (pathogenic antigens such as viruses and bacteria).

In the upper respiratory tract, goblet cells produce mucous which covers the ciliated epithelium and acts as a protective barrier to block adherence of microbial and other foreign particles to epithelial cells. The epithelial cells of the lower respiratory tract are covered in a thin layer of liquid surfactant, which as well as serving to block the adherence of foreign particles to epithelial cells and maintaining the stability of the alveoli, contains several non specific factors which defend the lower airways, including $alpha_1$-

antitrypsin, lysozyme, interferon, lactoferrin and trace amounts of C_3, C_4 and C_6 of the complement system (Clarke & Pavia, 1988).

The inhaled particles and organisms attached to the mucous in the upper respiratory tract are removed mechanically by ciliary movement, coughing and sneezing. By contrast, the lower respiratory tract most of the clearance of small particulate matter and microorganisms relies on the phagocytic system. Alveolar macrophages (discussed in more detail in 1.4.2a) are the principal scavengers of the alveoli. The inhaled particles which are phagocytosed by alveolar macrophages may then experience one of several fates (Reynolds, 1985, Gee, 1970):

i) Complete degradation within the phagosome of the macrophage, which prevents interaction of intact antigenic material with the elements of the specific immune response located beneath the respiratory epithelium.

ii) Macrophages containing undegraded material may migrate up the distal airways to reach the mucociliary escalator for elimination. Again this allows inhaled material to bypass the specific immune system.

iii) Alternatively, the macrophages may migrate into the interstitium and peribronchial lymphatics. In this case, engulfed material has the potential of interacting with cells of the specific immune system (discussed later, 1.4).

1.3 PARTICLE DEPOSITION

The fate of inhaled particles is determined by their dimension (Turner-Warwick, 1978).

The hairs of the nasal mucosa trap the majority of particles over 20μm, as well as up to 50% of those with a diameter of >5μm. Particles of 10μm and over will also lodge in the trachea and become trapped on the mucociliary blanket. They are then eliminated by swallowing or expectoration. Smaller particles (3-5μm diameter) tend to be deposited further down the bronchial tree, but again are cleared by ciliary action. Usually, the only particles which reach the alveolar space and hence are dependent on alveolar macrophages for clearance, are 1-2μm in diameter (Brain & Valberg, 1979). This group of particles includes the organic dusts responsible for the various syndromes of Extrinsic Allergic Alveolitis (EAA) (Austwick, 1966).

1.4 PULMONARY IMMUNE SYSTEM

The elements of the specific immune system form a second layer of defence which deals with organisms or particles that breech the non specific exclusion barriers.

T lymphocytes are essential for almost all lung immune reactions, including the stimulation of B lymphocytes to secrete antibodies, directly exerting cytotoxic activity, regulating the activity of other lymphocytes and stimulating the proliferation, recruitment and activation of inflammatory cells. The activation of T lymphocytes is an antigen specific event which requires antigen to be presented to T cells in the form of a peptide bound to

cell surface glycoproteins of the major histocompatibility complex (MHC) present on accessory cells such as dendritic cells and macrophages (Clevers et al, 1988, Davis & Bjorkman, 1988). Lymphocytes and mononuclear phagocytes are present at or near the air-tissue interface at all levels of the respiratory tract extending from the submucosa of the nasopharynx to the interstitium and alveolar spaces of the pulmonary parenchyma.

1.4.1 ORGANISATION OF PULMONARY LYMPHOID TISSUE

Lymphoid tissue within the lung falls into four main categories: i) lymph nodes ii) bronchus associated lymphoid tissue iii) lymphoreticular aggregates; and iv) Bronchoalveolar cells. The degree of structural organisation of lymphatic tissue decreases progressively from the upper to the lower respiratory tract.

i) *Lymph nodes*

Encapsulated lymph nodes are mainly located adjacent to large bronchi and are situated in the peribronchial tissues. They do not come into direct contact with the respiratory epithelium, but they receive lymphatic drainage both from the mucosa of the conducting airways and from the lung parenchyma (Morrow, 1971). These nodes are similar to those found elsewhere, containing antigen presenting cells, and T and B lymphocytes, required to react to foreign antigens.

ii) *BALT*

Small collections of lymphoid tissue may be found in the submucosa of the transbronchial tree. These are referred

to as bronchus associated lymphoid tissue (BALT) (Bienenstock et al, 1973a,b) and are the pulmonary equivalent of the Peyer's patches in the gut. The formation of BALT is thought to be largely dependent on antigenic stimulation, since it has been found in some patients with chronic pulmonary inflammation but not in the lungs of healthy individuals (Pabst, 1992). It is however, present in the lungs of healthy rabbits and rats, and therefore its functional anatomy has been studied in these animals (Sminia et al, 1989). BALT has a structure of a conventional secondary lymphoid organ, with B cells in central follicles, surrounded by rims of T cells.

BALT has a specialised epithelium, referred to as follicle associated epithelium (FAE) (Bienenstock et al, 1973a, Chamberlain et al, 1973, Bienenstock & Johnston, 1976), which consists of a single layer of flattened, nonciliated epithelial cells, and is devoid of glandular and goblet cells. This specialised epithelium seems to be capable of actively transporting, potentially antigenic materials, up to the size of bacteria, into the lymphoid follicle (Tenner-Racz et al, 1979, Fournier et al, 1977). It has also been suggested that alveolar macrophages, transported by the ciliated epithelium, enter the BALT via the FAE (Lipscomb et al, 1981). BALT is particularly prominent at branchings in the tracheobronchial tree, where there is increased turbulence of airflow, increasing the probability for particle deposition (Bienenstock et al, 1973a).

iii) *lymphoreticular aggregates*

Small collections of lymphoid cells are present in the bronchi, interlobular septa and pleura and are also found in the alveolar interstitium. Like BALT they tend to appear between the bronchial epithelium and the accompanying pulmonary artery. However, they are not as well organised into lymphoid follicles (Kradin 1985). These structures are particularly prominent in persons with heavy exposure to dusts and atmospheric pollutants (Kradin, 1985).

iv) Bronchoalveolar cells

Bronchoalveolar cells are those cells retrieved from distal lung tissue by bronchoalveolar lavage (BAL) and are assumed to be representative of the cells present within air spaces of alveoli and peripheral bronchioles. BAL cells from normal subjects consist of 80-85% macrophages, 10-15% lymphocytes and 5% neutrophils and eosinophils (Reynolds, 1987). Of the lymphocytes, the vast majority are T cells (Hunninghake et al, 1981, Reynolds, 1987), with a similar ratio of $CD4^+$:$CD8^+$ cells to that found in the peripheral blood (Costabel et al, 1984). During acute and chronic inflammatory processes of lung parenchyma the total yield of all cell types is increased and the percentages of lymphocytes and/or polymorphonuclear leukocytes are generally increased, while the percentage of macrophages is proportionately decreased (Crystal et al, 1984a,b).

1.4.2 MONONUCLEAR CELLS IN THE LUNG

a) Pulmonary macrophages

Different kinds of macrophage have been recognised in the lung and play a variety of important roles (Brain, 1988, Sibille & Reynolds, 1990) including antigen processing and presentation to T cells, as well as phagocytosis and killing of microbial organisms.

The pulmonary alveolar macrophage is the best characterised and most extensively studied, simply because it can be easily recovered by bronchoalveolar lavage. The alveolar macrophage is the resident phagocyte of the alveolar space (DuBois, 1986) and like all macrophages it possesses Fc receptors for IgG (Reynolds, 1975a) and has a greater number of receptors for IgG_1 and IgG_3 than IgG_2 and IgG_4 (Naegel, 1984). Since alveolar macrophages also possess a receptor for the activated component of complement, C3b (Reynolds, 1975a), antigen, antibody and complement complexes can also interact with the C3b receptor, resulting in optimal phagocytosis and killing. IgE receptors are also expressed by the alveolar macrophage (Joseph et al, 1980, Melewicz et al, 1982) and are particularly abundant on these cells from asthmatic subjects (Joseph et al, 1983) More recently, alveolar macrophages have been shown to express an Fc receptor for IgA (Sibille et al, 1989).

During phagocytosis alveolar macrophages may release into the lung tissue, large amounts of toxic oxygen metabolites, believed to be directly involved in bacterial killing, and arachadonic acid metabolites (reviewed in

Sibille & Reynolds, 1990, Holtzman, 1991). Thromboxane A_2 (TXA_2) and LTB_4 appear to be the major arachadonic acid metabolites produced by alveolar macrophages and are potent proinflammatory mediators. TXA_2 is a constrictor of pulmonary vessels and airways and LTB_4 is a potent chemoattractant for neutrophils and eosinophils and increases vascular permeability. PGD_2 a bronchoconstrictor, is also secreted from alveolar macrophages. However, given that it is not desirable to have hypersensitivity responses in the lung air spaces, the secretion of PGE_2 by alveolar macrophages, which can suppress different cell functions like the proliferation of lymphocytes and the chemotaxis of neutrophils, serves to counteract the proinflammatory properties of TXA_2 and LTB_4. Indeed the alveolar macrophages are thought to play an important role in the maintenance of homeostasis at the airway surface, by inhibiting the inductive phase of local T cell responses to incoming antigens (Holt, 1986). The exact mechanism of inhibition is not clear but may involve the secretion of lymphostatic mediators, such as prostoglandin E_2, defective processing and presentation of antigen or deficient IL1 production (Holt 1986). The inhibitory role of the alveolar macrophage is particularly effective at high macrophage/T cells ratios as occurs in vivo on the alveolar surface (Holt, 1978). However, the situation can be readily reversed by pathogenic antigens, which induce inflammation and an influx of mononuclear cells from the peripheral blood (Holt, 1978).

In contrast, to the alveolar macrophages, interstitial

macrophages are located in the connective tissue of the lung and are not directly exposed to airborne particles. The interstitial macrophages are obtained from minced lung explants but are not recovered by BAL (Bowden & Adamson, 1972). In vitro evidence suggests that tissue macrophages are also capable of inhibiting T cell proliferation and therefore appear to provide another layer of protection against immune responses to inhaled antigens (Holt et al, 1988). Pulmonary macrophages resident in the intravascular structures are also found in human lungs (Dehring & Wismar, 1989). These cells are morphologically different from blood monocytes and adhere firmly to the endothelial cells. In experimental models these macrophage appear to remove particles and bacteria from the circulation (Warner et al, 1987). Finally lung macrophages are also present in the lumen of the upper respiratory tract. Some of these macrophages are likely to correspond to alveolar macrophages transported by the mucociliary escalator. However, a certain number of these appear to be resident cells of the airways adhering tightly to the epithelial cells (Brain et al, 1984). They could help in the removal of debris present in the large airways.

b) Dendritic cells

Dendritic cells, characterised by their dendritic morphology and intense staining for class II MHC antigen, are now thought to be the principal antigen presenting cells in respiratory tissues (Holt et al, 1988). These cells are found in large numbers and are widely distributed in the upper and lower respiratory tract (Soler et al, 1989). In the upper airways, the endogenous

dendritic cell population appears intimately associated with the epithelial basement membrane, with class II rich dendritic processes reaching upwards between epithelial cells to the airway lumen (Holt et al, 1988). Tissue macrophages in the lung wall are capable of inhibiting T cell proliferation induced by antigen-bearing dendritic cells in vitro (Holt et al, 1988). The close proximity of dendritic cells and tissue macrophages within the micro-environments in the airway wall suggests that such interactions are likely to occur in vivo (Holt et al, 1988).

As both T lymphocytes and antigen presenting cells are present in the alveolar lumen, BALT, lung interstitium and draining lymph nodes, sensitisation of T cells could occur in any of these sites (McDermott et al, 1982). However, current evidence suggests that most primary immune responses against inhaled antigens are initiated in the draining lymph node (Hance, 1991). Once antigen reactive cells are generated in the intrathoracic nodes they migrate from the lymph nodes to the lung and thereby may allow secondary immune response to occur locally in the lung (Hance, 1991).

1.4.3 MAST CELLS

The largest numbers of mast cells in the human body are found at the interface of the internal and external environments, such as skin, conjunctiva and mucosal surfaces, such as the respiratory and gastrointestinal tracts. Mast cells are widely distributed in the

respiratory tract (McDermott et al, 1982) and have been estimated to be present in human lung at a concentration of between 1 and 7 x 10^6 cells per gram of lung tissue (Wasserman, 1980).

Two distinct types of human mast cell have been described based on neutral protease content of their granules. MC_{TC} cells contain chymase and tryptase whereas MC_T cells contain only tryptase (Schwartz & Huff, 1991). MC_T cells are the predominant type of mast cell in the lung, particularly alveolar septae, and in the small intestinal mucosa, while the MC_{TC} cells are the predominant type found in the skin and in the gastrointestinal submucosa (Irani et al, 1986).

Immunological activation of mast cells results in the release of preformed granule associated mediators which include histamine. Histamine is responsible for vasodilation, contraction of smooth muscle, increased mucous secretion and increased epithelial permeability in the lung characteristic of a type I hypersensitivity reaction. Membrane derived lipid mediators, usually arachadonic acid metabolites are synthesised and released from mast cells following degranulation, to mediate the late phase of immediate hypersensitivity (Schwartz & Huff, 1991). The characteristics of the late phase of immediate hypersensitivity include bronchial smooth muscle contraction, enhanced bronchial mucous secretion and an influx of eosinophils and neutrophils. In addition an array of different cytokines are produced by mast cells including IL1, IL3, IL4, IL5 IL6, IFNγ and GM-CSF

(Schwartz & Huff, 1991), which extends their role as effector cells of immediate hypersensitivity reactions, to include an effect on the growth and regulation of many cells types.

Mast cells may play a role in the pathogenesis of chronic inflammatory conditions such as EAA, by increasing vascular permeability, sufficiently to allow increased access of inflammatory cells to the interstitial tissues, thus aiding the development of granulomas (Haslam et al, 1987)

1.5 SPECIFIC IMMUNE RESPONSES IN THE LUNG

1.5.1 HUMORAL IMMUNITY

All major immunoglobulin isotypes are present in bronchial secretions (Daniele, 1988a). However, as at other mucosal surfaces, IgA predominates, usually exceeding levels of IgG. The proportion of IgG is lowest in the upper respiratory tract and rises progressively towards the lower respiratory tract (Kaltreider, 1976b, Young & Reynolds, 1984). In bronchoalveolar lavage fluid, IgG considerably exceeds IgA. The bulk of IgA, IgM and IgE in bronchial secretions is probably synthesised locally, while IgG is derived predominantly by transudation from serum (McDermott et al, 1982, Daniele, 1988).

The IgA in bronchial secretions neutralises respiratory viruses and toxins, agglutinates microbial organisms for efficient removal, blocks the uptake of antigens and inhibits the adherence of microbes to mucosal surfaces (Reynolds et al, 1975a). Since IgA antibody opsonises

poorly and is an ineffective activator of the complement cascade, this enables it to fulfill these roles with limited tissue damage (Reynolds et al, 1975b). IgG and IgM antibodies are important components of the immunologic defence of the mucosal surface of the respiratory tract. They agglutinate particulate antigens, activate complement, and neutralise certain bacteria and viruses (Reynolds et al, 1975b). IgE antibody assists in host resistance to certain parasitic infections (Befus et al, 1982). In addition, there are IgE receptors on alveolar macrophage (Joseph et al, 1980, Melewicz et al, 1982) and bridging of IgE receptors on mouse alveolar macrophages causes the release of leukotriene C_4 (Rankin, 1982). Thus, in previously sensitised individuals, inhaled allergens may initiate an inflammatory response directly within the airways, thereby increasing bronchial epithelial permeability and allowing allergens and particles to gain access to the submucosal immune system.

1.5.2 CELL MEDIATED IMMUNITY

T lymphocytes are central to the development of any cell mediated immune response. T lymphocytes (especially $CD4^+$ T cells) regulate other T cells as well as B cells, NK cells and many non lymphoid cells. T cells are found throughout the pulmonary lymphoid tissue including the lymph nodes, BALT, lymphoreticular aggregates and BAL fluid. T cells in BAL fluid have been the most extensively studied since they can be easily recovered. 10% of cells recovered by BAL are lymphocytes and of these 60% are T cells (Costabel et al, 1984, Costabel et al, 1985, Agostini et al, 1988).

T lymphocytes express receptors for antigen (TCR) that are associated in a molecular complex to CD3 surface proteins. Most $CD3^+$ cells express a disulphide linked heterodimer formed by α and ß chains that recognise antigen in an MHC-restricted fashion. Cell surface expression of CD4 and CD8 "accessory" molecules divide mature αß T cells into distinct subsets, the expression of these molecules being mutually exclusive (Reinherz & Schlossman, 1980). $CD4^+$ and $CD8^+$ cells interact with different classes of major histocompatibility complex (MHC) molecules. $CD8^+$ cells respond to antigen complexed with MHC class I molecules (Rosenstein et al, 1989) and $CD4^+$ T cells react to antigens bound to MHC class II molecules (Swain, 1983).

$CD4^+$ lymphocytes play a major role in delayed type hypersensitivity responses (DTH) in the lung. Immune $CD4^+$ cells recognise microbial antigens displayed on the surface of infected class II positive phagocytes and secrete IFNγ and other cytokines to activate macrophages to kill intracellular pathogens. This process can initiate acute and chronic inflammatory responses including granuloma formation and is characteristic of a variety of lung disease including tuberculosis, EAA and sarcoidosis (duBois et al, 1991). $CD4^+$ cells also play a role in regulating B cells, NK cells and T cells in the lung (Lipscomb, 1989, duBois et al, 1991). The majority of $CD4^+$ cells in the lung have the memory phenotype consistent with chronic exposure to antigen (Saltini et al, 1990). In addition in diseases associated with chronic inflammation, such as tuberculosis, it is the local lung

population of $CD4^+$ cells, with the "memory" cell phenotype, that secrete IFN-γ and are therefore likely to play a major role in the local cell mediated immune response to M.tuberculosis (Barnes et al, 1989).

$CD8^+$ lymphocytes play a major role in controlling viral infections in the lung, due to their cytotoxic capabilities (Kaltreider, 1991). In addition, it is now clear that $CD8^+$ lymphocytes may play a role in controlling many lung infections originally considered to be largely a function of $CD4^+$ lymphocytes, such as Listeriosis and tuberculosis (Lipscomb, 1989). Once activated, $CD8^+$ cells can secrete IFNγ which can activate macrophages to kill intracellular pathogens. In addition, $CD8^+$ cells may specifically lyse infected macrophages that cannot be activated by lymphokines. Lysis of these macrophages would release microorganisms that could be phagocytosed and killed by activated macrophages (Muller et al, 1987, DeLibero et al, 1988).

a) Gamma Delta T cells

A small population of T cells express a TCR formed of gamma (γ) and delta (δ) chains. A range of between 2-8% of lymphocytes from BAL fluid are $\gamma\delta$ T cells (Balbi et al, 1990). In the human lung, as in the peripheral blood, the majority of $\gamma\delta$ T cells express the disulphide form of the $\gamma\delta$ T cell receptor (Balbi et al, 1990). These are thought to be the "memory/activated" population of $\gamma\delta$ T cells, since the majority of them also express CD45RO antigen (Miyawaki et al, 1990).

The functions of $\gamma\delta$ T cells are unknown but may include

cytolytic activity secretion of multiple cytokines and helper activity for immunoglobulin production. Evidence that γδ T cells might be involved in pulmonary immunity has come from studies which showed an increased proportion of these cells in chronic inflammatory diseases of the lung such as farmers' lung (Trentin et al, 1990) and sarcoidosis (Balbi et al, 1990). In addition, recent evidence suggests that γδ cells may be involved in specifically suppressing IgE responses to protein antigens presented via intact non inflamed epithelial surfaces in the respiratory tract, suggesting that they may provide another layer of protection against hypersensitivity reactions in the lung (McMenamin et al, 1991).

b) Natural killer cells

Up to 40% of lymphocytes derived from BAL fluid express neither T cell nor B cell antigens. It is likely that some of these cells are natural killer (NK) cells (Robinson et al, 1984).

Natural killer (NK) cells are a distinct subpopulation of non-T, non-B lymphocytes, that are able to lyse target cells without prior sensitisation or MHC restriction (Herberman et al, 1975a,b). The receptors mediating this recognition, remain elusive (Robertson & Ritz, 1990). In addition to spontaneous cell mediated cytotoxicity, NK cells mediate antibody dependent cellular cytotoxicity (ADCC), via the CD16 molecule (FcγIII) (Robertson & Ritz, 1990).

NK cells have been implicated in several activities in vivo, including destruction of tumour cells, resistance to viral infections, and regulation of haematopoiesis and antibody production (Herberman & Ortaldo, 1981, Trinchieri, 1989, Arai et al, 1983). Lung NK cells have been shown to limit pulmonary metastases in animal models (Wiltrout et al, 1985) and could therefore be important mediators of tumour defence in the human lung. NK cells may also play an important role in a protective immune response to viral infections in the lung such as influenza (Stein-Streilein & Guffee, 1986). IFNγ producing NK cells may also be involved in defence against bacterial infections in the lung, by providing a stimulus for macrophage activation (Bancroft et al, 1987).

Although NK cells are found throughout the lung, the cells capable of mediating spontaneous cytotoxicity appear to be restricted to the lung interstitium and are $CD16^{+}$, (FcγIII receptor) (Weissler et al, 1987). Most NK cells in the alveoli and larger airways express CD57 (HNK1) and are functionally inactive (Robinson et al, 1984, Weissler et al, 1987). Thus NK cell activity is compartmentalised to the interstitium of the lung rather than the air spaces. Again this may reflect the regulatory mechanisms designed to prevent immune responses from interfering with gas exchange in the alveoli. In addition, these findings also provide evidence that not all immunocompetent cells in the human lung can be obtained by BAL and should encourage caution in the interpretation of lavage fluid studies in the evaluation of interstitial lung disease.

1.6 HYPERSENSITIVITY STATES

When an immune response acts inappropriately and causes tissue damage, it is described as a hypersensitivity reaction. The classification of hypersensitivity responses, introduced by Coombs & Gell (1963), is now nearly 30 years old. While the actual reactions are now known to be more complex than those originally described, the Coombs & Gell scheme of four hypersensitivity states, still offers a reasonable framework. The lung is one of the principal tissues in which such reactions occur.

Type I reactions in the lung

Asthma is characterised by reversible obstruction of the airways and is usually assumed to be a manifestation of a type I hypersensitivity reaction localised to the lung (Jansen et al, 1991). The classic descriptions of asthma focus on the immediate asthmatic response (IAR) occurring 10-20 minutes after antigen challenge and characterised by submucosal oedema and vessel dilation, increased mucous production and smooth muscle contraction. The IAR is a result of the release of various preformed mediators from sensitised mast cells, including histamine and is mediated by antibodies of the IgE class (Ishizaka & Ishizaka, 1975, 1984).

In some patients the IAR is followed by a late asthmatic reaction (LAR). The clinical pattern of this LAR is marked by a slowly progressive bronchial obstruction, starting three to four hours after allergen inhalation and reaching its maximum after seven to eight hours (Jansen et al, 1991). In the LAR, polymorphonuclear and mononuclear

cell infiltrates are superimposed on the more classic features of increased vascular permeability, smooth muscle contraction and increased bronchial mucous production. The precise mechanisms by which the LAR is induced are not yet known but may involve the newly formed mediators of mast cells such as prostaglandins and leukotrienes (Schwartz & Huff, 1991).

Type II and III reactions in the lung

These reactions both involve IgG or IgM antibody. Although, Type II reactions involve antibodies directed to auto-antigens on the surface of specific cells or tissues, whereas Type III reactions are due to the formation of immune complexes containing extrinsic antigens, the pathogenic mechanisms leading to tissue injury are similar. These involve activation of complement, which can damage tissue either directly, or via the attraction neutrophils, into the area.

In the lung, the best example of Type II hypersensitivity is Goodpastures syndrome, which is characterised by intra-alveolar haemorrhage (Dixon, 1968, Holdsworth et al, 1985), with linear deposits of IgG on glomerular and alveolar basement membranes. Thus far, the components basement membrane to which antibody binds, has not been formally identified.

Type III hypersensitivity in the lung is typified by idiopathic pulmonary fibrosis (reviewed in Crystal, 1984a). It presents clinically with dyspnoea and cough in patients in their fifth decade of life. The course of the disease is progressive and usually fatal within an average

of 3-6 years from the onset of symptoms. Lung histology varies with the stage of disease; an early alveolitis characterised by accumulation of mainly neutrophils and macrophages mixed with small numbers of lymphocytes and eosinophils, is followed by thickening and fibrosis of alveolar walls, with an increase in interstitial collagen. Immunofluoresence studies have shown immunoglobulin and complement deposition in the alveolar wall. In addition, immune complexes have been found in the blood and BAL of patients with marked alveolitis. Although the antigens in the immune complexes have not been identified, components of the alveolar wall, such as type I collagen have been proposed as candidates (Crystal, 1984a).

Type IV reactions in the lung

Type IV reactions, also referred to as cell mediated or delayed hypersensitivity responses, are so named because sensitised T cells can mediate reactions that develop over several hours without involvement of either circulating antibody or complement. Tissue damage involves the production of cytokines by T cells which can activate other cells, particularly macrophages, to be cytotoxic and thereby cause tissue damage.

The classic example of Type IV hypersensitivity in the lung is tuberculosis, in which cell mediated immunity against tuberculin accounts for many of the pulmonary histologic findings (Schatz et al, 1979, Lipscomb, 1989).

Sarcoidosis, a systemic granulomatous disease of unknown aetiology (Crystal et al, 1984b) is also an example of a

disease caused by a type IV hypersensitivity response. Almost all organs of the body can be affected, but the respiratory tract is most commonly involved and this is the major cause of the morbidity and mortality. BAL in untreated patients with active disease reveals an alveolitis dominated by lymphocytes and macrophages (Campbell et al, 1985, Semenzato et al, 1985, Semenzato, 1991). The T cell population in the lung of sarcoid patients is characterised by increased percentages of $CD4^+$ cells and an increased CD4:CD8 cell ratio.

Extrinsic allergic alveolitis is a lung disease in which there is evidence of both Type III and Type IV mediated hypersensitivity. The greater classification of the pathology of this disease should help in understanding the mechanisms of normal and hypersensitivity responses in the lung.

1.7. EXTRINSIC ALLERGIC ALVEOLITIS

Extrinsic allergic alveolitis (EAA) is a pulmonary hypersensitivity disease caused by the repeated inhalation of naturally occurring environmental antigens (reviewed in Schatz et al, 1977, Roberts & Moore, 1977, Richerson, 1983, Salvaggio, 1990). The antigens include bacterial, fungal and animal proteins, as well as a variety of chemical antigens. Immunologically, it has features of both Type III and Type IV hypersensitivity reactions.

1.7.1 CLINICAL FEATURES

The classic descriptions of EAA focus on the characteristic acute symptoms of the disease which occur some four to eight hours after exposure to the antigen and which usually resolve within 24-48 hours. There are both respiratory and systemic symptoms (Campbell, 1932, Reed, 1965, Fink et al, 1968). Typical respiratory symptoms are a dry cough, breathlessness and chest tightness, (but without wheeze or sputum production). The systemic symptoms include fever, sweating, myalgia, arthralgia and a flu-like sensation. The main feature on clinical examination is the presence of fine crepitations throughout the lung fields.

Some patients also report more immediate symptoms, which occur less than 40 minutes after antigen exposure. In pigeon breeders, these include symptoms such as cough, wheezing, sneezing and watering of eyes shortly after entering the loft (Banham 1987). Most of these patients go on to develop typical acute symptoms occurring 4-8 hours after exposure. It is unclear whether these immediate

symptoms represent a non specific irritant effect of dust, or whether they are mediated via an immediate type hypersensitivity response involving IgE or IgG_4 antibody (Van Wijk et al, 1987, Pelikan & Pelikan-Filipek, 1983).

A proportion of patients develop a more chronic disease, in which the presence of symptoms is not necessarily related to antigenic exposure. Its features include chronic breathlessness, a persistent productive cough, tiredness, anorexia, weight loss and in children, failure to thrive (Boyd, 1982). Progressive pulmonary fibrosis and respiratory disability eventually develop. In contrast to these insidious manifestations, the disease may also present in a fulminant manner; Barrowcliffe & Arblaster (1968) described a case of a young man who died within weeks of initial exposure to mouldy hay.

Therefore it is clear that EAA is not a uniform disease, but rather a complex syndrome with differences between subjects in clinical presentation and subsequent course. As a result, Fink (1983) classified the disease into three forms; acute, subacute and chronic.

Fink classification

The <u>acute</u> form of EAA manifests as recurrent episodes of typical respiratory and systemic symptoms which develop 4-8 hours after exposure and resolve within 24-48 hours. Pulmonary function tests typically show restrictive ventilatory defect with impaired gas diffusion but may return to normal between episodes. The relationship between symptoms and antigen exposure is most apparent in

this form of the disease.

The chronic form is characterised by the insidious development of respiratory symptoms such as persistent dyspnoea and productive cough. Chest X-rays show changes of pulmonary fibrosis while pulmonary function tests show a range of restrictive and obstructive defects. There is no clear evidence of the disease necessarily progressing from the acute to the chronic form in individual patients and some patients will present for the first time with established disability due to the chronic form of the disease without ever having experienced symptoms directly related to antigen exposure (Fink et al, 1968).

The subacute form is similar to the chronic form in that dyspnoea and cough develop insidiously, but there are usually also episodes of discrete chills and dyspnoea occurring after antigenic exposure as in the acute form. This form is often reversible and symptoms improve when antigenic exposure is discontinued. It is therefore an intermediate form between the acute and chronic forms of the disease.

1.7.2 CAUSATIVE AGENTS

The airborne particles which can cause alveolitis are in the order of one micron in diameter (Austwich 1966). This is the correct size to reach and settle in the alveoli (Brain & Valberg, 1979) and it is at the alveolar level that the disease process is most evident (Hensley et al, 1969).

The number of dusts which are known to cause EAA form a

list which is increasing with greater awareness of the disease (Table 1). Due to the widespread occurrence of the thermophilic Actinomycetes, the spores of these organisms are most frequently the causative agent of alveolitis, while the popularity of keeping different bird species has similarly resulted in widespread exposure to avian antigens (Hendrick et al, 1978).

1.7.3 PATHOLOGY

The first feature of EAA is a neutrophilic alveolitis (Barrowcliffe & Arblaster, 1968, Fournier et al, 1985) occurring within 24 hours of exposure to antigen, and in some cases, accompanied by vasculitis (Barrowcliffe & Arblaster, 1968, Ghose et al, 1974). Several days after inhalation challenge, the alveolitis becomes lymphocytic in nature and this is often held to be the classical feature of acute EAA (Costabel et al, 1984, 1985, Leatherman et al, 1984, Mornex et al, 1984, Semenzato et al, 1986).

The chronic lesion of EAA becomes more evident after repeated exposure to antigen and is characteristically associated with the development of interstitial granulomas comprised of lymphocytes, foamy macrophage and fibrosis (Seal et al, 1968, Hensley et al, 1969, Chmelik et al, 1974). Intra-alveolar buds were described as a prominent feature and consist of masses of loose connective tissue located within alveolar spaces and attached to the alveolar wall by a thin stalk (Kawanami et al, 1983). Vasculitis appears to be an unusual feature at this stage of the disease, present in only two of 18 cases of chronic

EAA reported by Kawanami (1983)

Bronchiolitis is also a common feature of the pathology of EAA (Seal et al, 1968) and some authors favour the term "Extrinsic allergic bronchiolo-alveolitis".

1.7.4 IMMUNOPATHOGENESIS

There is evidence of both immune complex and cell mediated mechanisms in the pathogenesis of EAA. It is thought that immune complexes may play a role in the early phases of disease, whereas cell-mediated immunity may be involved in the later and more prolonged stages of disease. Most of the studies to explore the pathogenesis of EAA have used farmers' lung and pigeon breeders' disease as models.

a) Humoral immunity

The association of serum antibody with EAA was first described by Pepys et al (1961), when the serum of a farmer with disease was shown to precipitate an extract of thermophilic Actinomycetes grown from mouldy hay. Serum precipitins against pigeon serum were then described in subjects with interstitial lung disease caused by exposure to pigeons (Reed et al, 1965). It is now clear that high titres of specific serum antibody directed against the offending organic dust are found in patients with all types of EAA (Pepys, 1969, Fink et al, 1971). These antibodies are primarily complement-fixing IgG antibodies (Fink et al, 1969a, 1971, Caldwell et al, 1973, Moore & Fink, 1975, Marx & Flaherty, 1976), but low amounts of specific serum IgA and IgM antibody have also been demonstrated (Patterson et al, 1976a, 1976b). The presence

of serum precipitins does not necessarily signify clinical disease, as these are present in up to half of exposed, but asymptomatic subjects (Fink et al, 1971, Barboriak et al, 1973, Moore & Fink, 1975, Roberts et al, 1976, DoPico et al, 1976). However, symptomatic subjects, generally have higher specific serum antibody levels than their asymptomatic counterparts (Boyd 1978, McSharry et al, 1984) and the severity of symptoms correlates well with antibody titre (Boyd, 1978).

There are also increased concentrations of IgG, IgM and IgA antibodies in BAL fluid of symptomatic and asymptomatic subjects with EAA (Reynolds et al, 1977, Patterson et al, 1979, Calvanico et al, 1980). Interestingly, specific IgA antibody levels were usually higher in the BAL of symptomatic subjects with PBD, whereas the IgG titre did not differ between symptomatics and asymtomatics (Patterson et al, 1979). Studies of IgG subclasses in BAL fluid have reported increases in total IgG_4 subclass levels in symptomatic subjects with PBD compared with asymptomatics, (Calvanico et al, 1980), but no difference in the levels of specific IgG_4 antibody between the two groups (Kitt et al, 1986).

The development of the symptoms of EAA follows a similar time course to the local Arthus reaction which occurs in the skin of subjects with EAA challenged with appropriate antigens. Although this suggests that immune complex reactions might be involved in EAA, the significance of these Arthus type cutaneous reactions remains unclear. Some studies indicated that both symptomatic and

asymptomatic individuals were skin test positive (Hargreave & Pepys, 1972, Moore et al, 1974, Schatz et al, 1976), but others found that the reactions were largely confined to subjects with alveolitis and high titre IgG antibodies to avian antigens (McSharry et al, 1983).

The role of immune complexes is also challenged by the fact that lung biopsies from patients with EAA usually show interstitial pneumonitis, alveolitis and bronchiolitis, with a primarily mononuclear infiltrate and granuloma formation, rather than the haemorrhagic necrosis, neutrophilic infiltration and vasculitis (Emanuel et al, 1964, Seal et al, 1968, Chemlik et al, 1974), which would be more characteristic of Arthus reaction. In addition, immunofluoresence studies on lung biopsies from patients with alveolitis rarely demonstrate the presence of immune complexes and complement deposition (Turner-Warick et al, 1971, Meijer et al 1971, Barrios et al, 1979). However it should be noted that examination of acute cases of EAA early after challenge provides some evidence for vasculitis (Barrowcliffe & Arblaster, 1968, Ghose et al, 1974) and neutrophil infiltrates (Fournier et al, 1985), raising the possibility that a local Arthus reaction still may be present in these patients.

Intradermal skin testing of patients with EAA inducing antigens may also induce an immediate (15 min) wheal and flare reaction (Hargreave & Pepys, 1972, McSharry et al, 1983). Although both symptomatic and asymptomatic individuals may demonstrate this reaction (Hargreave & Pepys, 1972, McSharry et al, 1983), symptomatic subjects

have higher grades of reaction (McSharry et al, 1983). The basis of the reaction is uncertain, as its size correlates with serum IgG rather than IgE antibody titres, against the test antigen (McSharry et al, 1983). Despite this, levels of specific reaginic IgG_4 antibody to pigeon dropping extracts were not found to be increased in either BAL fluid or the serum of subjects with PBD (Kitt et al, 1986). Thus, as there is no correlation between IgE antibody and disease, there is no evidence to suggest a role for a Type I hypersensitivity reaction in the pathogenesis of EAA.

b) Cellular immunity

Phenotypic Assessment

Histopathological studies which revealed mononuclear cell infiltrates and granuloma formation in the lung (Hensley et al, 1969, Chmelik et al, 1974, Seal et al, 1968) suggested a role for cell mediated immunity in the pathogenesis of pigeon breeders disease.

Total white cells recovered by BAL in subjects with EAA are increased on average fivefold, mainly due to a rise in the number of T lymphocytes (Costabel et al, 1984, Leatherman et al, 1984, Costabel et al, 1985). Similar increases in both the white cells and lymphocytes recovered by BAL have however been found in asymptomatic subjects (Leatherman et al, 1984). A peripheral blood leucocytosis was also reported in symptomatic subjects with PBD who were acutely symptomatic at the time of blood sampling (Reed et al, 1965). However, most of the other studies using peripheral blood have failed to report total

WBCC and so it is not certain whether leucocytosis is a general feature (Costabel et al, 1984, Leatherman et al, 1984, Costabel et al, 1985)

Investigations to determine the phenotype of lymphocytes in the lungs of subjects with extrinsic allergic alveolitis reported a predominance of $CD8^+$ cells in the BAL fluid of symptomatic subjects compared with controls (Costabel et al, 1984, Agostini et al, 1984, Leatherman et al, 1984, Costabel et al, 1985, Semenzato et al, 1986,). However, this was not restricted to symptomatic subjects, as similar reversed CD4:CD8 cell ratios were reported in the BAL fluid of similarly exposed, but asymptomatic subjects (Leatherman et al, 1984, Semenzato et al, 1986).

The results of studies which have looked at the phenotype of cells in the peripheral blood have been conflicting. Two studies reported reduced CD4:CD8 ratios in symptomatic subjects with EAA, compared with controls (Costabel et al, 1984, Costabel et al, 1985), while another three studies found no difference (Leatherman et al, 1984, Agostini et al, 1984, Semenzato et al, 1986). Unfortunately, the two groups that did find reduced CD4:CD8 lymphocyte ratios in the peripheral blood of symptomatic subjects with EAA, did not include a group of asymptomatic subjects in their studies. As a result it is not known whether these changes reflect the symptomatic status of the subjects or whether they reflect exposure to a given antigen capable of inducing EAA in some subjects, regardless of symptoms (Costabel et al, 1984, Costabel et al, 1985).

A number of factors may be responsible for the conflicting results of analysis of CD4:CD8 ratios in the peripheral blood of subjects with EAA. One possibility is that these varied results, reflect the use of small heterogeneous subject groups with various syndromes of EAA, ranging from Farmers' lung to mushroom workers' lung. It is also possible that differences in the time between blood sampling and the last exposure to antigen, in these studies, may have affected the results. Several studies have shown a predominance of $CD8^+$ cells over $CD4^+$ cells, after recent antigen exposure, which was found to decline with antigen avoidance in both BAL fluid (Trentin et al, 1988, Costabel et al, 1984) and in the peripheral blood (Costabel et al, 1984) such that the CD4:CD8 cell ratio in the peripheral blood returned to normal 5 days after antigen exposure (Trentin et al 1988). In addition, it is possible that different groups were looking at subjects with EAA, that were at different stages of disease, since recent evidence has suggested that within a given syndrome of extrinsic allergic alveolitis there can be different cellular profiles depending on the stage of disease (Reynolds et al, 1989). These workers showed that subjects with chronic PBD had a reduced BAL lymphocyte subset CD4:CD8 cell ratio, while those with the acute PBD had a predominance of BAL $CD4^+$ cells.

i) NK cells

Studies aimed at determining whether NK cells are involved in the pathogenesis of EAA have revealed increased percentages of $CD57^+$ ($HNK1^+$) cells in BAL fluid

(Agostini et al, 1984, Semenzato et al, 1986, Semenzato et al, 1988) and in the peripheral blood (Trentin et al, 1986) of subjects with EAA compared with controls. However, similar findings were also reported for asymptomatic subjects in the one study that included them (Semenzato et al, 1986). An increase in NK cell activity (assessed in a K562 target cells assay) of peripheral blood mononuclear cells, associated with the rise in the level of $CD57^+$ cells, was also reported in many of these studies (Trentin et al, 1986, Semenzato et al, 1986, Semenzato et al,1988). Although all these investigators concluded that the cells under examination were NK cells, it is now known that only 50% of cells that express CD57 are actually NK cells (Lanier et al, 1983). Thus it is possible that at least some of these cells may have been non MHC restricted T cells.

ii) Expression of activation markers

Increased proportions of lymphocytes expressing activation markers, such as Class II antigen (Mornex et al, 1984, Costabel et al, 1985, Trentin et al, 1990), the interleukin 2 (IL2) receptor and VLA-1 (Very Late Activation) antigen (Trentin et al, 1990) have been found in the BAL fluid of symptomatic subjects with EAA compared with controls. It has been shown that both $CD4^+$ and $CD8^+$ lymphocytes account for the increase in the expression of class II on lymphocytes (Costabel et al, 1985). However, using UCHL1 as a marker for "activated/memory" $CD4^+$ cells (Akbar et al, 1988), Johnson et al (1989) revealed increased expression of UCHL1 on lymphocytes in the lungs of both symptomatic and asymptomatic pigeon breeders.

Johnson et al, (1989) also showed that when symptomatic pigeon breeders were relavaged after isolation from pigeons for three weeks, there was a significant reduction in the proportion of lymphocytes expressing UCHL1. Very few studies have looked at the levels of activation markers expressed on the lymphocytes in the peripheral blood of subjects with EAA. The few studies that have, used class II expression as a marker of lymphocyte activation but did not find any differences between symptomatics and controls. (Mornex et al, 1984, Costabel et al, 1985).

The use of cytokines as important indicators of the presence of an active immune response was utilised by Reynolds (1989) who reported increased levels of IL-1 and IL-2 in BAL fluid of symptomatic and asymptomatic pigeon breeders compared with controls. In addition, the same study showed that asymptomatic pigeon breeders had higher levels of soluble IL-2 receptor (IL-2R), expressed in terms of lymphocytes per ml of BAL fluid, compared with their symptomatic counterparts (Reynolds, 1989). He suggested that excess soluble IL-2R, on a cellular basis, in BAL fluid may be a mechanism by which some pigeon breeders remain asymptomatic. No difference in the absolute levels of soluble IL-2R were found in this study.

iii) Other inflammatory cells

Little is known about the contribution of alveolar macrophages to the alveolitis of EAA. Due to the influx of T lymphocytes into the lungs of subjects with EAA, the percentages of macrophages derived by BAL are reduced from

around 80% to about 20% of BAL cells (Costabel et al, 1984, Costabel et al, 1985, Haslam et al, 1987). However it is possible that these cells become more active since increased phagocytic and bactericidal activity of these cells occurs in rabbits after sensitisation and challenge with M.faeni (Stankus et al, 1978). In addition, the percentages of macrophages expressing RFD1 (associated with interdigitating cells) and RFD7 (associated with mature macrophages) are increased in both symptomatic and asymptomatic pigeon breeders compared with controls (Johnson et al, 1989). However, similar levels of class II antigens, expressed on macrophages from subjects with EAA and normal controls (Costabel et al, 1986). Therefore, further studies are needed to document the functional status of the alveolar macrophages in EAA.

Mast cells rarely exceed 0.5% of cells in BAL, in normal controls and it has been reported that mast cells may be as much as 10 fold higher in the BAL of subjects with EAA (Haslam et al, 1987). In addition, these cells showed signs of degranulation and mast cell counts fell after removal of the subjects from exposure, despite a persistent increase in the percentages of lymphocytes (Haslam et al, 1987). It has been suggested that the increased numbers of mast cells may be the result of the activity of lymphokines such as IL3, released by activated T cells, which can promote mast cell recruitment and maturation.

Functional Assessment

Only a limited number of studies have investigated the role of cell mediated immunity in the pathogenesis of EAA.

An initial study by Caldwell et al (1973) demonstrated peripheral blood lymphocyte sensitivity to pigeon serum by measuring macrophage migration inhibition factor (MIF) in subjects with PBD. However, subsequent studies found that lymphocytes from both symptomatic and asymptomatic subjects, with various different syndromes of EAA, released MIF (Fink et al, 1975), leucocyte migration inhibitory factor (LIF) and leucocyte mitogenic factor (LMF) (Girard et al, 1978) when incubated with the appropriate antigen, although lymphokine release was more common in lymphocytes from symptomatic subjects.

Lymphocyte sensitivity to antigens in EAA, as measured in an in vitro proliferation assay, using pigeon serum and pigeon droppings as antigens, has been shown in many studies in both symptomatic and asymptomatic pigeon breeders (Schatz et al, 1976, Girard et al, 1978, Moore et al, 1980). Girard et al (1978) also looked at the subjects with farmers lung and hypersensitivity to exotic woods and similarly found that lymphocytes from both symptomatic and asymptomatic subjects responded to the relevant disease associated antigens. However, Hansen & Penny (1974), could only detect lymphocytes sensitivity in their symptomatic pigeon breeders. In the few studies that included lymphocytes from a control population of normal healthy individuals, no sensitivity has been found (Girard et al, 1978, Hansen & Penny, 1974).

Therefore, the results of the functional studies are inconclusive and nothing has been done for many years despite the antigens not having been identified.

1.7.5. ANIMAL MODELS OF EAA.

The variabilty and limitation of clinical studies has engendered the use of animal models to study the immunopathogenesis of EAA.

These studies have used both defined antigens such as ovalbumin and proven aetiologic agents of disease, such as pigeon serum or Micropolyspora faeni. In most experimental models there is an additional need for an adjuvant (Richerson et al, 1971, Richerson, 1972, Joubert et al, 1976, Bernardo et al, 1979), mitogen (Moore et al, 1975) or other non specific inflammatory agent to induce disease. As a result, it has been suggested that under clinical conditions, an acute inflammatory reaction initiated by endotoxin contamination of inhaled dusts, plays an essential role in the induction of EAA (Burrel & Rylander, 1981).

Animal models have allowed longitudinal analysis of the changes in lung cell populations after controlled laboratory exposure to an antigen. This has confirmed the clinical impression that the first pathological feature is an early polymorphonuclear infiltrate, occurring within 4 hours after inhalation challenge (Bernardo et al, 1979) and accompanied by the presence of chemotactic factors in BAL (Yoshizawa et al, 1982, Yoshizawa et al, 1988). 48-96 hours after challenge, a mononuclear cell infiltrate

(Bernardo et al, 1979, Richerson, 1971, Joubert et al, 1976) ensues and resolution of the alveolitis occurs after 6 days (Richerson 1971).

An unusual feature of animal models is that repeated inhalation challenge with antigen following acute disease, fails to induce chronic EAA and indeed, the alveolitic lesions eventually disappear (Richerson 1978, Ratajczak et al, 1980, Richerson et al, 1981). This clearing of disease is antigen specific, but is not associated with decreased antibody responses or decreased antigen specific lymphocyte blastogenesis (Richerson et al, 1981, Ratajczak et al, 1980). This phenomenon may be the counterpart of chronically exposed human subjects who are asymptomatic, despite the presence of precipitating antibody (Do Pico et al, 1976) and lymphocyte sensitivity (Semenzato et al, 1986). Interestingly, desensitisation in experimental animals can be prevented if the repeated aerosol challenge with antigen is accompanied by muramyl dipeptide, suggesting that non-specific activation of T cells is also required for maintenenance of the disease (Chedid et al, 1978, Richerson et al, 1982).

Further studies of pigeon-sensitivity in monkeys have suggested that animals which became symptomatic lacked a population of antigen-specific suppressor T cells in their peripheral blood were present in asymptomatic animals (Keller et al, 1982). Moreover, depression of suppressor activity by low dose total body irradiation was accompanied by the appearance of clinical disease (Keller et al, 1982). The same group have presented functional

evidence of suppressor cells in BAL fluid of asymptomatic humans (Keller et al, 1984), although this has been challenged by others who found that both symptomatics and asymptomatics had a population of suppressor cells (Semenzato et al, 1986). These apparently conflicting results may reflect the different experimental design.

Attempts to produce lung lesions in animals by means of passive transfer or selective immunisation with antibodies, have resulted in minimal pulmonary lesions such as infiltrates characterised only by mild haemorrhage and neutrophilia (Johnson & Ward, 1974, Richerson, 1972, Roska et al, 1977). In contrast, experimental alveolitis in rabbits, can be transferred with lymphocytes (Bice et al, 1976). This has been confirmed more recently in a guinea-pig model of EAA, where transfer of cultured lymph node or spleen cells from sensitised animals could transfer susceptibility (Schuyler et al, 1987). Antibody containing serum could not transfer susceptibility in this model.

Further evidence that mast cells may be important in the development of EAA have come from a study in mast cell deficient mice. Passive transfer of mast cells to these mice increased the severity of EAA-like lesions which developed after transnasal administration of antigen (Takizawa et al, 1989).

1.7.6 CONCLUSIONS

There is clinical evidence of both antibody and cell mediated immunity in the pathogenesis of EAA but the

relative contributions are inconclusive. Functional studies are poor and inconclusive. Animal studies favour cell mediated immunity, but show that specific immune responses, are alone not sufficient to induce disease and that an adjuvant is needed. Animal models also indicate that specific down-regulation of the immune system, may control these responses.

The aim of this thesis was therefore to reassess the role of CMI in the pathogenesis of EAA, by performing parallel functional and phenotypic studies of peripheral blood lymphocytes from well defined groups of patients divided on the basis of their symptoms and antibody status. To do this, PBD was used as a model of EAA in general.

1.8. PIGEON BREEDERS DISEASE

1.8.1 HISTORICAL NOTE

Hypersensitivity to avian proteins was first described by Plessner in 1960, in workers employed in plucking duck and goose feathers. Subsequently, similar symptoms have been reported following exposure to parakeets (Pearsall, 1960) budgerigars (Hargreave et al, 1966), chickens (Korn et al, 1968 and turkeys (Boyer et al, 1974).

PBD was first described in the "gaveurs de pigeons", people who force fed pigeons in Paris and who had daily contact with thousands of birds (Renon, 1897). However, it was not until the report by Reed et al (1965) that the clinical and serological findings were correlated and the association of the disease with pigeons was proven by

controlled challenge with pigeons. The disease was subsequently reported worldwide (Boyd, 1975, Molina, 1976).

1.8.2 OCCURRENCE AND INCIDENCE

In 1968, the American Racing Pigeon Union reported that there were approximately 75,000 breeders in the United States, 180,000 in England and 250, 000 in Belgium (Fink et al, 1968). Estimates of the incidence of disease vary from 0-22%, depending on the population studied (Elgefors et al, 1971, Christensen et al, 1975, Boyd 1975, Banham et al, 1986, Fink et al, 1972).

1.8.3 ANTIGENS ASSOCIATED WITH PBD

Symptomatic and asymptomatic pigeon breeders have precipitating antibodies to a variety of pigeon materials, including droppings, serum (Barboriak et al, 1965, Reed et al, 1965), egg white, feathers (Fink et al, 1968) and egg yolk (Reed et al, 1965). Pigeon serum is a good source of antigen for use in serological tests and inhalation provocation tests (Hargreave & Pepys, 1972, Hendrick et al, 1980), but pigeon droppings have been considered as a more clinically relevant source of antigen as they are found in the natural loft environment (Fredericks, 1978). However, pigeon droppings are hygroscopic and bulky, do not desiccate easily and are normally removed from the loft regularly to maintain overall cleanliness. Furthermore, many pigeon fanciers experience symptoms in circumstances, (eg shows) where excreta are kept to a minimum. Boyd (1978) has suggested that pigeon bloom, a

waxy substance which coats the feathers and which is readily dispersed in the loft environment is a more likely source of antigen. This was later confirmed to be highly antigenic by radioimmunoassay (Banham et al, 1982).

Pigeon serum gammaglobulin and albumin were shown to be the major antigens in pigeon serum (Fink et al, 1969b). Specific serum antigens belonging to the gammaglobulin fraction were found which did not have antigenic identity to the serum proteins of other avian species (Faux et al, 1971, Sennekamp et al, 1981) and as a result, pigeon serum gammaglobulin was considered to be the specific antigen associated with PBD. This antigen was further demonstrated to be more immunogenic in man than pigeon serum albumin in a quantitative precipitin test (Fink et al, 1969b).

Pigeon droppings were also shown to be a major antigen source by inhalation provocation tests (Reed et al, 1965) and were later shown to contain both globulin and albumin (Barboriak et al, 1965, Edwards et al, 1970). In a series of papers, Berrens et al (1972 a-c) fractionated pigeon droppings extract (PDE) proteins and showed that the major antigen had gamma-mobility on immunoelectrophoresis, and was later shown to be IgA (Fredericks & Tebo, 1980). Prior to this, IgA derived from pigeon droppings had been described as XPGG, since it cross reacted with the main serum gamma-globulin antigen (Edwards et al, 1969). My study used both pigeon serum and pigeon droppings as antigen sources.

1.8.4 WHY STUDY PBD?

PBD is one of the most extensively studied of the disease syndromes of EAA. The population at risk is easily identifiable and very willing to participate in laboratory studies, enabling both symptomatic and asymptomatic individuals to be studied. This allows detailed study of the clinical, immunological and physiological changes associated with disease. In addition, sources of antigen which can produce symptoms of PBD in both humans (Reed et al, 1965) and animals (Barboriak et al, 1976, Fink et al, 1970) have also been identified, allowing controlled laboratory challenges and longitudinal time course experiments to explore the full spectrum of the disease process.

As the clinical and immunological aspects of all syndromes of alveolitis are the same, PBD is a useful model for studying the immunopathogenesis of the disease process which could equally apply to the more individual syndromes of EAA listed in Table 1. In addition, the complex nature of the disease process, involving both the cellular and humoral arms of the immune response make it an ideal model to study hypersensitivity lung diseases in general and wider aspects of pulmonary and mucosal immunology.

1.9 SCOPE OF THIS THESIS

This thesis explores the role of the cellular immune response in the pathogenesis of acute PBD (Fink et al, 1983), that is PBD associated with systemic and respiratory symptoms, 4-8 hours after contact with pigeon

antigen. The studies use peripheral blood to gauge the types of responses which might also be occurring in the lung. Because of the difficulties involved in obtaining BAL, peripheral blood was used to enable a much larger population of pigeon breeders to be studied.

The third chapter looks at the total and differential WBCC and lymphocyte subsets in the peripheral blood of pigeon breeders grouped according to symptoms, presence of serum antibody to pigeon antigen and whether or not they are acutely symptomatic at the time of blood sampling. The aim was to determine whether there were any changes in the peripheral blood and whether these changes reflected the symptomatic status of the subjects.

In the fourth chapter, symptomatic pigeon breeders who have severe longstanding PBD are selected for additional phenotypic and functional analysis of NK cells in their peripheral blood. The same subjects are then assessed for the level of $\gamma\delta$ T cells and cells expressing "memory" markers in their peripheral blood in the fifth and sixth chapters respectively, in an attempt to determine whether these cells are involved in the pathogenesis of disease.

Finally the function of T cells in the peripheral blood of pigeon breeders with severe longstanding PBD is explored in a lymphocyte proliferation assay (chapter 7). The results of this study lead to the studies of the final chapter which explore the possibility that endotoxin or some other mitogenic agent might be involved in the pathogenesis of PBD.

TABLE 1

Varieties of Extrinsic Allergic Alveolitis (Schatz & Patterson, 1983, Daniele, 1988b)

CLINICAL SYNDROME	SOURCE	TYPICAL ANTIGENS
	Bacteria thermophilic actinomycetes	
Farmer's lung	Mouldy hay	Micropolyspora faeni T. vulgaris
Humidifier lung	Contaminated air conditioning	T. candidas Naegleria gruberi (Anderson et al, 1985)
Bagassosis	Sugar cane bagasse	T. saccharii
Mushroom worker's lung	Mouldy compost	T. vulgaris Mushroom spores (Nakazawa & Tochigi 1989)
	Fungi	
Maple bark stripper's lung	Mouldy maple bark	Cryptostroma corticale
Suberosis	Mouldy cork	Penicillium frequentans
Sauna taker's lung	Contaminated water	Pullularia
Sequoisis	Mouldy wood dust	Pullularia
Saxophonist's lung	Contaminated mouthpiece	Candida albicans (Lodha et al, 1988)
Summer-type pneumonitis	Home environment	Trichosporon cutaneum (Yoshida et al, 1988)
Cheese washer's lung	Mouldy cheese	Penicillium caseii
Dry rot alveolitis	Contaminated moist wood	Merulius lacrymans
Malt worker's lung	Malt	Aspergillus clavatus
	Animal Antigens	
Pigeon fancier's lung		Avian protein
Budgerigar fancier's lung		Avian protein
Rat lung		Rat serum protein
Pituitary snuff user's lung		Bovine and porcine protein
	Chemicals	
Isocyanate alveolitis	Industrial exposure	Toluene di-isocyanate
Paulis reagent pneumonitis		Sodium diazobenzene sulphate
Pyrethrum pneumonitis	Insecticide	Pyrethrum
	Unidentified Antigen	
Coffee worker's lung		Coffee dust
Thatched roof lung		Thatch

CHAPTER 2
MATERIALS AND METHODS

2.1 SUBJECTS

2.1.1 DIAGNOSTIC CRITERIA FOR ACUTE PIGEON BREEDERS' DISEASE

Patients were selected by respiratory physicians with experience in EAA, on the basis of the criteria of Banham et al 1986 which "requires at least one typical delayed respiratory symptom (6-12 hours after exposure) of dyspnoea, cough or chest tightness, together with at least one of the systemic symptoms of fever, sweating or arthralgia, such symptoms occurring on at least three occasions".

2.1.2 PIGEON FANCIERS

The pigeon breeders used in this study were derived from two main sources.

a) Participants at pigeon fanciers conventions

(Winter Gardens, Blackpool, 1989 and 1990)

i) Subjects derived from the *Blackpool 1989 pigeon fanciers convention* were volunteers who approached a medical stand at the convention. Many were seeking advice on what they considered were pigeon-exposure related diseases but many others were asymptomatic, interested volunteers. The population was probably biased in favour of symptomatic subjects and would not represent the status of the general population who keep birds.

The subjects were divided into four groups based on their serum antibody status to pigeon antigen and on the basis of a positive or negative diagnosis of acute PBD as

determined by a self administered symptom based questionnaire (Appendix), using the symptomatic criteria of Banham et al 1986, as outlined above.

ii) Subjects derived from *Blackpool 1990 pigeon fanciers convention* were volunteers selected on the basis that they were acutely symptomatic at the time of blood sampling in addition to being diagnosed as having acute PBD as determined by a questionnaire (Appendix), administered by an experienced respiratory physician, using the symptomatic criteria of Banham et al 1986 (see above, 2.1.1). All subjects had a positive serum antibody titre to pigeon antigen.

b) Patients attending a respiratory outpatients clinic (Royal Infirmary, Glasgow).

Subjects from this source had well described PBD, with a positive serum antibody titre to pigeon antigen along with chest radiographic appearances and restrictive pulmonary defects consistent with PBD.

None of the pigeon breeders used in this thesis were taking oral steroids either at the time of study or in the preceding six months.

2.1.3 CONTROL SUBJECTS

Normal healthy laboratory workers were used as control subjects.

Further details of individual groups of pigeon breeders

and controls used in this thesis, including their demographic details, are given in chapter 3.2.1.

2.2 PREPARATION AND ANALYSIS OF PIGEON DERIVED ANTIGENS

2.2.1 PIGEON ANTIGENS

a) Pigeon Serum

Pigeon serum was obtained from healthy laboratory-housed pigeons (Yorkhill Hospital, Glasgow), by exsanguination of the wing vein, sterilised by 0.2μm Millipore filtration (Millipore S.A., France) and stored at -20^{o}C until use. Both pooled and individual serum samples were collected.

i) Ammonium Sulphate Fractionation

In order to separate the globulin component of pigeon serum (PS), the pH of a solution of cold, saturated ammonium sulphate was adjusted to pH 6.5 by the addition of a few drops of 0.1M NaOH just before use and then added dropwise to a sample of pigeon serum with constant stirring. 1ml of $(NH_3)_2SO_4$ was added for every 2mls of PS giving a resultant saturation of 33%, which was optimum for avian globulin separation (Hebert, 1974). After stirring for 30 minutes, the suspension was centrifuged at 400g for 30 minutes at 4^{o}C and the supernatant (Fraction A) collected and stored at 4^{o}C. The precipitate was reconstituted to the volume of the original serum sample with saline and then the above precipitation steps repeated followed by centrifugation at 400g at 4^{o}C for 30 minutes. The precipitate (Fraction B), was then reconstituted to a quarter of the volume of the original

serum sample and both samples were then dialysed in phosphate buffered saline (PBS) at 4°C over 2 days with frequent changes of buffer. The solutions were then sterilised by Millipore filtration to 0.2μm (Millipore) and an estimation of protein content determined by Lowry, see below (2.2.2).

b) Pigeon serum albumin

Lyophilised pigeon serum albumin (Sigma plc) was reconstituted in PBS to a concentration of 1mg/ml and stored at -20°C until use.

c) Pigeon Droppings

Fresh pigeon droppings were collected into sterile glass universals and stored at -20°C until use. Pigeon droppings were thawed and extracted in PBS with 0.2% sodium azide (PBS/azide). The protease inhibitors; 1mM phenylmethylsulfonyl fluoride (PMSF), (Sigma) and 1uM pepstatin (Protein Research Foundation, Japan) were included in the PBS/azide extraction solution in one experiment. PMSF and pepstatin were diluted in dimethyl formamide and 50% ethanol respectively. 100mls of the PBS/azide solution was used to extract 50g of pigeon droppings by constant stirring for 24 hours at 4°C. After centrifugation at 3000rpm for 60 minutes, the soluble proteins were extracted by pipetting off the supernatant and then centrifuging for a further 30 minutes at 3000rpm to maximise the removal of fine sediment. The green suspension was then dialysed against PBS at 4°C with repeated changes of buffer,

followed by millipore filtration using a 5μm filter followed by a 0.8μm filter (Millipore SA, France) to remove particles. The suspension was then concentrated using an Amicon Dyaflow concentrator PM 10 (Amicon Ltd. Stonehouse, Great Britain) to approximately 10mls and then sterilised by filtration.

2.2.2 LOWRY METHOD OF PROTEIN QUANTIFICATION

A mixture of 1% copper sulphate pentahydrate and 2% sodium tartrate in equal proportions was made freshly on the day and 1ml of this was added to 50mls of 2% sodium carbonate in 0.1N NaOH on the day of use (solution 1). A series of 5 standards of bovine serum albumin (BSA, Fraction V, Sigma) in PBS was made from 100μg/ml to 20μg/ml, to a final volume of 1ml in sterile glass tubes. A tube containing 1ml of PBS was used as a negative control. Appropriate dilutions of test samples were made in 1ml aliquots, such that they would have similar protein concentrations to the standards, using ultraviolet absorption (280nm) readings as a rough indication of protein content. Usually three different dilutions of test samples were assayed.

5mls of solution 1 was added to all tubes and mixed thoroughly. After incubating for 10 minutes at room temperature, 0.5ml of Folin Ciocalteau's reagent (Sigma) diluted 1:2 in distilled water was added to each tube and then mixed thoroughly. After 30 minutes the absorbance of each protein solution at 750_{nm} was read in a

spectrophotometer.

Using the BSA standards a plot of protein content (μg) against E750nm was drawn. The protein content of the test samples was then calculated using the standard curve. If a test sample was too concentrated it was diluted in PBS and a correction factor used to calculate the final result.

2.2.3 ANALYSIS OF PROTEINS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

The discontinuous gel system of Laemmi 1970 was used.

a) 7.5% Separating / Resolving Gel

A 7.5% gel was used for the separation of proteins in the approximate molecular weight range between 40-250 KDa.

A solution of 1.88mls of a 40% Bis Acrylamide solution, 2.5mls of 1.5M Tris hydroxymethyl aminomethane (Tris) pH8.8, 100μl of 12% sodium dodecyl sulphate (SDS) and 5.52 mls of water was added to a Buchner flask mixed thoroughly and then degassed for 2 minutes to remove oxygen. Immediately after adding 50μl of 10% ammonium persulphate and 5 μl of Temed, the catalyst for the polymerisation reaction, the solution was poured into the space between the glass plates on the casting stand, using a needle and syringe. The gel was then immediately overlayed with a small layer of water-saturated isobutanol, to exclude oxygen, an inhibitor of polymerisation and to reduce the meniscus forming on the

gel surface. The gel was then allowed to set for 1 hour at room temperature before the butanol solution was carefully poured off and the top layer of the gel rinsed with distilled water which was removed with a piece of filter paper before the addition of the stacking gel. The stacking gel was added immediately to avoid the resolving gel drying out.

b) 5% Stacking gel

A 5% acrylamide solution was used to concentrate the proteins before separation and to increase the resolution of bands.

A solution of 0.75mls of 40% Bis /Acrylamide, 0.68mls of 0.65M Tris/HCL pH6.8, 0.05mls of 12% SDS and 4.52 mls of water was added to a Buchner flask mixed and degassed for 2 minutes. Immediately after adding 50 μl of 10% ammonium persulphate and 5.8μl of Temed , the mixture was than poured onto the resolving gel with the comb, which generates separate wells, ready to be pushed firmly into position once the gel had been poured. The gel was then allowed to set for 1 hour before the comb was removed and the samples applied.

c) Sample preparation

Samples and standards including a sample containing various proteins of known molecular weight (high MW standards, Sigma) were diluted in an equal volume of sample buffer containing the reducing agent: ß2-mercaptoethanol and glycerol to increase the viscosity of the sample (50% glycerol, 0.25M Tris HCL, 5% SDS, 5% ß2-mercaptoethanol, 0.025% bromophenol blue and water). The

samples were then heated at 95°C for 5 minutes, to allow protein reduction before being applied to the wells of the gel under the electrode buffer (30.3g Tris, 14.4g Glycine, 1g SDS in 1 litre water, pH 8.3).

d) Running conditions

Gels were run in the electrode buffer at a constant voltage of 200V for approximately 30 minutes, until the marker dye had reached the bottom of the gel.

e) Fixing and staining

The gel was carefully removed from the glass plates and soaked in fixative/destaining solution (30% ethanol, 5% glacial acetic acid, and water) for 30 minutes before being transferred to staining solution (45% ethanol, 10% glacial acetic acid, 0.1% PAGE Blue and water) for 2 hours. The gel was then destained in fixative/destaining solution and could be preserved in this until it was photographed.

2.3 DETECTION OF SERUM ANTIBODY TO PIGEON DERIVED ANTIGENS

2.3.1 COLLECTION OF PIGEON BREEDERS' SERUM SAMPLES FOR ENZYME-LINKED IMMUNOSORBENT ASSAY

Serum samples collected at pigeon fanciers conventions were frozen on dry ice before being transferred to a -20°C freezer, until use. Serum samples from hospital out patients were immediately frozen at -20°C until use.

2.3.2 ENZYME-LINKED IMMUNOSORBENT ASSAY

The indirect microplate enzyme immuno- assay method of Voller et al (1976) was employed using Titertek microtitration strips (Flow Laboratories ,Irvine).

The wells of the plate were coated with 20μg/ml of pigeon serum in carbonate buffer 0.1M, pH 9.6 at 100ul per well overnight at 4°C. The plate was washed three times by immersion in wash buffer PBS 0.1M pH 7.2 containing 0.5% Tween-20 (PBS-T). The test and control sera, diluted 1 in 100 in PBS-T, were added in duplicate at 100ul per well and incubated for 1 hour at room temperature. The plates were washed three times as before and 100ul of alkaline phosphatase conjugated rabbit anti-human IgG (ICN Immunobiologicals) used at 1 in 1000 dilution in PBS-T was added to each well and incubated for a further 1 hour and 30 minutes. After a further three washes, a substrate solution of p-nitrophenyl phosphate (104 phosphate substrate, Sigma) at 2.5 mg/ml in 10% diethanolamine pH9.6 was added at 100ul per well. After approximately 30 minutes, when sufficient colour had developed in the positive control samples, the absorbance of each well at 405nm, was read using a spectrophotometer (Multiskan Flow Laboratories). Values were calculated from the standard curve made up of the absorbance readings of serially doubly diluted, reference, high titre serum , quantified as having an absolute level of 100ug/ml of IgG antibody to pigeon globulin antigen in a quantitive precipitin test (Boyd, 1975).

2.4 ANALYSIS OF CELL-MEDIATED IMMUNITY

2.4.1 COLLECTION OF BLOOD AND MONONUCLEAR CELL SEPARATION

Peripheral blood mononuclear cells, for functional studies, were prepared from heparinised venous blood from patients and normal volunteers by density gradient separation. Blood was collected into plastic universals, containing 10 units of preservative free-heparin (Leo Laboratories, Bucks, UK) per 1ml of blood and two volumes of blood were layered over three parts Lymphoprep (Nycomed Pharma As, Norway) before centrifuging at 400g for 35 minutes at room temperature. Interface cells were then removed using a pasteur pipette and washed twice with buffered culture medium (RPMI 1640, Gibco BRL, Paisley, Scotland) containing 200 units/ml penicillin, 200 μg/ml streptomycin and 2mM L-glutamine.

Blood for flow cytometric analysis was collected into tubes containing EDTA and analysed within 24 hours.

2.4.2 WHITE BLOOD CELL COUNT AND MONONUCLEAR CELL COUNT

All WBCC were performed in a Coulter counter (Coulter Electronics, England). Mononuclear cell counts were performed in a haemocytometer (Improved Neubauer, Weber, England).

2.4.3 FLOW CYTOMETRY

A Becton Dickinson flow cytometer (FACScan) was used.

a) Differential cell count by flow cytometry

LeucoGATE reagent (Becton and Dickinson, Belgium) which consists of a mixture of MAbs to the common leucocyte antigen CD45 and the monocyte specific antigen CD14 was used to stain the first aliquot of whole blood from each subject. The light scatter and fluorescence parameters of the cells in this tube allowed SimulSET software (Becton Dickinson) to analyse data from the flow cytometer and to determine the percentages of lymphocytes, neutrophils and monocytes .

b) Lymphocyte subset analysis

The optimal settings on the flow cytometer, for demonstrating peripheral blood lymphocytes were; forward scatter: detector E00 and amplifier 1.23 on a linear scale, side scatter: detector 325 and amplifier 1.00 on a linear scale. For FL1 (FITC): detector 535 and the amplifier on a log scale and FL2 (PE): detector 458 and amplifier on a log scale. The thresholds were all set at 52 with compensation FL1-FL2 = 0.6% and FL2-FL1 = 23.2%.

LeucoGATE analysis allowed gating of consistently more than 95% of the total lymphocytes with less than 10% contamination by debris, granulocytes and monocytes. Fluorescence was standardised using a control reagent, NAME (Becton Dickinson, Belgium) in order to determine the level of nonspecific background staining of cells with either Fluorescein isothiocyanate (FITC) or Phycoerythrin (PE) labelled mouse monoclonal immunoglobulins with no known specificity.

c) Antibody reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-CD3

(Dakopatts, Denmark) and phycoerythrin (PE)-conjugated anti-CD3 (Coulter Immunology, Florida) were used as pan T cell markers. FITC-conjugated anti-CD4, FITC-conjugated anti-CD8, both from Coulter Immunology (Florida) and PE-conjugated anti-CD8 (Becton-Dickinson,CA) were used to identify the appropriate T cell subset. A combination of FITC-conjugated anti-CD4 and PE-conjugated 2H4 (Coulter Immunology) was used to identify $CD45R^{+}4^{+}$ T cells, while $CDw29^{+}4^{+}$ T cells were identified using a similar combination of FITC-conjugated anti CD4 and PE-conjugated 4B4 (Coulter Immunology).

FITC conjugated anti-TCRδ1 (T cell sciences, Cambridge MA.) was used to stain all $\gamma\delta$ T cells and FITC conjugated anti-δTCS-1 (T cell sciences) used to stain the subpopulation of $\gamma\delta$ T cells expressing the nondisulphide linked form of the $\gamma\delta$ TCR.

B cells were identified using PE-conjugated anti-CD19 (Dakopatts).

Cells expressing the NK cell markers CD56 and CD16 were identified using PE-conjugated anti-CD56 (Coulter Immunology) and PE-conjugated anti Leu11c (Becton Dickinson) respectively.

b) Immunofluorescence staining

5μl of all of the above antibodies were used except for the antibody specific for CD19 which was used at 10μl and the cocktails of antibodies to CD4 and CD45R or CDw29 which were used at 20μl. The antibodies were used alone or in combinations, where both antibodies were added

together.

A whole blood method of labelling and lysing cells was used. 100ul of whole blood was mixed and incubated at 37°C with the appropriate amount of each antibody in separate tubes. Erythrocytes were then removed by lysis through the addition of 2mls of FACS Lysing solution (Becton and Dickinson, Belgium) diluted 1 in 5 in distilled water. After incubation at room temperature for 10 minutes in the dark (to prevent quenching of the fluorochrome) and centrifugation at 300g for 10 minutes, the lysing reagent and cell debris were removed and the cell pellet washed in 2mls of PBS containing 0.2% sodium azide. After centrifuging for a further 10 minutes at 300g, the PBS was pipetted off and the samples fixed in 200ul of 1% paraformaldehyde in PBS pH7.4.

i) Additional step for Gamma Delta cells:

Due to the low numbers of $\gamma\delta$ cells, 100ul of PBS containing 0.2% bovine serum albumin (Fraction V, Sigma) with 0.1% sodium azide (PBS-BSA-azide buffer) was added to 100μl of whole blood prior to the addition of 5ul of anti gamma delta MAb and the mixture incubated for the longer period of 30 minutes with a mix after 15 minutes, in order to optimise specific binding while reducing the possibility of non-specific binding. After the incubation period, the tubes were then washed with 2mls of PBS/azide and centrifuged for 10 minutes at 300g at 4°C.

2.4.4 LYMPHOCYTE CULTURES

Lymphocytes were cultured with various pigeon proteins

and with Pokeweed mitogen (PWM) (Gibco BRL, Paisley) as a positive control. The culture systems had to be standardised in a number of experiments and these are described in detail in Chapters 7 and 8. Only the outline of the assays is given here. Peripheral blood mononuclear cells (PBMC), from Ficoll, were resuspended in RPMI 1640 (Gibco BRL), supplemented with penicillin (200 units/ml), streptomycin (200μg/ml) and L-glutamine (2mM) (Gibco BRL, Paisley). PBMC were cultured in 0.1ml volumes in conical bottomed microtitre plates (Titertek, Flow Laboratories) at a cell concentration of $1x10^5$/well. Various concentrations of pigeon antigens and mitogen were added in a volume of 25μl and each well was given an appropriate volume of heat inactivated FCS (Gibco BRL, Paisley and later Biological Industries, Glasgow), each tested for low intrinsic mitogenic activity, such that each well would contain 10% FCS. The plates were sealed with Titertek non- toxic films (Flow Labs., Irvine) and incubated at 37^oC in 5% CO_2 in an air humidified incubator (Amersham) for 6 days. 6 hours before harvesting, the plates were pulsed with 1μCi of ^3H-Thymidine (Amersham) and after reculture, cell bound DNA was harvested using a multi-cell harvester (Titertek Cell Harvester, Skatron, Norway). Cell extracts were placed in scintillation fluid (LKB, Scintillation Products, Loughborough, England) and radioactivity measured in a scintillation counter (Packard, United Technologies, USA). Cultures were performed in triplicate and the results are presented as the mean counts per minute per well.

2.4.5 NATURAL KILLER CELL ASSAY

a) Target Cells

The target cells were K562, a human erythroleukaemic cell line. The target cells were maintained in continuous culture in RPMI 1640, with 200 units/ml penicillin, 200 μg/ml streptomycin, 2mM L-glutamine and 10% heat inactivated fetal calf serum ("complete" RPMI). The cells were subcultured routinely every 2 days and 24 hours prior to being used in a cytotoxic assay.

b) Labelling of target cells

An aliquot of culture medium containing $3x10^6$ target cells was centrifuged at 400g for 5 minutes and the supernatant discarded to leave a cell pellet which was then incubated with 0.2ml [^{51}Na] sodium chromate (specific activity 20 MBq/ml, Amersham plc., Aylesbury, U.K.) at 37°C for 1 hour. The cells were then washed six times with "complete" RPMI. The final concentration was then adjusted to $2x10^4$/ml.

c) Cytotoxic Assay

The cytotoxic assay was carried out in V-bottomed microtitre plates (Flow labs, Rickmansworth, U.K.) at effector-to-target cell ratios of 100:1, 50:1 and 25:1. To achieve these ratios, 0.1ml of each of the patients mononuclear effector cell concentrations ($2x10^6$, $1x10^6$ and $5x10^5$ per ml) were incubated with 0.1ml of PBMC at a concentration of 2x10/ml. Effector cells and target cells were incubated together for 4hours at 37°C in an atmosphere of 5% CO_2.

Two controls were included: the first estimated the spontaneous release (SR) of isotope from 0.1ml labelled target cells that had been incubated with 0.1ml of medium alone without effector cells; and the second estimated the total activity (TA) by completely lysing 0.1ml target cells by the addition of 0.1ml of 5 N NaOH. All tests and controls were carried out in quadruplicate.

Following the incubation, 0.1ml of cell-free supernatant were removed from each well and counted for 3 minutes in a gamma counter (LKB 1282 Compugamma, Milton Keynes, U.K.) to assess radio-isotope release. The results were expressed as a percentage lysis according to the following formula:

$$\text{percentage lysis} = \frac{\text{mean c.p.m. of sample} - \text{mean c.p.m. of SR}}{\text{mean c.p.m. of TA} - \text{mean c.p.m. of SR}} \times 100$$

2.5 STATISTICAL METHODS

Data expressed as "mean±SEM" are normally distributed. Data expressed as median and range are not normally distributed. Comparison of two sets of normally distributed data was made using Student's t test. However, if the two sets of data were not normally distributed then a non-parametric Mann-Whitney U test was used. Comparison of more than two sets of normally distributed data was made using a Scheffé's test. Comparison of more than two sets of data that were not

normally distributed was made using a Kruskal and Wallis test.

A paired t test was used to compare total and differential WBCC, lymphocyte subpopulations (chapter 3) and $CD56^+$ cells (chapter 4) before and after overnight incubation.

A comparison of the number of subjects in each group with CD4:CD8 ratios; <1.0, $1.0 \leqslant r \leqslant 2.5$, >2.5, was made using a Chi-squared test.

A P value of <0.05 was considered to be significant in all tests.

CHAPTER 3

PERIPHERAL BLOOD LEUCOCYTE PHENOTYPES AMONG PIGEON BREEDERS

3.1 INTRODUCTION

In an attempt to determine the role of cell mediated immunity in the pathogenesis of EAA, several studies have looked for changes in the phenotypes of leucocytes in the BAL fluid and in the peripheral blood of subjects with EAA. Symptomatic subjects with EAA (Costabel et al, 1984, Leatherman et al, 1984, Agnostini et al, 1984, Costabel et al, 1985, Semenzato et al, 1986) and in some cases also asymptomatic subjects (Leatherman et al, 1984, Semenzato et al, 1986) were shown to have a predominance of $CD8^+$ cells in their BAL fluid compared with controls. However phenotypic analysis of cells in the peripheral blood has revealed conflicting results. Reduced CD4:CD8 ratios in symptomatic subjects with EAA were reported in two studies (Costabel et al, 1984, Costabel et al, 1985), while other groups found no differences (Leatherman et al, 1984, Agnostini et al, 1984, Semenzato et al, 1986). A contributory factor to the variability may have been the use of heterogeneous subject groups with various forms of EAA, ranging from Farmer's lung to Mushroom worker's lung and the grouping together of subjects at different stages of disease.

The present study used pigeon breeders disease as a well defined model to study EAA in general. The first part of this chapter looks at the total and differential WBCC and lymphocyte subsets in the peripheral blood of 131 pigeon breeders grouped into well defined categories.

The second part of the study analysed the cellular phenotypes of peripheral blood leucocytes in an

additional group of pigeon breeders, chosen on the basis of those patients, shown by the initial study, to have cellular changes. This smaller study group allowed a more detailed phenotypic analysis of peripheral blood leucocytes.

Pigeon breeders in group 1 were taking low dose inhaled steroids (not more than 200μg of beclomethasone daily) ~~to~~ alleviate the symptoms of PBD.

3.2 EXPERIMENTAL PROTOCOL

3.2.1 SUBJECTS

131 pigeon breeders and 21 controls were included in the first part of this study and 17 pigeon breeders and 17 controls in the second part. Respiratory physicians with experience in EAA placed the pigeon breeders in seven groups, according to their symptoms, antibody status and whether or not they were acutely symptomatic at the time of blood sampling. The symptomatic criteria were that of Banham et al (1986) (outlined in 2.1.1). All pigeon breeders except those in groups 1 and 8 were volunteers, attending pigeon fanciers' conventions and were diagnosed on the basis of a questionnaire (Appendix). Subjects in groups 1 and 8 were hospital outpatients attending a respiratory clinic at Glasgow Royal Infirmary. Two control groups of normal healthy individuals with no avian exposure, were also included (groups 7 and 9). Relevant clinical details of these groups are listed in Tables 3.1 and 3.8.

GROUP 1

These subjects were symptomatic and antibody positive, as well as having restrictive pulmonary function defects and chest radiographic appearances consistent with EAA. These were subjects with more severe longstanding disease than the equivalent symptomatic antibody positive subjects selected from pigeon fanciers' conventions (Groups 2 and 3).

GROUP 2

These symptomatic, antibody positive subjects were

acutely symptomatic at the time of blood sampling and were therefore valuable in terms of analysing the cellular changes associated with a symptomatic episode.

GROUPS 3-6

Subjects in groups 3, 4, 5 and 6 were split into four groups depending on their antibody and symptomatic status.

Group 3 comprised symptomatic, antibody positive subjects who differed from group 2 subjects in that they were not acutely symptomatic at the time of blood sampling.

Group 4 comprised asymptomatic, antibody positive subjects. This group was important in terms of establishing whether alterations in the cellular phenotype required the presence of an active specific immune response.

Group 5 comprised subjects who had symptoms suggestive of acute PBD, but lacked specific antibody to pigeon antigen. This group of subjects enabled analysis of whether the cellular changes were due to the presence of respiratory disease or whether they were a specific result of a hypersensitivity response to pigeon antigen.

Group 6 comprised asymptomatic, antibody negative pigeon breeders.

GROUP 7

The control group comprised normal healthy laboratory workers from the Department of Immunology, Western Infirmary, Glasgow, who had not been exposed to avian

antigen. None of these subjects were smokers. The median age of the subjects was 34 years with the range from 21 to 58 years.

GROUPS 8 AND 9

Group 8 subjects, selected after the initial analysis of groups 1-7, comprised an additional 17 symptomatic antibody positive subjects with severe longstanding PBD similar to group 1 subjects.

Group 9 subjects comprised 17 controls selected from the same population as the controls comprising group 7 in the previous study group. The median age of the subjects was 31 years of age and the age range was from 21 to 56 years.

The smaller study groups allowed a more detailed analysis of peripheral blood leucocytes and permitted direct comparison between a patient and control group for a larger number of parameters.

3.2.2 GENERAL PROTOCOL

Total and differential WBCC and evaluation of the percentages of $CD19^+$, $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes were determined in peripheral blood cell samples by flow cytometry. Subjects in Groups 8 and 9 were also evaluated for levels of $CD3^+8^+$ cells in their peripheral blood by two colour flow cytometry, to distinguish between $CD3^+8^+$ T cells and $CD3^-8^+$ NK cells. Absolute numbers of B cells, T cells and T cell subsets were derived from the total lymphocyte count. The serum was

collected and used to measure antibody to pigeon-derived antigens by ELISA.

3.3 RESULTS

3.3.1 LEVELS OF SPECIFIC IgG ANTIBODY TO PIGEON ANTIGEN AMONG PIGEON BREEDERS

The mean antibody titre of group 2 symptomatic antibody positive subjects, was significantly higher than that of group 3 (symptomatic, antibody positive subjects) and group 4 (asymptomatic antibody positive subjects), (Table 3.1).

3.3.2 THE EFFECT OF AN OVERNIGHT INCUBATION AT ROOM TEMPERATURE ON PERIPHERAL BLOOD LEUCOCYTES COLLECTED IN EDTA.

The logistical problems of travelling 250 miles to a pigeon fanciers' convention to collect a large number of peripheral blood samples meant that peripheral blood samples collected from subjects in groups 2-6 had to be held overnight at room temperature before the cellular analysis was performed. However the samples from hospital patients (groups 1 and 8) and control subjects (groups 7 and 9) were analysed immediately and therefore I assessed whether an overnight incubation would affect peripheral blood leucocytes. Thus, total and differential WBCC and the percentages of $CD3^+$, $CD19^+$, $CD4^+$ and $CD8^+$ cells in the peripheral blood of additional pigeon breeders and controls were determined before and after a 24 hour incubation period at room temperature.

The results in Tables 3.2 - 3.4 indicate that the total and differential WBCC and lymphocyte subsets are not significantly altered after an overnight incubation. This ensures that any differences between the patient groups were not likely to have been caused by a difference in the method of blood collection and analysis.

3.3.3 CELLULAR ANALYSIS OF PERIPHERAL BLOOD IN PIGEON BREEDERS

Group 1

These symptomatic, antibody positive pigeon breeders with severe, longstanding PBD had normal WBCC, normal percentages and absolute numbers of neutrophils and lymphocytes of both B and T cell subsets (Tables 3.5 and 3.6). However, these subjects had the lowest percentages of $CD4^+$ cells, the highest percentages of $CD8^+$ cells and the lowest CD4:CD8 lymphocyte ratio of all groups. 36% of subjects in this group had reversed $CD4^+$:$CD8^+$ lymphocyte ratios, compared with only 10% in the control group (Table 3.7). However, this was not significantly different from the control group or the other symptomatic antibody positive groups (groups 2 and 3). Figure 3.1 shows that the reversed CD4:CD8 cell ratios in both the pigeon breeders and controls were due to increased percentages of $CD8^+$ cells.

Group 2

These symptomatic antibody positive pigeon breeders who were symptomatic at the time of blood sampling had

significantly higher WBCC than controls or pigeon breeders who were symptomatic and antibody positive, but who were not displaying symptoms at the time of blood sampling (Group 3), ($P<0.005$, $P<0.05$ respectively, Table 3.5). This increase in the WBCC was reflected in an increase in the absolute number of both neutrophils and lymphocytes, although only the absolute number of lymphocytes was significantly higher than the control group ($P<0.02$, Table 3.5). The increase in the absolute number of lymphocytes comprised mainly $CD3^+$ T-cells ($P<0.05$ Table 3.6). B cell numbers were unchanged. The increase in the absolute number of T cells comprised both $CD4^+$ and $CD8^+$ cells, whose counts were significantly higher than those in control subjects or subjects in group 3, ($P<0.01$ and $P<0.02$, respectively, Table 3.6). However, the proportions of all cell types were normal.

Group 4

Subjects who were asymptomatic but antibody positive had normal WBCC as well as normal percentages and absolute numbers of neutrophils and lymphocytes (Table 3.5). The percentages and absolute numbers of B and T cells of both CD4 and CD8 positive phenotypes were also normal (Table 3.6). Although the median $CD4^+$:$CD8^+$ T cell ratio was not significantly different from that of the controls , 31% of subjects in this group had a CD4:CD8 T-cell ratio >2.5 compared with only 14% in the control group (Table 3.7). Figure 3.1 shows that the altered ratio in these pigeon breeders was due to an increase in the percentages of $CD4^+$ cells and a decrease in the percentage of $CD8^+$ cells.

Group 5

Symptomatic, but antibody negative subjects in group 5 had significantly higher WBCC than the control group ($P<0.05$, Table 3.5). This was reflected in increased absolute numbers of all cell types studied, although only the absolute numbers of $CD3^+$ T cells were significantly different from the controls ($P<0.05$ Table 3.6). The percentage of all cell types was normal.

Groups 3 and 6

Despite being symptomatic and having specific serum antibody, subjects in group 3 had normal cellular profiles as did subjects in group 6, the asymptomatic antibody negative pigeon breeders (Tables 3.5 and 3.6).

Group 7 : controls

The absolute numbers and percentages of cells and the median and range of CD4:CD8 T cell ratios in the peripheral blood of control subjects fell within the normal established ranges (England & Bain, 1976, Simmons et al, 1974, Costabel et al, 1984, 1985, Hannet et al, 1992).

3.3.4 FURTHER PHENOTYPIC ANALYSIS OF PERIPHERAL BLOOD LEUCOCYTES IN PIGEON BREEDERS

The results above (3.3.3) indicated that subjects with severe longstanding PBD had increased percentages of $CD8^+$ cells and decreased percentages of $CD4^+$ cells compared with controls. However, as the differences were not statistically significant, I decided to repeat the

studies in an additional group of pigeon breeders with similar symptoms of PBD (group 8) to those in group 1 and in a further group of controls (group 9). In addition, double staining immunofluoresence was used to determine levels of $CD3^{+}8^{+}$ T cells and $CD3^{-}8^{+}$ NK cells.

The results of this study showed no difference in the WBCC and the percentages and absolute numbers of neutrophils and lymphocytes between pigeon breeders and controls (Table 3.8). As shown in the previous study, the percentages and absolute numbers of T cells, B cells and $CD4^{+}$, $CD8^{+}$ and $CD3^{+}CD8^{+}$ cell subsets were also similar in the patient and control groups (Table 3.9), as were the $CD4^{+}$:$CD8^{+}$ cell ratios (Table 3.10). However, as before 41% of pigeon breeders had a reversed $CD4^{+}$:$CD8^{+}$ T cell ratio compared with only 19% in the control group (Table 3.11). This was again due to increased percentages of $CD8^{+}$ cells.

In an attempt to establish whether the increased percentage of $CD8^{+}$ cells in pigeon breeders with a reversed CD4:CD8 ratio was due to either $CD3^{+}CD8^{+}$ T cells or $CD3^{-}CD8^{+}$ NK cells, double staining immunofluoresence was used to detect CD3 and CD8 antigens on cells. The number of $CD3^{+}CD8^{+}$ cells was expressed as a percentage of the total number of $CD8^{+}$ cells, for 4 pigeon breeders with reversed $CD4^{+}$:$CD8^{+}$ ratios and 6 pigeon breeders with "normal" $CD4^{+}CD8^{+}$ cells ratios ($1.0 \leqslant r \leqslant 2.5$), all from group 8 and in 10 controls from group 9. The results (Table 3.12) show that all three groups had similar proportions of $CD3^{+}CD8^{+}$ and $CD3^{-}CD8^{+}$ cells, indicating

that the increased percentage of $CD8^+$ cells was probably due to both $CD3^+CD8^+$ T cells and $CD3^-CD8^+$ NK cells.

3.3.5 THE EFFECT OF AGE ON THE DISTRIBUTION OF PERIPHERAL BLOOD LEUCOCYTES IN PIGEON BREEDERS

The median age of the patients in groups 1, 3, 4 and 8 was significantly higher than that of the controls in groups 7 and 9 (Tables 3.1 and 3.8), while the age range of both control groups was also much narrower than those of the patient groups (Tables 3.1 and 3.8).

To determine if these age differences could have accounted for the phenotypic differences reported above, I examined whether the cellular indices of the PBD patients in groups 1 and 8 were influenced by their age. However, the results revealed no age effects on the phenotypes of peripheral blood leucocytes in these subjects.

3.4 CONCLUSIONS

The results of this chapter showed that two separate groups of symptomatic, antibody positive pigeon breeders, with severe longstanding PBD, had a greater proportion of subjects with reversed $CD4^+$:$CD8^+$ ratios than two groups of controls. The reversed ratios were due to increased percentages of $CD8^+$ cells. Although these differences were not statistically significant, the fact that they were found in two separate groups strengthens these findings. Further analysis revealed that both $CD8^+$ T cells and NK cells contributed to the overall rise in $CD8^+$ cells.

A different group of pigeon breeders, who were acutely symptomatic at the time of blood sampling and with positive antibody titres to pigeon antigen (group 2), displayed increased WBCC. This increase was reflected in raised absolute numbers of neutrophils, lymphocytes, CD3 positive T cells, CD4 and CD8 positive cells. B cell numbers were unchanged in this group and in all other groups. Increased WBCC were not found in symptomatic antibody positive pigeon breeders, who differed from group 3 subjects in that they were not showing symptoms at the time of blood sampling (group 3). They had normal cellular profiles. The difference seemed to reflect the symptomatic status of the subjects at the time of blood sampling which suggests that acute exacerbations lead to alterations in peripheral blood leucocytes.

Subjects in Group 2 also had significantly higher antibody levels compared with groups 3 and 4, the other

two community based, antibody positive subject groups.

This study also showed that symptomatic pigeon breeders without serum antibody to pigeon antigen (group 5) had statistically significant increases in WBCC compared with control subjects. The significance of this is not known but it is possible that the subjects in this group actually had Organic Dust Toxic Syndrome and not PBD (see 9.1).

An increased number of asymptomatic antibody positive subjects (group 4), had high CD4:CD8 T cell ratios (>2.5) in their peripheral blood compared to the controls. Their raised numbers of $CD4^+$ cells might reflect the presence of an active humoral immune response.

These findings demonstrate that phenotypic analysis of cells in the peripheral blood of pigeon breeders within well defined symptom groups, reveals differences between groups at varying stages of disease, and also differences in some groups when compared to control cell profiles. This study emphasises the need to categorise pigeon breeders into well defined groups before attempting to evaluate their laboratory profiles.

Group	1	2	3	4	5	6	7
n	11	27	41	36	8	8	21
M:F	11:0	26:1	38:3	34:2	7:1	8:0	13:8
median age (range)	50* (32-90)	46* (20-75)	48* (27-79)	49* (16-69)	50 (35-71)	45 (37-55)	34 (21-58)
smokers	0	0	4	5	8	7	0
Ab (titre)	+ (44±5)	+ (54±6)**	+ (34±5)**	+ (25±3)**	-	-	-
symptoms	Yes	Yes	Yes	No	Yes	No	Controls

Ab (titre) serum IgG antibody positive or negative mean ± SEM (μg/ml).
* Significant difference between groups 1,2,3,4 versus group 7. ($P<0.05$ groups 1 & 3 and $P<0.01$ groups 2 & 4)
** Significant difference between group 2 versus groups 3 and 4, $P< 0.05$.
symptoms Symptomatic criteria of Banham et al (1986).
M:F Male to female ratio

TABLE 3.1

The number of subjects, the ratio of males to females and the symptomatic, antibody and smoking status of six groups of pigeon breeders and one group of controls.

	WBCC (cmm^{-1})	
	0	24
C	6379	5855
C	5376	5993
C	5978	7062
C	7199	6889
C	6848	6710
Mean±SEM	6356±321	6502±243

C - control
P - pigeon breeder
0 - analysed immediately
24 - analysed after a 24 hour incubation period

TABLE 3.2

Peripheral blood WBCC before and after a 24 hour incubation at room temperature.

	% Neutrophils		% Lymphocytes	
	0	24	0	24
P	53	70	39	23
P	42	37	52	57
P	57	53	32	36
C	48	42	47	52
Mean±SEM	50±3	51±7	43±4	42±8

C - control
P - pigeon breeder
0 - analysed immediately
24 - analysed after 24 hour incubation

TABLE 3.3

Percentages of neutrophils and lymphocytes in peripheral blood samples before and after a 24 hour incubation at room temperature.

	CD19		CD3		CD4		CD8	
	Positive cells							
	0	24	0	24	0	24	0	24
P	4	3	86	86	18	20	51	55
P	-	5	76	76	45	47	21	23
P	10	7	64	65	23	26	39	44
C	10	7	70	74	41	39	23	17
C	22	24	65	63	41	44	20	21
Mean±SEM	12±4	9±4	72±4	73±4	34±5	35±5	31±6	32±7

C - control
P - pigeon breeder
0 - analysed immediately
24 - analysed after 24 hour incubation

TABLE 3.4

Percentages of lymphocyte subsets in peripheral blood samples before and after a 24 hour incubation at room temperature.

Group	WBCC	Neutrophils	Lymphocytes
1	6990 ± 1160	47 ± 6 (3483 ± 987)	45 ± 6 (2992 ± 755)
2	8576 ± 406*	56 ± 2 (4859 ± 321)	36 ± 2 (3390 ± 382)*
3	6424 ± 259*	53 ± 1 (3416 ± 201)	40 ± 2 (2526 ± 133)
4	7093 ± 434	52 ± 1 (3787 ± 342)	40 ± 1 (2732 ± 145)
5	9225 ± 1330*	57 ± 4 (5726 ± 1257)	38 ± 4 (3453 ± 316)
6	7862 ± 690	55 ± 3 (4386 ± 515)	39 ± 3 (2256 ± 244)
7	5407 ± 353*	56 ± 1 (3042 ± 238)	36 ± 1 (1909 ± 146)*
group differences	2 and 7 2 and 3 5 and 7	No Difference	(2 and 7)

* Group value differs from another group
values are mean ± SEM
() mean absolute numbers (cmm^{-1})

TABLE 3.5

WBCC, mean percentages and (absolute numbers) of lymphocytes and neutrophils in the peripheral blood of six groups of pigeon breeders and one group of controls.

Group	$CD19^+$	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+$: $CD8^+$
1	11±2 (374±187)	70±3 (1950±352)	33±3 (893±239)	31±3 (908±246)	1.2 (0.5-3.0)
2	9±1 (321±52)	75±1 (2518±275)*	40±2 (1347±183)*	28±2 (958±117)*	1.6 (0.6-2.7)
3	12±1 (403±78)	75±2 (1894±100)	42±1 (809±54)*	27±2 (523±53)*	1.5 (0.3-4.0)
4	14±1 (387±31)	75±1 (2078±119)	45±1 (918±119)	24±1 (523±46)	1.9 (0.6-5.3)
5	13±2 (463±86)	79±1 (2729±284)*	48±2 (1276±105)	25±2 (737±156)	2.2 (1.1-2.9)
6	13±2 (409±74)	77±2 (2256±244)	43±3 (991±135)	27±2 (583±64)	1.7 (1.0-2.7)
7	13±1 (238±24)	71±2 (1337±142)*	41±2 (673±58)*	24±2 (404±39)*	1.5 (0.9-4.2)
group differences		(2 and 7) (5 and 7)	(2 and 3) (2 and 7)	(2 and 7) (2 and 3)	

* group value differs from another group
values are mean ± SEM, or in the last column median and (range).

TABLE 3.6

The mean percentages and (absolute numbers) of lymphocyte subsets and the median and (range) of ratios of $CD4^+$:$CD8^+$ cells in the peripheral blood of six groups of pigeon breeders and one group of controls.

GROUP	N	number and (percentage) of subjects with CD4 : CD8 ratio					
		<1.0		1≤r≤ 2.5		>2.5	
1	11	4	(36)	6	(55)	1	(9)
2	27	7	(26)	17	(63)	3	(1)
3	41	3	(7)	32	(78)	6	(15)
4	36	4	(11)	21	(58)	11	(31)
5	8	0	(0)	6	(75)	2	(25)
6	8	1	(12)	5	(63)	2	(25)
7	21	2	(10)	16	(76)	3	(14)

N - number of subjects in each group
r - ratio

TABLE 3.7

The numbers and percentages of subjects in six groups of pigeon breeders and one control group with a $CD4^+$:$CD8^+$ cell ratio >2.5, between 2.5 and 1.0 and <1.0.

Group	Age	Ab.titre	WBCC	Neutrophils	Lymphocytes
Pigeon breeders 8	48* 13-71	47 ± 7	7838±665	60±5 (4965±761)	30±4 (2136±196)
Control 9	31 21-56		6486±520	60±2 (4024±471)	33±2 (2009±131)

values are mean ± SEM except for age which are median and range
() - absolute numbers (cmm^{-1}) WBCC (cmm^{-1})
* - significant difference between pigeon breeder and control group
Ab.titre - antibody titre ($\mu g/ml$)

TABLE 3.8

The mean ages, WBCC and percentages and absolute numbers of neutrophils and lymphocytes in the peripheral blood of 17 subjects with PBD and 17 controls.

Group	$CD19^+$	$CD3^+$	$CD4^+$	$CD8^+$	$CD3^+CD8^+$
Pigeon breeders 8	11±1 (238±41)	64±3 (1367±133)	36±3 (762±91)	30±2 (647±71)	23±2 (521±63)
Control 9	11±1 (207±30)	68±2 (1420±95)	38±2 (770±51)	29±2 (611±65)	25±2 (561±82)

values are mean ± SEM
() - absolute numbers (cmm^{-1})

TABLE 3.9

The mean percentage and absolute numbers of lymphocyte subsets in the peripheral blood of 17 subjects with PBD and 17 control subjects.

Group	$CD4^+$: $CD8^+$
Pigeon breeders 8	1.3 (0.5 - 2.6)
Control 9	1.4 (0.6 - 2.2)

values are medians
() - ranges

TABLE 3.10

The median ratio and the (range of ratios) of $CD4^+$:$CD8^+$ cells in the peripheral blood of 17 subjects with PBD and 17 controls.

Group	N	number and (percentage) of subjects with CD4 : CD8 ratio		
		<1.0	1.0≤r≤2.5	>2.5
Pigeon breeders 8	17	7 (41)	9 (53)	1 (6)
Controls 9	16	3 (19)	13 (81)	0 (0)

N - number of subjects in each group
r - ratio

TABLE 3.11

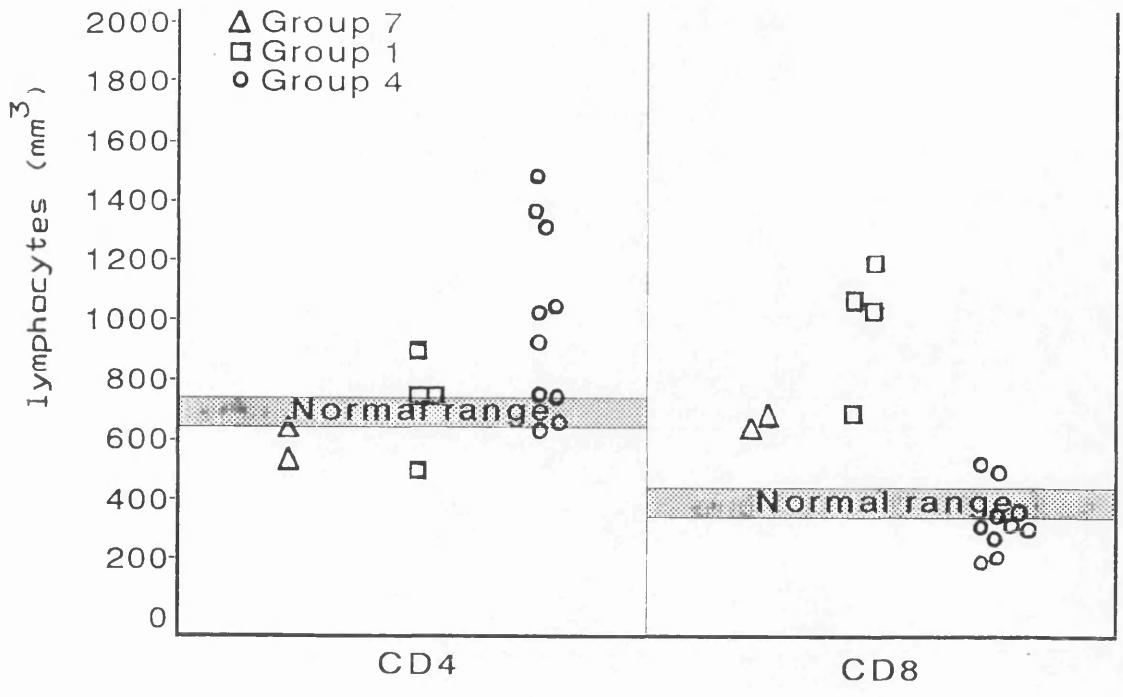
The number and percentage of subjects in a group of pigeon breeders with PBD and a group of controls with a $CD4^+$:$CD8^+$ cells ratio >2.5, between 1.0 and 2.5 and <1.0.

% $\frac{CD3^+CD8^+}{CD8+}$		
Pigeon breeders (r)	Pigeon breeder (n)	Controls
80 (39-93)	79 (63-95)	84 (59-92)

"normal" - $CD4^+$ cells predominate over $CD8^+$ cells.
(r) - reversed
(n) - "normal"

TABLE 3.12

The median and (range) of percentages of $CD3^+CD8^+$ cells expressed as a percentage of the total number of $CD8^+$ cells in the peripheral blood of 4 pigeon breeders with reversed $CD4^+$:$CD8^+$ cell ratios, 6 pigeon breeders with "normal" $CD4^+$:$CD8^+$ cell ratios and 10 controls.



normal range - range of values detected in group 7 controls

Figure 3.1

The absolute number of $CD4^+$ and $CD8^+$ cells in the peripheral blood of 2 controls (Group 7) and 4 pigeon breeders (Group 1) with reversed $CD4^+$:$CD8^+$ cell ratios and 10 pigeon breeders with $CD4^+$:$CD8^+$ cell ratios >2.5 (Group 4).

CHAPTER 4

PHENOTYPIC AND FUNCTIONAL STUDIES OF NATURAL KILLER CELLS IN PIGEON BREEDERS

4.1 INTRODUCTION

NK cells kill a variety of target cells without prior stimulation or MHC restriction, either directly or via FcR (reviewed by, Robertson & Ritz, 1990). As NK cells are activated non-specifically by cytokines such as IL2 and IFNγ (Trinchieri et al, 1984, London et al, 1986) their number and activity may be a marker of the overall level of the immune response in a given disease process, as well as a possible indicator of the involvement of non-specific cellular immunity. In addition, the release of TNFα and IFNγ by activated NK cells has implicated a role for them in the pathogenic process of chronic inflammation in rheumatoid arthritis (Hendrich et al, 1991) and in the destructive phases of graft versus host disease (Mowat & Felstein, 1987).

Previous reports on the number and activity of NK cells in EAA have been conflicting , which may reflect the use of poor NK cell markers (Agostini et al, 1984, Semenzato et al, 1986, Semenzato et al, 1988, Trentin et al, 1986). It is now known that no single surface antigen identifies all human NK cells unambiguously. The CD57 (HNK-1, Leu7) antigen is known to be expressed by only 50-60% of peripheral blood NK cells and only 50% of peripheral blood lymphocytes that express CD57 are actually NK cells (Lanier et al, 1983). CD56 (NKH-1), the neural cell adhesion molecule (NCAM 1) (Lanier et al, 1989) and CD16 (Leu 11), the low affinity receptor for the Fc region of IgG (FcγRIII) (Perussia et al, 1983a, Perussia et al, 1983b, Lanier et al, 1986), are now two of the most extensively used NK cell markers. However,

although CD56 is expressed by virtually all human peripheral blood NK cells (Griffin et al, 1983, Hercend et al, 1985), it is also expressed on a small proportion of $CD3^+$ T cells which are sometimes referred to as "non-MHC restricted T cells" (Lanier et al, 1986, Schmidt et al, 1986). In addition, CD16 is also expressed on some macrophages and granulocytes (Perussia et al, 1983a, Perussia et al, 1983b, Perussia et al, 1984).

The first part of this chapter uses PBD as a model of EAA and measured levels of $CD56^+$ cells in the peripheral blood of pigeon breeders in groups 1-6 and in controls (group 7), described in chapter 3. The second part of this chapter performed a more detailed analysis of NK cell markers in a smaller group of subjects with PBD (group 8) and controls (group 9), described in chapter 3, in an attempt to differentiate between $CD3^+$ "non MHC restricted T cells" and $CD3^-$ true NK cells, expressing CD56 or CD16. Finally the functional activity of the peripheral blood NK cells from these patients was measured.

4.2 EXPERIMENTAL PROTOCOL

This study involved the pigeon breeders and controls used in chapter 3 (Groups 1-9). The first part of the study examined the level of $CD56^+$ cells in the peripheral blood of subjects in Groups 1-7. The second part used two colour flow cytometry to detect the levels of $CD16^+$ and $CD56^+$ cells in the peripheral blood of subjects in groups 8 and 9 and the co-expression of CD3 and CD8 on each population of cells. The cytotoxic activity of peripheral blood mononuclear cells from subjects in groups 8 and 9 were also determined in an in-vitro cytotoxic assay using K562 target cells.

4.3 RESULTS

4.3.1 THE EFFECT OF A 24 HOUR INCUBATION AT ROOM TEMPERATURE ON THE LEVEL OF $CD56^+$ CELLS IN PERIPHERAL BLOOD STORED IN EDTA.

As explained in chapter 3, peripheral blood samples collected from subjects in Groups 2-6 had to be incubated for 24 hours at room temperature before any phenotypic analysis of peripheral blood lymphocytes was performed. Therefore, in order to assess whether this had any affect on the levels of $CD56^+$ cells, peripheral blood samples were collected in EDTA from an additional 4 subjects. The levels of $CD56^+$ cells in these samples were then measured before and after the blood had been incubated at room temperature for 24 hours. As shown in Table 4.1, there was a slight reduction in the level of $CD56^+$ cells in the peripheral blood after a 24 hour incubation at room temperature, but this was not statistically significant.

4.3.2 LEVELS OF $CD56^+$ CELLS IN THE PERIPHERAL BLOOD OF PIGEON BREEDERS AND CONTROLS.

The percentages and absolute numbers of $CD56^+$ and $CD16^+$ cells were not normally distributed and so median values and ranges are shown.

As shown in Table 4.2, symptomatic antibody positive patients (group 1), who had restrictive pulmonary function defects and chest radiographs consistent with EAA and the symptomatic antibody positive subjects (group 2) , who were acutely symptomatic at the time of

blood sampling, had a significant increase in absolute numbers of circulating $CD56^+$ cells compared with the control group (group 7) ($P<0.05$ and $P<0.02$, respectively). No significant differences were found in the numbers of $CD56^+$ cells in other groups, nor in the percentages of these cells.

4.3.3 THE COEXPRESSION OF CD56, CD16, CD3 AND CD8 ANTIGENS ON PERIPHERAL BLOOD LYMPHOCYTES IN PBD

The following experiments attempted to characterise further the $CD56^+$ cells which were found to be increased in the pigeon breeders comprising group 1 of the previous study. This was done using the additional group of pigeon breeders (group 8) and controls (group 9), described in chapter 3. Coexpression of CD3 and CD8 on $CD56^+$ and $CD16^+$ cells was examined. Figure 4.1 shows the typical flow cytometer profiles obtained by two-colour flow cytometry analysis of CD56 (NKH1), CD16 (Leu11), CD3 and CD8 antigen on peripheral blood lymphocytes in both pigeon breeders and controls.

a) $CD3^+CD56^+$ T cells and $CD3^-CD56^+$ NK cells and the expression of CD8 on $CD56^+$ cells.

As shown above, the median absolute number of $CD56^+$ cells was higher in the group of pigeon breeders (group 8), compared with the controls (group 9) (Table 4.3). However, unlike the previous findings, the difference was not significant. In addition, the median percentage of $CD56^+$ cells was higher in the peripheral blood of the pigeon breeder and control groups in this study (21% and

20%, respectively), compared with the median percentages of these cells detected in group 1 and group 7, described previously (14% and 11% respectively). The percentages and absolute numbers of $CD3^+CD56^+$, $CD8^+CD56^+$ and $CD8^-56^+$ cells were similar in both the pigeon breeders and the controls. Although the pigeon breeders had slightly increased percentages and absolute numbers of $CD3^-56^+$ cells compared with controls, the differences were not statistically significant (Tables 4.3 and 4.4).

b) $CD3^+CD16^+$ T cells and $CD3^-CD16^+$ NK cells and the expression of CD8 on these cells

A median of 19% of peripheral blood lymphocytes were $CD16^+$ in pigeon breeders compared with 11% in the control group and there was an increased absolute number of $CD16^+$ cells in the pigeon breeders (Table 4.5). However due to a wide scatter of values, these differences did not reach significance. As very few $CD16^+$ cells expressed CD3 in either the pigeon breeders or controls, pigeon breeders also had an increased median percentage and absolute number of $CD16^+CD3^-$ cells compared with controls (Table 4.5, 18%, $412cmm^{-1}$ and 10%, $187cmm^{-1}$, respectively). An increase in the percentage and absolute number of $CD8^-$ $CD16^+$ cells was also found, while the percentage and absolute numbers of $CD8^+CD16^+$ cells were almost identical to control group levels (Table 4.6). In consequence, the proportion of $CD16^+$ cells which expressed CD8, was significantly lower in pigeon breeders compared with controls (25% vs 46%,

P<0.05).

4.3.4 THE EFFECT OF AGE ON THE LEVEL OF CELLS EXPRESSING CD56 AND CD16 ANTIGENS IN THE PERIPHERAL BLOOD.

As noted earlier (3.3.5), the median ages of patients in groups 1, 3, 4 and 8 were significantly higher than the median age of the control groups 7 and 9 (Tables 3.1 and 3.8).

In order to determine whether the age differences between pigeon breeders and controls could have caused the differences in the percentages of $CD56^+$ and $CD16^+$ cells, I correlated the ages of subjects in group 8 with the percentages of $CD56^+$ and $CD16^+$ cells in their peripheral blood.

The results of this analysis revealed that the ages of subjects in group 8 did not correlate with the levels of $CD56^+$ and $CD16^+$ cells in their peripheral blood.

4.3.5 CYTOTOXIC ACTIVITY OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM SUBJECTS WITH PBD AND NORMAL HEALTHY CONTROLS

The in vitro cytotoxic activity of peripheral blood mononuclear cells from 11 pigeon breeders from group 8 and 10 controls from group 9 is shown in Figure 4.2. No differences in the levels of NK activity were recorded between patients and controls at any effector to target cell ratios.

4.4 CONCLUSIONS

The results of this study revealed increased percentages and absolute numbers of $CD56^+$ cells in the peripheral blood of pigeon breeders with severe longstanding PBD (group 1) and symptomatic pigeon breeders who were acutely symptomatic at the time of blood sampling (group 2) compared with controls (group 7) and other groups of pigeon breeders with less severe disease. Similar differences were found in an additional group of pigeon breeders (group 8) with similar disease to subjects of group 1 and another group of controls (group 9), although in this study they did not reach significance. In addition, higher percentages of $CD56^+$ cells were found in both group 8 and group 9 compared with the levels detected in group 1 and group 7 of the previous study. The inconsistencies in the two sets of results may reflect sampling variation. Further phenotypic analysis showed that the $CD56^+$ cells in these pigeon breeders were mainly $CD3^-CD56^+$ NK cells, rather than $CD3^+56^+$ " non-MHC restricted T cells" and were both $CD8^+$ and $CD8^-$. Analysis of $CD16^+$ cells in these subjects also revealed increased percentages and absolute numbers of $CD16^+$ cells compared with controls and these were $CD8^-CD16^+$. These were also probably not T cells, as there were only small numbers of $CD3^+CD16^+$ cells in either pigeon breeders or controls.

Despite the increased percentages of $CD16^+$ cells (and to a lesser degree $CD56^+$ cells) in the peripheral blood of subjects with severe PBD, this was not associated with increased cytotoxic activity of their peripheral blood

mononuclear cells. It is possible that these cells may play a role in the pathogenesis of PBD as inflammatory mediators given that activated $CD16^+$ NK cells produce inflammatory cytokines such as TNFα and IFNγ. They may also be involved in an antibody dependent cellular cytotoxicity (ADCC) reaction in the lung.

	% CD56$^+$	
	0	24
P	13	11
P	18	16
P	13	5
C	28	19
Mean ± SEM	18.0 ±3.5	13.0 ±3.1

P - pigeon breeder
C - control

TABLE 4.1

The percentage of CD56$^+$ cells in the peripheral blood of 3 subjects with PBD and 1 control before and after a 24 hour incubation at room temperature.

Group	n	Symptoms	Antibody	%CD56+ cells	Ab.no.CD56+ cells
1	11	+	+	14 (5-47)	407* (30-2218)
2	27	+	+	14 (3-33)	349* (181-1918)
3	41	+	+	13 (1-37)	260 (29-957)
4	36	-	+	11 (1-37)	313 (31-1258)
5	8	+	-	8 (1-17)	226 (45-452)
6	8	-	-	9 (4-13)	227 (114-532)
7	21	-	-	11 (2-24)	198 (21-618)

Group - group number as given in chapter 3
n - number of subjects in a given group
symptoms - of acute PBD as defined by Banham et al 1986
* - group value differs from control group $P<0.05$
Ab.no - absolute number (cmm^{-1})

TABLE 4.2

Median values and (ranges) for the percentage and absolute number of $CD56^+$ cells in the peripheral blood of pigeon breeders (Groups 1-6) and controls (Group 7).

	$CD56^+$	$CD56^+ CD3^+$	$CD56^+ CD3^-$	$\frac{CD56^+ CD3^+}{CD56^+}$
pigeon breeders				
%				
median	21	4	13	17
range	5-57	0-8	2-50	0-71
Ab.no				
median	419	106	374	
range	30-930	0-415	42-1037	
controls				
%				
median	20	5	10	35
range	10-53	0-21	5-30	0-53
Ab.no				
median	331	95	199	
range	106-1579	0-626	112-894	

Ab.no - absolute number (cmm^{-1})

TABLE 4.3

The median percentage and absolute number of $CD56^+$, $CD56^+CD3^+$ and $CD56^+CD3^-$ cells and the levels of $CD56^+CD3^+$ cells expressed as a percentage of the total number of $CD56^+$ cells, in the peripheral blood of 12 subjects with PBD and 13 controls.

	$CD56^+ CD8^+$	$CD56^+ CD8^-$	$\frac{CD56^+ CD8^+}{CD56^+}$
Pigeon breeders			
%			
median	8	8	46
range	4-17	4-38	31-56
Ab.no			
median	216	234	
range	75-518	75-726	
Controls			
%			
median	8	9	44
range	4-24	6-30	36-58
Ab.no			
median	172	205	
range	94-715	101-894	

Ab.no - absolute number (cmm^{-1})

TABLE 4.4

The median percentage and absolute number of $CD56^+CD8^+$ and $CD56^+CD8^-$ cells and the level of $CD56^+CD8^+$ cells expressed as a percentage of the total number of $CD56^+$ cells in the peripheral blood of 8 subjects with PBD and 9 controls.

	$CD16^+$	$CD16^+ CD3^+$	$CD16^+ CD3^-$	$\frac{CD16^+CD3^+}{CD16^+}$
Pigeon breeders				
%				
median	19	0	18	0
range	5-54	0-8	4-55	0-30
Ab.no				
median	394	0	412	
range	106-1065	0-136	85-2177	
Controls				
%				
median	11	0	10	0
range	6-32	0-3	5-29	0-17
Ab.no				
median	206	0	187	
range	112-954	0-89	90-864	

Ab.no - Absolute number (cmm^{-1})

TABLE 4.5

The median percentage and absolute number of $CD16^+$, $CD16^+CD3^+$ and $CD16^+CD3^-$ cells and the level of $CD16^+CD3^+$ cells expressed as a percentage of the total number of $CD56^+$ cells in the peripheral blood of 12 subjects with PBD and 13 controls.

Patients	$CD16^+ CD8^+$	$CD16^+ CD8^-$	$\frac{CD16^+ CD8^+}{CD16^+}$
Pigeon breeders			
%			
median	5	16	25*
range	1-12	4-40	7-57
Ab.no			
median	120	310	
range	21-232	66-1659	
Controls			
%			
median	6	6	46
range	2-10	3-26	22-56
Ab.no			
median	103	110	
range	52-220	56-775	

* - P<0.05 vs controls
Ab.no - absolute number (cmm^{-1})

TABLE 4.6

The median percentage and absolute number of $CD16^+CD8^+$ and $CD16^+CD8^-$ cells and the level $CD16^+CD8^+$ cells expressed as a percentage of the total number of $CD16^+$ cells in the peripheral blood of 11 subjects with PBD and 12 controls.

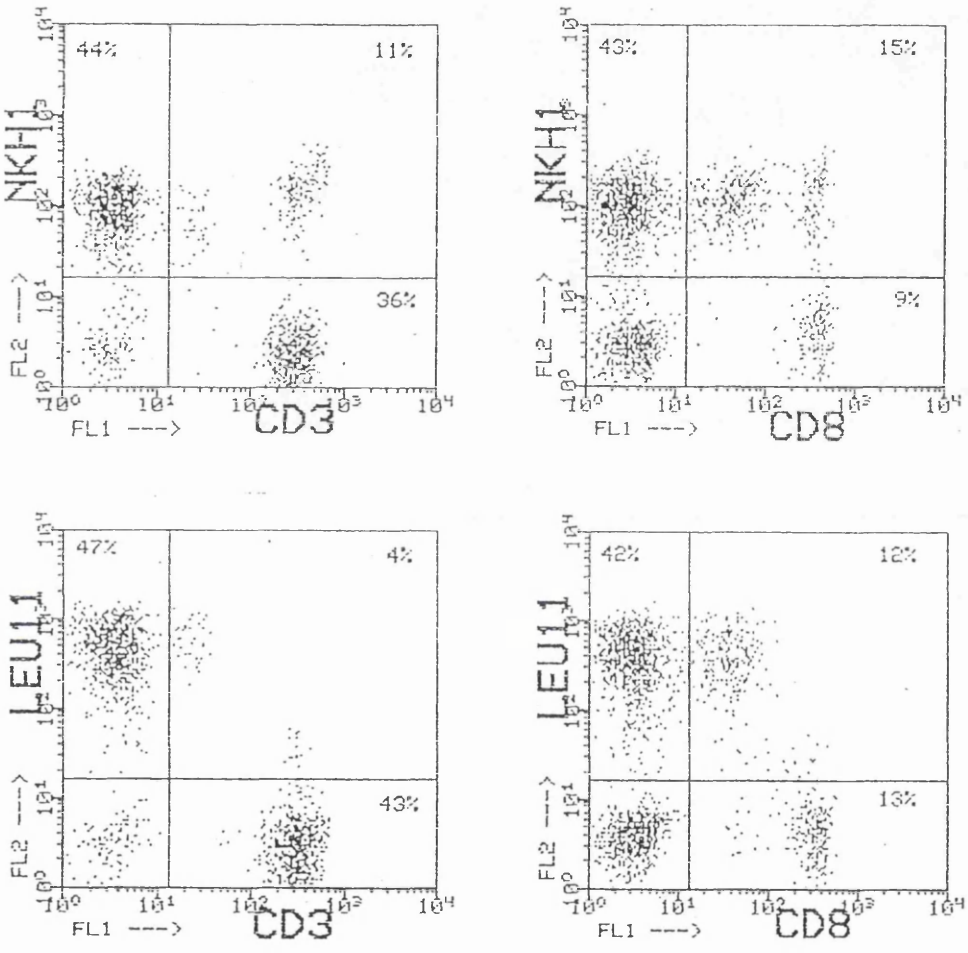


FIGURE 4.1

Two-colour flow cytometry analysis of CD56 (NKH1), CD16 (Leu11), CD3 and CD8 antigen on peripheral blood lymphocytes in a representative subject.

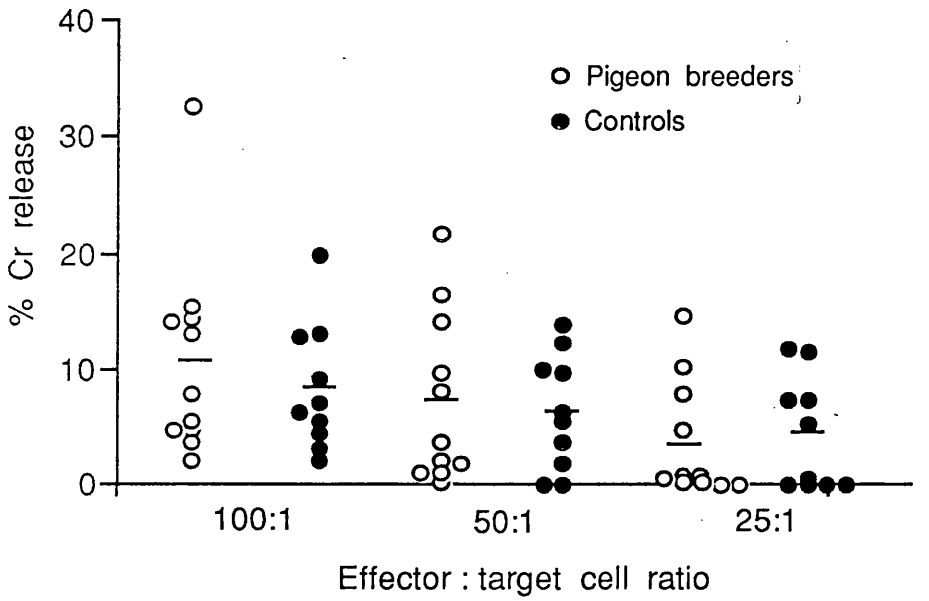


FIGURE 4.2

Cytotoxic activity of peripheral blood mononuclear cells at effector to K562 target cell ratios of 100:1, 50:1 and 25:1 in 11 subjects with PBD and 10 controls.

CHAPTER 5

PHENOTYPIC STUDIES OF GAMMA DELTA T CELLS IN PIGEON BREEDERS

5.1 INTRODUCTION

A subset of T cells (1-5%) express TCR$\gamma\delta$ (Lew et al, 1986, Weiss et al, 1986). In humans, two major $\gamma\delta$ T cell subsets have been identified, namely the Vδ1 subset, which express Vδ1 chains, recognised by δTCS1 mAb (Wu et al, 1987) most of which are non covalently associated with Vγ1 chains (Casorati et al, 1989, Bottino et al, 1988) and the Vδ2 subset, which express Vδ2 chains, recognised by BB3 mAb (Ciccone et al, 1988) most of which are linked by disulphide bridges to Vγ2 chains (Cassorati et al, 1989, Bottino et al, 1988, Borst et al, 1989, Parker et al, 1990). In humans, 90% of $\gamma\delta$ T cells in the peripheral blood (Falini et al, 1989) and the majority of $\gamma\delta$ T cells in the lung (Balbi et al, 1990) are $BB3^+$.

It has been suggested that $\gamma\delta$ T cells may protect epithelial surfaces (Janeway et al, 1988, Janeway, 1988, Raulet, 1989). In support of this idea, increased numbers of $\gamma\delta$ T cells have been reported in coeliac disease (Halstensen et al, 1989) and in chronic inflammatory diseases of the lung such as farmers' lung (Trentin et al, 1990) and sarcoidosis (Balbi et al, 1990). In addition, recent evidence suggests that $\gamma\delta$ T cells may be involved in specifically suppressing IgE responses to protein antigens presented via intact non inflamed epithelial surfaces in the respiratory tract, suggesting that they may provide another layer of protection against hypersensitivity reactions in the lung (McMenamin et al, 1991).

To investigate the possibility of the involvement of $\gamma\delta$ T

cells in the inflammatory immune response in PBD, this chapter measured total $\gamma\delta$ T cell levels and determined $\gamma\delta$ T cell subsets, in the peripheral blood of subjects with severe, longstanding PBD and controls. In addition the coexpression of CD8 antigens with the $\gamma\delta$TCR was also determined since intraepithelial $\gamma\delta$ T cells appear to be predominately $CD8^-$ (Halstensen et al, 1989).

5.2 EXPERIMENTAL PROTOCOL

One and two colour flow cytometry was used to quantify percentages of all $\gamma\delta$ T cells using Mab TCRδ1 (Band et al, 1987), their nondisulphide-linked subpopulations using MAb δTCS1 (Wu et al, 1987, Faure et al, 1988) and the coexpression of CD8 on these cells, in the peripheral blood of 12 patients with severe, longstanding PBD (group 8, chapter 3) and 14 controls (group 9, chapter 3). In addition, absolute numbers of cells were derived from the total lymphocyte counts.

5.3 RESULTS

The percentages and absolute numbers of $\gamma\delta$ T cells (TCRδ1$^+$) and the subpopulation of these cells expressing the nondisulphide-linked form of the $\gamma\delta$ TCR (δTCS1$^+$) were not normally distributed and so median values and ranges are shown. Figure 5.1 shows the typical FAC's profiles obtained by two-colour flow cytometry analysis of TCRδ1, δTCS1, CD3 and CD8 on peripheral blood lymphocytes in a representative subject.

5.3.1 LEVELS OF $\gamma\delta$ T CELLS

Pigeon breeders had significantly fewer $\gamma\delta$ T cells than controls (1.5% vs 6%, respectively, $P<0.01$, Table 5.1). This was associated with a significantly lower absolute number of TCRδ1$^+$ cells in the pigeon breeders compared with the controls, $P<0.05$ (Table 5.1). As anticipated, all TCRδ1 staining cells coexpressed CD3 (Figure 5.1).

Similar percentages and absolute numbers of δTCS1$^+$ cells were found in the peripheral blood of pigeon breeders and controls (0.4% and 0.2%, respectively, Table 5.1). In addition the percentages of $\gamma\delta$ T cells which were δTCS1$^+$ were not significantly different in pigeon breeders and controls (16% and 2% respectively, Table 5.1).

5.3.2 EXPRESSION OF CD8 ON $\gamma\delta$ T CELLS

The percentages and absolute numbers of both TCRδ1$^+$CD8$^+$ and TCRδ1$^+$CD8$^-$ cells were significantly lower in the peripheral blood of pigeon breeders compared with

controls ($P<0.02$ and $P<0.05$, respectively, Table 5.2). As a result, there was no difference in the proportion of $\gamma\delta$T cells which expressed CD8 in pigeon breeders and controls (Table 5.2). Due to the relatively low numbers of δTCS1$^+$ cells, subpopulations of δTCS1$^+$ cells coexpressing CD8 were not quantified.

5.3.3 THE EFFECT OF AGE ON THE LEVELS OF $\gamma\delta$ T CELLS

As was discussed in chapter 3 (3.3.5) and chapter 4 (4.3.4), the median ages of patients in group 8 were significantly higher than the median ages of the control group 9 (Table 3.8).

In order to determine whether this could have accounted for the differences in the levels of $\gamma\delta$ T cells, I examined whether the percentage of $\gamma\delta$ T cells in pigeon breeders was affected by their age. No correlation was found between age and the percentage of $\gamma\delta$ T cells in these subjects.

5.4 CONCLUSIONS

This study found decreased percentages and absolute numbers of $\gamma\delta$ T cells in the peripheral blood of subjects with severe longstanding PBD compared with control subjects. The percentages of both $CD8^+$ and $CD8^-$ $\gamma\delta$ T cells were reduced and therefore similar proportions of $\gamma\delta$ T cells expressed CD8 antigen in the peripheral blood of pigeon breeders and controls. The percentages and absolute numbers of the subpopulation of $\gamma\delta$ T cells expressing the nondisulphide-linked receptor (δTCS-1) were similar in the peripheral blood of patient and control groups, although the levels were very low, as reported earlier (Falini et al, 1989).

These results suggests that $\delta TCS\text{-}1^-$ cells are leaving the blood, as their absolute number decreased, while $\delta TCS\text{-}1^+$ cells numbers stayed the same.

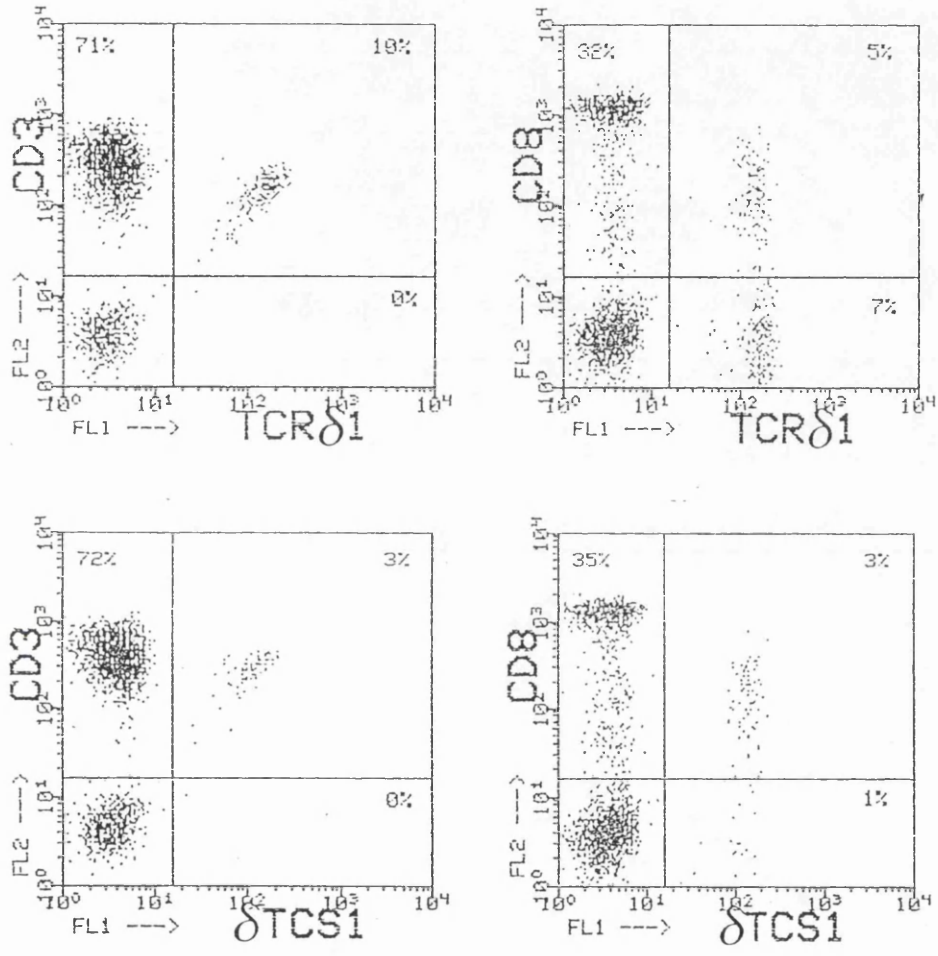


FIGURE 5.1

Two colour flow cytometry analysis of TCRδ1, δTCS1, CD3 and CD8 antigen on peripheral blood lymphocytes in a representative subject.

	TCRδ1+		δTCS1+		% δTCS1+ / TCRδ1+
	%	Ab.no	%	Ab.no	
Pigeon breeders					
	1.5*	50*	0.4	9	16
	(0-11.0)	(0-415)	(0-2.0)	(0-207)	(0-56)
Controls					
	6.0	90	0.2	5	2
	(1.7-12.0)	(34-328)	(0-3.5)	(0-86)	(0-41)

* - value differs significantly from the corresponding control value
Ab.no - absolute number (cmm^{-1})

TABLE 5.1

The median and (range) of percentages and absolute numbers of total $\gamma\delta$ T cells ($TCR\delta1^+$) and the subpopulation of $\gamma\delta$ T cells expressing the nondisulphide linked form of the TCR ($\delta TCS1^+$) and the median and range of percentages of $\delta TCS1^+$ cells expressed out of the total number of $\gamma\delta$ T cells in the peripheral blood of 12 subjects with PBD and 14 controls.

	TCRδ1+ CD8+		TCRδ1+ CD8-		TCRδ1+ CD8+ / TCRδ1+
	%	Ab.no	%	Ab.no	
Pigeon breeders					
	0.5*	13*	1.0*	25*	23
	(0-3.0)	(0-51)	(0-7.0)	(0-119)	(0-83)
Controls					
	1.6	34	4.0	52	27
	(0.5-5.0)	(9-149)	(1.1-12.0)	(23-311)	(12-63)

* - value differs significantly from the corresponding control value
Ab.no - absolute number (cmm^{-1})

TABLE 5.2

The median and (range) of percentages and absolute numbers of $TCR\delta1^+CD8^+$ and $TCR\delta1^+CD8^-$ cells and the percentage of $TCR\delta1^+CD8^+$ cells expressed as a percentage of the total number of $TCR\delta1^+$ cells in the peripheral blood of 12 subjects with PBD and 14 controls.

CHAPTER 6

EXPRESSION OF ACTIVATION AND/OR MEMORY MARKERS BY T CELLS IN PBD

6.1 INTRODUCTION

Human T cells can be divided into two populations based on their expression of isoforms of the leucocyte common antigen (CD45), a high molecular weight (180-220 KDa) membrane glycoprotein expressed on cells of haematopoietic origin (Sanders et al, 1988a). Differential expression of these alternately splicing exons (A, B and C) leads to low and high molecular weight isoforms of CD45 (Streuli et al, 1987). Most of the work on the subdivision of these cells has been based on $CD4^+$ T cells. $CD4^+$ T cells expressing the high molecular weight isoform of CD45 (CD45RA) and recognised by anti-CD45RA antibodies, do not respond to recall antigens and do not provide help for B cell responses (Morimoto et al, 1985b). In contrast, $CD4^+$ T cells expressing the low molecular weight isoform (CD45RO) and recognised by anti-CD45RO antibodies, respond by proliferation to soluble recall antigens and provide help for pokeweed mitogen or antigen induced immunoglobulin synthesis (Smith et al, 1986, Akbar et al, 1988).

After in vitro stimulation, $CD45RA^+$ cells acquire CD45RO and lose CD45RA (Akbar et al, 1988). This, together with the ability of $CD45RO^+$ T cells to respond to recall antigens, supports the view that the $CD45RA^+$ subset represent "naive" cells, whilst the $CD45RO^+$ subset contains "memory" $CD4^+$ T cells. Given more recent findings that $CD45R^-$ ("memory") cells can revert to $CD45R^+$ ("naive") cells in mice, it is possible that the two populations of cells may better be defined as "activated" and "resting" respectively (Bell &

Sparshott, 1990), although they will not be referred to as such in this thesis. The switch in expression of low to high molecular weight isoforms of CD45 is usually accompanied by the expression of novel adhesion molecules, one of which is CDw29 (VLA ß) (Sanders et al, 1988b). Therefore a substantial proportion of $CD45RO^+$ cells can be identified using anti-CDw29 antibodies (Sanders et al, 1988b, Morimoto et al 1985a, Serra et al, 1988). More limited data suggests that the same distinctions may also hold true for CD8 (Merkenschlager et al, 1989) and $\gamma\delta$ T cells (Hayward et al, 1989).

A large number of studies have found increased proportions of $CD45RO^+$ memory $CD4^+$ T cells in a variety of human chronic inflammatory disease, such as rheumatoid arthritis (Emery et al, 1987, Lasky et al, 1988), tuberculosis (Barnes et al, 1989) and coeliac disease (Halstensen et al, 1990). Increased levels of "memory" cells have also been reported in the BAL fluid of symptomatic and asymptomatic pigeon breeders (Johnson et al, 1989) but levels in the peripheral blood have not been studied.

Therefore, the aim of this chapter was to quantify total numbers of $CD45RA^+$, and $CDw29^+$ cells and the levels of $CD45RA^+CD4^+$ and $CDw29^+CD4^+$ cells in the peripheral blood of subjects with PBD and control subjects, to determine whether there were any changes and if so, whether these changes mirrored the previous findings of increased numbers of "memory" T cells in the lung (Johnson et al, 1989).

6.2 EXPERIMENTAL PROTOCOL

Two-colour flow cytometry was used to quantify the percentage of $CD4^+$ T cells co-expressing the $CD45RA^+$ and $CDw29^+$ markers. The pigeon breeders used had severe longstanding PBD and were the same as those used in chapters 3, 4 and 5 (group 8). The controls were also the same as those studied in chapters 3, 4 and 5 (group 9).

6.3 RESULTS

Figure 6.1 shows the typical FACS profile obtained by two- colour flow cytometry analysis of CD45RA, CDw29 and CD4 on peripheral blood lymphocytes in both pigeon breeders and controls. It is clear from these profiles that there are $CD45RA^-$ lymphocytes and lymphocytes which have varied expression ("low" to "high") of CD45RA. $CD45RA^+$ were gated to include both the "CD45RA-low" and "CD45RA-high" population of lymphocytes. Expression of CDw29 on lymphocytes varied between "low" and "high" with no $CDw29^-$ population, consistent with previous reports (Sanders et al, 1988a). Therefore $CDw29^+$ lymphocytes were gated to exclude the "CDw29-low" population of lymphocytes.

6.3.1 EXPRESSION OF CD45RA

A median of 72% of peripheral blood lymphocytes were $CD45RA^+$ in subjects with PBD compared with a median of only 60% in controls ($P<0.05$, Table 6.1). There was also a higher absolute number of $CD45RA^+$ cells in the peripheral blood of pigeon breeders compared with those in the control group although this difference was not statistically significant (Table 6.1).

When the expression of CD4 by $CD45RA^+$ cells was examined by two-colour flow cytometry, no significant differences between the percentage and numbers of $CD45RA^+CD4^+$ or $CD45RA^+CD4^-$ populations were detected in controls and subjects with PBD (Table 6.2). However, most of the increase in total $CD45RA^+$ cells in subjects with PBD appeared to be due to an increase in the $CD45RA^+CD4^-$

subset, which was 57% in subjects with PBD compared with 46% in controls. In contrast, the percentage and absolute number of $CD45RA^+CD4^+$ cells in subjects with PBD were virtually identical to controls (18%, $334cmm^{-1}$ and 15%, $288cmm^{-1}$, respectively Table 6.2). The proportion of $CD4^+$ cells which co-expressed CD45RA was also similar (Table 6.2).

6.3.2 EXPRESSION OF CDw29

Similar percentages and absolute numbers of $CDw29^+$ lymphocytes were recorded in subjects with PBD and controls (70%, $1412cmm^{-1}$ and 65%, $1297cmm^{-1}$, respectively, Table 6.1). In addition, similar percentages and absolute numbers of $CDw29^+CD4^+$ and $CDw29^+CD4^-$ cells were found in subjects with PBD and controls (Table 6.3). The proportion of $CD4^+$ cells which coexpressed CDw29 was also similar (Table 6.3).

6.3.3. RATIO BETWEEN "NAIVE : MEMORY" T CELLS

The median ratio of $CD45RA^+$:$CDw29^+$ cells ("naive:memory") in the peripheral blood of subjects with PBD and controls was identical (0.9, Table 6.4). Ratios of $CD45RA^+CD4^+$:$CDw29^+CD4^+$ ("naive:memory" $CD4^+$ T cells) in the peripheral blood of patients and controls were also similar (0.5 and 0.7, respectively, Table 6.4).

6.3.4 THE EFFECT OF AGE ON $CD45RA^+$ AND $CDw29^+$ CELLS IN THE PERIPHERAL BLOOD

As previously mentioned, the median ages of pigeon breeders (group 8) used in this study, were significantly higher than those of the controls (group 9).

In order to determine whether the age differences between the patients and controls would have had an effect on the levels of $CD45RA^+$ and $CDw29^+$ cells reported in this study, I correlated the ages of subjects in group 8 with the levels $CD45RA^+$ and $CDw29^+$ cells in their peripheral blood.

The results of this analysis revealed that the ages of subjects in group 8 did not correlate with the levels of $CD45RA^+$ and $CDw29^+$ cells in their peripheral blood.

6.4 CONCLUSIONS.

The results of this study showed that subjects with severe longstanding PBD have significantly higher median percentages and higher absolute numbers of $CD45RA^+$ cells in their peripheral blood than controls. Analysis of the expression of CD4 on these cells, did not reveal any significant differences in the percentages of $CD45RA^+CD4^-$ cells and $CD45RA^+CD4^+$ cells, although the increased percentage of $CD45RA^+$ cells was due mainly to increased levels of $CD45RA^+CD4^-$ cells. The proportion of $CD4^+$ T cells expressing CD45RA was similar in pigeon breeders and controls.

No differences in the percentages and absolute numbers of $CDw29^+$ were found in the peripheral blood of patients compared to controls. Analysis of the expression of CD4 on these cells revealed no differences in the percentages of either $CDw29^+CD4^-$ cells or $CDw29^+CD4^+$ T cells.

Similar ratios of $CD45RA^+$:$CDw29^+$ lymphocytes and $CD45RA^+$:$CDw29^+$ $CD4^+$ T cells were recorded in the peripheral blood of subjects with PBD and controls, suggesting that the balance of "naive:memory" cells in the peripheral blood is not altered in subjects with PBD.

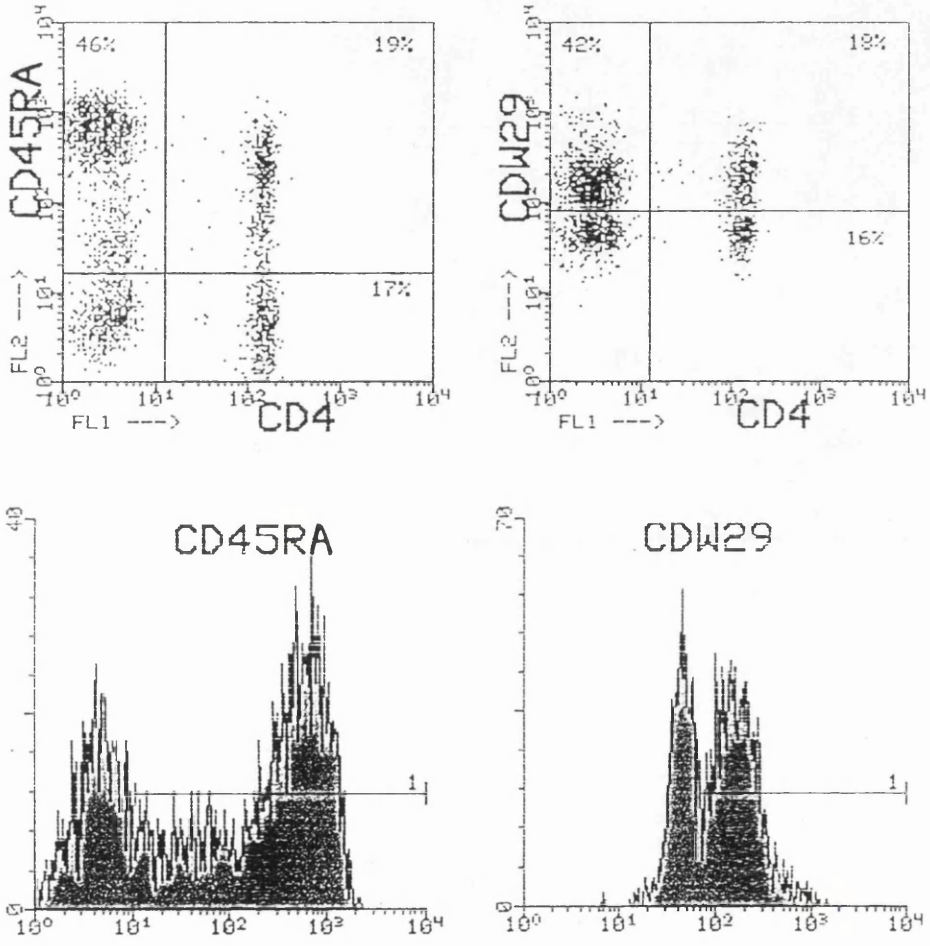


FIGURE 6.1

Two-colour flow cytometry analysis of CD45RA, CDw29, and CD4 antigen on peripheral blood lymphocytes in a representative subject.

	CD45RA$^+$		CDw29$^+$	
	%	Ab.no	%	Ab.no
Pigeon breeders				
	72*	1560	70	1412
	(49-98)	(900-7671)	(39-86)	(800-8500)
Controls				
	60	1218	65	1297
	(54-75)	(794-2235)	(42-80)	(470-2121)

* value differs significantly from control value
Ab.no absolute number (cmm^{-1})

TABLE 6.1

The median and (range) of percentages and absolute numbers of CD45RA$^+$ cells and CDw29$^+$ cells in the peripheral blood of 12 subjects with PBD and 14 controls

	$CD45RA^+CD4^+$		$CD45RA^+CD4^-$		$\frac{\%\ CD45RA^+CD4^+}{CD4^+}$
	%	Ab.no	%	Ab.no	
PIGEON BREEDERS	18	334	57	1259	36
	(4-31)	(82-1890)	(31-73)	(582-6531)	(16-100)
CONTROLS	15	288	46	945	38
	(7-28)	(154-605)	(30-64)	(503-1907)	(20-66)

Ab.no absolute number (cmm^{-1})

TABLE 6.2

The median and (range) of percentages and absolute numbers of $CD45RA^+CD4^+$ and $CD45RA^+CD4^-$ cells and the percentage of $CD45RA^+CD4^+$ cells expressed as a percentage of total $CD4^+$ cells in the peripheral blood of 12 subjects with PBD and 14 controls.

	$CDw29^+CD4^+$		$CDw29^+CD4^-$		$\frac{\%\ CDw29^+CD4^+}{CD4^+}$
	%	Ab.no	%	Ab.no	
PIGEON BREEDERS	22	494	44	1066	73
	(16-39)	(261-2384)	(19-64)	(347-6166)	(37-85)
CONTROLS	23	441	38	756	61
	(11-35)	(123-716)	(26-60)	(347-1405	(28-74)

Ab.no absolute number (cmm^{-1})

TABLE 6.3

The median and (range) of percentages and absolute numbers of $CDw29^+CD4^+$ and $CDw29^+CD4^-$ cells and the percentage of $CDw29^+CD4^+$ cells expressed as a percentage of the total number of $CD4^+$ cells in the peripheral blood of 12 subjects with PBD and 14 controls.

	$2H4^+$: $4B4^+$	$2H4^+$ $CD4^+$: $4B4^+$ $CD4^+$
PIGEON BREEDERS	0.9 (0.7-1.8)	0.5 (0.2-1.6)
CONTROLS	0.9 (0.7-1.7)	0.7 (0.3-2.4)

Table 6.4

The median and (range) of ratios of $2H4^+$:$4B4^+$ cells and $2H4^+CD4^+$:$4B4^+CD4^+$ cells in the peripheral blood of 12 subjects with PBD and 14 controls.

CHAPTER 7

FUNCTIONAL ACTIVITY OF PERIPHERAL BLOOD LYMPHOCYTES IN PIGEON BREEDERS

7.1 INTRODUCTION

Studies of specific cell mediated immunity in PBD have been stimulated because of the mononuclear cell infiltrates which are found in the lungs of affected subjects. Most studies have reported that peripheral blood mononuclear cells (PBMC) from both symptomatic and asymptomatic pigeon breeders proliferated in response to either pigeon serum protein (Moore et al, 1980, Schatz et al, 1976) or pigeon dropping extract (PDE) (Schatz et al, 1976, Girard et al, 1978). However, one study did suggest that a significant response only occurred with PBMC from symptomatic pigeon breeders stimulated with pigeon serum or PDE (Hansen & Penny, 1974). In addition, those studies which examined a normal control population found no proliferative response of control PBMC to pigeon droppings (Hansen & Penny, 1974, Girard et al, 1978) or pigeon serum (Girard et al, 1978), unless there was a history of exposure to birds other than pigeons, (Schatz et al, 1976).

The variability in these results and the fact that different groups used different culture conditions, makes it difficult to determine the role of specific cell mediated immunity in PBD. In addition, these studies were done more than 10 years ago and have not been repeated. The aim of this chapter was to establish a reliable lymphocyte proliferation assay using pigeon serum as an antigen since this is the most complete antigen source and is capable of inducing symptoms of PBD in symptomatic breeders after controlled aerosol exposure (Reed et al, 1965, Hargreave et al, 1972). This assay would then be

used to compare the proliferative response by PBMC from subjects with severe longstanding PBD and controls cells to pigeon serum. This would also indicate whether lymphocyte proliferation would be a useful monitor for clinical staging of PBD. My intention was then to try and identify the antigen or antigens causing proliferation.

7.2 EXPERIMENTAL PROTOCOL

PBMC were obtained from subjects with severe longstanding PBD, with similar symptoms of PBD to subjects in group 1 and group 8 of the previous studies (chapters 3-6). Controls were from the same population used to select controls for the previous chapters.

PBMC were stimulated with whole pigeon serum and two fractions of pigeon serum; "globulin" and "albumin rich", separated by a 33% ammonium sulphate precipitation procedure. A commercial source of pigeon albumin was also used in the lymphocyte proliferation assay.

Stimulation indices were calculated by dividing the mean counts per minute (cpm) ^{3}H Thymidine incorporation of three stimulated cultures with the mean cpm of the appropriate unstimulated cultures.

7.3 RESULTS

7.3.1. ESTABLISHMENT OF OPTIMAL CULTURE CONDITIONS FOR MEASURING PROLIFERATIVE RESPONSES TO PIGEON SERUM

a) Effects of varying the concentrations of PBMC and pigeon serum protein.

To establish the optimal concentration of pigeon serum, doubling dilutions of pigeon serum were used to obtain pigeon serum protein concentrations ranging from 775μg of protein per well to 3μg of protein per well, based on the range of concentrations which had been used in previous studies (Hansen & Penny, 1974, Moore et al, 1980, Schatz et al, 1976). These dilutions were incubated with PBMC at concentrations of $5x10^4$ or $1x10^5$ cells per well and in this series of experiments an incubation period of 6 days was used, as previous work had suggested this to be the optimal time, (Schatz et al, 1976). In the first experiment, using 1 pigeon breeder and 1 control, it was immediately established that PBMC from both the pigeon breeder and control proliferated in response to pigeon serum (Figure 7.1). Therefore to establish further the optimal concentration of PBMC and pigeon serum protein, PBMC from 3 controls and 1 subject with PBD were used and the data pooled, since it was easier to get cells from controls, (Figure 7.2).

It is clear from Figure 7.2 that when cells were cultured in the presence of pigeon serum, a dose dependent proliferative response occurred at both concentrations of responder cells. However, the responses were usually

greater using $1x10^5$ cells per well, compared with cells at a concentration of $5x10^4$ cells per well, apart from at the highest concentration of pigeon serum protein (775μg/well), when the stimulation index was similar for the two concentrations of PBMC. Maximum stimulation was achieved when $1x10^5$ cells were incubated with 388μg of pigeon serum protein per well, although concentrations of pigeon serum protein as low as 6μg of protein per well with $1x10^5$ cells per well induced significant stimulation.

b) Time course for the proliferative response of PBMC to pigeon serum

I next examined whether six days was the optimal incubation period for maximum proliferation. To do this, $1x10^5$ PBMC per well, were cultured for 3, 6 or 8 days with a range of concentrations of pigeon serum protein which covered the optimal dose established above (13μg to 412μg per well).

The results summarised in Figure 7.3, indicate that at day 3, SI's were less than 5 over the range of concentrations of pigeon serum protein used. On day 6, significant stimulation of PBMC occurred in wells containing between 103μg and 412μg of pigeon serum protein per well. At day 8, only wells containing pigeon serum protein at a concentration of 412μg per well had a stimulation index of greater than 5 and no significant response was seen with any other concentration. Thus, an incubation period of 6 days was chosen as being optimal for proliferation of PBMC to pigeon serum protein.

A fuller time course covering every day between 3 and 8 days was not feasible due to the shortage of cells and pigeon serum. However, a limited study looking at 4 and 6 day incubation periods showed that only a small degree of proliferation had appeared by day 4 and confirmed that 6 days was the optimal incubation period (data not shown).

7.3.2 THE PROLIFERATIVE RESPONSE OF PBMC FROM SYMPTOMATIC PIGEON BREEDERS AND CONTROLS TO PIGEON SERUM

As the preliminary experiments indicated that both controls and subjects with PBD proliferated in response to pigeon serum, it was important to determine whether there was any quantitative difference between these groups. Therefore, PBMC from 6 subjects with PBD and 6 controls were cultured at $1x10^5$ cells per well with pigeon serum for 6 days, using the range of concentrations of pigeon serum protein found to be stimulatory in the previous experiment (775μg/well to 6μg/well).

From Figure 7.4, it can be seen that the mean stimulation indices, are not significantly different using PBMC from subjects with PBD and controls, across the range of concentrations of pigeon serum used in this assay. It would therefore appear that pigeon serum is equally mitogenic to PBMC from subjects with PBD and controls.

The proliferative responses of PBMC from subjects with

PBD and controls to pokeweed mitogen were also compared and found to be similar (SI's of 10.1±2 and 13.8±8.3, respectively)

7.3.3 THE MITOGENICITY OF OTHER SERUM SAMPLES

a) Mitogenicity of several different batches of pigeon serum

As the previous experiments used pigeon serum from the same pigeon which was housed in the animal house at Yorkhill Hospital in Glasgow (PS1), it was necessary to establish whether other pigeon serum samples were also mitogenic for both controls and subjects with PBD.

Two additional serum samples were collected from pigeons also housed in the animal house at Yorkhill Hospital (PS2- 3), while a fourth serum sample was obtained from a different animal house (Stirling University) by pooling serum from four pigeons (PSS). The four serum samples were tested at four concentrations (675μg, 338μg, 168μg and 84μg, per well) on the PBMC of 2 subjects with PBD and 1 control.

Figure 7.5 and 7.6. shows that all four batches of pigeon serum were mitogenic to PBMC from subjects with PBD and controls, respectively. Indeed all four samples induced much higher responses by control PBMC than by PBMC from the two pigeon breeders. However, on this occasion, control PBMC also responded better to PWM (SI 81±7 cpm) compared with the PBMC from pigeon breeders (7±3), probably due to the lower background counts of control cells (139±15 and 1632±260, cpm). The actual counts

recorded for PWM and pigeon serum were similar for the control and the two subjects with PBD.

b) Mitogenicity of chicken serum

As this mitogenic effect on normal PBMC seemed to be a property of all pigeon serum, I decided to examine whether serum from another avian species has similar properties. I chose to look at the proliferative response of PBMC from 2 subjects with PBD and 1 control, incubated for 6 days, with concentrations of chicken serum protein similar to those used in the experiments with pigeon serum.

The results summarised in Figure 7.7 show that chicken serum failed to stimulate any PBMC at any of the concentrations used, despite a normal response by the same cells to PWM

7.2.4 FRACTIONATION OF PIGEON SERUM

The above studies have shown that although pigeon serum stimulates proliferation of PBMC, this is not specific to subjects with PBD. I next investigated whether the mitogenicity of pigeon serum was masking a specific response of pigeon breeders' PBMC to individual components of pigeon serum. As specific antibody to pigeon serum globulin and albumin has been found in pigeon breeders (Fink et al, 1969b) and because they are common components of several sources of crude pigeon material (Edwards et al, 1970), I fractionated pigeon serum into these two fractions, using a 33% ammonium sulphate precipitation method.

a) Protein yield

After precipitation with 33% ammonium sulphate, 114mg of globulin were obtained from 20ml of pigeon serum, which had a total protein content of 28mg per ml. The protein which remained in solution amounted to 400mg (Albumin rich). From this it can be concluded that there was approximately 5.7mg of globulin per ml of pigeon serum, or that 20% of this pigeon serum protein was globulin. The recovery of protein from this extraction was 92%. The "globulin" and "albumin rich" fractions and the original serum sample were run on a zonal electrophoresis gel which is shown in Figure 7.8. The gel shows that one of the fractions was a relatively pure globulin fraction ("globulin") and the other contained the rest of the serum proteins, including albumin ("albumin rich").

b) Proliferation assay

The "globulin" and "albumin rich" fractions and the original pigeon serum sample from which they were derived, were incubated at a range of concentrations with PBMC from subjects with PBD and controls. The concentrations of the fractions used were based on the amounts estimated to be present in a mitogenic dose of the whole pigeon serum.

Figure 7.9 shows the mean stimulation indices for the proliferation of PBMC from 5 controls and 4 subjects with PBD stimulated with whole pigeon serum. As before, significant stimulation of PBMC from subjects with PBD and controls was achieved at all four concentrations of

pigeon serum. However, none of these PBMC responded to any concentration of the "globulin" fraction (Figure 7.10).

The albumin rich fraction induced significant stimulation of PBMC from both controls and subjects with PBD (Figure 7.11). This was maximal at a concentration of 45μg of "albumin rich" protein per well for PBD cells and at 90μg/well for controls. The level of proliferation was lower than that obtained when the same PBMC were stimulated with pigeon serum.

Similar results were obtained with another pigeon serum sample which was fractionated into "globulin" and "albumin rich" fractions (data not shown).

These results indicate that the mitogenic component of pigeon serum was present in the "albumin rich" fraction, but no specific antigen could be identified.

7.3.5 ANTIGENICITY OF PURIFIED PIGEON SERUM ALBUMIN

Specific antibodies to pigeon serum albumin have been detected in the peripheral blood of pigeon breeders (Fink et al, 1969b). Since the previous studies did not determine whether the mitogenicity of pigeon serum was masking a specific response to pigeon albumin, the final experiments examined whether a specific response could be obtained using a commercial source of pigeon serum albumin. A sample of this extract was run on a zonal electrophoresis gel to determine its purity and this showed that it did not contain any major contaminants. (Figure 7.12). Various concentrations of albumin were

used in proliferation assays, using concentrations likely to be found in pigeon serum as an approximate guide. PBMC from three subjects with PBD and four controls were tested with this protein.

Figure 7.13 shows that concentrations of pigeon serum albumin ranging from 1μg to 140μg per well failed to stimulate PBMC from either subjects with PBD or controls. Therefore, pigeon serum albumin is unlikely to be the antigen responsible for the cellular response detected in subjects with PBD.

7.4 CONCLUSIONS

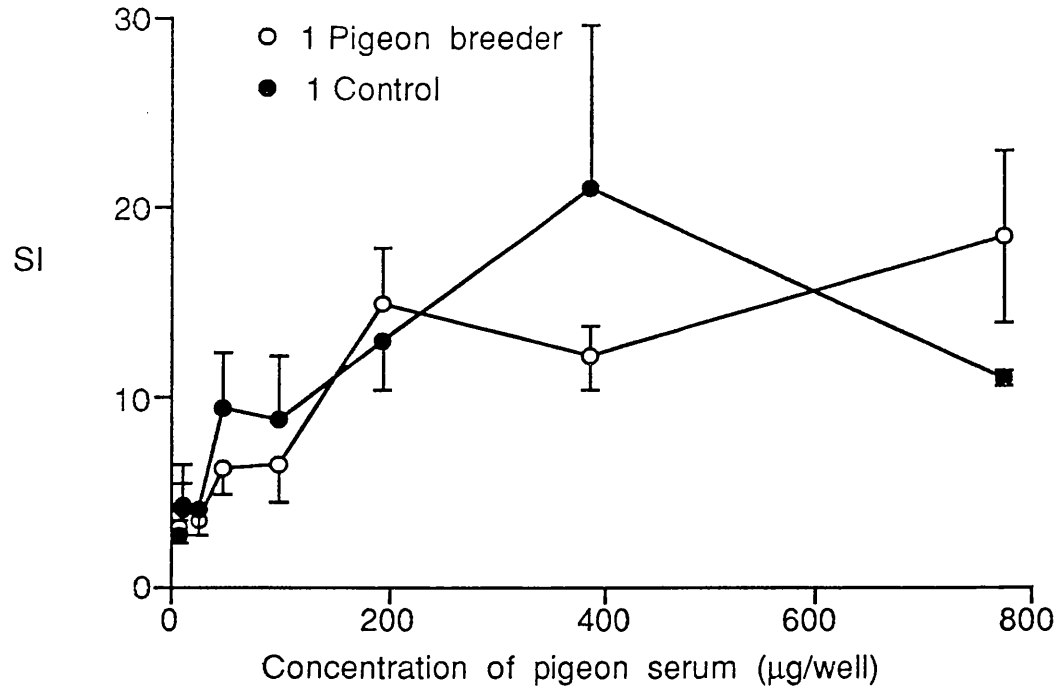
This study revealed that pigeon serum stimulated proliferation by PBMC over a wide range of concentrations, although a concentration of approximately 350μg per well was optimal. These responses were maximal at 6 days of culture and occurred with PBMC from both controls and from subjects with PBD.

All of the batches of pigeon serum from a range of pigeons housed in different animal houses showed this mitogenicity, but chicken serum failed to stimulate PBMC from either subjects with PBD or controls.

A 33% ammonium sulphate fractionation of pigeon serum into a "globulin" and an "albumin rich" fraction revealed that at least some of the mitogenic activity in pigeon serum was present in the "albumin rich" fraction, although this fraction was unable to induce the same level of stimulation of PBMC as that achieved when using whole pigeon serum. The "globulin" fraction did not cause proliferation by PBMC from either subjects with PBD or controls and is therefore unlikely to be the antigen involved in the cellular response in PBD. Using a commercial source of pigeon serum albumin, no proliferative response by PBMC from either subjects with PBD or controls was found and therefore albumin is also unlikely to be the antigen causing the cellular immune response in PBD and also not the mitogen.

Therefore these results do not support a role for antigen specific CMI to pigeon serum in PBD. However they could suggest that pigeon serum contains a non-specific mitogen

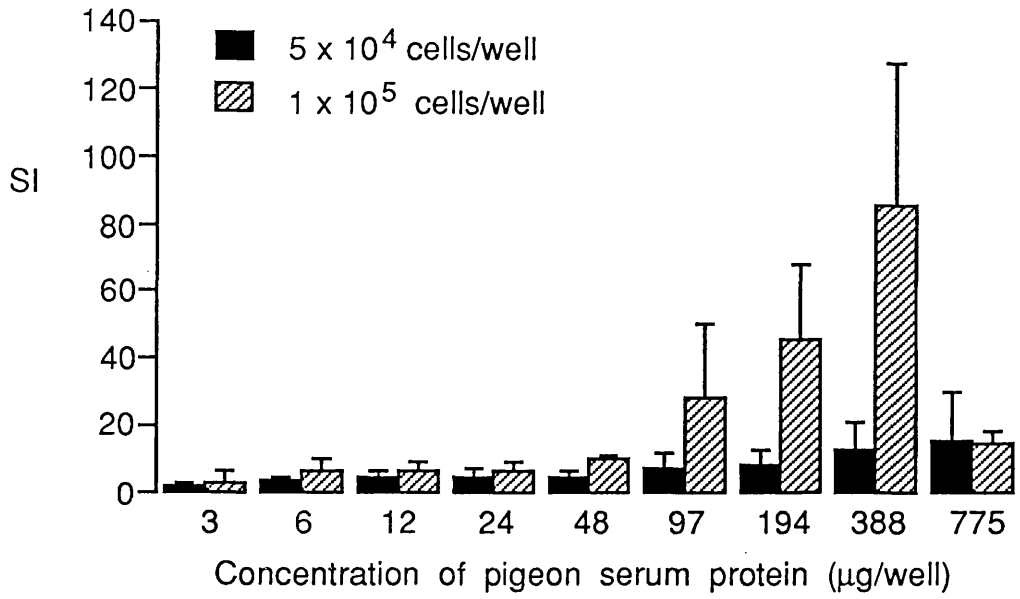
and it is conceivable that stimulation of lymphocytes by this agent could play some role in the pathogenesis of the disease. The next chapter will try to examine this in more detail.



SI - stimulation index
SEM - standard error of the mean
Bars represent the mean SI + 1 SEM

FIGURE 7.1

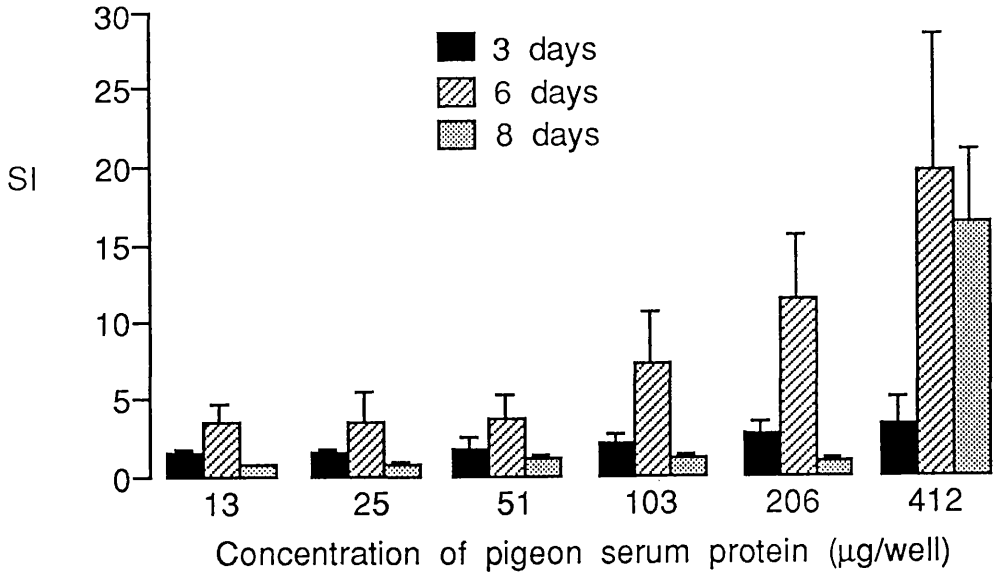
The proliferative response of PBMC from 1 pigeon breeder and 1 control incubated for 6 days with a range of concentrations of pigeon serum protein.



SI - stimulation index
SEM - standard error of the mean
Bars represent the mean SI + 1 SEM

FIGURE 7.2

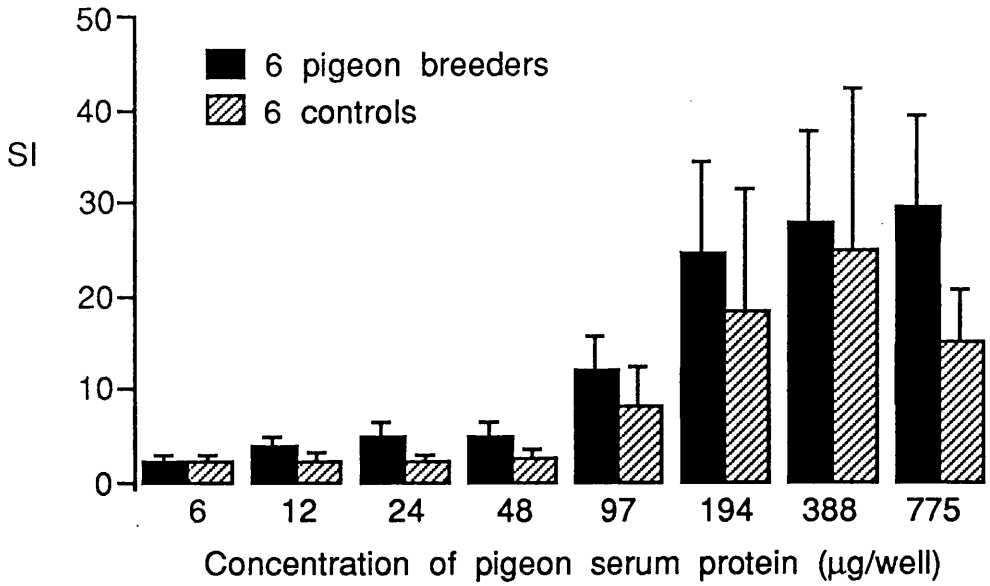
The proliferative response of PBMC at concentrations of $1x10^5$ and $5x10^4$ cells per well incubated with a range of concentrations of pigeon serum protein for 6 days.



SI - stimulation index
SEM - standard error of the mean
bars represent the mean SI + 1 SEM

Figure 7.3

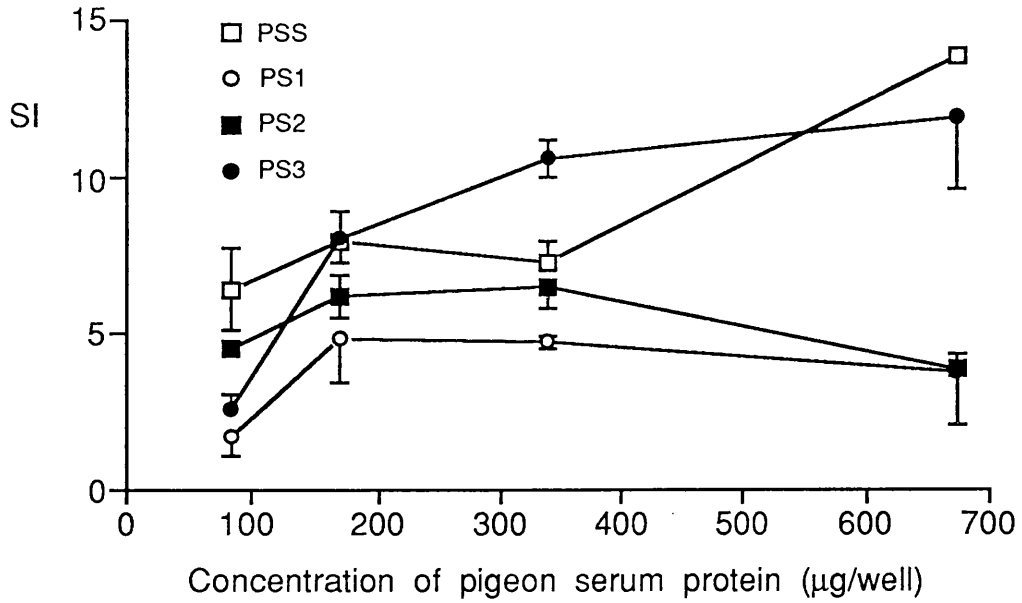
Time course for the proliferative response of PBMC from 3 controls incubated with a range of concentrations of pigeon serum protein.



SI - stimulation index
SEM - standard error of the mean
bars represent the mean SI + 1 SEM

Figure 7.4

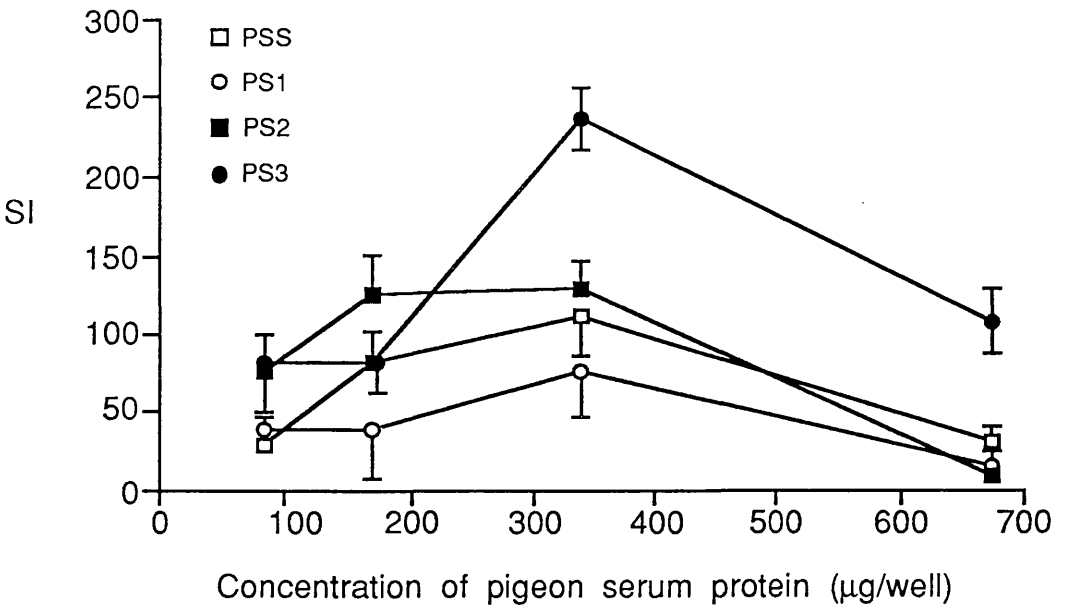
The proliferative response of PBMC from 6 pigeon breeders and 6 controls incubated with a range of concentrations of pigeon serum protein.



SI - stimulation index
SEM - standard error of the mean
bars represent the mean SI + 1 SEM

Figure 7.5

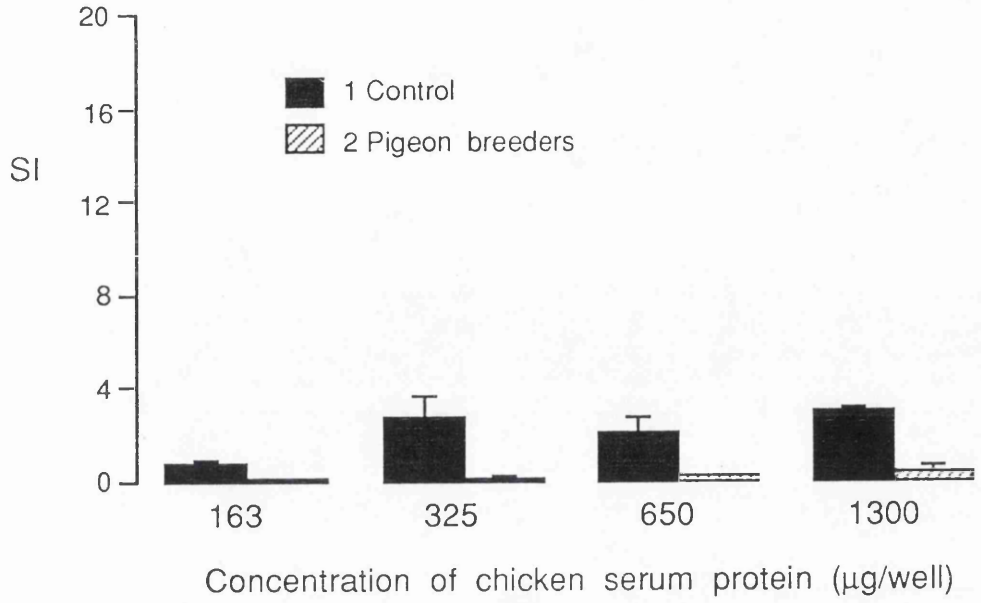
The proliferative response of PBMC from 2 subjects with PBD incubated with four different batches of pigeon serum over a range of concentrations.



SI - stimulation index
SEM - standard error of the mean
bars represent the mean SI + SEM

Figure 7.6

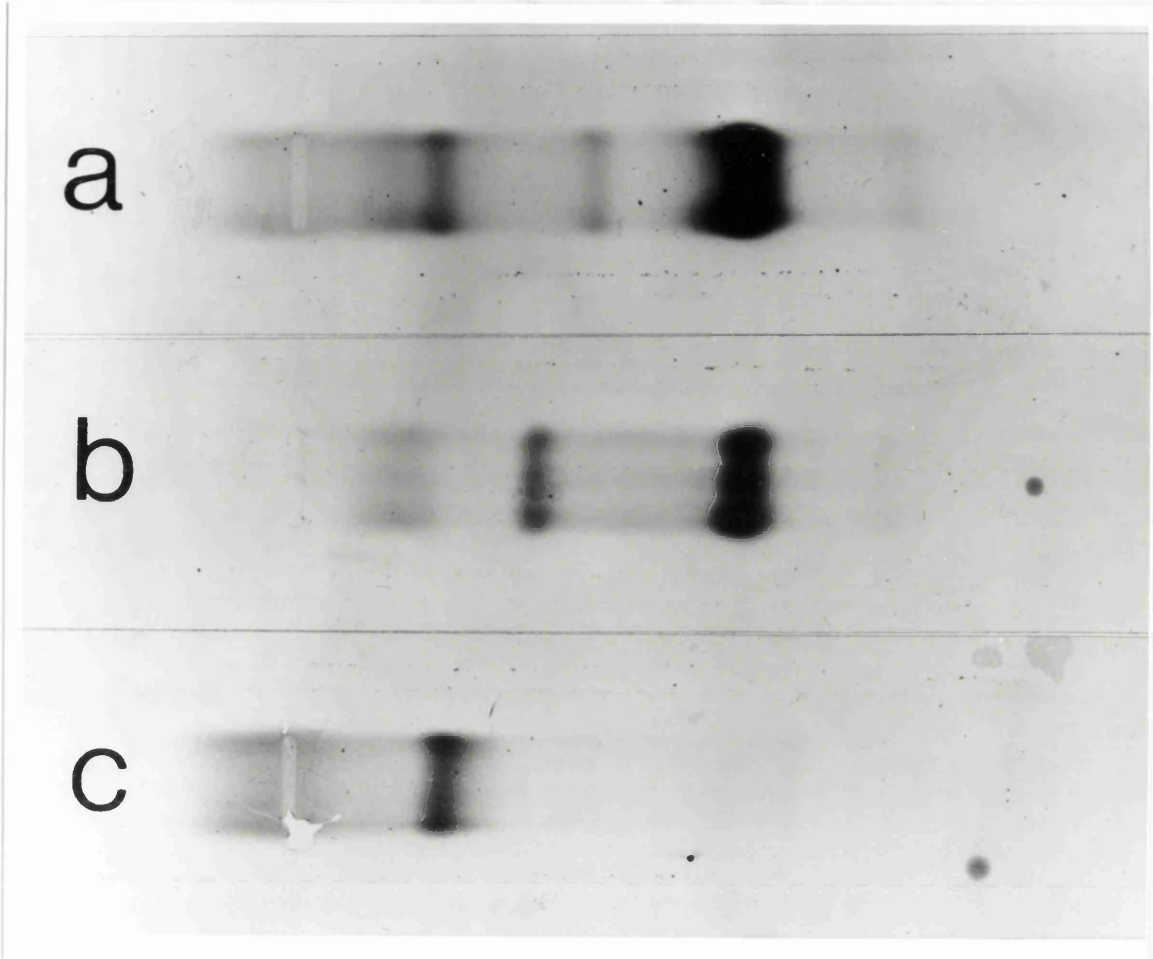
The proliferative response of PBMC from 1 control incubated with four different batches of pigeon serum over a range of concentrations.



SI - stimulation index
SEM - standard error of the mean
bars represent the mean SI + SEM

Figure 7.7

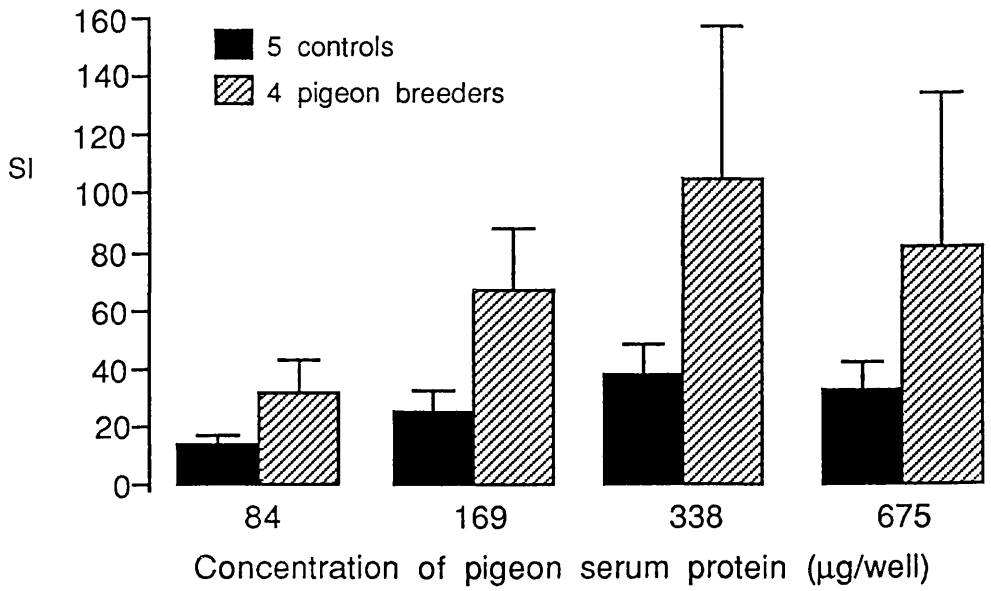
The proliferative response of PBMC from 2 pigeon breeders and 1 control incubated with a range of concentrations of chicken serum protein.



a - whole pigeon serum
b - "albumin rich" fraction
c - "globulin" fraction

Figure 7.8

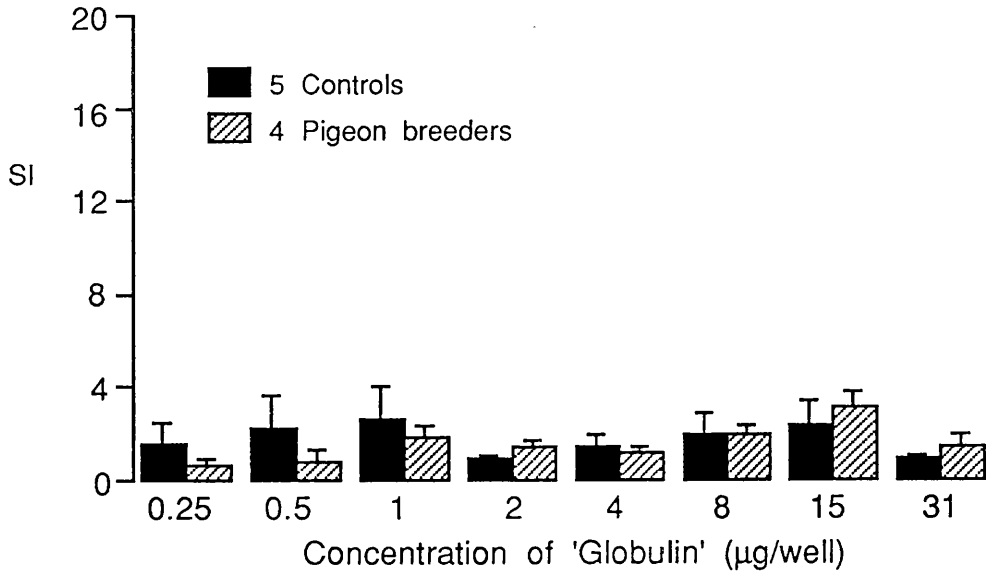
A zonal electrophoresis gel showing the protein content of whole pigeon serum and the "globulin" and "albumin rich" fractions derived from it.



SI - stimulation index
SEM - standard error of the mean
bars represent the mean SI + 1 SEM

Figure 7.9

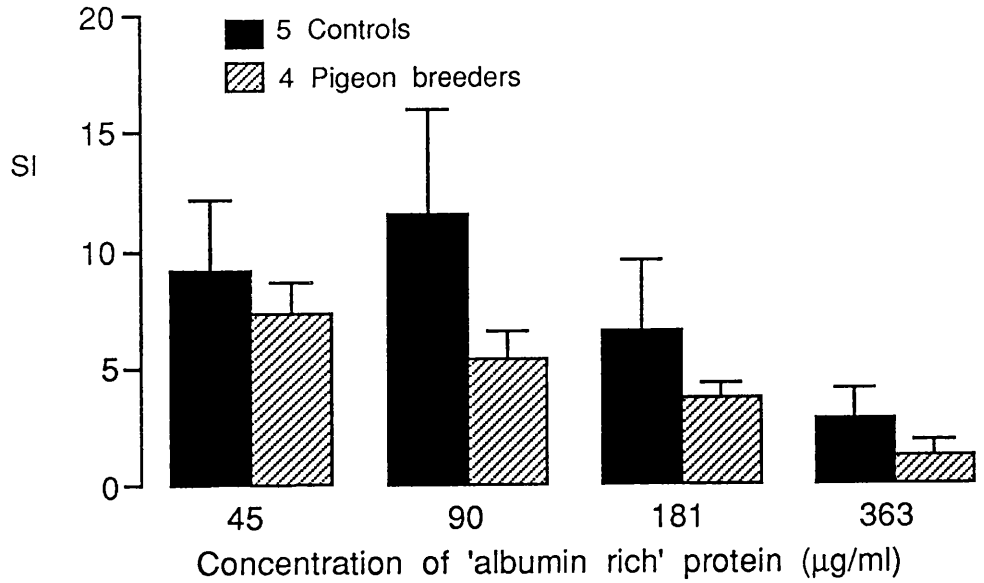
The proliferative response of PBMC from 4 pigeon breeders and 5 controls incubated with a range of concentrations of pigeon serum protein



SI - stimulation index
SEM - standard error of the mean
bars represent the mean SI + 1 SEM

Figure 7.10

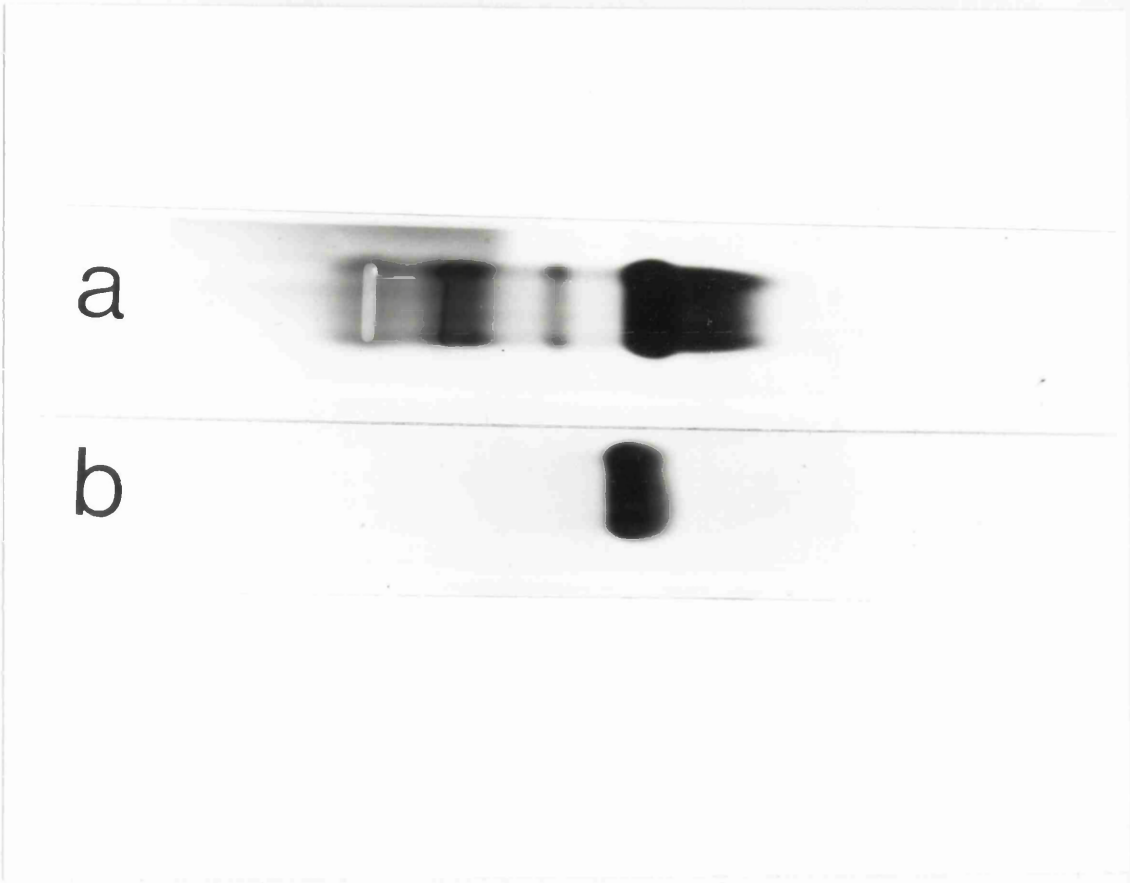
The proliferative response of PBMC from 4 pigeon breeders and 5 controls incubated with a range of concentrations of the "globulin" fraction of pigeon serum



SI - stimulation index
SEM - standard error of the mean
bars represent the mean SI + 1 SEM

Figure 7.11

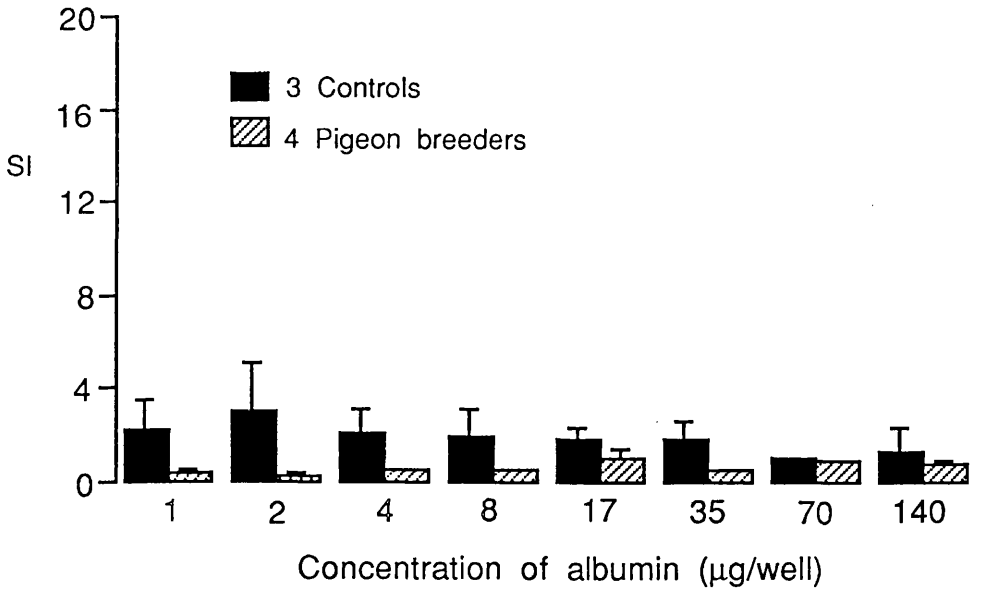
The proliferative response of PBMC from 4 pigeon breeders and 5 controls incubated with a range of concentrations of the "albumin rich" fraction of pigeon serum.



a - whole pigeon serum
b - pigeon serum albumin

Figure 7.12

A zonal electrophoresis gel showing the protein content of whole pigeon serum and a commercial source of pigeon serum albumin.



SI - stimulation index
SEM - standard error of the mean
bars represent the mean SI + 1 SEM

Figure 7.13

The proliferative response of PBMC from 4 pigeon breeders and 3 controls incubated with a range of concentrations of pigeon serum albumin.

CHAPTER 8

ANALYSIS OF THE MITOGEN PRESENT IN PIGEON SERUM

8.1 INTRODUCTION

The previous chapter indicated that pigeon serum contained a mitogenic agent. Since in most animal models of EAA an additional inflammatory or mitogenic component is necessary to initiate and maintain disease, it is conceivable that stimulation of lymphocytes by this agent could play some role in the pathogenesis of PBD (Richerson et al, 1971, Richerson, 1972, Joubert et al, 1976, Bernardo et al, 1979, Moore et al, 1975). This chapter attempts to identify this agent and explore its potential importance in PBD.

The first part of this study examines the possibility that endotoxin is the mitogenic agent in pigeon serum. Endotoxin was chosen because of the suggestions that it might play a role in the pathogenesis of other lung diseases, such as byssinosis (Neal et al, 1942, Cinkotia et al, 1977, Castellan et al, 1984, Rylander et al, 1985) and humidifier fever (Rylander et al, 1978, Rylander & Haglind, 1984, Muittari et al, 1980). In addition, Burrel and Rylander (1981) suggested that endotoxin might play a role as an initiator of the acute inflammatory reaction preceding the onset of EAA. The second aim was to determine whether the mitogenic element of pigeon serum could have a role in the pathogenesis of PBD. This was examined by investigating the mitogenicity of pigeon droppings, as they are a more clinically relevant antigen.

8.2 EXPERIMENTAL PROTOCOL

PBMC used in this study were obtained from subjects with severe longstanding PBD, with similar symptoms of PBD to the pigeon breeders used in the previous chapter.

The molecular weight of the mitogenic agent in pigeon serum was established by dialysis and its thermostability determined by heating pigeon serum. Endotoxin levels in pigeon serum were measured in a Limulus lysate assay. To explore the possibility that the mitogenic agent was endotoxin, Polymyxin B, a known inhibitor of endotoxin, and purified LPS were used. Concentrations of Polymyxin B used were based on the principle that one molecule of polymyxin B binds to one monomer unit of LPS (Lipid A) and therefore 3.5μg of polymyxin B should bind to 10μg of LPS (Morrison & Jacobs, 1976). Finally, the proliferative response of PBMC to a protein extract from pigeon droppings (PDE) was examined. All of the cultures were incubated for 6 days unless otherwise stated.

8.3 RESULTS

8.3.1 THE EFFECT OF TEMPERATURE AND DIALYSIS ON THE MITOGENICITY OF PIGEON SERUM

a) **Thermostability**

Two different batches of pigeon serum were heated for one hour at 65°C and then cultured at four different concentrations (775μg, 388μg, 194μg and 97μg per well) with PBMC from 1 control.

The results for one batch of pigeon serum are summarised in Figure 8.1 and indicate that the mitogenicity was not affected by heating to a temperature of 65°C for one hour. Similar results were obtained with the other batch of pigeon serum (data not shown).

b) **Effect of dialysis**

Two different batches of pigeon serum were dialysed against PBS using dialysis tubing with a molecular weight cut off of 10KDa. After dialysis, the pigeon serum was reconcentrated to the original volume and four concentrations of dialysed and non-dialysed samples from each batch of pigeon serum were incubated with PBMC from 1 control.

The results for one of the batches are summarised in Figure 8.2. These show that dialysis had no effect on the mitogenicity of pigeon serum sample and therefore the mitogenic agent is likely to have a molecular weight of more than 10KDa. Similar results were obtained with the other batch (data not shown).

8.3.2 LEVELS OF ENDOTOXIN IN PIGEON SERUM

The levels of endotoxin in 1 sample of pigeon serum were measured in a Limulus lysate assay. Pigeon serum, with a total protein content of 30mg per ml, was found to contain 5pg of endotoxin per ml. Therefore mitogenic amounts of pigeon serum per well (approximately 350μg protein) would contain approximately 0.06pg of endotoxin.

8.3.3 MITOGENIC EFFECTS OF LPS

The aim of this experiment was to establish whether the level of endotoxin found in pigeon serum could have been responsible for its mitogenic effects. PBMC were incubated with the pigeon serum above or with concentrations of commercially obtained LPS, at concentrations covering the quantity present in mitogenic amounts of pigeon serum, (0.015pg to 12pg of LPS per well). In addition, as a positive control, LPS was also used at the higher concentrations which are generally considered to be mitogenic in vitro (0.1μg to 1000μg per well).

Figure 8.3 shows that PBMC from 2 controls did respond to the pigeon serum containing 5pg of endotoxin per ml, described above. However, the results of the proliferative response of PBMC from 1 control, summarised in Tables 8.1 and 8.2 show that PBMC failed to respond to LPS at any of the concentrations used, over a six day period. The other control also failed to respond (data not shown). PWM used as a positive control induced significant stimulation of PBMC (SI 72), indicating that the conditions for the proliferation of lymphocytes were

suitable.

Therefore this experiment failed to determine whether LPS accounts for the mitogenic effects as the positive control for the experiment did not work.

8.3.4 THE EFFECT OF POLYMYXIN B ON THE MITOGENICITY OF PIGEON SERUM

As an alternative approach, I examined whether the mitogenic effect of pigeon serum could be inhibited by Polymyxin B. Polymyxin B, an inhibitor of endotoxin, was added to cultures of PBMC stimulated with 340μg per well of pigeon serum protein per well. Concentrations of Polymyxin B from 0.04μg to 5μg per well were used, which would have been sufficient to inhibit quantities of LPS from 0.12μg to 15μg of LPS per well. As high concentrations of Polymyxin B had been reported to be toxic to macrophages (Stokes et al, 1989), the proliferative response of PBMC to pokeweed mitogen, in the presence of polymyxin B, was used as a control.

The results of these studies using PBMC from 2 subjects with PBD and 1 control are summarised in Figures 8.4 and 8.5. These show that Polymyxin B had no effect on the proliferative response of PBMC from either pigeon breeders or controls cultured with pigeon serum or pokeweed mitogen. Unfortunately, because of the failure to obtain a positive response with LPS, it was not possible to confirm that Polymyxin B would have blocked the mitogenic effect of endotoxin if present in pigeon serum.

8.3.5 THE EFFECT OF A PROTEIN EXTRACT FROM PIGEON DROPPINGS ON PBMC

To determine whether the mitogenic component of pigeon serum is important in the pathogenesis of PBD, this study looked for its presence in pigeon droppings. Pigeon droppings were chosen as the likely medium through which this mitogenic component could be inhaled from the loft environment. This study examined the proliferative response of PBMC from subjects with PBD and controls to pigeon dropping extract.

Protein from 100g of fresh pigeon droppings was extracted in PBS, followed by centrifugation to remove insoluble particles. After dialysis, concentration and filter sterilisation the protein content of the resulting 20mls of protein solution was 23mg per ml. The sample was then run on a 7.5% SDS-Page gel. Figure 8.6b shows that faint bands of protein were seen of molecular weight 116KDa, 60KDa, 45KDa and 35KDa.

To determine the effect of this extract on PBMC, a range of concentrations of this were incubated with PBMC from 2 subjects with PBD and 3 controls. Table 8.3 shows that no response of PBMC was apparent at any of the concentrations of PDE used in this assay. In fact, at some concentrations of PDE, the SI was below 1 and so it is possible that the PDE was toxic to the PBMC. Using pigeon serum as a positive control, significant proliferation of PBMC from the 2 subjects with PBD and the 3 controls was achieved (mean SI of 19±8 and 69±29 respectively).

In a second experiment, I attempted to improve the yield of intact protein obtained from pigeon droppings, by including the enzyme inhibitors, phenylmethylsulfonyl fluoride and pepstatin in the extraction process. However similar yields of protein were recovered (280mg per 100g of pigeon droppings) and as before, four bands of protein with molecular weights of 116KDa, 60KDa, 45KDa and 30KDa were obtained (Figure 8.6a).

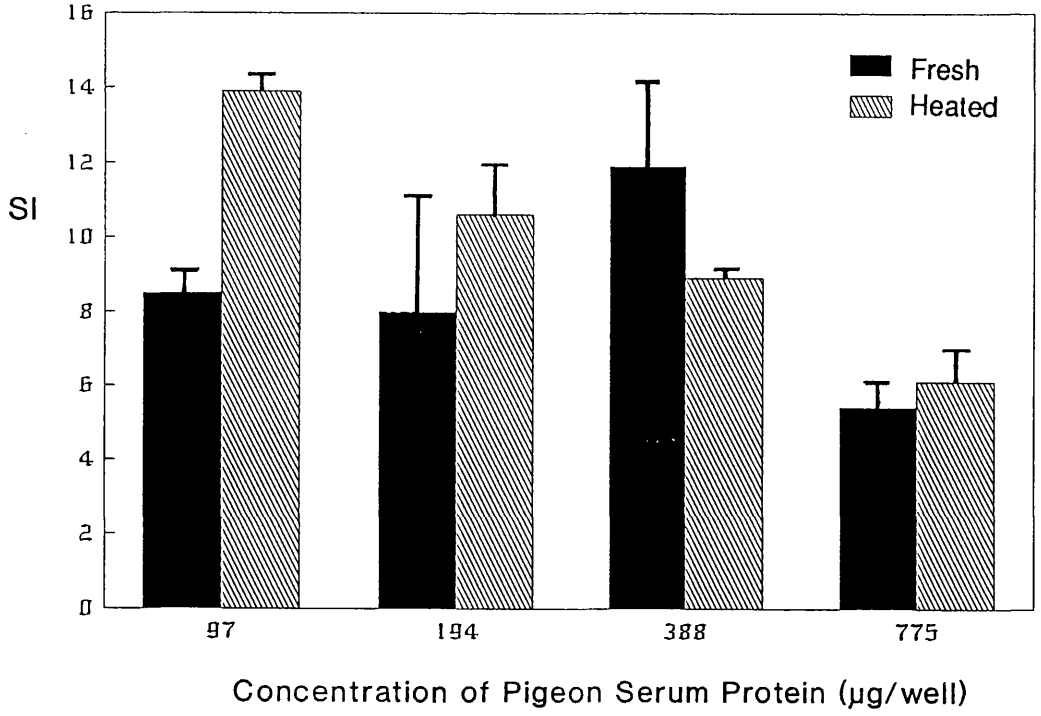
This extract was added to PBMC, from 5 controls, both at concentrations used in the first experiment and at lower dilutions, in an attempt to avoid possible toxic effects. The results in Table 8.4 show that no response to PDE was obtained from PBMC, over the range of concentrations used. Again using pigeon serum as a positive control, the lymphocytes from these subjects responded with a mean SI of 30±6.

8.4 CONCLUSIONS

This study showed that the mitogenic agent in pigeon serum was thermostable at 65^{o}C and was likely to have a molecular weight of more than 10KDa.

Measurement of the level of endotoxin in pigeon serum in a Limulus Lysate assay revealed that it contained 5pg of endotoxin per ml. However the study failed to determine whether the mitogenic agent was endotoxin.

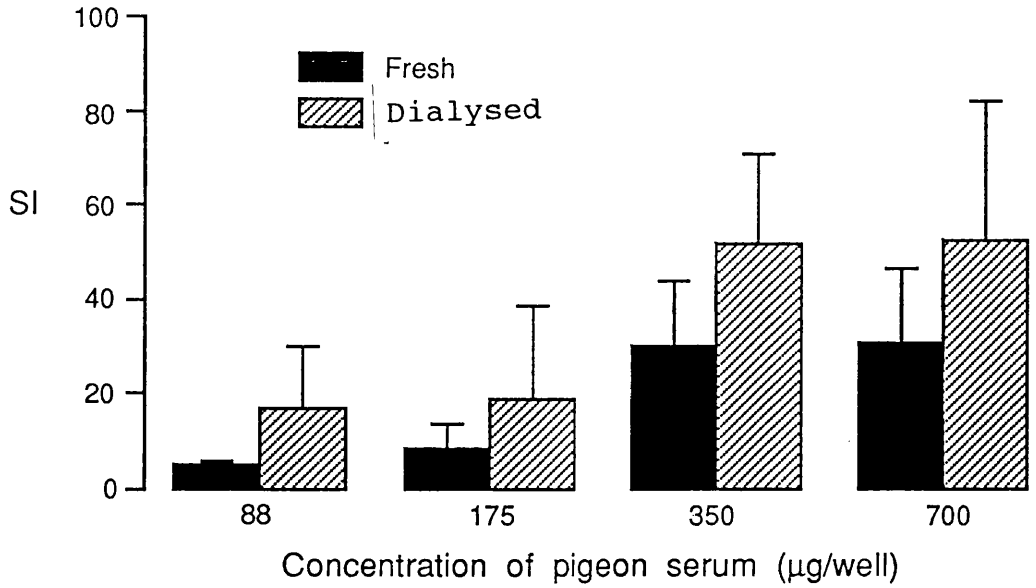
Analysis of the protein content of pigeon droppings revealed that it contained four main proteins, of molecular weight 116KDa, 60KDa, 45KDa and 35KDa. The soluble constituents of pigeon droppings were neither antigenic or mitogenic to PBMC and therefore the mitogenic agent of pigeon serum is either not a constituent of pigeon droppings or is not present in sufficient amounts to be mitogenic in vitro. It remains to be determined if the mitogenic agent is present in another pigeon material.



SI - stimulation index
SEM - standard error of the mean
bars represent the mean SI + 1 SEM

FIGURE 8.1

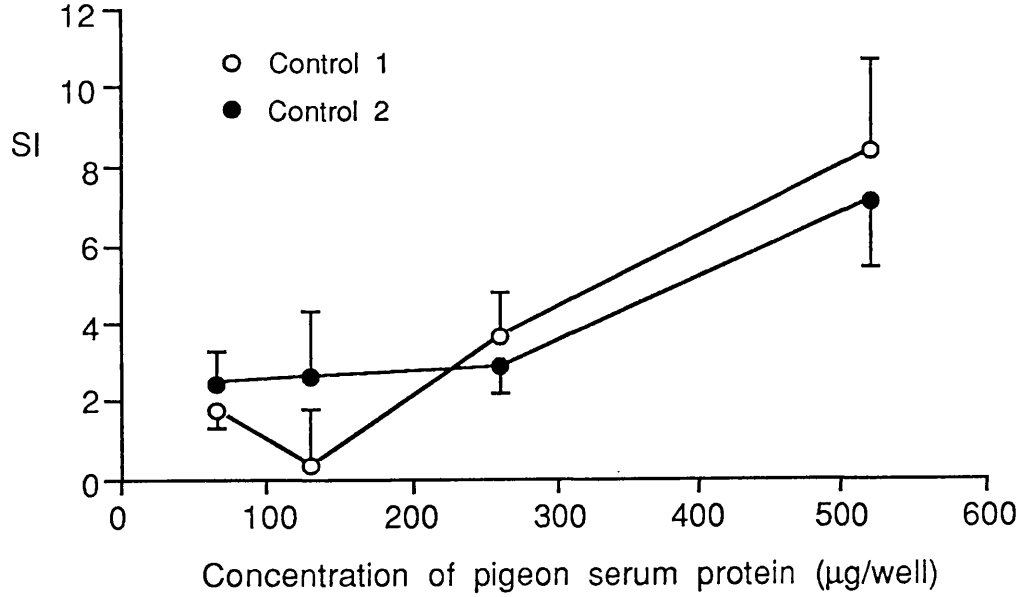
The proliferative response of lymphocytes from 1 control incubated with a range of concentrations of "fresh" and "heated" (65^{o}C) pigeon serum.



SI - stimulation index
SEM - standard error of the mean
bars represent the mean SI + 1 SEM

FIGURE 8.2

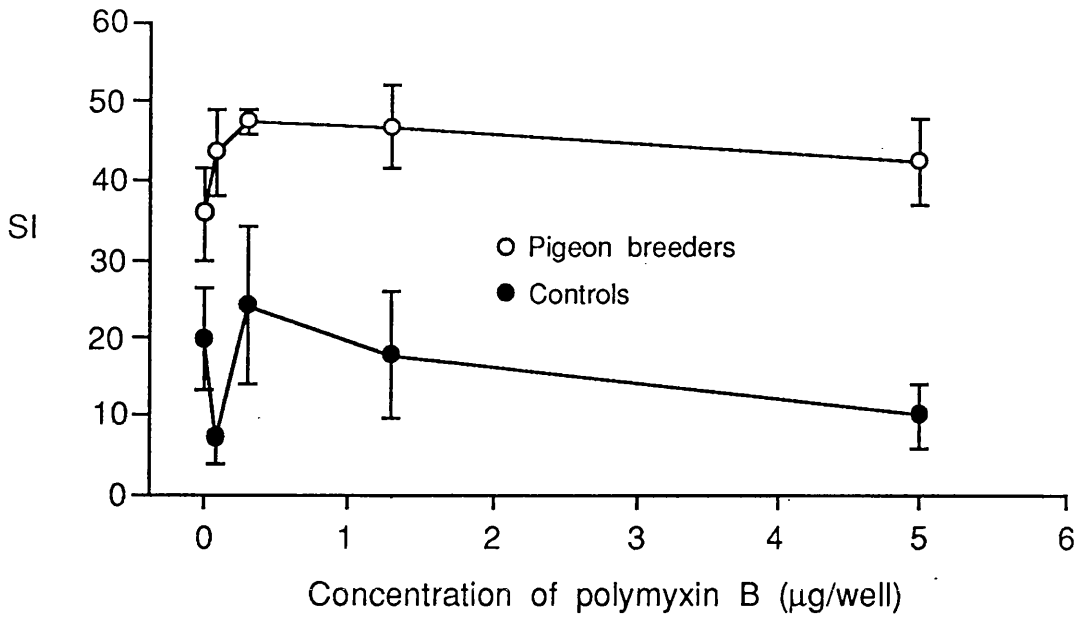
The proliferative response of lymphocytes from 1 control incubated with a range of concentrations of "fresh" and "dialysed" pigeon serum.



SI - stimulation index
SEM - standard error of the mean
points represent the mean SI + 1 SEM

FIGURE 8.3

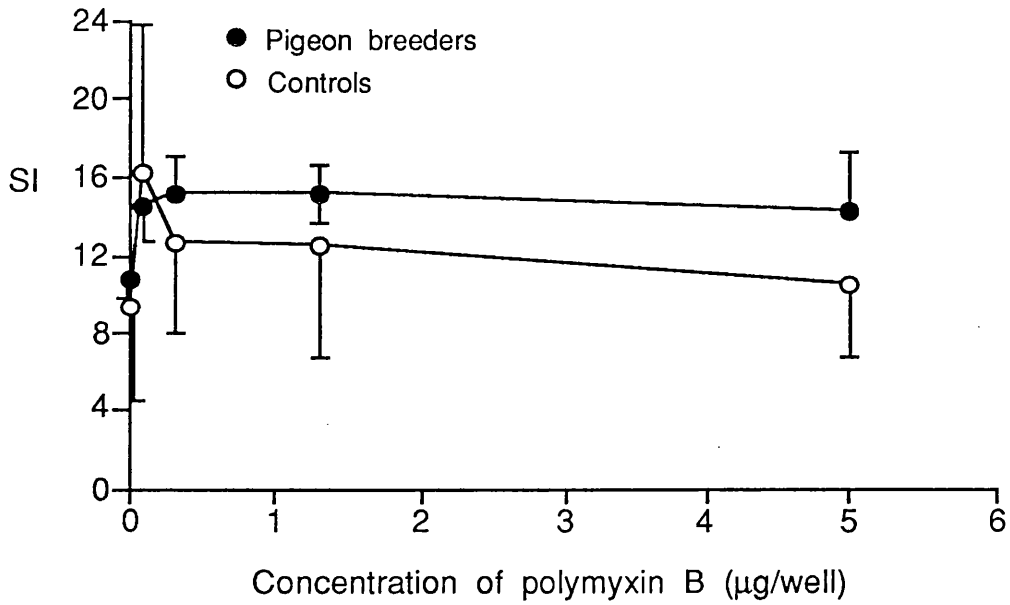
The proliferative response of lymphocytes from 2 controls incubated with pigeon serum containing 5pg of endotoxin per ml



SI - stimulation index
SEM - standard error of the mean
points represent the mean SI + 1 SEM

FIGURE 8.4

The effect of a range of concentrations of Polymyxin B on the proliferative response of lymphocytes from 2 subjects with PBD and 1 control incubated with pigeon serum



SI - stimulation index
SEM - standard error of the mean
points represent the mean SI + 1 SEM

FIGURE 8.5

The effect of a range of concentrations of Polymyxin B on the proliferative response of lymphocytes from 2 subjects with PBD and 1 control incubated with pokeweed mitogen.

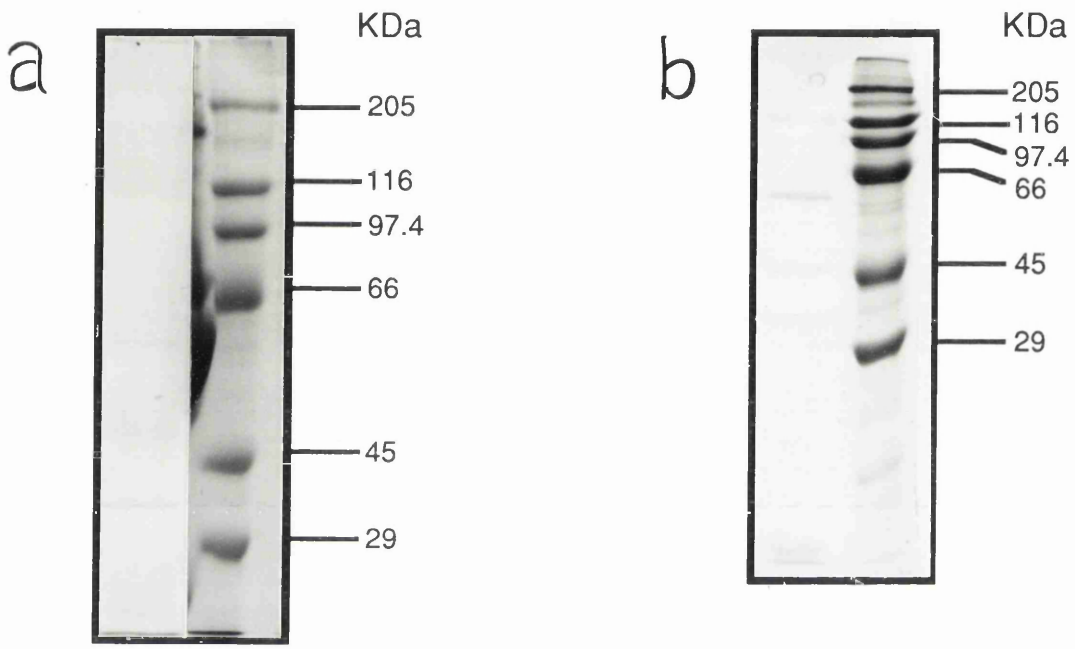


FIGURE 8.6 a/b

7% SDS polyacrylamide gel. Lanes contain (from left to right) whole PDE, markers, whole PDE, markers. Gel stained with PAGE blue. Anode is at the bottom.

Incubation time	pg LPS/well 12	1	0.25	0.06	0.015
1 day	1.2±0.1	1.2±0.1	2.0±0.1	1.6±0.1	1.8±0.4
2 days	1.8±0.5	0.8±0.1	1.8±0.1	0.6±0.1	0.7±0.1
3 days	1.2±0.2	0.6±0.1	2.1±0.2	1.4±0.2	2.2±0.3
4 days	0.9±0.2	1.7±0.1	0.8±0.2	1.5±0.1	0.9±0.1
6 days	-	1.3±0.2	1.8±0.3	1.7±0.8	1.6±0.5

SI - stimulation index
SEM - standard error of the mean
- - not done
figures are the mean SI ± 1 SEM

TABLE 8.1

Time course of the proliferative response of lymphocytes from 1 control incubated with a range of concentrations of LPS.

Incubation time	µg LPS/well					
	1000	100	25	10	1.2	0.1
1 day	1.3±0.4	1.7±0.1	1.3±0.1	1.7±0.3	1.0±0.1	1.0±0.2
2 days	1.0±0.4	1.3±0.2	0.8±0.1	1.3±0.1	2.1±0.9	1.0±0.3
3 days	1.1±0.1	0.5±0.7	0.8±0.1	0.7±0.1	1.5±0.1	0.4±0.1
4 days	0.6±0.1	0.9±0.3	0.8±0.03	1.7±0.2	0.7±0.1	1.5±0.1
6 days	0.5±0.03	0.9±0.4	0.8±0.2	0.5±0.1	1.1±0.2	1.4±0.2

SI - stimulation index
SEM - standard error of the mean
figures are the mean SI ± 1 SEM

TABLE 8.2

Time course of the proliferative response of lymphocytes from 1 control incubated with a range of concentrations of LPS.

	µg PDE/well							
	2	5	10	21	42	85	170	340
pigeon breeder	1.6±0.9	1.1±0.9	0.8±0.4	1.1±0.8	0.6±0.5	0.9±0.9	2.1±0.1	0.9±0.9
controls	0.5±0.1	1.0±0.1	0.7±0.2	0.3±0.02	0.5±0.4	0.6±0.5	0.3±0.1	0.1±0.1

SI - stimulation index
SEM - standard error of the mean
figures are the mean SI ± 1 SEM

TABLE 8.3

The proliferative response of lymphocytes from 2 subjects with PBD and 3 controls incubated with a range of concentrations of PDE.

	µg PDE/well							
	0.005	0.011	0.022	0.045	0.09	0.19	0.38	0.75
Incubation time								
6 days	1.4±0.1	2.0±04	1.0±0.4	1.3±0.1	1.3±0.3	1.2±0.1	0.9±0.2	0.7±0.1

	µg PDE/well							
	1.5	3	6	12	25	50	100	200
Incubation time								
6 days	0.7±0.2	0.7±0.1	0.6±0.1	0.5±0.05	0.6±0.1	0.7±0.1	0.4±0.1	0.3±0.07

SI - stimulation index
SEM - standard error of the mean
figures are the mean SI ± 1 SEM

TABLE 8.4

The proliferative response of lymphocytes from 5 controls incubated with a range of concentrations of PDE.

CHAPTER 9

GENERAL DISCUSSION

Group 1 pigeon breeders differed from the other pigeon breeders in that they were taking low dose inhaled steroids. The effect of low dose inhaled steroids on the phenotypes of peripheral blood cells is likely to be minimal but has not been formally studied. As a result, there is a slight possibility that the changes in the phenotypes of peripheral blood cells, such as increased percentages of $CD45RA^{+}$ cells and decreased percentages of $\gamma\delta$ T cells, found in group 1 pigeon breeders, may be a steroid effect. Future studies should look at a similar group of pigeon breeders who are not taking steroids.

The initial view of EAA was that it was an immune complex mediated disease, involving a Coombs and Gell (1963) Type III hypersensitivity reaction. However, it became apparent that many pigeon breeders had specific serum antibody, without clinical symptoms of disease. Furthermore, histopathological studies revealed that lymphocytic cellular infiltrates and granuloma formation occurred in the lungs. These observations prompted studies to explore the hypothesis that cell mediated immunity has a role in the pathogenesis of EAA.

Previous studies designed to explore the role of cell mediated immunity in the pathogenesis of EAA have assessed the phenotype or function of cells in small heterogeneous subject groups, often with different syndromes of EAA. As a result, the findings of these studies were variable and inconclusive. The aim of this thesis was to use PBD as a model of EAA and to perform phenotypic and functional studies of well defined categories of patients.

Peripheral blood was used in these studies as its ready availability enabled a larger population of pigeon breeders to be studied.

9.1 PERIPHERAL BLOOD LEUCOCYTE PHENOTYPES AMONG PIGEON BREEDERS

Chapter 3 examined whether there were alterations in total and differential WBCC and T and B cell numbers which correlated with disease.

Increased WBCC were recorded in the peripheral blood of symptomatic antibody positive pigeon breeders who were acutely symptomatic at the time of blood sampling (group 2). This was reflected in increased numbers of neutrophils, total lymphocytes and $CD4^+$ and $CD8^+$ cells. B cell numbers were unchanged. However, Group 3 pigeon breeders with similar symptoms, but who were not acutely symptomatic at the time of blood sampling, had normal WBCC. These results suggest that acute exacerbations of PBD lead to a transient leucocytosis. This non-specific consequence of acute inflammation has been reported previously in symptomatic subjects with PBD, who had similar symptoms of fever and dyspnoea, at the time of blood sampling (Reed et al, 1965). In the lung, a transient polymorphonuclear cell infiltrate follows inhalation challenge (within 24 hours) (Fournier et al, 1985) which is followed several days later by a lymphocytic alveolitis (Costabel et al, 1984, Leatherman et al, 1984, Agnostini et al, 1984, Costabel et al, 1985, Semenzato et al, 1986). A leucocytosis is also found in the lung, within 24 hours of inhalation challenge with pigeon serum, in animal models of EAA. This consists of an early accumulation of neutrophils (Bernardo et al, 1979), followed by a mononuclear cell infiltrate 48 to 96 hours later (Bernardo et al, 1979). Therefore it is tempting to speculate that the increased numbers of leucocytes found in peripheral blood of subjects in group 2 are being recruited to the lung. This could be tested by analysing cells from the peripheral blood and BAL in parallel, at a selected time interval after inhalation

challenge. However, given the invasive nature of BAL, it is unlikely that repeated BAL sampling during the course of an acute symptomatic episode would be permitted and therefore repeated blood sampling would be the only way to monitor cellular changes during a longitudinal time course study.

Symptomatic pigeon breeders without serum antibody to pigeon antigen also had significantly increased WBCC compared with control subjects. An increased WBCC in these individuals fits with the hypothesis that these subjects may have Organic Dust Toxic Syndrome, since increased WBCC have been recorded in subjects with this disease (May et al, 1986, doPico, 1986). Organic Dust Toxic Syndrome, presents with similar symptoms to PBD (eg, fever, myalgia, chest tightness etc) several hours after exposure to organic dusts (doPico, 1986). However, it differs from EAA in that it is not associated with precipitating antibody and the pulmonary function tests and chest radiographic appearances of affected subjects, are usually normal. Potential etiological agents are dusts containing mould and bacteria, endotoxins, or other unidentified agents. However a complicating factor in my group of patients is that they were all smokers. As smoking has an inhibitory effect on the antibody response to inhaled antigens (Boyd et al, 1977, McSharry et al, 1985), this could have inhibited the antibody response in my subjects. A population of non-smoking symptomatic, antibody negative pigeon breeders would have been useful in my study. However it is important to emphasise that the increased WBCC count which I found was not itself

due to the increase seen in smokers (Miller et al, 1982, Robertson et al, 1983), as although all PBD subjects with leucocytosis were smokers, 7/8 of the asymptomatic antibody negative pigeon breeders (group 7) were also smokers and yet they did not have significantly increased WBCC.

Two groups of antibody positive pigeon breeders with severe longstanding disease who had restrictive PFT's and typical chest radiographic appearances had evidence of reversed CD4:CD8 ratios compared with controls. This was due to increased numbers of $CD8^+$ cells. Although the difference was not statistically significant in individual groups, similar findings were made in both studies suggesting that this is a genuine abnormality in at least a proportion of subjects with PBD. The variability in the CD4:CD8 ratios in subjects within the same group raises the question of the clinical significance of a reversed CD4:CD8 ratio in individual patients. One study showed that reversed CD4:CD8 ratios in peripheral blood and BAL fluid were a characteristic of symptomatic pigeon breeders with recent antigen exposure, while those who were studied more than 5 days after their last antigen exposure had normal CD4:CD8 ratios (Costabel et al, 1984). This would suggest that the length of time between blood sampling and the last exposure to antigen may affect the cellular profile, a factor which was not controlled for in my subjects with severe longstanding PBD. It is possible that the generally less severe disease exhibited by group 2 pigeon

breeders who were acutely symptomatic at the time of blood sampling may explain why they did not also have reversed CD4:CD8 ratios. A recent study by Reynolds et al (1989) showed that only subjects with chronic PBD had reduced CD4:CD8 ratios, while those with acute PBD showed no difference. Therefore it is possible that some of my subjects with severe longstanding PBD, have a mixed form of acute or chronic and acute PBD, similar to the subacute PBD, defined by Fink (1983). It would be important to analyse peripheral blood cells in distinct groups of pigeon breeders with acute, chronic and subacute forms of the disease.

Reduced CD4:CD8 ratios have previously been reported in BAL fluid of subjects with EAA, (Costabel et al, 1984, Leatherman et al, 1984, Agnostini et al, 1984, Costabel et al, 1985, Semenzato et al, 1986). However the results of studies analysing the levels of CD4 and CD8 cells in the peripheral blood have been conflicting. Two studies found reduced CD4:CD8 ratios in symptomatic subjects (Costabel et al, 1984, Costabel et al, 1985), while others found no abnormalities (Agostini et al, 1984, Leatherman et al, 1984, Semenzato et al, 1986). These conflicting results may reflect the fact that they did not distinguish between subjects with various syndromes of EAA and that the time between blood sampling and the last exposure to antigen was not controlled for in these studies.

Studies that have looked at the levels of CD4 and CD8 cells in asymptomatic subjects have also reported

similar reversed CD4:CD8 cells ratios in BAL fluid (Leatherman et al, 1984, Semenzato et all, 1986) but not in the peripheral blood (Leatherman et al, 1984, Semenzato et al, 1986). This is consistent with my own study, which found no difference in the levels of CD4 and CD8 cells in the peripheral blood of asymptomatic, antibody negative pigeon breeders (group 6). However, an increased proportion of asymptomatic, antibody positive subjects (group 4) had high CD4:CD8 ratios (>2.5) in their peripheral blood compared with controls. This was due mainly to increased numbers of $CD4^+$ cells in these subjects. Increased numbers of $CD4^+$ cells might be responsible for the presence of an active humoral immune response in these subjects. This could be tested by determining the functional activity of these cells in an in vitro antibody production assay. It is not clear whether these asymptomatic pigeon breeders have had or will develop clinical symptoms of disease. However, the pattern of no pathology, but high antibody is similar to that described in animal models of EAA, in which repeated inhalation challenge with antigen, fails to induce chronic EAA and any acute alveolitic lesions disappear (Richerson et al, 1978, Ratajczak et al, 1980, Richerson et al, 1981). This clearing of disease (waning) is antigen specific, but is not associated with decreased antibody responses or decreased antigen lymphocyte blastogenesis (Richerson et al, 1981, Ratajczak et al, 1980). Therefore it is possible that some of these asymptomatic antibody positive subjects have the equivalent of waning disease. However, others may be at

an early subclinical stage of disease and may still progress to develop clinical symptoms. A longitudinal time course study on these asymptomatic antibody positive pigeon breeders involving the collection of peripheral blood at defined intervals, together with clinical assessment of disease would be useful in resolving this question.

The level of specific antibody did not correlate with any of the cellular changes mentioned above. However, symptomatic antibody positive pigeon breeders who were acutely symptomatic at the time of blood sampling (group 2) had significantly higher antibody levels than equivalent symptomatic patients who were not acutely symptomatic (group 3) or asymptomatic antibody positive pigeon breeders (group 4). In general, symptomatic pigeon breeders have higher antibody titres than their asymptomatic counterparts (Boyd 1978, McSharry et al, 1984), which would explain the difference in antibody titres between symptomatic and asymptomatic pigeon breeders. The difference between group 2 and group 3 subjects may reflect the fact that group 2 subjects are more likely to have a symptomatic episode (Boyd, 1978).

Taken together, these initial results suggested that there are changes in peripheral blood leucocytes associated with some groups of pigeon breeders depending on their symptoms. In addition, these results reveal that even within a group of symptomatic antibody positive pigeon breeders, there can be differences in their peripheral blood leucocyte profiles which may reflect

differences in the stage of disease. My results also suggest that factors such as the symptomatic state at the time of blood sampling and the time between blood sampling and the last exposure to pigeon antigen, may affect the phenotypes of cells in the peripheral blood. Therefore future studies should attempt to monitor cellular changes at frequent intervals after antigen challenge in subjects within a given syndrome of EAA at a defined stage of disease in both BAL fluid and in the peripheral blood. The results of my study show that despite some positive changes, there are few significant differences in the phenotypes of peripheral blood leucocytes in any of the groups of pigeon breeders and therefore peripheral blood T cell analysis is of limited clinical value in PBD.

9.2 ANALYSIS OF NK CELLS IN PIGEON BREEDERS

Levels of NK cells were analysed in the peripheral blood of pigeon breeders to determine the extent to which non-specific immunity may play a role in the pathogenesis of PBD. In addition, given that the proliferation of these cells is sensitive to IL2 and IFNγ (Anegon et al, 1988), their levels may also be indicative of the level of the immune response. It is also possible that these cells may play a role in the pathogenesis of PBD, as the production of IFNγ and TNFα by activated NK cells has been implicated to play a role in the pathogenic process of chronic inflammation in rheumatoid arthritis (Hendrich et al, 1991) and in the destructive phase of graft versus host disease (Mowat & Felstein, 1987).

Levels of $CD56^+$ cells were analysed in the peripheral blood as a means of assessing NK cell numbers. CD56 (NCAM1), is expressed by virtually all human NK cells (Griffin et al, 1983, Hercend et al, 1985) and by a small proportion of "non MHC restricted T cells" (Lanier et al, 1986a, Schmidt et al, 1986). Thus, true NK cells can be defined phenotypically as $CD56^+CD3^-$ lymphocytes (Robertson & Ritz, 1990). In my initial study, double staining was not used to detect the expression of CD3 on $CD56^+$ cells and therefore the findings served simply to highlight any possible differences which could be analysed in more detail later.

This analysis revealed increased absolute numbers of $CD56^+$ cells in symptomatic antibody positive pigeon breeders who were acutely symptomatic at the time of blood sampling compared with controls. This was not present in those patients who were not acutely symptomatic at the time of blood sampling and so this feature paralleled the presence or absence of leucocytosis in these groups.

The two groups of symptomatic antibody positive pigeon breeders with severe longstanding PBD, were also found to have increased absolute numbers of $CD56^+$ cells in their peripheral blood compared with their controls. However, the difference was only statistically significant in one of the groups. The inconsistencies in the two sets of results may reflect sampling variation, which reaffirms the previous findings of variation in the CD4:CD8 ratios

in individual pigeon breeders with severe longstanding PBD. Again, this raises the question of the clinical significance of raised levels of $CD56^+$ cells in individual pigeon breeders. Levels of $CD8^+$ cells did not correlate with levels of $CD56^+$ cells and therefore $CD8^+$ and $CD56^+$ cells may be playing separate roles in the pathogenesis of PBD.

Normal levels of $CD56^+$ cells were detected in the peripheral blood of the other four groups of pigeon breeders, indicating that $CD56^+$ cells appeared to be specifically raised only in subjects with severe longstanding PBD. Thus, it is possible that these lymphocytes may play a role in the pathogenesis of PBD.

To determine whether the raised levels of $CD56^+$ cells were true NK cells, two-colour flow cytometry was used to differentiate true $CD3^-CD56^+$ T cells from $CD3^+CD56^+$ T cells in the additional group of symptomatic subjects with severe longstanding PBD. This revealed no statistically significant differences in the percentages of either $CD3^+CD56^+$ T cells or $CD3^-CD56^+$ NK cells in pigeon breeders, although there was a slightly increased absolute number of $CD3^-CD56^+$ NK cells. This suggests that some of the pigeon breeders within this group have increased numbers of $CD56^+$ NK cells. Analysis of the expression of CD8 on the $CD56^+$ cells in these subjects revealed no difference in the percentages or absolute numbers of $CD8^+CD56^+$ or $CD8^-CD56^+$ cells. Despite the rise in the level of NK cells in some of these pigeon breeders, this was not associated with increased

cytotoxicity of these cells against K562 target cells in vitro. This would suggest therefore that if NK cells play a role in the pathogenesis of PBD, then it may not involve their natural cytotoxic capabilities.

Additional analysis to determine the expression of CD16 on cells in the peripheral blood of these subjects (groups 8 and 9) was also carried out. CD16 is the $Fc\gamma_{III}$ receptor and is thought to be solely responsible for the antibody dependent cellular cytotoxicity of NK cells (Perussia et al, 1984, Trinchieri et al ,1984). This analysis revealed increased absolute numbers and percentages of $CD16^+$ cells in pigeon breeders (group 8) compared with controls (group 9). However, due to the wide range of values in both groups, the differences were not statistically significant. The increase in the percentage of these cells in the symptomatic antibody positive pigeon breeders was due to $CD3^-CD16^+$ cells and therefore NK cells. These were also shown to be predominantly $CD8^-$ cells and there was no correlation between the numbers of $CD16^+$ cells and $CD8^+$ cells in individual pigeon breeders. It is possible that $CD16^+$ NK cells could become activated in PBD by interacting with specific IgG antibody bound to pigeon antigen associated with the surface of a cell in the lung. Activation of these cells could result in release of various cytokines such as IFNγ and TNFα (Anegon et al, 1988), which could be involved in the chronic inflammatory response in the lung. Another possibility is that these cells mediate antibody dependent cellular cytotoxicity (Perussia et al, 1984). Future studies could assess the functional

activity of these cells in an ADCC assay. Indeed this could be done using pigeon erythrocytes as targets as they express antigen on their surface which is recognised by IgG from antibody positive pigeon breeders.

I did not examine NK cells in the lung tissue itself. However, it has been shown that increased levels of $CD57^+$ cells in the lung are mirrored by an increase in the levels of these cells in the peripheral blood (Semenzato et al, 1986). If the same phenomenon is also true of NK cells in general then my study suggests that pigeon breeders with severe longstanding PBD have increased levels of $CD16^+$ NK cells and $CD56^+$ NK cells in their lungs, although this would contradict the findings of a number of studies which found normal levels of $CD16^+$ cells in BAL fluid of subjects with EAA (Agostini et al, 1984, Semenzato et al, 1986, Semenzato et al, 1988). However, it should be noted that BAL fluid is inappropriate for assessing $CD16^+$ cells in the lung, as these cells are largely confined to the lung interstitium itself (Weissler et al, 1987). This emphasises the need to look at all areas of the lung directly.

9.3 $\gamma\delta$ T CELLS IN PIGEON BREEDERS

In view of the evidence that $\gamma\delta$ T cells might play a role in chronic inflammatory diseases of the lung such as sarcoidosis (Balbi et al, 1990) and Farmer's lung (Trentin et al, 1990) and due to their proposed role in the protection of epithelial surfaces (Janeway et al, 1988, Janeway, 1988, Raulet, 1989), their possible

involvement in PBD was investigated in subjects with severe longstanding PBD (group 8).

Pigeon breeders with severe longstanding PBD were found to have significantly lower percentages of $\gamma\delta$ T cells in their peripheral blood compared with controls (group 9). This result contrasts with reports of increased levels of $\gamma\delta$ T cells in the lungs of farmers with EAA (Trentin et al, 1990), perhaps indicating that the lower numbers of $\gamma\delta$ T cells in the blood are secondary to an accumulation of these cells in the lung. Although this needs parallel examination of peripheral blood and BAL samples, it should be noted that, in sarcoidosis, increased levels of $\gamma\delta$ T cells are found both in the peripheral blood and in the lung (Balbi et al, 1990).

I attempted to examine, indirectly, the possible relationship between peripheral blood and lung $\gamma\delta$ T cells by looking for expression of markers which have been associated with $\gamma\delta$ T cells in mucosal tissues. $\gamma\delta$ T cells in the gut of humans are predominately $CD8^-$ (Halstensen et al, 1989), but my study showed that percentages of both $CD8^+$ and $CD8^-$ $\gamma\delta$ T cells were reduced in the peripheral blood of subjects with severe longstanding PBD. Thus if the reduction in peripheral blood $\gamma\delta$ T cells does reflect recruitment into the lung, then both $CD8^+$ and $CD8^-$ $\gamma\delta$ T cells may be involved in PBD. In coeliac disease, the increased numbers of $\gamma\delta$ intraepithelial lymphocytes are predominantly $CD8^-$ (Halstensen et al, 1989). However, the significance of these findings is unclear, as the functional capabilities of $CD8^+$ and $CD8^-$

$\gamma\delta$ T cells are not known.

A second marker δTCS-1, is found on a small number of peripheral blood $\gamma\delta$ T cells (Bottino et al, 1988) but on most gut $\gamma\delta$ T cells (Spencer et al, 1989). In my study, both subjects with PBD and controls had very low levels of δTCS-1$^+$ T cells and I was unable to discern any difference in these subsets between the groups. These results are consistent with the possibility that the decreased number of $\gamma\delta$ T cells in PBD, is due to loss of the other subset which expresses the disulphide-linked form of the $\gamma\delta$ TCR and which accounts for more than 90% of all $\gamma\delta$ T cells in the peripheral blood (Bottino et al, 1988). It is possible that the δTCS1$^-$ cells are leaving the peripheral blood, perhaps as a result of recruitment of these cells into the lung. Similar results have been reported in sarcoidosis, where the number of δTCS-1$^+$ in the peripheral blood and the lung remained the same while the total number of $\gamma\delta$ T cells increased (Balbi et al, 1990).

Therefore, future studies should look at peripheral blood in parallel with BAL using MAb to detect both the disulphide linked (BB3) and nondisulphide forms (δTCS1) of the $\gamma\delta$ T cell receptor. In addition, future studies could use experimental models of lung inflammation to see whether increased levels of these cells in the lung are associated with lung pathology.

9.4 "ACTIVATED" AND/OR "MEMORY" CELLS IN PBD

In an attempt to determine the extent to which a specific immune response is involved in the pathogenesis of PBD, I examined the levels of "naive" or "memory" T cells in the peripheral blood of subjects with severe longstanding PBD compared to controls. Double staining immunofluoresence was used to phenotype $CD4^+$ cells into "naive" and "memory" cell populations based on their respective expression of CD45RA and CDw29.

This study revealed increased percentages of $CD45RA^+$ cells in the peripheral blood of subjects with PBD compared with controls. This was due mainly to an increased percentage of $CD45R^+CD4^-$ cells. In contrast, similar percentages of $CDw29^+$ and $CDw29^+CD4^+$ cells were found. CDw29 (VLA ß) (Sanders et al, 1988b), has been used to identify a population of T cells similar to that defined by CD45RO (UCHL1) (Beverly, 1987). Although its expression does not overlap exactly with that of CD45RO, CDw29 is present on a population of $CD4^+$ cells roughly reciprocal to that of CD45RA (Morimoto et al 1985b). Thus my results indicate that subjects with PBD have a normal proportion of "memory" $CD4^+$ T cells in their peripheral blood. It is interesting that the rise in the level of $CD45RA^+$ cells was not accompanied by a decrease in the level of $CDw29^+$ cells given the roughly reciprocal expression of these markers (Morimoto et al, 1985b). This suggests that there is an increase in $CD45RA^+CDw29^+$ cells in these pigeon breeders. $CD45RA^+CDw29^+$ cells may represent newly activated cells.

It is not clear whether the $CD45RA^{+}CD4^{-}$ cells are "naive" or "memory" cells, since there is only limited evidence to suggest that expression of CD45 isoforms on other cells has the same functional relevance (Merkenschlager et al, 1989). These $CD45RA^{+}CD4^{-}$ cells could be $CD8^{+}$ T cells or NK cells, which were shown to be increased in earlier studies (chapters 3 and 4). It is unlikely that these $CD45RA^{+}$ cells are $\gamma\delta$ T cells or B cells, given the low levels of these cells in the peripheral blood, although it is possible that $CD8^{+}$ T cells NK cells, B cells and $\gamma\delta$ T cells all contribute. Therefore, future studies should use double staining immunofluoresence using MAb's to CD45RA in conjunction with CD8 and MAb's to NK cell markers such as CD56 and CD16 to determine the nature of these $CD45RA^{+}CD4^{-}$ cells. In addition, analysis of the levels of $CD45RO^{+}$ cells (UCHL1) in these subjects would also provide a useful comparison to the levels of "memory" cells detected by CDw29.

The finding of increased percentages of $CD45RA^{+}$ cells in the peripheral blood of subjects with PBD contrasts with the decreased proportion found in other chronic inflammatory conditions, such as rheumatoid arthritis (Emery et al, 1987) and multiple sclerosis (Rose et al, 1985, Morimoto et al, 1987). In addition, increased levels of the reciprocal population of $UCHL1^{+}$ T cells were found in the lungs of both symptomatic and asymptomatic pigeon breeders by Johnson et al (1989). Again it would be important to determine whether

increased levels of $CD45RA^+$ cells in the peripheral blood is reflective of an increase or decrease in the level of these cells in the lung. There is some evidence to suggest that levels of these cells in the lung do not reflect their respective levels in the peripheral blood. Barnes et al (1989) found increased levels of $CD4^+CDw29^+$ cells and normal levels of $CD4^+CD45RA^+$ cells in the pleural fluid of subjects with tuberculous pleuritis but found no change in the levels of these cells in the peripheral blood.

9.5 FUNCTIONAL ACTIVITY OF PERIPHERAL BLOOD LYMPHOCYTES IN PBD

The results discussed above have shown that there are changes in the the phenotypes of peripheral blood lymphocytes in pigeon breeders with severe longstanding PBD compared with controls, which suggests that cell mediated immunity may play a role in the pathogenesis of PBD. However, these experiments did not study the stimulus for these changes and the remainder of my thesis attempted to investigate whether antigens extracted from pigeon material could provoke cell mediated immune responses by lymphocytes from pigeon breeders in vitro.

The presence of specific IgG and IgA antibody to pigeon antigen in symptomatic and asymptomatic pigeon breeders (Patterson et al, 1976a,b), is consistent with the presence of antigen-reactive T_H cells in the peripheral blood of antibody positive pigeon breeders. However, antibody levels do not correlate with disease,

suggesting a possible role of cell mediated immunity perhaps directed at the same or related antigens. In my first experiments I used crude pigeon serum as a source of antigen, since pigeon breeders have antibodies to several antigenic constituents of this (Barboriak et al, 1965, Reed et al, 1965) and it is the most commonly used source of pigeon antigen. In my hands, pigeon serum stimulated lymphocytes from subjects with PBD and controls. This mitogenic activity of pigeon serum has not previously been reported, possibly because earlier studies either used concentrations of pigeon serum protein much lower than those found to have optimal mitogenic activity in my study (Hansen & Penny, 1974, Moore et al, 1980, Schatz et al, 1976) or did not correct their counts for background stimulation of the cells in culture without pigeon serum, therefore making their results inconclusive (Girard et al, 1978).

In my study 350μg of pigeon serum protein per well (100μl) was optimal for lymphocyte proliferation. However, given that a recent study showed that dust collected from one cubic metre of a pigeon loft contained on average, only tens of ng of pigeon antigen (Anderson et al, 1991), it is likely that the sensitising dose of antigen required in vivo, is much less than that required for in vitro sensitisation in my study .

Because of the mitogenic effect in controls, I attempted to use purified antigen to look for specific cell mediated immunity. As there were no previous reports using purified antigens in this system, I selected pigeon

globulin and albumin, as these contain several antigenic components found in a variety of pigeon derived material including pigeon serum, pigeon droppings and crop and saliva fluid (Edwards et al, 1970). My study used pigeon serum as a source of these antigens, as it was readily prepared and had a higher protein content than pigeon droppings.

To explore the possibility that the mitogenic activity of pigeon serum was masking an underlying antigenic response, pigeon serum was fractionated into a "globulin" and an "albumin rich" fraction, using a 33% ammonium sulphate precipitation step. The fraction which was predominantly globulin, failed to induce a proliferative response in lymphocytes from either pigeon breeders or controls. In contrast, the fraction containing the other serum proteins, of which albumin was one of the main components, was found to possess significant mitogenicity, although with significantly reduced activity, compared with the original serum sample. In order to determine whether this mitogenicity was masking a specific response of lymphocytes to pigeon serum albumin, the other main antigen involved in a humoral immune response, I obtained a commercial source of relatively pure pigeon serum albumin. This was shown to have neither antigenic nor mitogenic activity.

Although this study was unable to identify any specific response of lymphocytes from pigeon breeders to pigeon serum antigens, the presence of T dependent antibody responses to pigeon antigen (Patterson et al, 1976a,b) in

pigeon breeders indicates the presence of antigen specific T cells. It is possible that these cells are compartmentalised in the lung and would therefore not be detected in sampling of peripheral blood. To explore this hypothesis, lymphocytes from BAL fluid could be incubated with pigeon serum or purified antigens from pigeon serum. Another explanation could be that the T cells which responded to pigeon antigen have been tolerised. Holt & McMenamin (1989) presented evidence to suggest a distinct hierarchy in the immune response to aero-allergens in relation to relative susceptibility to tolerance induction via low grade inhalation exposure: IgE>DTH>IgG>IgA. A similar hierarchy of tolerance susceptibility has been suggested in oral tolerance (Mowat 1987). This would also explain the lack of specific IgE antibody to pigeon antigen in pigeon breeders. It is possible that T cells are initially required to initiate the inflammatory process and induce the production of specific IgG antibody to pigeon antigens. Many of the T cells may then become tolerised, leaving non-specific immune responses, such as NK cells to perpetuate the inflammatory process, perhaps through their release of IFNγ and TNFα.

Given the proposed role for a mitogen in the pathogenesis of EAA as an initiator of an acute inflammatory reaction preceding the onset of EAA (Burrel & Rylander, 1981), I attempted to identify the nature of the mitogenic agent in pigeon serum, by testing out the hypothesis that it could be endotoxin. Endotoxin was chosen since it has been shown to have a role in the pathogenesis of other

respiratory disorders such as byssinosis (Cavagna et al, 1969, Pernis et al, 1961, Cinkotia et al, 1977, Castellan et al, 1984, Rylander et al, 1985) and humidifier fever (Westphal & Luderitz, 1961, Rylander et al, 1984, Muittari et al, 1980). The endotoxin content of one pigeon serum sample was measured in a Limulus lysate assay and found to contain 5pg/ml of endotoxin. To determine whether this level of endotoxin could have been responsible for the mitogenic activity of pigeon serum, a commercial source of LPS was obtained and used in the proliferation assay, in quantities equivalent to those that would be contained in a mitogenic dose of pigeon serum. Lymphocyte proliferation was not induced by mimicking concentrations of endotoxin. However, since I was also unable to obtain lymphocyte stimulation using recommended concentrations of endotoxin known to induce proliferation of lymphocytes, the results of these experiments must remain inconclusive. It is possible that the culture conditions for endotoxin induced stimulation were not appropriate. If more time had been available, various concentrations of LPS over a fuller time course would have been tested to obtain a positive control response at recommended concentrations of LPS, before attempting to use the lower concentrations that would be present in a mitogenic dose of pigeon serum.

As an alternative approach, I examined the effects of Polymyxin B, a known inhibitor of endotoxin, on the mitogenic response to serum. The concentrations of Polymyxin B that I used were based on the findings of

Morrison & Jacobs (1976) who reported that 3.5μg of Polymyxin B inhibits approximately 10μg of LPS and on the level of endotoxin measured in the Limulus lysate assay. Using these conditions, I could not find any inhibitory affect of Polymyxin B on the proliferation of lymphocytes induced by pigeon serum. However, as I was unable to induce proliferation by adding exogenous LPS, these findings need confirmation by proving that the doses of Polymyxin B that I used would inhibit known quantities of LPS, in my hands. Therefore I am not able to exclude completely the possibility that endotoxin is the mitogenic agent in pigeon serum.

Whatever is the mitogen in pigeon serum, is it likely to have a role in the pathogenesis of PBD ? One way of examining whether the mitogenic agent in pigeon serum was likely to have a role in the pathogenesis of PBD was to determine whether it was found in a source of pigeon material commonly found in the loft environment, such as pigeon droppings. Pigeon serum proteins have been shown to be excreted in pigeon droppings (Edwards et al, 1969) so pigeon serum and droppings, contain many antigens in common.

The protein derived from pigeon droppings in this thesis, was shown by SDS Page gel electrophoresis, under reducing conditions, to consist of 4 main protein bands; 116KDa, 60KDa, 45KDa and 30KDa, similar to that found by Calvanicco, (1986). Since the major component of PDE has been found to be intestinal IgA, (Goudswaard et al, 1977), then it is possible that some of the protein bands

could have represented the heavy and light chains of this protein.

The protein extract from pigeon droppings was neither antigenic nor mitogenic to lymphocytes. These results are in contrast with previous studies which have shown that lymphocytes from symptomatic pigeon breeders (Hansen & Penny, 1974) and occasionally also from asymptomatic pigeon breeders (Schatz et al, 1976, Girard et al, 1978), proliferated in response to PDE. However, the results from the latter two studies were not convincing, as the levels of stimulation were low and the background counts were not taken into account in the study by Girard et al (1978). All the studies did however agree with my findings that there were no mitogenic effects of PDE on control lymphocytes (Schatz et al, 1976, Girard et al, 1978, Hansen & Penny, 1974).

The lack of mitogenicity in pigeon droppings does not rule out the possibility that it is present in some other form of pigeon material, such as pigeon bloom, a fine waxy substance which coats the feathers and is readily dispersed in the loft environment (Banham et al, 1982) or in crop or saliva fluid (Edwards et al, 1970). Therefore, future studies could look for its presence in these sources of pigeon antigen. However, the problem with using these sources of pigeon antigens is that they are difficult to collect and large quantities would be required, to obtain sufficient amounts of antigen.

Chicken serum was incubated with PBMC to determine whether serum from other avian species was mitogenic. My

study could not detect any mitogenic effect of chicken serum on peripheral blood lymphocytes. This is interesting, since birds such as chickens and turkeys, which do not fly are not associated with significant clinical difficulties (Boyd et al, 1982, Elman et al, 1968). Boyd et al (1982), suggested that the lack of clinical difficulties associated with chickens was possibly due to their feathers not being well developed and the bloom content being much lower than that seen in pigeons. However, this study suggests that mitogenicity of bird proteins may be an important factor. Future studies should look at serum from other bird species in relation to the incidence of EAA caused by these birds to determine whether the mitogenicity theory holds true.

Animal models of EAA also support the role for a mitogen in the pathogenesis of EAA. In most experimental models, there is a need for an adjuvant (Richerson et al, 1971, Richerson, 1972, Joubert et al, 1976, Bernardo et al, 1979), mitogen (Moore et al, 1975) or other non specific inflammatory agent to induce disease. In addition, repeated inhalation challenge with antigen following acute disease fails to induce chronic EAA and indeed the alveolitic lesions disappear, unless a non specific stimulus, such as muramyl dipeptide, is also given (Chedid et al, 1978, Richerson et al, 1982). These studies suggest that a mitogen may be important not only for initiating disease but also in maintaining it.

Alveolar macrophages play a major role, maintaining immunological homeostasis at the airway surface by

inhibiting the inductive phase of local T cell responses to incoming antigens (Holt, 1986). This situation can be readily reversed by pathogenic antigens which induce inflammation and an influx of mononuclear cells from the peripheral blood (Holt, 1978). Therefore, it is possible that a mitogen is required in EAA, to induce a non specific inflammatory response in the lung, resulting in macrophage infiltration from the peripheral blood, which will be capable of presenting pigeon antigen to T cells and thereby induce a specific cell mediated immune response.

Future studies should therefore look at the phenotypes of peripheral blood cells and BAL cells in parallel, in subjects with well defined symptoms, at similar stages of disease and at known time intervals after antigen exposure. In addition, controlled inhalative challenge studies would be useful, using various purified pigeon antigens and possibly also mitogens such as LPS to explore the nature of the stimulus required to induce the peripheral blood cell changes.

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APPENDIX

QUESTIONNAIRE

PIGEON FANCIERS SURVEY

NAME: UNIT NO:

ADDRESS: DATE:

GPS NAME AND ADDRESS: D.O.B:

INSTRUCTIONS: Please tick appropriate answer, or write in space provided when indicated.

If in doubt answer "No"

Pigeon Data:

1. Do you keep pigeons at present? YES/NO

   If NOT when did you stop and why?

2. How long have you kept pigeons? ____YRS/____MTHS

3. Have you kept pigeons continuously?____ or

   periodically?____

4. How many pigeons do you generally keep? ____

5. Have you ever been in regular contact with birds? YES/NO

   If yes, please give details:

6. How many hours, on average, do you spend in contact with the pigeons during the season?_____

7. Do you ever wear a mask when in contact with pigeons? YES/NO

   Has this helped your chest?

Symptom Data:

8. When in contact with your pigeons have you EVER noticed:

   a. Shortness of breath ------

   b. Wheezing in your chest ------

   c. A dry cough ------

   d. Sneezing, blocked or running nose ------

   e. Itchy/runny eyes ------

   f. Tightness in chest ------

9. Have the above symptoms EVER been severe enough to make you come away from pigeons YES/NO

10. How long after coming into contact with pigeons do these symptoms occur?

    less than ten minutes ------

    ten to thirty minutes ------

    more than thirty minutes ------

11. Do these symptoms occur:

    occasionally (once per 3 months) ------

    sometimes (once per 2 weeks) ------

    usually (at least twice per week) ------

12. How long have you had them? ------

13. Several hours (4 - 8) AFTER being in contact with pigeons have you EVER experienced attacks of, or persistent:

    a. Shortness of breath ------

    b. Wheeze ------

    c. Shivering and/or feeling cold ------

d. Sweating ------

e. Fever or temperature ------

f. Dry cough ------

g. Tiredness or aching muscles ------

h. Tightness in chest ------

14. How long is it before you feel well again? ------

15. Have you ever noticed "flu-like" feelings in the evening and at night which have gone by the following morning? YES/NO

16. How long do these symptoms occur?

occasionally (once per 3 months) ------

Sometimes (once per 2 weeks) ------

Usually (at least twice per week) ------

17. How long have you had them? ------

18. Are these symptoms different -

a. at the weekend WORSE ----
SAME ----
BETTER ----

b. after attending a pigeon show WORSE ----
SAME ----
BETTER ----

c. after a holiday away from home WORSE ----
SAME ----
BETTER ----

d. after cleaning out the loft WORSE ----
SAME ----
BETTER ----

Respiratory Data

19. Do you USUALLY cough first thing in the morning in winter? YES/NO

20. Do you USUALLY cough during the day in the winter? YES/NO

21. Do you cough like this on most days for as much as three months each year? YES/NO

22. Do you USUALLY bring up any spit of phlegm from your chest first thing in the morning in winter? YES/NO

23. Do you USUALLY bring up any spit or phlegm from your chest during the day in the winter? YES/NO

24. Do you bring up spit like this on most days for as much as three months each year? YES/NO

25. Do you ever get sgort of breath when walking with others of your own age, on level ground? YES/NO

26. During the past three years have you ever had any chest illnesses which have kept you from your usual activities for more that a week? YES/NO

27. Have you ever had -

a. heart trouble _____

b. bronchitis _____

c. pneumonia/pleurisy _____

d. tuberculosis _____

e. asthma _____

f. other chest illnesses _____

28. Have you ever had any illness requiring a period in hospital? YES/NO

Give details:

29. Do you require regular medicines for any condition? YES/NO

a. What is the condition?

b. What are the medicines?

30. Have you ever worked in:

a. a coal mine? YES/NO

b any other mine? YES/NO

c. a quarry? YES/NO

d. a foundry? YES/NO

e. a pottery? YES/NO

f. a cotton, flax of hemp mill? YES/NO

g. with asbestos?

31. How many cigarettes/cigars do you smoke per day? ____

33. How long have you smoked? ____

34. If you have ever smoked,

a. How long did you smoke for? ____ yrs

b. How many did you smoke per day? ____

c. When did you stop? ____

Allergies

35. Have you ever suffered from

a. hay fever YES/NO

b. asthma YES/NO

c. Eczema/Dermatitis YES/NO

d. Were you chesty as a child? YES/NO

36. What did you suspect causes the/these condition/s in your case?

37. Does any relative have any of the conditions listed above?