Clinical and Molecular Biological Studies

in

Recurrent Aphthous Stomatitis

A Thesis Presented by

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Summary

Recurrent aphthous stomatitis is the most common condition affecting the oral mucosa. It is characterised by painful, recurrent oral ulcers which appear singly or in crops and heal spontaneously. Recurrent aphthae have a multifactorial aetiology, although the basic defect suggested in many published investigations an immunological imbalance caused by a micro-organism. The aim of these studies was to research different aspects of the pathogenesis and therapeutic features of recurrent aphthous stomatitis.

The investigations in this thesis were carried out in two groups of aphthous patients, who attended the Oral Medicine Clinic in different periods of time and the data were analysed in a different way in each group. The first group of 252 patients, were studied prospectively and a second group of 213 patients were initially studied retrospectively. Haematological investigations and therapeutic effects of vitamins and minerals were studied and were compared with a control group. For the first time in Glasgow Dental Hospital and School, a special computer program for oral ulceration was applied for the collection and analysing of the data in one of the groups of aphthous patients.

Food allergens as environmental factors were tested in 295 patients with aphthae as well as 100 control subjects. Although the results were unable to prove the involvement of food additives in recurrent aphthous stomatitis, reviewing those patients with a positive allergic reaction showed that in many of these patients avoiding the allergens may potentially improve their condition.

Investigation of haematological deficiencies also did not support a correlation of these deficiencies with recurrent aphthous stomatitis. But after replacement therapy, during the follow-up time available, 41 per cent of patients showed some improvement in the severity of their ulceration.

In reviewing the literature, symptomatic therapy was found to be ineffective at inducing remission of the disease. However, it is the only treatment for many patients for decreasing the severity of the disease. A topical cream of 5 amino-salicylate was examined in a double-blind trial with cross-over. However, the effect of this cream could not be distinguished by this trial, but in a double-blind study without a cross-over, a minor effect of the drug was found.

In support of the involvement of viruses in the aetiology of recurrent aphthous stomatitis, the nested PCR and assays of ELISA and IFA were employed. Results of PCR investigations showed that HHV-6 DNA was present in 29 per cent of aphthous lesions. Using ELISA, specific IgG antibodies against HHV-6 were detected in 96.7 per cent of all serum samples with no significant difference between aphthous patients, oral lichen planus or control subjects. Specific IgM antibodies against HHV-6 was found in a higher prevalence rate in aphthous samples compared with the two other groups: a significant difference of $p=0.01$ was found between sera of aphthous patients compared with healthy controls.

HCMV and VZV DNA were not detected in aphthous samples. Also serological findings did not show any significant increase in the prevalence of specific IgG antibodies against these two viruses. Serum IgM antibodies against HCMV were

positive in a small number of samples with no difference between groups and IgM antibody against VZV was not positive in any serum samples. These data fail to show that recurrent aphthous stomatitis can be a manifestation of VZV or HCMV infection or reactivation. However, the possibility of involvement of HHV-6 is raised by the present studies.

The possible involvement of *Mycobacterium paratuberculosis* was examined by the nested PCR investigations. Although mycobacterial DNA was detected only in four biopsy samples of aphthous patients and in none of the oral lichen planus patients or controls, this difference was not significant and more research is necessary to confirm such involvement.

Publications and communications

Ghodratnama, F., Wray, D., Felix, D H. and Gibson, J. (1996) Haematological deficiencies in recurrent aphthous stomatitis (RAS). *Journal of Dental Research* **75,** 1170.

Ghodratnama, F., Wray, D. and Riggio, M. (1996) Evaluation of viral aetiology of recurrent aphthous stomatitis, using molecular biological techniques. *Journal of Dental Research* **75,** 1137.

Ghodratnama, F., Felix, D H. and Wray, D. (1997) A trial of topical mesalazine in recurrent aphthous stomatitis. *Journal of Dental Research* **76,** 236.

Ghodratnama, F., Bagg, J. and Wray, D. (1997) Antibodies to human herpesvirus-6, varicella zoster and cytomegalovirus in recurrent aphthous stomatitis. Submitted to British Society of Dental Research, April 1997.

Ghodratnama, F., Riggio, M P. and Wray, D. (1997) Search for human herpesvirus-6, human cytomegalovirus and varicella zoster virus DNA in recurrent aphthous stomatitis tissue. *Journal of Oral Pathology and Medicine* **26:** 192-197.

Preface

The work in this thesis was carried out in Department of Oral Surgery and Oral Medicine, Dental School, Glasgow University under supervision of Professor David Wray.

These studies represent original work carried out by the author. Where use has been made of material provided by others, due acknowledgement has been made in the text.

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Glossary of Abbreviations

5 ASA.	5 Amino-Salicylate
ADCC.	Antibody Dependent Cellular Cytotoxicity
ATCC.	American Type Culture Collection
B-cell.	Bursa Equivalent Cell
BPC	British Pharmaceutical Codex
C.	Complement
CD.	Cluster of Differentiation
ELISA.	Enzyme-Linked Immunosorbent Assay
Hb.	Haemoglobin
HCMV.	Human Cytomegalovirus
HHV-6.	Human Herpes Virus type 6
HIV.	Human Immunodeficiency Virus
HLA.	Human Leukocyte Antigen
IFA.	Immunofluorescent Assay
Ig.	Immunoglobulin
KD.	Kilo Dalton
MCV.	Mean Cell Volume
OD.	Optical Density
PCR.	Polymerase Chain Reaction
POD.	Peroxidase
RAS.	Recurrent Aphthous Stomatitis
Reference P/N	Human Ig specific for virus in Tris buffer solution
RPM.	Round Per-Minute
T-cell.	Thymus-Dependent Cell
TCR.	T Cell Receptor
VZV.	Varicella Zoster Virus

Contents

Chapter one. General Introduction and Literature Review

Although recurrent aphthous stomatitis (RAS) is the most common disease of the oral mucosa, it is one of the least understood diseases of the oral cavity (Vincent and Lilly, 1992). Recurrent aphthous stomatitis is a disorder characterised by recurring, self-healing ulcers confined to the oral mucosa (Wray, 1982a; Greenberg, 1984).

There is an increasing body of evidence on different aspects of recurrent aphthous stomatitis. Since the aetiologic factors are unknown, laboratory procedures to confirm the diagnosis of recurrent aphthous stomatitis have not been established. However, an autoimmune or viral aetiology are possible factors (Pedersen and Hornsleth, 1993).

Occasionally recurrent aphthous stomatitis is a manifestation of other syndromes, such as Behçet's syndrome, inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (Landesberg et al. 1990; Endre, 1991), and some immunodeficiency syndromes, including infection with human immunodeficiency virus (Scully and Porter, 1989; Porter and Scully, 1993).

1.1. Historical Review

Hippocrates (460-370 B.C.) is credited with the first use of the word "aphthai" accorded to the prodromal burning sensation, to describe an oral condition, although the lesions described were probably those of thrush infection. (Sircus et al. 1957) Similarly Jourdain-Berchillet (1849) discussed "aphthae" in great detail but was again clearly describing cases of thrush. Indeed, the Oxford English Dictionary defines the word aphtha as "the infantile disease thrush". (Farmer, 1958)

The first clinical description of the recurrent oral ulcerations which are now recognised as recurrent aphthous stomatitis was by Von Mikulicz and Kummel in 1888 (Mikulicz and Kummel, 1912; Farmer, 1958) and the first description in the English language was by Sibley in 1899 (Farmer, 1958).

Early publications on recurrent aphthous stomatitis were mainly descriptive and because of the diversity seen in the clinical presentations and apparent aetiologies, a large number of synonyms arose to describe the condition (Table 1.1). Sutton, in 1911 was the first to distinguish the major formm of recurrent aphthous stomatitis and termed it "peradenitis mucosa necrotica recurrens" although this has now been supplanted by the term "major aphthous stomatitis" (Cooke, 1961; Lehner, 1968). In 1960, Cooke described another type, "herpetiform stomatitis", which was named not on the basis of aetiology but on clinical appearance.

1.2. Clinical Features

Patients with recurrent aphthous stomatitis are classified in to three categories, depending on the clinical features of the lesions: minor ulcers (Mikulicz's aphthae), major ulcers (periadenitis mucosa necrotica recurrens, Sutton's aphthae, stomatitis aphthous recurrens cicatricicans) and herpetiform ulcers (Lehner, 1968; Lehner, 1969c ; Wray, 1982a; Greenberg, 1984; Axell and Henricsson, 1985; Scully and Porter, 1989; Pedersen, 1993).

Table 1.1. Synonyms which have been used to describe the Clinical entity of RAS (Sircus et al. 1957)

Acute aphthous stomatitis
Canker sores
Dyspeptic ulcers
Fragmentary Behçet's syndrome
Habitual solitary aphthous ulcer
Maculofibrinous stomatitis
Mikulic's aphthae
Periadenitis mucosa necrotica recurrens
Recurrent aphthous stomatitis
Ulcus necroticum mucosae oris
Ulcus neuroticum mucosae oris
Vesicular stomatitis

Minor ulcers are rounded and usually less than 10 mm in diameter and heal within 4-14 days without any scars. Major ulcers are usually over 10 mm in diameter, often with irregular borders and scar on healing. The herpetiform ulcers are rounded, well-demarcated small ulcers of one to two mm which usually appear in crops of 10-100. The herpetiform ulcers may occasionally occur in a keratinized area. These ulcers heal without scars within 4-14 days (Wray, 1982a; Greenberg, 1984; Pedersen, 1993).

Approximately 80-85 per cent of patients with a diagnosis of recurrent aphthous stomatitis show minor ulcers, 7-10 per cent major ulcers and 7-10 per cent herpetiform ulcers. (Wray, 1982c; Wray, 1984; Lamey and Lewis 1991)

The incidence of recurrent aphthous stomatitis varies from 15 per cent to more than 50 per cent (Brody and Silverman, 1969; Donatsky, 1973), and the prevalence from five to sixty-six per cent (Donatsky, 1973; Scully and Porter, 1989; Porter and Scully, 1991) in selected population samples. However, many authors have reported an incidence of 20 per cent with a prevalence of two per cent (Sircus et al. 1957; Farmer, 1958; Axell and Henricsson, 1985; Porter and Scully, 1991; Pedersen et al. 1992; Taylor et al. 1992; Hunter et al. 1993; Pedersen and Ryder, 1994). The disease appears to have higher prevalence in females than in males (Sircus et al. 1957; Farmer, 1958; Brody and Silverman, 1969; Donatsky, 1973).

The first appearance of ulcers is usually in childhood or the teenage years. The lesions rarely occur, for the first time, after the age of 50 (Farmer, 1958; Lehner, 1968; Brody and Silverman, 1969; Donatsky, 1973; Scully and Porter, 1989). However, 10 per cent of females have their first attack over the age of 50 (Sircus et al. 1957;

Farmer, 1958), whereas this trend is not seen in the male population (Farmer, 1958). In males, ulcers become severe in the first and third decades, in contrast to females, where they are more severe in the second and third decades (Farmer, 1958). Recurrent aphthous stomatitis patients may suffer from ulcers for different periods (weeks, months or years) and in some severe cases ulcers remain indefinitely (Brody and Silverman, 1969; Donatsky, 1973; Pedersen, 1993).

The lesions may appear asymptomatically or begin with either premonitory tingling, a tenseness, hyperaesthesia, a burning sensation or pain, a roughening or a raw sensation of the mucosa, for a period of up to 24 hours at a site where the lesion will develop (Farmer, 1958; Stanley, 1972)

In the pre-ulcerative stage, a macule or slightly raised papule appears, which may become slightly indurated. A single lesion (or several pinhead nodules) gradually develops a superficial membrane or coating, which is surrounded by a dusky red inflammatory halo. (Farmer, 1958; Brody and Silverman, 1969; Stanley, 1972; Lennette and Magoffin, 1973; Sciubba, 1984) The eventual shape of the lesion is determined by its site. Those on the lip or cheek mucosa are rounded or slightly elongated, while those in the vestibule or sulcus or on the floor of mouth may be markedly elongated or linear (Farmer, 1958; Brody and Silverman, 1969; Stanley, 1972).

Recurrent aphthous stomatitis is characterised by recurrent, self-limiting, painful, single or multiple ulcers of the non-keratinized oral and pharyngeal mucosa, and the

tongue (Farmer, 1958; Lennette and Magoffin, 1973; Pedersen, 1993). Pain varies in severity between individuals (Stanley, 1972). Aphthae occur on non-keratinized oral mucosa such as buccal and labial mucosa, lateral and ventral aspect of the tongue, floor of mouth and soft palate, but occasionally ulcers enlarge to keratinized mucosa (Vincent and Lilly, 1992). Affected sites may be different in different recurrent aphthous stomatitis patients. Lips and buccal mucosa are the most common sites for major, minor and herpetiform aphthous ulcers, but pharyngeal involvement showed a highly significant association with major aphthae (Lehner, 1968). Although the hard palate and gingivae are very rare sites, these in addition to the floor of the mouth, are more common in herpetiform ulcers (Lehner, 1968).

In the ulcerative stage, the central superficial membrane changes to blanching necrosis. During the first to second days, this blanched area or membrane sloughs, and a shallow well-defined ulcer is left behind (Stanley, 1972). A grey-white or yellowish fibrinous exudate or clot then forms over the floor of the ulcer (Farmer, 1958; Graykowski et al. 1966; Stanley, 1972; Pedersen, 1993). The small and painful ulcer may continue to enlarge for 4 to 6 days, until it reaches its maximum size. Small ulcers may coalesce to form major lesions. Pain decreases when the covering fibrinous clot in the ulcer develops. After a period of time, between 4 to 35 days, the ulcer heals, without a clinically observable scar. However, in a very small percentage, in major lesions with a long healing period, a scar remains for many years (Stanley, 1972).

Bagan et al. (1991) have reported that patients with minor recurrent aphthous stomatitis have the lowest rate of recurrence, number and duration of the lesions. Healing time for major recurrent aphthous stomatitis was higher than minor recurrent aphthous stomatitis and herpetiform ulcers.

1.3. Histopathologic Features

1.3.1. Light Microscopy

Histologically, an aphthous ulcer is covered by fibrinious slough, and inflammatory cell migration has filled the underlying tissues (Lehner, 1969c; Marsland and Brown, 1975; Sciubba, 1984).

In the premonitory stages of recurrent aphthous stomatitis, the underlying connective tissue shows no marked inflammatory reaction (Stenman and Heyden, 1980). As the reaction become more severe, the vacuolation of individual supra-basal cells in the oral epithelium can be distinguished (Stenman and Heyden, 1980). In later stages of premonitory lesions, in addition to extensive distribution of vacuolated cells throughout the spinous cells, the oral mucosa becomes thicker, due to a slight hypertrophy (Stenman and Heyden, 1980). These vacoulated cells may subsequently coalesce to form intraepithelial vesicles (Stanley, 1972; Stenman and Heyden, 1980). Such early disruption of epithelium and vesicle formation has also been reported by others (Farmer, 1958; Brody and Silverman, 1969).

The earliest cellular infiltration into the submucosa in the pre-ulcer stage of a lesion appears to consist mainly of lymphocytes with some plasma cells, mast cells and

eosinophils (Dolby and Allison, 1969). Although there is a difference in the amount of eosinophilic cells in different specimens (Stanley, 1972), the lymphocytes predominate until frank ulceration is established when polymorphonuclear leukocytes become more predominant in the areas subjacent to the ulcer. However, the lymphocytes remain quantitatively the most common cell type in the periphery of the lesion and in the deeper aspects (Graykowski et al. 1966; Brody and Silverman, 1969; Stanley, 1972). After about 48 hours of ulceration the mast cell population in the subepithelial region, reduces (Dolby and Allison, 1969). Polymorphonuclear leukocyte migration, prevascular infiltration, oedema and hyperaemia are also seen (Stanley, 1972; Sciubba, 1984).

In an other report from sections through one and three day old lesions, variable numbers of haemorrhagic foci of extravasated erythrocytes have been demonstrated. These foci coalesced and occurred in the centre and lateral to the ulcer, that is in region of connective tissue papillae. The bottom of the ulcers consisted of a superficial exudate with clotted fibrin, numerous red blood cells, neutrophilic granulocytes and cellular debris, while beneath this layer, the infiltrate consisted of lymphocytes, macrophages, and neutrophilic granulocytes. As the infiltrates spreads laterally, lymphocytes become more numerous. Foci of perivascular round-cell infiltration were also seen in the underlying lamina propria and submucosa. (Sciubba, 1984) Similar observations were made on 7-day old aphthae (Sciubba, 1984).

In the healing stage, granulation tissue, which contains numerous immature capillaries lined by endothelial cells and surrounded by delicate fibrous tissue, underlies the

fibropurulent plaque of the ulcer (Stanley, 1972). In advance of the period of healing, the granulation tissue undergoes collagenisation and some chronic inflammatory cells, particularly plasma cells, may persist in the deeper layer of the lamina propria (Stanley, 1972). These findings might support a viral or\and an immunological aetiology of recurrent aphthous stomatitis (Stenman and Heyden, 1980).

The inflammatory cell expression in the connective tissue, underlying the ulcer, is a later occurrence in the RAS-lesion (Stenman and Heyden, 1980). In a study on 43 patients with minor recurrent aphthous stomatitis and 15 healthy individuals as controls, there was no significant difference between mononuclear cells in ulcer specimens and healthy mucosa of non-RAS individuals. However, mononuclear cell counts in the lamina propria of clinically unaffected mucosa of both active and remission stages of recurrent aphthous stomatitis were significantly lower than mucosal biopsies from controls and ulcerative areas of recurrent aphthous stomatitis patients (Pedersen et al. 1992).

In the subepithelial region of aphthous lesions which are more than 48 hours old, the mast cell population is significantly reduced, and does not support an immediate hypersensitivity reaction as an aetiological factor (Dolby and Allison, 1969; Brody and Silverman, 1969). More often, plasma cells and eosinophils are found in the older lesions (Lehner, 1969c).

However there are no pathological indices to diagnose recurrent aphthous stomatitis by light microscopy, but most findings show inflammatory cell expression in the

connective tissue in the ulcerative stage. This may be found in any non-specific ulcerative disease, but reduced numbers of mononuclear cells in the oral mucosa of recurrent aphthous stomatitis patients indicate a cell immune deficiency in these patients which may be due to an autoimmune or viral aetiology.

1.3.2. Electron Microscopy

Electron microscopy confirms the light microscopic findings (Lehner and Sagebiel, 1966; Lehner, 1969c). Degeneration of the prickle cells can be seen on electron microscopy as a prominent feature in aphthous lesions and macrophage-like cells and T-lymphocytes have been reported to be attached to these degenerating cells (Lehner, 1969c; Honma, 1976). In addition, intra-nuclear inclusion bodies and intra-cytoplasmic phagosome-like bodies were observed (Lehner and Sagebiel, 1966). The intra-nuclear inclusion bodies were also found in the epithelial cells of herpetiform ulcer biopsies. The size of these spherical shaped bodies has been reported to be from 500 to 650 millimicrons in diameter (Lehner and Sagebiel, 1966; Lehner, 1969c). The type and shape of these inclusion bodies may vary in different cells and they have been observed in many diseases, particularly those of viral, neoplastic or autoimmune aetiology (Sapp and Brooke, 1977). Intra-cytoplasmic phagosome-like bodies were found in all ulcers, at sites with severe intra-cellular separation, mononuclear infiltration and at the epidermal portion of the basement membrane. Their size differs from 350 to 650 millimicrons in diameter. (Lehner and Sagebiel, 1966; Lehner, 1969c; Luzardo Baptista, 1975) Also mitochondria were frequently swollen with loss of internal cristae, in the cells containing the inclusion bodies and in epithelial cells

adjacent to the inflammatory cells (Lehner and Sagebiel, 1966; Lehner, 1969c; Luzardo Baptista, 1975). The phagosome-like bodies seen in aphthous ulcers including the herpetiform type, are like the early invasive destructive process of mononuclear cells upon antigen-containing epithelial cells in the delayed type of hypersensitivity reactions (Lehner and Sagebiel, 1966).

Sterologic study of biopsy specimens of one to seven day old oral ulcers from minor, herpetiform, Behçet's syndrome and non-ulcerated oral mucosa of aphthous patients confirmed that the infiltration of inflammatory cells at the ulcer's centre is different in composition from that in the lateral border region (Schroeder et al. 1983). Monocytes/macrophages are a constant and large infiltrate component in this infiltrate composition. Also blast-forming T-lymphocytes are present and small, non-active lymphocytes are a rare component (Schroeder et al. 1983). Neutrophilic granulocytes, monocytes/macrophages, and medium-sized lymphocytes each provided almost one third of the infiltrate population in central region of the ulcer, while in the region lateral to the ulcer medium-sized lymphocytes provided two thirds and neutrophilic granulocytes and monocytes/macrophages together the rest of the infiltrate population (Schroeder et al. 1983).

Merckel cells have been demonstrated in the oral cavity by electron microscopy. This finding may suggest that catecholamines released from these cells by psychogenic stimuli would, through local vasoconstriction, trigger epithelial necrosis and hence lead to ulceration of the oral mucosa (Wilgram, 1972).

Electron microscopic investigation of recurrent aphthous stomatitis failed to show recognisable micro-organisms such as mycoplasma or L-form organisms. However a viral aetiology could not be excluded on the basis of finding intra-nuclear inclusion bodies.

1.3.3. Immunofluorescence and Immunohistochemistry

Direct and indirect immunofluorescent staining of sections from recurrent aphthous stomatitis both *in vivo* and *in vitro*, indicated binding of IgG and IgM in the cytoplasm of autologous prickle cells in biopsies obtained from four patients with minor aphthae and two patients with major aphthae. Indirect staining of normal oral mucosa with sera from these patients failed to show any immunoglobulin binding (Lehner, 1969c). Immunofluorescent staining appears in biopsies from patients with minor aphthae and major aphthae, but negative results were obtained with herpetiform ulcers (Lehner, 1969c). However immunohistochemical studies (for IgG, IgM, IgA, and C3) of biopsies obtained from aphthous lesions and normal mucosa of patients with and without a history of recurrent aphthous stomatitis were positive for all samples (Sciubba, 1984). The labelled cells occurred singly or in clusters in the lower, and upper stratum spinosum but rarely in the basal and superficial layer (Sciubba, 1984). This appearance might be due to non-selective and non-specific binding of a whole battery of immune components including IgA, IgG, IgM, C1q and C3 by stratum spinosum (Malmstrom et al. 1983; Sciubba, 1984).

On the other hand, Malmstrom et al (1983) observed no IgG, IgM, IgA or fibrinogen fluorescence in the mucosa of recurrent aphthous stomatitis patients, using direct immunofluorescence. However 15 of 16 biopsies showed deposits of C3 along capillary walls (Malmstrom et al. 1983).

A strong fluorescent reticular network was observed at the site of the ulcer and connective tissue beneath the ulcer. This binding was positive only with immunoglobulin A, while other ulcerative lesions from other patients selected as controls were negative (Brody and Silverman, 1969).

By using highly specific IgG, IgM, and IgA conjugates, direct staining of the basement membrane zone in 14 aphthous lesions with IgG and in 16 lesions with C3 was reported (Donatsky and Dabelsteen, 1977). C3 was also demonstrated in 11 of 12 experimental oral wounds but not in normal mucosa. When the sections were stained indirectly, it was difficult to determine the degree of staining due to non-specific staining of the connective tissue. Direct staining of the prickle cells was not seen with any isotype of immunoglobulin in patients or controls (Donatsky and Dabelsteen, 1977). Indirect staining , however, revealed cytoplasmic immunoglobulin deposition in the prickle cells with IgG, IgA and IgM in aphthous lesions, experimental oral wounds and normal oral mucosa. The distribution of end point titres in the sera of these three groups showed that most immunoglobulin was directed against oral mucosa in the group with recurrent aphthous stomatitis. These investigations (Donatsky and Dabelsteen, 1977), therefore, could not confirm the finding of Lehner (1969c) with respect to the direct immunofluorescent staining of

prickle cells, but showed staining of the basement membrane zone. Furthermore, they demonstrated only quantitative immunofluorescence. In addition, they felt the IgA staining of connective tissue demonstrated by Brody and Silverman (1969) was non-specific. (Donatsky and Dabelsteen, 1977)

In another study direct immunofluorescence of biopsies and peripheral blood of patients with recurrent aphthous stomatitis showed a high incidence of IgM and IgE bearing lymphocytes and polymorphonuclear leukocytes in aphthous lesions and an increase in the number of IgE-coated lymphocytes in the peripheral blood of these patients (Bays et al. 1977). Direct fluorescence also showed that vascular fluorescence with C3 was positive in approximately one third of the biopsies from recurrent aphthous stomatitis patients (Van Hale et al. 1981). Forty-five per cent (10 of 22 patients) of specimens demonstrated fluorescence along the basement membrane zone (Van Hale et al. 1981). In this group of patients only two cases showed positive basement membrane zone deposition of conjugated anti-sera to immunoglobulin (one each of IgG and IgM) (Van Hale et al. 1981). In this study, the pattern of immunofluorescence findings was similar to those in certain other oral diseases such as lupus erythematosus, bullus and cicatrical pemphigoid, desquamative gingivitis and lichen planus (Van Hale et al. 1981).

In a study of peripheral T lymphocytes sub-populations in 30 patients with recurrent aphthous stomatitis and 29 sex and age matched recurrent aphthous stomatitis free controls, the CD4+ percentage was significantly lower in the patients than in the control group, whereas CD8+ cells and the CD4+/CD8+ ratio did not differ

significantly between patients and controls (Pedersen et al. 1991) Also, these findings were observed by other investigators (Kayavis et al. 1987; Savage et al. 1988; Landesberg et al. 1990). Although in similar studies, it has been reported that the number CD4+ (T-helper) cells in recurrent aphthous stomatitis patients did not differ in either active or non-active stages of the disease compared with controls (Pedersen et al. 1992). In this study, the CD8+ cell percentage was significantly higher in both unaffected and ulcer areas of the recurrent aphthous stomatitis patients in the active stage of the disease as compared with controls (Pedersen et al. 1992). The CD4:CD8 ratio also was reported to be lower than previous reported in both unaffected and ulceration areas of recurrent aphthous stomatitis patients in the active stage of ulceration. In peripheral blood the ratio of CD4+/CD8+ lymphocytes was decreased due to a significant increase of CD8+ cells (Kayavis et al. 1987; Savage et al. 1988; Pedersen et al. 1989). In addition, it has been reported that in the peripheral blood of all patients with severe aphthous ulceration, the number of T-helper/inducer cells (Cdw29) was increased and the number of T-suppresser/inducer cells (CD45R) decreased during both remission and early periods of ulceration (Savage et al. 1988; Landesberg et al. 1990). Helper/inducer T-lymphocytes of peripheral blood help B cells in the antibody producing process. The CD45R subset appears to represent a "naive" cell whereas the Cdw29 subset is a memory cell. (Savage et al. 1988)

The natural killer cells (CD45RA+) were significantly higher in peripheral blood of patients with recurrent aphthous stomatitis (Pedersen et al. 1991) while natural killer cell activity was not significantly different between patients with recurrent aphthous

stomatitis and controls during early active disease and two weeks after remission of ulcers (Pedersen and Pedersen, 1993).

By applying three-colour immunofluorescence, Pedersen and Ryder (1994) observed an increase in the fraction of the T cell receptor γδ+ cells in patients with active recurrent aphthous stomatitis. They also reported changes in natural killer cell subsets: an increase in the percentage of CD16+/CD56- and CD16-/CD56+ in patients with active recurrent aphthous stomatitis compared with those in controls and also an increase in the percentage of CD56+/CD8- cells in active compared with inactive patients. There were, however, no significant differences in the CD57+ figures (Pedersen and Ryder, 1994).

T-lymphocyte subsets in the oral mucosa, in general, were significantly lower in patients with recurrent aphthous stomatitis in active and inactive stages, compared with the control group. However CD4+ percentages were not significantly different between the two groups of controls and patients with recurrent aphthous stomatitis (Pedersen et al. 1992). CD8+ percentage cells in lesions from recurrent aphthous stomatitis patients were significantly increased during the active process as compared with controls (Pedersen et al. 1992; Pedersen, 1993).

There are therefore, no exact pathogonomic features which can be demonstrated in recurrent aphthous stomatitis by histological methods. Immune complex and immunoglobulin deposition may be an epiphenomenon secondary to inflammation. Histological features mainly strengthen the hypothesis that immunological factors are

involved in the pathogenesis of recurrent aphthous stomatitis. However, studies on T-lymphocytes in peripheral blood and oral mucosa raise the possibility of an autoimmune and / or micro-organism aetiology.

1.4. Differential Diagnosis

Superficial ulceration of the oral mucosa is common in diseases of the oral cavity. A diagnosis is made by the analysis of multiple factors, including the lesion's location, size, grouping, onset, patient's age, involvement of other systems of the body and course of the disease (Chole and Domb, 1979).

The diagnosis of recurrent aphthous stomatitis depends on clinical features. It is necessary to differentiate recurrent aphthous stomatitis from other conditions which may have a similar presentation. Disorders, such as erosive lichen planus, bullous lesions, erythma multiforme and oral Crohn's disease can be distinguished from recurrent aphthous stomatitis clinically and in some complex cases histologically or by immunofluorescence. (Wray, 1982a)

Recurrent aphthous stomatitis is often misdiagnosed as a recurrent herpes simplex virus infection. An acute viral stomatitis can be distinguished by virus culture or other virology methods. Besides, recurrent herpes simplex infections, generally affect the vermilion border only, although recurrent intra-oral herpes infection is often difficult to distinguish from herpetiform type of recurrent aphthous stomatitis. However there are some clinical differences between these two groups. In herpetiform recurrent aphthous stomatitis, crops of ulcers (up to 100) involve the non-keratinized mucosa

buccally and sublingually. In contrast, recurrent intra-oral herpes tends to be more localised on keratinized areas such as attached gingivae and hard palate. In case of any doubt, viral culture, cytology, or histology can be applied in the differential diagnosis. (Wray, 1982a)

Recurrent aphthous stomatitis can be a feature of Behçet's syndrome, although Behçet's syndrome is a rare condition, in contrast with recurrent aphthous stomatitis which is extremely common. Behçet's syndrome has many features: oral ulceration, erythma nodosum, phlebitis, peripheral arthritis and sacroileitis. The diagnosis of Behçet's syndrome can be confirmed when two or more major criteria (oral, genital, ocular or cutaneous involvement) or a combination of major and minor (vascular, neurological, skeletal or intestinal) manifestations are present (Wray, 1982a). The distribution of symptoms can be associated with a genetic component such as, an increased frequency of HLA-B_5 and B_{27}. However complete evidence for an immunogenetic basis for recurrent aphthous stomatitis has not been established (Wray, 1982a).

1.5. Actiologic Factors

It has been postulated that the aetiology of recurrent aphthous stomatitis is unknown and controversy has existed in the literature about the importance of various factors in the pathogenesis of recurrent aphthous stomatitis.

In this thesis they will be divided into: genetic factors, exogenous environmental factors, host or endogenous environmental factors, immunological factors and micro-organisms associated with recurrent aphthous stomatitis.

1.5.1. Genetic Factors

a) Inheritance patterns

Ship (1965) in a study on a large population of students, could not show a definitive genetic background on susceptibility to recurrent aphthous stomatitis. However. it was not possible to rule out a genetic relationship in those subjects with a positive parental background and those with a negative parental background.

In one study, 12 sets of monozygotic twins and seven sets of dizygotic twins with a positive family history of recurrent aphthous stomatitis were investigated to identify any inheritance patterns involved in the aetiology of the disease (Miller et al. 1977). Results have shown that, identical twins, with a positive family history, develop aphthous lesions more than non-identical twins (91.5 per cent versus 57 per cent) (Miller et al. 1977). This difference, like conditions in other diseases may suggest polygenetic inheritance with environmental or infectious components modifying the expression of the disease (Miller et al. 1977).

The proportion of recurrent aphthous stomatitis positive individuals is higher when either the father (33.8 per cent) or mother (52.5 per cent) are positive than when neither parent is positive (8.9 per cent). The incidence of the disease was thus increased in individuals with a positive family history (Miller et al. 1980). On the basis

of these findings, also, it was not possible to define the precise pattern of genetic transmission. (Miller et al. 1980)

Given these, inheritance patterns in recurrent aphthous stomatitis show that factors other than inheritance may have accounted for some components of the disease expression.

b) HLA tissue typing

In recent years, there has been an extensive search for association between specific diseases and antigens of the major HLA histocompatibility locus (Katz, 1977). HLA associated diseases can be categorised in to different groups. The major groups are: autoimmune diseases, malignancies, disease with a metabolic component or diseases with unknown aetiology. Until now, no certain role of HLA has been identified in the development of autoimmunity in related diseases. One of the early hypothesis for HLA disease associations is that, the cell surface HLA molecule could act as a receptor for a specific virus and provide a way for it to infect the cell, thus making individuals carrying a particular gene susceptible. Several instances have been described in the mouse where H-2 molecules can bind specifically to viruses (e.g. semliki forest virus and adenovirus type II). No specific examples are known in man for HLA being a viral receptor. However, it is clear that cell surface markers in man, such as CD4, can act in this capacity (e.g. as receptor for the human immunodeficiency virus, HIV). This hypothesis would explain the genetic and environmental component in the aetiology of many diseases (Ollier and Symmons, 1992). For instance, individuals who have the genetic background of HLA-B_{15} and

HLA-DQw_3 appear to be predisposed to HSV-induced erythma multiforme (Scully, 1993).

Susceptibility to many oral diseases was reported to be associated with HLA antigens. HLA-A_3, HLA-B_5 and HLA-B_8 were more frequent in patients with lichen planus than in controls (Saurat et al. 1977), HLA-B_5 was increased in Behçet's syndrome (Erosy et al. 1977), recurrent herpes labialis appeared to have an increased frequency of HLA-A_1 (Russell and Schlaut, 1977), and the prevalence of HLA-B_{12} was found to increase in recurrent aphthous stomatitis (Challacombe et al. 1977b). The HLA-B_{12} type was present in 43 of 100 patients with the disease compared to 22 of 100 controls (Challacombe et al. 1977b). However in a similar study of 66 patients and 100 controls, no differences in incidence were found (Dolby et al. 1977). Also HLA-B_{12} was related to the muco-cutaneous type of Behçet's syndrome, which has the same clinical features as recurrent aphthous stomatitis (Lehner et al. 1979a). Recurrent aphthous stomatitis also showed an increased frequency of the HLA type A_2 (Lehner et al. 1979a). Disease susceptibility may segregate with the HLA haplotype (Dolby et al. 1977). In a study in different populations, the increased HLA-DR_7 and a decreased HLA-B_5 was indicated to be related to recurrent aphthous stomatitis (Gallina et al. 1985). However, Dolby et al. (1977) were unable to find such a relation between recurrent aphthous stomatitis and HLA antigens. In this study, only HLA-A phenotypes were investigated whereas, the possible involvement of other phenotypes such as HLA-B_{12} could not be excluded.

1.5.2. Exogenous Environmental Factors

a) Mucosal injury

Clinicians have long suspected that trauma can initiate lesions in patients with a history of recurrent aphthous stomatitis (Sircus et al. 1957; Farmer, 1958; Graykowski et al. 1966). In a study by Wray, et al (1981) there was no clinical and histological difference between mechanical ulcerated lesions and spontaneous ulcers. This phenomenon also has been observed in Behçet's syndrome, where intradermal injection causes formation of pustules and mucosal injury initiates mouth ulcers (Wray et al. 1981). Patients with Behçet's syndrome also show an increased sensitivity to inflammatory mediators such as histamine, which will induce pustules when inoculated intradermally. In Ehlers-Danlos syndrome the oral tissues are also more sensitive to injury presumably due to defective synthesis of collagen (Wray et al. 1981).

b) Allergy and food sensitivity

Allergy affects 10 per cent of the population and is usually genetically determined (Porter and Scully, 1990; Scully and Cawson, 1993)). Atopic allergy depends on production of specific IgE antibodies which bind to mast cells and basophils. Antigen binding to the surface IgE causes degranulation of mast cells with release of mediators such as histamine and leukotrienes. Patients with atopic allergies are also possibly more prone to develop allergy or anaphylaxis in response to drugs, however, some normal individuals can produce IgE antibodies but without ill-effect (Porter and Scully, 1990). There is no reliable evidence that immediate-type hypersensitivity reactions produce oral mucosal disease and there is no oral counterpart of eczema.

Furthermore, no oral diseases have been proved to have any significant direct association with atopic allergy, although patients with allergic orofacial granulomatosis and erythma migrans may have a higher prevalence of atopy than the normal population (Porter and Scully, 1990). Ship in 1960 claimed a complete remission in seven patients with recurrent aphthous stomatitis when placed on an elemental diet, but failed to confirm this later in a series of six patients (Ship et al. 1962). Moreover, patients with recurrent aphthous stomatitis have been shown to display increased serum antibodies to cow's milk proteins (Taylor et al. 1964) and other food antigens (Thomas et al. 1973) compared to normal, but not when compared to patients with other oral ulcerative conditions. (Thomas et al. 1973). However, it is possible that local mucosal breakdown leading to absorption of increased amounts of food antigens was the cause of the increased incidence of food antibodies (Thomas et al. 1973).

Certain foods, especially walnuts, chocolate or citrus fruits, appear to precipitate ulcers in a few patients with aphthous stomatitis, but although there may be a slightly increased prevalence of allergic disease associated with recurrent aphthous stomatitis, there is little evidence for an allergic mechanism (Eversole et al. 1982; Porter and Scully, 1990).

In a *in vitro* study on recurrent aphthous stomatitis patients (Wray et al. 1982) the leukocytes from 60 patients with recurrent aphthous stomatitis were tested for histamine release in response to environmental and food allergens. Of the sixty patients tested, nine patients showed a significant release to only food-stuffs. Fourteen

patients had a positive reaction *in vitro* to both foods and environmental allergens, and four showed a significant release of histamine in response to respiratory allergens alone. The remaining, thirty-three patients showed a negative or insignificant release of histamine to all antigens tested. In this study eight patients gave a history of food allergy but only two of these patients showed a positive histamine release reaction to food allergens. However in twenty-one of the patients who had a positive histamine release to certain foods, there was no correlation between the clinical history and the results of the *in vitro* assay (Wray et al. 1982). Therefore there was no evidence for a correlation between atopic allergies and recurrent aphthous stomatitis.

Recently, in a study on patients with recurrent aphthous stomatitis, 22 patients had undergone dermatological patch testing to investigate a possible allergic component (Nolan et al. 1991a). In 20 patients at least one allergen was identified. Benzoic acid and cinamonaldehyde were positive in most of the patients (Nolan et al. 1991a). After dietary advice on avoidance of allergens, patients were followed up for a period of six months to six years. Eight of 20 patients with identified allergens had no further ulceration despite being followed up for average of three years. Of the 12 other patients an 80 per cent improvement on average was reported (Nolan et al. 1991a).

Recurrent aphthous stomatitis is one of the oral manifestations of gluten sensitivity (coeliac disease) (Ferguson et al. 1980; Hunter et al. 1993). However a gluten-free diet has been effective in causing remission of ulcers in some patients with recurrent aphthous stomatitis in whom coeliac disease had been specifically excluded (Walker et

al. 1980; Wray, 1981). Aphthous ulcers rarely have been reported as an allergic reaction to foreign bodies (Gordon and Gordon, 1974).

c) Psychological factors

Sircus et al (1957) stated that mental stress precipitated attacks of ulceration in 21 per cent of patients; they described psychological factors as aggravating mechanisms rather than the cause of the disease. Other authors have also drawn these associations (Farmer, 1958; Ship et al. 1961; Ship et al. 1962; Graykowski et al. 1966). Whether such factors are of primary aetiological significance or a result of the ulceration has not been established. Ship et al, (1967) however reported finding significant differences between aphthous stomatitis patients and controls when both groups were evaluated by the Cornell medical index as to depression, tension inadequacy, anxiety and anger.

In the study of Pedersen (1989a), psychological stress was evaluated by means of the social readjustment rating scale (SRRS) and a visual analog scale (VAS). In this study, the evaluation was carried out on 22 patients with minor recurrent aphthous ulceration after the onset of new ulcers (0-8 days), as well as in an ulcer-free stage. However, it was not possible to find any positive association between psychological stress as a wide entity and recurrence of recurrent aphthous stomatitis.

Although the basis of the immunological changes associated with psychological stress is unknown, the nervous system might inhibit lymphoid function or induce immunosuppression by causing the increased release of immunosuppressive

corticosteroids, catecholamines, prostaglandins or opioids (Wilgram, 1972; Porter et al. 1990).

d) Smoking habits

Some reports have indicated a potential inhibitory effect of smoking on the occurrence of recurrent aphthous stomatitis, (Chellemi et al. 1970; Shapiro et al. 1970) and others (Sallay and Banoczy, 1968) have shown a significantly reduced level of keratinization of the oral mucosa in patients with recurrent aphthae, 94 per cent of whom were non-smokers.

There seemed to be different qualities between different tobacco habits regarding reduction in the frequency of episodes. Pipe smoking caused the most reduction in the frequency of recurrent aphthous ulceration (Axell and Henricsson, 1995). Gardy et al (1992) documented the positive therapeutic effect of smokeless tobacco used in the symptomatic alleviation of aphthous lesions. The biologic mechanism behind the negative association between tobacco habits and the frequency of recurrent aphthous stomatitis is not clear, and multidisciplinary longitudinal studies are needed if meaningful data are to be obtained (Axell and Henricsson, 1995). However increased keratinization of the mucous membrane to resist the formation of recurrent aphthous stomatitis in the mouth is most likely to be the main mechanism (Axell and Henricsson, 1995). For instance a negative correlation between keratinization of oral mucosa and aphthae in patients with leukoplakia has been pointed out (Axell and Henricsson, 1995). In a preliminary trial, nicorette tablets have had a beneficial effect on aphthous ulcers (Bittoun, 1991). Like cigarette smoking, smokeless tobacco use is

also associated with hyperkeratosis of the oral mucosa, but hyperkeratosis is topicaly localised to the mucosal area where the tobacco is held (Ernster et al. 1990; Grady et al. 1990; Grady et al. 1992). Because in the smokeless tobacco user, few components other than nicotine are systemically absorbed, nicotine may be one of the protective factor against aphthous ulcers (Grady et al. 1992).

In the study of salivary IgA levels in tobacco smokers, patients with minor aphthous ulceration and normal subjects, the level of salivary IgA remained within normal limits in both the ulcerative and non-ulcerative phases of aphthous ulcers, however in tobacco smokers there was a significant depression of salivary IgA concentration. It seems probable that salivary IgA has no definitive role in the pathogenesis of minor aphthae (Bennet and Reade, 1982). A similar study has been reported on the effect of smoking in ulcerative colitis, and the results shows that the IgA concentration in the intestinal fluid of smokers with ulcerative colitis was reduced compared with non-smokers (Srivastava et al. 1991). However most of the smoker patients with ulcerative colitis are only light smokers (Srivastava et al. 1991).

Given this, it may be deduced that decreased keratinization may play an important role in the pathogenesis of this disease. However the effect of smoking in prevention of ulcerative colitis does not support this hypothesis.

e) Micro-organisms

Several investigations into the occurrence of viruses, bacteria, fungi, mycoplasmas and protozoa in recurrent aphthous stomatitis have been carried out in an attempt to

implicate one of these micro-organisms in the pathogenesis (Farmer, 1958; Fraser-Moodie, 1960; Griffin, 1963; Barile et al. 1968; Ferguson, 1975; Donatsky et al. 1977; Studd et al. 1991).

Parasites

Toxoplasmosis has been associated with oral ulceration, although antibodies against this protozoa have not been shown to be increased in patients with recurrent aphthous stomatitis (Sircus et al. 1957).

Mycoplasmas

Despite attempts to isolate mycoplasmas from aphthous lesions, there is no evidence of an aetiological relationship between these organisms and recurrent aphthous stomatitis (Gordon et al. 1967; Lehner, 1972; Donatsky et al. 1977).

Fungi

No systematic mycological investigations exist on the occurrence of fungi in recurrent aphthous stomatitis, although *Candida albicans* cannot be consistently isolated from aphthous lesions (Donatsky et al. 1977).

Bacteria

The occurrence of fusiform bacteria and spirochaetes in smears from recurrent aphthous ulcers reported in early, preliminary studies and the observation that specimens from recurrent aphthous lesions produced pox-like formations not caused by viruses but by large numbers of fusiform-like organisms, resulted in discussion of a possible role for these organisms in recurrent aphthous stomatitis (Barile et al. 1968).

Recent bacteriological studies have not confirmed that fusiform bacteria or spirochaetes occur in specimens from recurrent aphthous lesions (Barile et al. 1968; Donatsky et al. 1977).

Pure cultures of a transitional L-form of *Streptococcus sanguis* were isolated from aphthous lesions of one patient with minor and two with major recurrent aphthae of total of three patients with the disease when the entire pseudomembrane was removed and material for examination scraped off from the base of the lesion (Barile et al. 1963). The pseudomembrane and saliva were reported to contain a mixed microbial flora. Blood obtained from culture during two exacerbation was found to be positive for the organism, whereas blood examined during remission was sterile. Scarred tissue from previous lesions of one patient was biopsied during a remission period and homogenised; on culture, this homogenised tissue showed the presence of L-form colonies (Barile et al. 1963). Later, a similar L-form organism was isolated from a biopsy specimen taken from a lesion of one patient with major aphthae (Graykowski et al. 1964). These authors (Graykowski et al. 1964) further reported that the bacterial parent of the transitional L-form was probably an alpha-haemolytic streptococcus and that the isolated L-form was able to produce ulcer-like lesions when injected into the flank of rabbits (Graykowski et al. 1964). An association between these streptococcal L-forms and recurrent major and minor aphthous stomatitis was suggested in the light of these preliminary findings (Barile et al. 1963; Graykowski et al. 1964).

This micro-organism was found in the majority of aphthous lesions (93 per cent), and less frequently in non-aphthous lesions (40 per cent) but also in post mortem oral mucosa (47 per cent). This micro-organism was primarily described as the occurrence of an L-form in the tissue sections (Stanley et al. 1964). Further, in a later review of the micro-biological aspects of *Streptococcus sanguis* in the pathogenesis of recurrent aphthous stomatitis, the micro-organisms in the sections were shown to be pleomorphic streptococci with diplococcoid or short chain structures (Barile et al. 1968). Isolation of L-forms from recurrent aphthous stomatitis has been repeated (Donatsky et al. 1977).

However, alpha-haemolytic streptococci, coagulase negative staphylococci and Neisseria species were together shown to be the predominant quantitative bacterial flora in cultures from abraded material or biopsies from recurrent aphthous lesions if the pseudomembrane was removed before the specimens were obtained (Donatsky et al. 1977).

A thorough microbiological examination of the morphology, biochemical properties, serological characteristics and lesion producing capacity of streptococcal isolates from recurrent aphthous ulceration concluded that the prototype, streptococcus, strain 2A was a *Streptococcus sanguis*, Lancefield group H, related to or identical with *Streptococcus sanguis*, American Type Culture Collection (ATCC) 10556. The skin lesion producing capacity of *Streptococcus sanguis*, strain 2A appeared to be related to the acid-soluble cell wall substances (Barile et al. 1968). However, this strain of streptococci, subsequently, has shown to be actually a strain of *Streptococcus mitis*

and antigenically is more like ATCC 10557 than ATCC 10556 (Hoover and Greenspan, 1983).

In conclusion, the high occurrence of oral *Streptococcus sanguis*, Neisseria and staphylococci in cultures obtained from major and minor recurrent aphthous stomatitis and the high occurrence of pleomorphic streptococci in the tissue sections might indicate an aetiological role for these bacteria and perhaps especially for S*treptococcus sanguis*. Further support of that hypothesis has been revealed in studies on the immunity against bacteria in recurrent aphthous stomatitis as discussed later.

Viruses

Many of oral mucosal ulceration, other than recurrent aphthous stomatitis, results from viral infection. Over the past years, many investigators have searched for a virus to explain the aetiology of recurrent aphthous stomatitis. The hypothesis of a viral aetiology is consistent with many features seen in this condition. The recurrent nature of the lesions is compatible with a virus infection (Gajdusek, 1977). However immunological features of recurrent aphthous stomatitis (section 1.6) have mainly referred to either autoimmunity or a viral pathogenicity (Pedersen, 1993). Hence, viruses are the strongest infective candidate for a role in the production of autoimmune disease (MacPhail et al. 1993). The immunological imbalances in recurrent aphthous stomatitis may be the result of viral involvement.

Viruses have never been isolated in aphthae of the major and minor type. However, viral isolates and antigens of adenovirus type 1 have been reported in a high

percentage of herpetiform ulcers (Nasz et al. 1971; Sallay et al. 1971a). In addition, adenovirus type 1 and herpes simplex virus caused blast transformation in a significant number of patients with recurrent aphthous stomatitis (Sallay et al. 1971b). These findings await confirmation. In herpetiform ulcers, inclusion bodies have been described by workers using electron microscopy (Lehner, 1969c) and some investigators have demonstrated degenerative changes histologically (Stenman and Heyden, 1980). These changes are however compatible with both a viral and an immunological aetiology (Sapp and Brooke, 1977; Stenman and Heyden, 1980).

Hooks (1978) reported that although viruses may be implicated in the aetiology of recurrent aphthous stomatitis and Behçet's syndrome, there are several considerations which make it hard to isolate a virus in these diseases.

Sallay et al. (1973) demonstrated isolation of two strains of Adenovirus type 1 from scraping samples of two patients suffering from herpetiform aphthous ulcers. By an immunoflurescent method, they also found adenovirus antigen in cell nuclei of ten patients with recurrent aphthous stomatitis including two patients in whom virus was isolated from the ulcers (Sallay et al. 1973).

Herpes simplex has not been cultured from aphthous ulcers. Also, specific fluorescence with herpes simplex immuno sera was not observed in recurrent aphthous stomatitis samples (Sallay et al. 1973). In an immunofluorescent method, using fluorescein-labeled monoclonal antibodies, herpes simplex type 1 was detected in two out of 30 patients with recurrent aphthous stomatitis (Ogawa et al. 1990).

However using an *in situ* hybridisation method, part of the herpes simplex virus genome was present in peripheral blood mononuclear cells (probably lymphocytes) of patients with the ocular and arthritic type of Behçet's syndrome and minor aphthous ulcers (Eglin et al. 1982). This was also confirmed in all three groups of recurrent aphthous stomatitis by applying the polymerase chain reaction (PCR), although there were differences in the proportions of those with the herpes simplex type 1 genome in the major and minor aphthous ulcer groups (Studd et al. 1991).

Varicella zoster virus (VZV) was detected in four out of 30 aphthous lesions by an immunofluorescent method (Ogawa et al. 1990). However Pedersen (1993) reported the presence of varicella zoster virus DNA by applying PCR in all 20 biopsies from aphthous lesions.

Human cytomegalovirus (HCMV) was not detected by immunofluorescence (Ogawa et al. 1990) and *in situ* hybridisation (Sun et al. 1996). But by applying PCR, three out of nine recurrent aphthous stomatitis patients were HCMV DNA positive in their biopsy samples (Sun et al. 1992; Sun et al. 1996).

Siegnel and Granich (1993) reported that recurrent aphthous stomatitis could be the oral manifestation of human herpes virus type 6 (HHV-6) reactivation, however they did not present enough evidence to support their hypothesis. Recently HHV-6 DNA was detected in saliva samples from two out of 12 (17 per cent) patients with recurrent aphthous stomatitis by the PCR method, although three per cent of healthy

subjects were also HHV-6 DNA positive. There was no significant difference between these two groups (Di Luca et al. 1995).

No virus has been consistently detected from recurrent aphthous stomatitis lesions, but combined clinical, immunopathological and virological observations on recurrent aphthous stomatitis seem to support a viral aetiology. With herpesviruses in particular having a propensity for latency and reactivation after primary infection, the theory of recurrent aphthous stomatitis being due to reactivation of one or more members of the herpesvirus family cannot be excluded.

1.5.3. Host or Endogenous Environmental Factors

a) Haematological deficiency

Although many studies suggest the relationship between haematological deficiencies and recurrent aphthous stomatitis (Hutcheon et al. 1978; Challacombe et al. 1983; Porter et al. 1988) there are also a number of investigations which denied such an association (Challacombe et al. 1977a; Olson et al. 1982). Because of full remission or improvement of ulceration after replacement of deficient factors, it seems that a full blood count including assays of haematinics is necessary to diagnose those haematological deficiencies which appear with a normal blood film (Wray et al. 1975; Challacombe et al. 1977a; Hutcheon et al. 1978; Challacombe et al. 1983; Tyldesley, 1983; Palopoli and Waxman, 1990).

Recurrent aphthous stomatitis, unlike glossitis and angular chielitis has been reported to occur infrequently with deficiencies of iron, folic acid and vitamin B_{12}. Thus,

deficiency of vitamin B_{12} is not generally recognised as a cause of recurrent aphthae, though isolated cases have been reported (Hjorting-Hansen and Bertram, 1968; Walker, 1973; Porter et al. 1988; Palopoli and Waxman, 1990).

Wray et al. (1975) reported in a series of 130 patients with recurrent aphthous stomatitis, that deficiencies of iron, folic acid and vitamin B_{12} were found in 17.7 per cent and that most patients with proven deficiency improved rapidly on replacement therapy. These findings have been supported in similar studies.(Hutcheon et al. 1978; Tyldesley, 1981)

Challacombe et al (1977a) however, reported later a series of 193 patients with recurrent aphthous stomatitis and found a lower incidence of deficiencies. They reported that anaemia did not play a primary aetiological role in most cases of recurrent aphthae on the basis of the low prevalence. Twenty per cent of patients in this series, however were sideropenic and the authors postulated that this was secondary to the oral ulceration.

In addition, those workers found that in this group of patients they were able to distinguish the ulcers occurring in these deficient patients on a clinical and haematological basis from major, minor and herpetiform ulcers and termed them pseudo-major, pseudo-minor and pseudo-herpetiform ulcers (Challacombe et al. 1977a). These lesions could not be distinguished clinically by Wray et al in their original series (1975) or in a subsequent study (Wray et al. 1978).

Iron deficiency has been identified to be increased in other ulcerative oral mucosa lesions compared with normal individuals (Challacombe et al. 1983).

Recently, perhaps because of the improvement in diet, folic acid deficiency has decreased and is now rarely diagnosed in recurrent aphthous stomatitis patients (Miller et al. 1994). In a younger population (age 7-16 years) of patients, haematological investigations have been reported to have the same pattern as in adults patients with recurrent aphthous stomatitis (Field et al. 1987).

b) Concurrent systemic disease

The oral manifestations of Behçet's syndrome can be very similar to those in recurrent aphthous stomatitis, especially when they occur as the earliest manifestation of this syndrome (Cooke, 1979).

In addition to the evidence associating recurrent aphthous stomatitis with haematological deficiencies, a consistent association has been shown with gastrointestinal disease. Recurrent aphthae are associated with various gastrointestinal disorders, especially Crohn's disease, ulcerative colitis and coeliac disease (Wray et al. 1975; Ferguson et al. 1980; Tyldesley, 1981; Plauth et al. 1991; Seo et al. 1992). In addition, an association with orofacial granulomatosis has been reported (Wiesenfeld et al. 1985; Field and Tyldesley, 1989; Bozkurt et al. 1992). Sircus et al (1957) however, believed that the incidence of recurrent aphthae among the population was so great that associations with gastrointestinal disease were probably of minor significance. This does not take into account, however, the clinical response seen in

recurrent aphthae patients when the underlying disease is treated (Wray et al. 1975; Lisciandrano et al. 1996).

Wray et al (1975) showed an incidence of 5.3 per cent of malabsorption and gluten sensitivity in their patients and later Ferguson et al (1975) reported that 8 of 33 patients with recurrent aphthous stomatitis had gluten sensitive enteropathy. Others subsequently have, however, been unable to confirm the high prevalence seen in the latter study (Ferguson et al. 1980; Rose et al. 1996). However, Walker et al (1980) showed a favourable response to gluten withdrawal in a small series of patients with recurrent aphthous stomatitis without enteropathy. This was strongly confirmed by other investigators (Wray, 1981; Hay and Reade, 1984).

Recently, it has been believed that there was no significant effect with gluten withdrawal and it has been suggested that in previous studies there were marked placebo responses in the condition (Hunter et al. 1993).

Aphthous ulcers can be one of the oral manifestations of immunodeficiency diseases such as AIDS. Aphthous-like ulcers may first be seen during the acute illness associated with HIV infection (Phelan et al. 1991; MacPhail et al. 1991; Ficarra, 1992; Scully and McCarthy, 1992; Porter and Scully, 1993; Muzyka and Glick, 1994; Weidle, 1996; Scott and Bender, 1996; Youle et al. 1996).

Recurrent aphthous stomatitis has also been reported as a muco-cutaneous manifestation of chronic fatigue syndrome (Krueger et al. 1987; Wahren and Linde, 1991; Rebora and Drago, 1994).

c) Endocrine influences

A clear relationship has been demonstrated in some women with recurrent aphthous stomatitis and their menstrual cycle, with ulceration being most troublesome in the luteal phase between ovulation and menstruation (Dolby, 1968a; McCartan and Sullivan, 1994). These patients usually notice clearing of their ulcers during pregnancy. In the study of Silverman and Shouse (1966) 50 per cent of the female patients with recurrent aphthous stomatitis were symptomatically improved during oestrogen administration. However their findings did not indicate the effect of endogenously secreted oestrogen and conjugated oestrogen substances administered in the form of an oral contraceptive on cornification of human oral epithelium (Silverman and Shouse, 1966).

Carruthers (1967) also treated patients with oral contraceptives and subsequently high dose oestrogen was effectively used (Bishop et al. 1967). Ferguson et al (1978) have used progestogens successfully in women with pre-menstrual aphthae but found them ineffective when no menstrual relationship existed. This is also supported by McCartan and Sullivan (1996) who tried oestrogen replacement therapy on 114 women with aphthous ulcers. In this trial, women with menstrual cycle associated aphthae were more likely to have complete or partial remission when receiving therapeutic ovarian hormones than those with unassociated aphthae. Also, women in whom lesions started or deteriorated during pregnancy, hormone therapy did not effect an improvement in the ulceration (McCartan and Sullivan, 1996).

In epithelium, two actions of oestrogen are to increase the rate of cellular proliferation and to promote keratinization. Such changes have been best identified in the vagina but oral mucosa also follows a similar trend (Ferguson, 1975). Keratinization of oral and vagina mucosa probably undergoes comparable changes during the menstrual cycle and pregnancy (Ferguson, 1975) although Silverman and Shouse (1966) did not detected these changes.

It is not yet clear, how sex hormones can effect different diseases. In *in vitro* studies of the differences in the susceptibility of mice to coxsackievirus B3 infection, it was reported that males generated an earlier and more powerful cytotoxic T cell response but a lower antibody response than females (Mims and White, 1984).

Sex hormones act via different surface receptors directly on T cells, especially CD8-positive cells. Oestrogen can increase the level of antibody production by inhibiting T suppresser activity. Progesterone, on the other hand, increases T suppresser function. Thus it is clear that hormones can influence the immune system and play a part in the higher frequency of autoimmunity in females. However, understanding of this role is still incomplete (MacPhail et al. 1993). It should be noted that, the administration of excess sex and cortocosteroid hormones enhanced the severity of viral infection and may depress immune responses, but adequate production of these hormones in the body is vital for normal resistance to infection. In case of imbalance in hormone production, excessive or widespread inflammatory responses could cause damage to host organs, and viral infections often shows much greater severity (Mims and White, 1984).

1.6. Immunological Factors

1.6.1 Humoral Immunity in Recurrent Aphthous Stomatitis

a) Total immunoglobulin levels in serum

The levels of immunoglobulin in serum from patients with recurrent aphthous stomatitis has had conflicting results in different studies. A decreased level of IgA was reported in six of eight sera from patients with recurrent aphthae (Brody and Silverman, 1969). Other studies have shown no differences between patients and controls for IgG, IgM or IgA (Thomas et al. 1973; Ben-Areh et al. 1976; Malmstrom et al. 1983) and no changes in relation to disease activity (Ben-Areh et al. 1976). Sun, et al (1986) measured serum IgG, IgM and IgA levels in oral aphthous patients during the various stages of the disease. IgG, IgM and IgA were significantly higher than the normal levels for healthy subjects. Serum IgG and IgM in patients were elevated during the early stage as compared with normal ($p<0.001$) (Sun et al. 1986). In the late stages IgA was also significantly increased but only IgG remained elevated in the convalescent stage, when IgA and IgM returned to normal. Significantly raised levels of IgG and IgA have been reported in sera from major aphthous stomatitis (Lehner, 1969a) although this has not been confirmed (Scully et al. 1979).

In a recent study of IgG subclasses, the majority of patients with minor recurrent aphthous stomatitis had no significant changes in serum levels of IgG1, IgG2, IgG3 or IgG4. However 8.4 per cent had marginally raised IgG1 levels and 1.4 per cent a significantly low level of IgG3 (Porter et al. 1992).

b) Total immunoglobulin levels in saliva

Salivary IgA levels have been reported as normal in patients with recurrent aphthous stomatitis and have not been shown to vary with disease activity (Wolf and Graykowski, 1979; Bennet and Reade, 1982). Raised IgG and IgA levels in stimulated mixed saliva have been reported, however, in Behçet's syndrome(Lehner, 1969a).

A significant reduction in IgA levels in stimulated parotid saliva has been reported in major aphthae, herpetiform ulcers and Behçet's syndrome (Scully et al. 1979). IgA secretion rates were also depressed in patients in arthritic, ocular and muco-cutaneous types of Behçet's syndrome. Lacrimal fluid IgA concentrations were increased in arthritic and ocular Behçet's syndrome, indicating there was not a generalised secretory abnormality in these patients (Scully et al. 1979). These data may indicate that IgA is necessary to protect the oral mucosa against disease.

c) Circulating immune complexes

It has been reported that, C9 and C-reactive protein are increased in Behçet's syndrome and recurrent aphthous stomatitis (Al-Bayaty et al. 1989). The higher levels of C9 and C-reactive protein in Behçet's syndrome might be important in the transition from the unifocal aphthous stomatitis to its systemic counterpart (Al-Bayaty et al. 1989). However, in patients with recurrent aphthous stomatitis, these changes in serum levels of complement throughout the course of the disease were not confirmed (Sun et al. 1986). Also, in various stages of recurrent aphthous stomatitis the levels of circulating immune complexes increased (Sun et al. 1986). The increased level of

circulating immune complexes is not directly correlated with the activity of the disease (Sun et al. 1986).

IgG circulating immune complexes were found in recurrent aphthous stomatitis and Behçet's syndrome although they were far more prominent in Behçet's syndrome, especially in the neuro-ocular and arthritic types. In recurrent aphthae immune complexes were at the highest level in herpetiform ulcers but these differences were not significant (Adinolfi and Lehner, 1976). Periods of remission were characterised by a switch from IgG or IgM complexes to IgA complexes and it has been hypothesised that immune complexes are not only of aetiological significance in Behçet's syndrome but are also important in determining the transition from recurrent aphthous stomatitis to Behçet's syndrome (Adinolfi and Lehner, 1976). Recently investigations have been carried out to identify the specificity of the complexed antibodies (Burton Kee et al. 1981). Preliminary results indicate that complexes cross react only within patient populations and not controls, implying the presence of an exogenous antigen (Burton Kee et al. 1981).

Furthermore, antigens from different damaged tissues may differentiate subsets of patients. It is hypothesised that this approach could eventually lead to the identification of antigens important in the aetiology of recurrent aphthous stomatitis and Behçet's syndrome (Burton Kee et al. 1981).

Cryoglobulins of the IgG, IgM or IgA class, with or without C3, were significantly found in recurrent aphthous stomatitis and Behçet's syndrome (Lehner et al. 1979b).

Cryoglobulins are abnormal plasma proteins (paraproteins) which are grouped with gamma globulin. They are characterised by precipitating, gelling or crystallising when serum or solutions of them are cooled. Cryoglobulins can play a pathogenic role in disease by complement activation, platelet aggregation and cytotoxic activity (Lehner et al. 1979b).

d) Circulating antibodies against micro-organisms

Mycoplasmas and parasites

Haemagglutinating antibodies to *Mycoplasma orale* were found in five of 33 patients with recurrent aphthae. No antibodies were found to *Mycoplasma laminus*. Control sera however showed a similar prevalence of antibodies against these mycoplasmas (Gordon et al. 1967).

Complement fixing antibodies to Toxoplasma were similar in patients and controls (Sircus et al. 1957).

Bacteria

Donatsky and Dabelsteen (1974b) examined the sera of recurrent aphthae patients and controls for the presence of humoral antibodies to *Streptococcus sanguis*, American type culture collection (ATCC) 10556 by means of a double layered immunoflurescent technique. Antibodies to the *Streptococcus sanguis*, strain 2A antigen were demonstrable in both populations, but the mean end point titres were significantly higher among the patients when compared to controls using this semiquantitive technique. They also reported that no antibodies against Neisseria were

detected in the same sera. Later Donatsky, (1976a; 1978) using class specific anti-human IgG, IgM and IgA fluorescent antibodies, reported that IgG antibodies were also significantly raised against *Streptococcus pyogenes* group A, strain M5 National Collection of Type Cultures (NCTC) 100065. The anti-*Streptococcus sanguis* antibodies did not change in relation to exacerbation of the disease. IgM and IgA antibodies against this organism, although present in patient sera, showed a similar distribution of end point titres to the distribution among sera from controls (Donatsky, 1976a; Donatsky, 1978). In addition, IgG, IgM and IgA antibodies against *Neisseria subflava* and IgG antibodies against Staphylococcus were often detected in patient sera but at the same concentration as that found in controls (Donatsky, 1978). IgM and IgA antibodies against Staphylococcus were present in only a few sera studied (Donatsky, 1978).

Viruses

Humoral immunity protects the host against reinfection by the same virus. Neutralizing antibody blocks the initiation of viral infection, probably at the stage of attachment or uncoating. Secretory IgA antibody is important in protecting against infection by viruses through the respiratory or gastrointestinal tracts (Jawetz et al. 1989). There are five different classes of immunoglobulins. The major serum antibodies are of IgG class. This class can in turn be divided into four subclasses, on the basis of the difference in the constant regions of their heavy chains. Much antiviral IgG is of the IgG3 subclass, while IgG1 responses are also particularly prominent in herpesvirus infections (Scully and Samaranayake, 1992).

IgM is the earliest serum antibody to appear in infections and is mainly confined to the vascular system. It has high affinity and this together with its early production, helps curb the spread of virus in the early stages. As the immune response matures, the IgM antibodies are gradually replaced by IgG. It is noteworthy that IgM antibodies are also the first to be detected in the foetus and cannot cross the placenta. Therefore the presence of antiviral IgM in sera suggests recent viral infection (Scully and Samaranayake, 1992).

Secretory IgA is the principal and most important immunoglobulin in secretions and on mucosal surfaces including oral, mainly in saliva. IgA antibodies are crucially important in resistance to infection of the mucosal surfaces (Scully and Samaranayake, 1992).

IgD and IgE constitute a very small proportion of immunoglobulins; IgD antibodies are present on B-cell surfaces while IgE is involved in allergic responses and defence against parasites. The roles of IgD and IgE are otherwise somewhat unclear (Scully and Samaranayake, 1992).

Specific antibodies play a critical role in modulating viral infections. They may interact with the virion itself, and either neutralize the virus or destroy the virus-infected host cell by a number of mechanisms. Viruses may well however, be intracellular and thus inaccessible to antibody (Scully and Samaranayake, 1992).

Adenovirus have been suggested to be of aetiological significance in recurrent aphthae although attempts to detect circulating antibodies have been carried out only for

herpetiform ulcers in patients, all of whom showed circulating antibodies to this virus. Antibodies in sera from healthy controls, however, were not examined (Sallay et al. 1971a).

Antibody titres against Herpes Simplex virus are not raised in sera from patients with recurrent aphthous stomatitis and end point titres do not vary with disease activity. Furthermore, such patients do not have positive skin tests to herpesvirus (Stark et al. 1954; Sircus et al. 1957).

In a preliminary study, serum IgG antibody against HCMV was positive in all patients with recurrent aphthous stomatitis and controls except one recurrent aphthous stomatitis patient in the active stage (Sun et al. 1996). In this investigation, the level of serum anti-HCMV IgG antibody was significantly higher in the remission stage compared with the active period of ulceration (Sun et al. 1996). In a study of two separate groups of patients with recurrent aphthous stomatitis, HCMV IgM antibody was significantly increased only in one of the groups, compared with healthy controls (Pedersen and Hornsleth, 1993). No explanation was made to indicate the two different results in the two groups of patients with almost the same stage of the disease. Also, there was no significant difference between patients and controls in their anti-HCMV IgG, M and anti-HCMV IgA (Pedersen and Hornsleth, 1993).

In another investigation on sera from five patients in the ulcerative stage of recurrent aphthous stomatitis and 10 to 14 days later, none of the patients had any difference in antibody concentration for adenovirus or influenza A, or B, para-influenza, or

respiratory syncytial viruses in paired sera (Pedersen, 1989b), while all patients had at least a four-fold difference in specific VZV antibody level between the two samples (Pedersen, 1989b). The author concluded that a four-fold or more difference in VZV antibody titres indicated a current infection (reinfection) and it showed VZV as a cause for oral ulceration in recurrent aphthous stomatitis (Pedersen, 1989b). Later on in a larger group of patients this finding was not confirmed (Pedersen and Hornsleth, 1993). In two groups of patients with recurrent aphthous stomatitis, 33 per cent (6/18) in first group and 51 per cent (9/17) of the patients in second group were serum anti-VZV IgM antibody positive and there was a significant difference between patients and controls in their VZV IgM values (Pedersen and Hornsleth, 1993). There was not any change, however between the first sample in the active period and the second one after 10-14 days of initiation of the ulcer (Pedersen and Hornsleth, 1993).

Anti Epstein-Barr virus IgG was not significantly different in recurrent aphthous stomatitis patients compared with controls (Pedersen and Hornsleth, 1993). Anti-Epstein Barr virus IgM also, did not seem to be different between patients and healthy controls (Pedersen and Hornsleth, 1993).

With respect to circulating antibody against HHV-6 virus, only anti HHV-6 IgG has been investigated in recurrent aphthous stomatitis patients. In this study the majority of recurrent aphthous stomatitis patients and healthy controls were sero-positive for anti HHV-6 IgG and there was no difference between patient groups and controls (Pedersen and Hornsleth, 1993). In addition, two of the HHV-6 IgG positive patients

were positive for HHV-6 IgA, while none of the controls were HHV-6 IgA positive (Pedersen and Hornsleth, 1993).

e) Circulating antibodies against tissue antigens

Oral mucosa

Patients with recurrent aphthous stomatitis have been reported to have circulating antibodies against saline extracts of human oral mucosal extract using haemagglutination, complement fixation, and microprecipitation (Lehner, 1964; Lehner, 1965; Lehner, 1967b). In addition, indirect immunofluorescence has revealed circulating antibodies of the G and M classes to allogenic foetal and oral mucosa (Donatsky and Dabelsteen, 1974a; Donatsky, 1976a; Donatsky and Dabelsteen, 1977; Donatsky, 1978). *In vivo* binding of these isotypes to oral mucosa, as demonstrated by direct immunofluorescence, has been reported (Lehner, 1969c), although this has not been confirmed (Donatsky and Dabelsteen, 1977). These antibodies appear to be able to bind complement (Lehner, 1969c; Donatsky and Dabelsteen, 1977; Donatsky, 1976a; Donatsky and Dabelsteen, 1974a; Donatsky, 1975; Lehner, 1969b). Antibody deposition is localised to the intra-cytoplasmic areas of the prickle cell layer of the oral epithelium (Lehner, 1969c). Such antibody deposition is not found in normal oral mucosa or experimentally induced oral wounds (Donatsky and Dabelsteen, 1977).

Organ and species specificity

Autoantibodies in the sera of patients with recurrent aphthous stomatitis exhibit a certain degree of organ specificity. Thus immune absorption studies of four sera showed that antibodies to foetal oral mucosa were absorbed by the mucosa from

foetal pharynx, larynx, oesophagus, conjuctiva, vagina and colon in addition to oral mucosa (Lehner, 1969b). Haemaglutinating antibodies, however, against human skin, colon and liver or antibodies detected by immunofluorescence against human skin and muscle were not found in sera from patients with recurrent aphthae (Lehner, 1964; Lehner, 1965; Donatsky and Dabelsteen, 1974a). Other antibodies such as nuclear, thyroid or gastric antibodies have not been shown in recurrent aphthous stomatitis (Addy and Dolby, 1972; Lehner, 1972). Immune absorption with the mucosa of guinea pig, mice or rabbits will absorb out haemaglutinating antibodies to foetal oral mucosa (Lehner, 1969b) and patients' sera with positive antibodies to adult human oral mucosa will cross-react with guinea pig oral mucosa using an immunofluorescent technique (Donatsky and Dabelsteen, 1974a).

It appears, therefore, that sera from patients with recurrent aphthous stomatitis contain auto-antibodies which have some tissue specificity but are not specific.

Antigenic cross-reactivity of antibodies

Antigenic cross-reactivity between adult and foetal human oral mucosa and *Streptococcus sanguis*, strain 2A was investigated by immunofluorescence (Donatsky, 1975). Absorption studies indicated that there was a significant cross-reactivity between *Streptococcus sanguis* and adult human oral mucosa and to a lesser extent with foetal mucosa (Donatsky, 1975). Subsequent immunoelectrophoretic analysis of these crude antigens mitigates against the possibility that the apparent cross-reactivity is due to bacterial contamination of the oral mucosal extracts (Donatsky, 1980) although antigenic determinants, important in the pathogenesis of recurrent aphthous

stomatitis, remain to be defined. However, this cross-reactivity between oral streptococcal and oral mucosal antigens was not supported by the findings of Hoover et al (1984).

Heat shock proteins are a group of highly conserved proteins found in eukaryotic and prokaryotic cells, including Gram-positive and Gram-negative micro-organisms (Hasan et al. 1995), therefore they may cause cross-reactivity between microbial and cellular host materials (Hasan et al. 1995). Recently, cross-reactivity between four peptides from the 65 KD mycobacteria heat shock protein and four peptides from the homologous sequence of the 60 KD human heat shock proteins has been reported to cause a lymphoproliferative response and subsequent to that oral ulceration in Behçet's syndrome (Fortune et al. 1996). The four peptides of mycobacteria increase the TCR-γδ subsets of cells. These cells are suggested to be involved in the pathogenesis of Behçet's syndrome and recurrent aphthous stomatitis (Pedersen and Ryder, 1994; Fortune et al. 1996). They increase the expression of heat shock proteins which cross react with host stress protein and cause tissue damage (Fortune et al. 1996). In a study on stimulation of peripheral blood mononuclear cells with the mycobacterial 65 KD heat shock protein, there was no statistically significant lymphoproliferative response toward this heat shock protein in recurrent aphthous stomatitis patients compared with control groups (Hasan et al. 1995). However, in T-cell epitope mapping using 29 overlapping peptides, derived from the mycobacterial 65 KD heat shock protein, only peptide 91-105 caused a significantly higher stimulation of lymphocytes in recurrent aphthous stomatitis patients compared with

controls (Hasan et al. 1995). The homologus 60 KD human mitochondrial peptide (amino acid 116-130) also yielded significant lymphocyte stimulation in specimens from recurrent aphthous stomatitis patients compared to controls (Hasan et al. 1995). This increase in T-lymphocytes responding to peptide 91-105 and to a lesser extent peptide 116-130 was found to be significant during ulceration, especially the prodromal stage, compared with remission (Hasan et al. 1995). The T cell epitope (91-105) was derived from the mycobacterial heat shock protein, that is highly conserved among Gram-positive bacteria which colonize in the oral cavity (Hasan et al. 1995). *Streptococcus sanguis* contains one or more B cell determinants (88-123) which overlap with the T cell epitope 91-105 (Hasan et al. 1995). Rabbit anti-sera against the 65-KD heat shock protein of *Mycobacterium tuberculosis* display a corresponding 65-KD band as well as with all six *Streptococcus sanguis* strains and *Streptococcus pyogenes* but not with *Streptococcus salivarius*. Also, by immunoblot analyses, a 65-70 KD band was found more frequently with *Streptococcus sanguis* KTH-2 or KTH-3 and IgA antibodies to the mycobacterial 65 KD heat shock protein in serum from patients with Behçet's syndrome than with sera from controls (Lehner et al. 1991).

In conclusion it is likely that the most cross-reactivity between microbial and cellular host material is that between heat shock proteins which are highly conserved. The increase of the 91-105 heat shock protein in oral mucosa may cause expansion of an auto-reactive T cell clone primed to the homologous peptide 116-130 which causes antigenic reaction and results in mucosal damage (Hasan et al. 1995).

f) Immunoglobulin deposits within the tissues

Immunoglobulins deposited within the tissues in patients with aphthous stomatitis and the presence of immunoglobulin-bearing intra-lesional leukocytes have been previously discussed in the section dealing with the histology of recurrent aphthous stomatitis. (Section 1.3.3)

1.6.2. Cell-Mediated Immunity in Recurrent Aphthous Stomatitis

The literature pertaining to the cellular immune response in recurrent aphthous stomatitis is extensive. Early studies were prompted by the intense lympho-monocytic infiltration seen on histological examination which is consistent with a delayed hypersensitivity reaction (Graykowski et al. 1966).

The association of recurrent aphthous stomatitis with immunologically related diseases and *in vitro* immunological studies strengthen the possibility of an immunopathogenesis for recurrent aphthae (Roy, 1977).

In addition, *in vivo* studies of delayed hypersensitivity further enhance this claim.

a) Cell-mediated immunity to mitogens

Lymphocytes from patients with recurrent aphthous stomatitis have been shown, on several occasions (Lehner, 1967a; Francis and Oppenheim, 1970; Martin et al. 1979), to proliferate normally *in vitro* when stimulated by mitogens such as phytohaemagglutinin (PHA) and conconcavalin A (con A).

Recently, however, depressed responses to these mitogens have been reported at the early stage of ulceration suggesting a non-specific immunodepression in these patients which may be an effect rather than a cause of the ulcerative lesions (Greenspan et al. 1981).

Exacerbation of Behçet's syndrome and recurrent aphthous stomatitis has been reported to occur after the ingestion of English and black walnuts in some patients (Marquardt et al. 1973). The meat of these nuts appears to be mitogenic to lymphocytes and chemotactic for mononuclear leukocytes *in vitro* in both patients and controls (Oppenheim et al. 1973). Basophils from all patients with clinically symptomatic unrelated allergies released a significant proportion of their histamine when exposed to mitogen containing extracts of black walnut meat. In contrast, non allergic subjects released little or none of their histamine (Oppenheim et al. 1973). The pellicle from these walnuts is rich in heat labile tannins which significantly depress lymphocyte responses (Oppenheim et al. 1973).

When walnuts are ingested they cause, within 48 hours, a 50 per cent reduction in lymphocytes proliferating *in vitro* to all stimulants in patients and controls (Marquardt et al. 1973). It was therefore suggested that a temporary depression of the functional T cell population by walnuts may indirectly allow B cells to produce more immunoglobulin and exacerbation of the disease (Marquardt et al. 1973).

b) Cell-mediated immunity against viruses

Both humoral and cellular components of the immune response are involved in control of viral infections. Virus-encoded proteins, usually capsid proteins, serve as targets for the immune response. Virus-infected cells may be lysed by cytotoxic T lymphocytes as a result of their recognition of viral polypeptides on the cell surface (Jawetz et al. 1989).

Patients with recurrent aphthous stomatitis have negative skin tests to herpes hominis (Stark et al. 1954). Herpesvirus and adenovirus have, however, been reported to induce blastogenesis of lymphocytes from patients with recurrent aphthae (Nasz et al. 1971; Sallay et al. 1971b). Assessing blastogenesis morphologically using light microscopy, these studies found transformation in 14 of 28 patients using herpesvirus and in 21 of 28 patients using adenovirus. The figures observed for controls were 3 of 12 and 4 of 21 respectively. On this basis the authors suggested that these viruses may be of aetiological significance in recurrent aphthous stomatitis. These findings await confirmation.

c) Cell-mediated immunity against bacteria

In vivo studies

Graykowski et al (1966) first reported that all of 30 patients with recurrent aphthous stomatitis showed a positive delayed hypersensitivity response after intradermal injection with *Streptococcus sanguis*, strain 2A, although this did not occur so consistently among controls. These authors were also able to produce aphthous-like lesions in laboratory animals experimentally by injecting them with this organism

(Graykowski et al. 1966). Later, Barile et al (1968) showed that 30 of 32 patients with recurrent aphthous stomatitis produced a skin reaction after intradermal injection with this organism; the maximum intensity of this reaction was seen after 24 to 48 hours. Only three of six patients with non-aphthous lesions and one of six healthy controls showed a similar response. Such intradermal challenge caused exacerbation of the stomatitis in four patients with minor recurrent aphthous stomatitis and in four patients with major aphthae, the sites of the skin tests necrosed and healed with scarring in a fashion similar to that seen with major aphthae in the mouth (Barile et al. 1968).

A pronounced delayed-type skin reaction to *Streptococcus viridians* has also been reported in a patient with recurrent aphthous stomatitis (Shore and Shelley, 1974), and skin reactions have also reported to be increased in association with recurrent aphthous stomatitis to several bacterial strains especially *Streptococcus viridans*, *Streptococcus pneumonia*, Streptococcus Lancefield group G, *Neisseria catarrhalis*, *Heamophilus influenzae*, *Staphylococcus aureus* and *Klebsiella pneumonia* (Sallay et al. 1971c; Shore and Shelley, 1974).

In vitro studies

In vitro findings with regard to the cellular immune response to bacteria among patients with recurrent aphthous stomatitis are conflicting.

By using leukocyte migration inhibition as a parameter of cellular immune function, several studies have shown that a hypersensitivity to *Streptococcus sanguis*, strain 2A

exists in recurrent aphthous stomatitis patients (Donatsky, 1976a; Donatsky, 1976b; Donatsky, 1978; Greenspan et al. 1981a). This could not be demonstrated among controls with other oral lesions or experimentally-induced oral wounds (Donatsky, 1976b). Moreover, several other bacteria tested, including *Neisseria subflava*, staphylococci, *Brucella abortus*, and *Escherichia coli* did not reveal any differences between patients and controls (Donatsky, 1976b; Donatsky, 1978). *Streptococcus pyogenes* (group A, strain M5, NCTC 100065) elicited a similar response to that of *Streptococcus sanguis*, strain 2A, but a correlation with disease activity was only seen with *Streptococcus sanguis*, strain 2A (Donatsky, 1976a; Donatsky, 1978).

When lymphocyte transformation was used to measure the cell-mediated response to the original isolate of *Streptococcus sanguis*, strain 2A in patients with recurrent aphthous stomatitis, a significant hypo-responsiveness was seen when compared with controls (Francis and Oppenheim, 1970). This work could not be reproduced by another group when the American type culture collection equivalent (ATCC 10556) was used (Martin et al. 1979).

More recently a hyporesponsiveness was seen in the proliferative response to *Streptococcus sanguis*, ATCC 10556 in patients with early lesions, although this group also showed a similar hyporesponsiveness with various mitogens (Greenspan et al. 1981b).

When the original study (Francis and Oppenheim, 1970) on lymphocyte transformation was carried out, the hyporesponsiveness to *Streptococcus sanguis*

appeared enigmatic, but recent evidence regarding immunoregulation makes these findings more plausible.

d) Cell-mediated immunity against tissue antigens

Oral mucosa

Increased lymphocyte transformation was induced by saline extracts of homogenates of oral mucosa in patients with minor aphthae and to a lesser extent in herpetiform ulcers (Lehner, 1967a). Lymphocyte transformation was regarded as positive when more than 400 counts per 10 minutes were observed. Lymphocyte transformation was positive in 10 of 19 patients with major or minor aphthae and only two of nine patients with herpetiform ulcers. A negative response was found in 12 normal controls and 13 patients with other oral ulcerative conditions. On the basis of data the author suggested that recurrent aphthous stomatitis was an autoimmune disease. More recently however, an enhanced lymphocyte proliferative response to oral mucosa could not be confirmed using a microtitre technique and indeed a temporary hyporesponsiveness at the early stage in recurrent aphthae patients was reported (Greenspan et al. 1981a).

Leukocyte migration inhibition has also been reported to be enhanced with oral mucosa in patients with recurrent aphthous stomatitis (Donatsky, 1976a; Donatsky, 1976b; Donatsky, 1978).

Further support for the involvement of cell-mediated immunity in the pathogenesis of recurrent aphthous stomatitis has been gained by demonstrating that lymphocytes

from patients with recurrent aphthae, but not controls, induce cytotoxicity in cultures of gingival epithelial target cells (Dolby, 1969; Rogers III et al. 1974; Rogers III et al. 1976). Tissue specificity of the cytotoxic response to oral epithelium was suggested by the lack of cytotoxicity to skin, vaginal or colonic mucosal cell (Dolby, 1970b; Dolby, 1972). This cytotoxicity was blocked by pre-incubation of the effector cells with rabbit anti-human lymphocyte serum (Dolby, 1970a) or the addition of hydrocortisone (Dolby, 1970b). Unfortunately, gingival epithelial target cells were used which is an area of the mouth generally not affected by aphthous lesions. The mechanism of cytotoxicity involved in these experiments is not entirely clear, although it was probably antibody independent. More recently however, raised antibody dependent cellular cytotoxicity (ADCC) has been demonstrated in patients with recurrent aphthous stomatitis using chicken red blood cell as targets (Greenspan et al. 1981a). These data indicated an increase in circulating killer (K) cells but did not address the question of whether ADCC was actually involved in the destruction of oral mucosal cells. In a study by Burnett and Wray (1985) in an *in vitro* experiment they showed that sera from recurrent aphthous stomatitis patients induced significantly more cytolysis than matched control sera. This cytolysis effect would be demolished by inactivating the sera at 56°C for 30 minutes. Also recurrent aphthous stomatitis mononuclear leukocytes showed no significant direct cytotoxicity. When recurrent aphthous stomatitis leukocytes combined with heat-inactivated autologous serum were compared with recurrent aphthous stomatitis leukocytes combined with heat-inactivated fetal calf serum (FCS), although no significant difference was observed between these two groups, in two of nine cases, recurrent aphthous

stomatitis leukocytes showed a greater cytolytic effect when combined with heat-inactivated autolougous serum than when combined with heat-inactivated FCS. These results suggested the presence of antibody-dependent cell-mediated cytotoxicity (ADCC) in some cases of recurrent aphthous stomatitis. (Burnett and Wray, 1985)

Other human tissues

A certain tissue specificity of the cell-mediated immune response is apparent in recurrent aphthous stomatitis. Although foetal human skin induced lymphocyte transformation in patients with recurrent aphthae, extracts of foetal human tissues from colon, striated muscle, salivary glands and liver only occasionally stimulated lymphocytes from these patients and a similar response was also seen in controls (Lehner, 1967a). In addition, extracts of kidney or uterus did not cause any significant leukocyte migration inhibition in lymphocyte cultures from patients with recurrent aphthae (Donatsky, 1976a; Donatsky, 1976b; Donatsky, 1978). Furthermore, no increased lymphocyte cytotoxicity was seen by patients lymphocytes toward skin, vaginal or colonic mucosal target cells (Dolby, 1972; Rogers III et al. 1974). No data have been reported with respect to species specificity of the cellular immune response.

1.6.3. Virus-Induced Immune Dysfunction

Some viruses, particularly the herpesviruses and HIV, can induce immune dysfunction and immune suppression. Herpesviruses suppress cellular immunity; mechanisms include inhibition of cytokine production and direct interaction of the virus with the

major histocompatibility complex components. Other responses, such as granulocyte function, antibody production and natural killer cell cytotoxicity are not impaired.

Environmental, genetic and hormonal factors appear to be involved in the pathogenesis of autoimmune disorders. No single micro-organism or mechanism can explain the varied phenomena of autoimmune disorders but evidence is accumulating that certain viral infections can be responsible by modifying or releasing sequestered cellular proteins, altering the immune system by polyclonal B-cell activation, so disturbing immunoregulation or releasing lymphokines, molecular mimicry, anti-idiotype antibodies, or immune complex formation. Autoantibodies may appear after some viral infections or vaccinations and may herald autoimmune disease. Viral infection may also exacerbate autoimmune diseases. Viral infections often disturb the host's immune system. Lymphocytes are, for example, often infected by HIV, hepatitis B, mumps or herpesviruses with profound consequences (Scully and Samaranayake, 1992).

Viruses may also change infected cells and thereby trigger autoantibody formation. Viruses, or their components (nucleic acids or antigens), may be found in autoimmune disorders. This may imply a pathogenic role or the virus may be a mere passenger. On the other hand, absence of these viral footprints does not exclude a role for viruses, which may have acted in a hit and run fashion (Scully and Samaranayake, 1992).

1.7. The Management of Recurrent Aphthous Stomatitis

The literature pertaining to the treatment of recurrent aphthous stomatitis is extensive. The majority of therapies used are empirical or symptomatic and the variety of therapeutic regimes reported indicate that none are adequately effective.

For the purposes of this review, proposed therapies for the treatment of recurrent aphthous stomatitis will be divided into those employed with the intention of reducing the number and severity of lesions and those used only to provide symptomatic relief.

1.7.1. Preventive Therapy

Vitamins and minerals

It has been reported that vitamin B_{12} injections in a patient with pernicious anaemia could resolve all oral manifestations of the disease including the recurrent aphthous stomatitis (Hjorting-Hansen and Bertram, 1968). The same effect of vitamin B_{12} was seen in other recurrent aphthous stomatitis patients with vitamin B_{12} deficiency (Walker, 1973).

In the study of Wray et al (1975) in a series of 130 patients with recurrent aphthous stomatitis, all 23 patients with proven deficiencies of iron, folic acid and vitamin B_{12} were improved or showed a remission of their ulceration with haematinic therapy (Wray et al. 1975).When this series was extended 87 per cent of deficient patients with recurrent aphthous stomatitis had a remission or marked improvement when the deficiency was treated (Wray et al. 1978). These studies were subsequently confirmed, when in a series of 200 recurrent aphthous stomatitis patients, 51 patients

were shown to have deficiencies of iron, folic acid or vitamin B_{12}. In this group of patients 67 per cent with deficiencies showed a remission or marked improvement with replacement therapy (Nally and Blake, 1975). In all of these groups of patients, (Nally and Blake, 1975; Wray et al. 1975; Wray et al. 1978) replacement with vitamin B_{12} was most effective while iron replacement was least effective. Also remission and improvement of the ulcers was demonstrated in other studies as a response to vitamin B_{12}, folate and iron therapy in haematological deficient recurrent aphthous stomatitis patients (Challacombe et al. 1977a; Hutcheon et al. 1978; Challacombe et al. 1983; Palopoli and Waxman, 1990). However in these reports a placebo effect was not considered but other workers showed that the systemic treatment with haematinics in the absence of detected deficiencies is ineffective (Sircus et al. 1957; Farmer, 1958). Although the combined therapy with aneurine hydrochloride and folic acid was reported to improve the severity of the ulcers in approximately half the examined subjects (Farmer, 1958; Roy, 1966). Recently, the use of vitamins B1, B2 and B6 was reported to be effective in treatment of recurrent aphthous stomatitis in whom vitamin B1, B2 or B6 deficiencies were diagnosed (Nolan et al. 1991b). These findings have not been repeated.

In a study of 63 patients with recurrent aphthous stomatitis, nine patients were shown to be deficient of zinc and in an open trial of systemic zinc sulphate the majority of patients were reported to improve (Merchant et al. 1977). A mild effect of systemic zinc was later reported in a double-blind trial when a significant improvement was seen in only a few patients (Merchant et al. 1981; Raeste et al. 1981). Subsequently in

another double-blind cross over study, there was no evidence to indicate the efficiency of zinc therapy on the incidence or severity of the ulceration. Also, there was no correlation between zinc status and response to therapy in any of the patients. (Wray, 1982b) Although there is a report which showed that systemic zinc therapy could prevent recurrent aphthous stomatitis in a patient with zinc and cellular immune deficiencies (Endre, 1991).

Hormones

There is a body of evidence which shows the relationship between oestrogen and changes in oral mucosa and the vagina (Ferguson, 1975). Also the relationship of recurrent aphthous stomatitis with the menstrual period and pregnancy prompted the use of oestrogen and oral contraceptives to treat recurrent aphthous stomatitis (Carruthers, 1967). High dose oestrogen was found to be effective for treating this subgroup of patients (Bishop et al. 1967). Progesterone in depot injection form, either medroxyprogestrone acetate or gestronal hexanoate have been used successfully in 10 women with pre-menstrual aphthae (Ferguson et al. 1978). Gestronal hexanoate was used in addition to medroxyprogestrone acetate as the former can not be converted *in vivo* to hydrocortisone, indicating that the effect seen was not due to a glucocorticoid metabolite of the progesterone used (Porter and Scully, 1990). In a preliminary study, anabolic corticosteroids in combination with lysine were reported to be effective in eliminating recurrence in eight patients (Papanayotou, 1972). However the potential side effects of treatment with anabolic corticosteroids has been stressed (Hyman, 1972). In a recent investigation, an effect of ovarian hormones on concurrent

aphthous stomatitis indicates that women with menstrual cycle associated aphthae are more likely to have remission or improve their ulcers when receiving therapeutic ovarian hormones (McCartan and Sullivan, 1996). Despite the effect of hormone therapy in a few cases, the ultimate usefulness of this treatment in recurrent aphthous stomatitis remains in doubt due to the undesirable side effects observed.

Dietary manipulation

Food allergy and sensitivity has been suggested to be important in the aetiology of recurrent aphthous stomatitis (See section 1.5.2). Some studies have shown a beneficial effect from withdrawing suspect foods from the diet (Ship, 1960; Spouge and Diamond, 1963; Copeman, 1978; Wray et al. 1982; Hay and Reade, 1984; Nolan et al. 1991a), but this has not been a universal finding (Ship et al. 1962; Eversole et al. 1982). Recently, the gluten fraction of flour has been implicated in causing ulceration in some people with recurrent aphthous stomatitis. Also the incidence of coeliac disease is increased in patients with recurrent aphthae (see section 1.5.3.b). The oral ulceration in such patients with malabsorbtion resolves in response to gluten withdrawal (Wray et al. 1975; Ferguson et al. 1975; Wray et al. 1978; Ferguson et al. 1980; Tyldesley, 1981; Wray, 1981). These findings, however, were not confirmed in other reports (Walker et al. 1980; Hunter et al. 1993). In addition, further groups of patients without gluten-induced enteropathy were reported to respond to a gluten free diet (Walker et al. 1980; Wray, 1981; Wright et al. 1986).

Lysine

Lysine is one of eight essential aminoacids in man. It has been found that lysine has the most inhibitory effect on viruses between other naturally occurring amino acids. During invasion of virus into the host cell, it alters the cell metabolism, causing the cell to synthesize virus-specific proteins high in arginine and low in lysine. From these data, investigators postulated that a high lysine/arginine ratio in cells might serve as a virus inhibitor. Recent studies concur with this hypothesis (Kagan, 1974; Little, 1984; Miller and Foulke, 1984). The lysine/arginine ratio could be increased naturally by eating a diet containing foods high in lysine and low in arginine, such as milk and meat, or decreased by a diet full of nuts, chocolate and wheat (Miller and Foulke, 1984). In a study by Wright (1994), the use of lysine as a prophylactic and treatment agent caused improvement in recurrence and healing of the aphthous ulcers. This result could be explained either by the effect of lysine on the replication of virus thus suggesting a viral aetiology of recurrent aphthous stomatitis, or by the withdrawing of wheat as a gluten-free diet in patients. This finding might therefore, be an explanation for those studies (Walker et al. 1980; Wray, 1981; Hay and Reade, 1984) which showed improvement in aphthous ulceration during a gluten-free diet in patients with normal jeojunal biopsies. Also, a trial of anabolic corticosteroids in combination with lysine was effective in causing remission of ulcers in recurrent aphthous stomatitis patients (Papanayotou, 1972). However it is not clear which part of the medication had the more significant effect.

Antihistamines

The mechanism whereby allergy, especially to foods, exerts its effects on susceptible patients with recurrent aphthae is unclear. IgE bearing lymphocytes are significantly increased in aphthous lesions (Bays et al. 1977) and mast cells are increased in tissue sections from the prodromal stages of recurrent ulcers (Lehner, 1969c). These mast cells degenerate within 48 hours of new ulcer formation (Dolby and Allison, 1969). Di-sodium cromoglycate, which prevents mast cell degranulation (Assem, 1973) is partially effective in reducing the pain and duration of aphthous lesions (Frost, 1973; Walker and Dolby, 1975; Dolby and Walker, 1975; Kowolik et al. 1978; Vaughan-William and Dolby, 1981). Antihistamines, however, though reported to be useful by some (Lehner, 1967b), have been shown to be without effect by others (Graykowski et al. 1966; Dolby, 1968b).

Immunopotentiating agents

Multiple smallpox and influenza A and B vaccinations were not effective in the treatment of recurrent aphthous stomatitis despite a few reports which showed some improvement in aphthous ulcers (Weichselbaum and Derbes, 1957; Graykowski et al. 1966; Miller and Foulke, 1984). Some of the preparations of human transfer factor given orally were reported to be effective in the treatment of recurrent aphthous stomatitis (Schulkind et al. 1984). Gamma globulin injections have proved to have no benefit in recurrent aphthous stomatitis (Fraser-Moodie, 1960; Graykowski et al. 1966). An initial report on the effectiveness of the immunopotentiator, levamisole, claimed this agent was effective in controlling recurrent aphthous stomatitis (Symoens

and Brugmans, 1974). In this study, levamisole was also effective in reducing the frequency of recurrent herpes infection (Symoens and Brugmans, 1974). Subsequent open and double-blind investigations into the use of levamisole have issued conflicting results (Olson et al. 1976; Meyer et al. 1977; Gier et al. 1978; Kaplan et al. 1978; Drinnan and Fischman, 1978; De cree et al. 1978; Olson and Silverman, 1978; Miller et al. 1978; Zissis et al. 1983). The therapeutic efficiency of this drug remains unclear.

Antiviral drugs

Similarity between clinical and immunohistochemical features of recurrent aphthous stomatitis and some viral diseases, especially those from herpesviruses, persuaded some investigators to try the effect of antiviral drugs in recurrent aphthous stomatitis. The effect of oral aciclovir was studied in recurrent aphthous stomatitis in a double-blind treatment trial (Wormser et al. 1988). In this study, 25 subjects who were under treatment for genital herpes simplex infection, received oral aciclovir, 400 mg daily for one year and 19 patients with the same condition of genital herpes simplex infection, received placebo for the same period of time and oral aciclovir only at the time of infection. All patients were self diagnosed to have at least one attack of recurrent aphthous stomatitis every year (Wormser et al. 1988). No significant difference was observed between the two groups of patients. Compared with patients' pervious history of attacks of the recurrent aphthous stomatitis lesions, 21 per cent of the group that received aciclovir intermittently had more attacks during the study, 32 per cent had fewer attacks, 32 per cent had the same number of attacks and 16 per cent were unevaluable for this comparison. Also frequencies observed in the group

treated continuously with aciclovir was approximately similar to the previous group where 24 per cent of the patients had more attacks, 20 per cent had fewer attacks, 48 per cent had the same number of attacks and eight per cent were unevaluable for this comparison (Wormser et al. 1988). In contrast, in another open study, a high dose of oral aciclovir was administered to find its efficiency in recurrent aphthous stomatitis patients (Pedersen, 1992). In this trial, that was designed on the basis of involvement of varicella zoster virus as an aetiology of recurrent aphthous stomatitis, eight otherwise healthy patients with severe recurrent aphthous stomatitis were treated with 800 mg oral aciclovir on a daily basis for one year. Ulcers disappeared in two patients totally, in four patients their ulcers improved dramatically and only in two cases did the medication prove ineffective (Pedersen, 1992). These findings were confirmed by an open study with a larger population of 30 recurrent aphthous stomatitis patients (Ozturkcan et al. 1996).

Immune-suppressive drugs

Systemic corticosteroids in the form of prednisone, at a dose of 40 mg per day, will suppress recurrent aphthous stomatitis entirely. The long term side-effects of such a regime are unacceptable and consensus among clinicians is that corticosteroids should only be used in patients with severe ulceration where the ulcers cannot be controlled by topical treatment. When used, systemic corticosteroids should be given for limited periods on an intermittent basis (Francis, 1970; Lehner, 1977; Dolby, 1973; Grossman and Sheagren, 1986; Burgess et al. 1990). Cyclophosphamide was reported to be of benefit in one patient (Francis, 1970), although azathioprine was found to be

unhelpful in a double-blind trial of patients with recurrent aphthous stomatitis (Eggleston and Nally, 1972).

Anxiolytic drugs

Since anxiety is one of the aetiologic factors in some patients with recurrent aphthous stomatitis (see section 1.5.2.c), reduction of anxiety can be a major step in the management of these patients. Low-doses of benzodiazepine medication such as alphazolam or lorazepam (0.5 to 2 mg) or diazepam (5 mg) prescribed daily for not more than two weeks was reported to be quite helpful in some cases (Burgess et al. 1990), but prolonged use of these medications is discouraged since they may lead to psychological dependence (Burgess et al. 1990).

Thalidomide, first used as a good hypnotic and sedative medication, was withdrawn from the market in 1961 because of its tetratogenic effects. Recent evidence suggests that, patients with Behçet's syndrome and severe aphthous ulcers in HIV-positive patients may benefit from thalidomide (Burgess et al. 1990; Radeff et al. 1990; Ghigliotti et al. 1993; Paterson et al. 1995; Schuler and Ehninger, 1995; Minor and Piscitelli, 1996; Youle et al. 1996; De Vincenzo and Burchet, 1996; Weidle, 1996). However a thalidomide-resistant HIV-associated case of oral ulceration has been reported. This patient was finally treated by granulocyte colony-stimulating factor (Manders et al. 1995).

1.7.2. Symptomatic Therapy

Symptomatic therapy is the main method of treatment for the majority of patients with recurrent aphthous stomatitis. The spectrum of agents used indicates a transient relief of symptoms. In general, symptomatic therapy is claimed to reduce the pain or duration of lesions but not the number or frequency.

Antibacterial agents

Regardless of the role of bacteria as primary aetiological agents in recurrent aphthous stomatitis, secondary infection of the ulcers from oral bacteria may prolong the ulceration and increase the discomfort experienced. In addition, oral hygiene is frequently, by necessity, neglected by the patients, which further increases the possibility of infection of the ulcers (Addy et al. 1974). For these reasons, and on a purely empirical basis, a range of anti-bacterial agents have been investigated for their efficiency in recurrent aphthous stomatitis. The use of topical chlortetracycline was initially reported in 1942 (Distelheim and Sulzberger, 1949). Since that time several studies have shown that oral tetracycline rinses are somewhat effective in decreasing the duration of the ulceration and reducing the associated pain but are ineffective in reducing recurrences of the oral lesions (Graykowski et al. 1966; Lehner, 1967b; Stanley, 1973; Graykowski and Kingman, 1978; Burgess et al. 1990). Chloramphenicol and oxytetracycline have not been proven to be clinically effective (Kutscher et al. 1953). Chlortetracycline oral suspension was reported to be most effective especially when combined with either topical or systemic corticosteroids (Distelheim and Sulzberger, 1949; Francis, 1970; Stanley, 1973). In addition,

tetracycline compresses have been reported to be more effective than an oral suspension of the antibiotic (Shore and Shelley, 1974). Topical tetracycline was reported to be the drug of choice in herpetiform ulcers (Cooke, 1960). Also, demeclocycline has been of some benefit in recurrent aphthous stomatitis patients in an open trial (Cottone and Langlais, 1977).

The commercial anti-microbial called Listerine antiseptic mouthrinse (Warner-Lambert Co.) was reported to be beneficial in the reduction of duration and severity of recurrent aphthous stomatitis (Meiller et al. 1991). The antiseptic, chlorhexidine gluconate reduced the incidence, duration and severity of ulcers when compared to placebo (Addy et al. 1974; Burgess et al. 1990) and the gel preparation of chlorhexidine was also effective (Addy et al. 1976). Oxygenating agents have been shown to be of no benefit in a double-blind study (Miller and Chalton, 1980) and it is discouraged since it has been reported to cause intra-oral ulceration (Burgess et al. 1990).

Topical anaesthetics

Topical lignocaine is the most widely used local anaesthetic. It provides partial anaesthesia of the mucous membranes for up to 30 minutes. In a comparison of various solutions of antihistamine or dyclonine hydrochloride with lignocaine a combination of dyclonine hydrochloride, 0.5 per cent in saline produced superior symptomatic relief (Ship et al. 1960; Dolby, 1973). Benzydamine hydrochloride (HCl) also, has been reported to have an analgesic effect on recurrent aphthous stomatitis lesions. (Yankell et al. 1981; Matthews et al. 1987)

Topical steroids

Hydrocortisone ointment was reported to show good results in lesions of recurrent aphthous stomatitis (Hillman, 1956; Morton, 1957). Late reports on the topical application of pellets of hydrocortisone hemisuccinate recorded approximately a 50 per cent reduction in the incidence of ulcers (Truelove and Morris-owen, 1958; Cooke and Armitage, 1960) and since then, a similar improvement in patients given topical triamcinolone acetonide in dental paste (BPC) has been reported (Zegarelli et al. 1959; Zegarelli et al. 1960; Rushton, 1962; Stoy, 1966; Brown et al. 1968; Macphee et al. 1968). Betamethasone-17-benzoate has also been reported to be effective by reducing the healing time of ulcers (Merchant et al. 1978). Triamcinolone 0.1 per cent in dental paste (BPC) and 2.5 mg tablets of hydrocortisone (BPC) are the most useful preparations (Roy, 1966; Annon.Editorial. 1974). Suppression of the adrenal cortex does not occur if the dosage is kept within reasonable limits (Lehner, 1967b; Lehner and Lyne, 1969; Lehner and Lyne, 1970). Corticosteroids such as desoxycorticosterone 0.25 per cent and fluocinonide 0.05 per cent appear to be effective for single ulcers (Pimlott and Walker, 1983; Burgess et al. 1990). Use of a corticosteroid in combination with an antibiotic and antifungal medication may be beneficial in some cases (Burgess et al. 1990). For numerous widely distributed ulcers use of a rinse such as dexamethasone elixir (0.5 mg dexamethasone per 5 ml) may be more effective (Burgess et al. 1990). Intra-lesional injection of triamcinolone acetonide recently was reported in major aphthous ulcers in AIDS patients (Friedman et al. 1994). Use of a double-layer film containing triamcinolone acetonide (SIM-990) for application on to the ulcers on the oral mucosa shows a good result in reduction

of the pain and healing time (Ishibashi et al. 1994). In addition, triamcinolone acetonide inhaler also has been reported to be of benefit in aphthous ulcers in HIV positive patients (Scott and Bender, 1996). A new topical adhesive containing, triamcinolone acetonide and Eudisper ointment was effective and well tolerated in recurrent aphthous stomatitis patients in a clinical trial (Sveinsson and Holbrook, 1993).

Other symptomatic therapies

Orabase (dental paste BPC) which is a methylcellulose based vehicle protects the ulcers but it has been shown to be of no value alone in the treatment of recurrent aphthous stomatitis (Rushton, 1962; MacPhee et al. 1968). However the combination of orabase and corticosteroids (Adcortyl in Orabase) was reported to be the topical medication of choice in the management of recurrent aphthous stomatitis (Lehner, 1967b). Bioadhesive patches made of pharmaceutical grade cellulose are reported to have a great effect on reducing the pain and healing time of the ulcers (Mahdi et al. 1996). Chemical cautery has long been used for symptomatic relief and to encourage healing (Antoon and Miller, 1980; Burgess et al. 1990), although damage to the tissue ensues and gangrene of the tongue has been reported after application of silver nitrate (Frost et al. 1978; Burgess et al. 1990). Astringent mouthwashes have also been used extensively, although in a double-blind trial of a zinc sulphate and zinc chloride mouth wash (BPC) was found to be less effective than placebo for symptomatic relief (Addy et al. 1974).

Despite some controversy about the effect of laser therapy in recurrent aphthous stomatitis, in some cases laser therapy could reduce the pain and healing time of the ulceration (Colvard and Kuo, 1991).

Recently, the effect of topical 5-amino salicylate in the treatment of Behçet's syndrome (Ranzi et al. 1986) and recurrent aphthous stomatitis (Bagg, 1985; Collier et al. 1992;) was investigated. Some of these reports indicate the efficiency of this medication in treatment of the mucosal ulceration in Behçet's syndrome and recurrent aphthous stomatitis (Ranzi et al. 1986; Collier et al. 1992) but in another report this effect was not found (Bagg, 1985).

1.8. Aims of Thesis

Aims of the present thesis are:

a) To investigate the current management of patients with recurrent aphthous stomatitis and epidemiologic aspects of the disease in Glasgow Dental Hospital and School.

b) To investigate the effect of a topical non-corticosteroid drug, 5 aminosalicylate cream in aphthous lesions.

c) To investigate the involvement of HHV-6, HCMV and VZV in the aetiology of recurrent aphthous stomatitis by the molecular biology technique, PCR, and serological experiments. Also, the presence of *Mycobacterium paratuberculosis* was investigated in aphthous lesions by the PCR technique.

Chapter Two. Audit of Diagnosis and Investigations in Patients with Recurrent Aphthous Stomatitis

2.1. Introduction

Audit is increasingly essential in medical subjects. Clinical audit, in addition to improving the quality of patient care, permits a more effective use of resources in other clinical studies and research. Those who employ medical audit, can use the information to identify areas of a particular clinical problem that may require improvement, then set realistic standards, discuss any noted discrepancies and finally implement necessary changes (Porter et al. 1993).

The aim of this study was to audit all information about each patient with recurrent aphthous stomatitis using a computer software package, and test the ability of the program to provide information for clinical investigations. In addition, this program allowed the clinician to monitor patients' improvement during different courses of medication, without the use of the patients' medical records.

As a result, all information about different aspects of recurrent aphthous stomatitis can be analysed and a comparison of these data with previous reports is possible. Also this analysis can assess the quality of the clinical management of the patients who attended the Oral Medicine Clinic.

2.2. Materials and Methods

2.2.1. Subjects

To investigate the clinical features of recurrent aphthous stomatitis, 252 patients who were referred to the Oral Medicine Clinic in Glasgow were personally reviewed and treated in the period from September 1995 to September 1996. In addition, a further

213 patients with recurrent aphthous stomatitis who were reviewed and treated in the Oral Medicine Clinic in the period of April 1993 till April 1994 were retrospectively studied and then prospectively reviewed for further investigations.

Added to these, 90 patients with normal oral mucosa, who attended the Oral Medicine Clinic for temporomandibular joint pain, were studied as a control group.

Also, 295 patients who initially were diagnosed as having recurrent aphthous stomatitis, were referred to the Contact Dermatitis Unit in the Royal Infirmary for identification of possible allergic components involved in the disease process. These patients were studied retrospectively. One hundred volunteers derived from dental staff and students were also tested for allergic reactions in the Oral Medicine Clinic as a control group. These data were part of a study conducted by Dr. S. Rees.

In all patients with recurrent aphthous stomatitis who are presented in this study, initially, oral lesions were not related to any systemic diseases on presentation, however the possibility of underlying systemic disease was investigated.

2.2.2. Clinical Observations and Investigations

At the first appointment, all information about medical history, history of the disease, clinical examination, and treatment plan, was recorded on a special form (Figure 2.1, 2.2). This information was then put in to a computer data base package specific to oral ulceration (Appendix I). Review appointments and further treatment and investigations were also recorded in the same manner.

This computer software (Oral Ulceration Software) was designed by the NHS Computer Office and Clinical Audit Unit in Glasgow Dental Hospital and School.

At the end of the study, information related to the first group of patients was extracted and compared with previous observations by other investigators on aphthous patients. (Details about other software packages used in this part will be explained in the statistical method section)

In first group of 252 patients, all information about full blood count, assays of ferritin, folic acid, vitamin B_{12} and anti-gliadin antibodies were recorded in the Oral Ulceration Software. Complementary tests were added according to the patient's needs, such as anti-intrinsic factor and anti-parietal cell antibodies. Replacement therapy and re-investigations for any persistent haematological deficiencies were also recorded.

In the second group of 213 patients, the haematological investigations were retrospectively studied and then patients were followed up to assess the effect of previous replacement therapy.

Venous blood was removed from all the patients and controls (90 patients with normal oral mucosa who were referred to Oral Medicine Clinic for temporomandibular joint pain) for haematological investigation (full blood count, serum ferritin, red blood cell folate and serum vitamin B_{12}).

Vitamin B_{12} deficiencies were diagnosed if the results were repeatedly below the normal range (Table 2.1). Patients with diagnosed deficiencies were questioned to identify a cause for the deficiency. Where clinical concern or doubt remained, patients were referred for medical investigation.

THE GLASGOW DENTAL HOSPITAL AND SCHOOL — DEPT. OF ORAL MEDICINE

SURNAME	CHRISTIAN NAME(S)	UNIT NUMBER
CONSULTANT	REFERRED BY	

C/O Recurrent Oral Ulcers

HPC

Age of Onset ☐☐ Duration ☐☐ Years ☐☐ Months

Size (mm) From ☐☐ To ☐☐ Average ☐☐

Number From ☐☐ To ☐☐ Average ☐☐

Time to Heal (in days) From ☐☐ To ☐☐ Average ☐☐

No. Ulcers/Month ☐☐

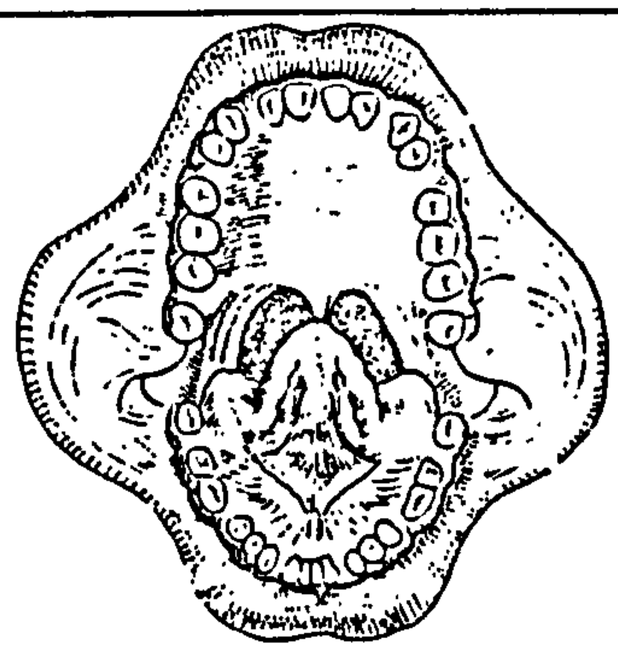
Sites Affected: inside lips ☐, buccal ☐, sulcus ☐, floor of mouth ☐, under tongue ☐, dorsum tongue ☐, soft palate ☐, other (specify) ☐, extra oral (specify) ☐

Precipitating Factors: trauma ☐, foods(specify) ☐, menses ☐, other (specify) ☐

Social History: married ☐, single ☐, other (specify) ☐, alcohol (units/wk) ☐☐, tobacco(amount/day) ☐☐

Relevant Medical History:

Drugs



PDH F/- ☐ -/F ☐ P/- ☐ -/P ☐

Regular attender Yes ☐ No ☐

Family History (Specify)

O/E

Ulcer Number (mark on map)	1	2	3	4	5	6	7	8	9	10
Size (mm)										
Pain (0,mild-1,mod-2,severe-3)										

Oral Hygiene good ☐ fair ☐ poor ☐

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Figure 2.1. First page of the special form provided for the first appointment of all patients with RAS. This form should be completed by the clinician.

Other Conditions

candidiasis (specify) ☐
angular cheilitis ☐
glossitis ☐
other (specify) ☐

Relevant Oral Signs:

Diagnosis

Recurrent Aphthous Stomatitis ☐
Minor ☐
Major ☐
Herpetiform ☐
Behcet's ☐
Other (specify) ☐

Treatment Plan

1. Investigations

FBC ☐
ferritin ☐
vit B_{12} ☐
folate ☐
glucose ☐
serum ☐
patch test ☐
biopsy ☐
ulcer diary ☐
other (specify) ☐

2. Therapy

		dose
none	☐	
chlorhexidine	☐	
tetracycline m/w	☐	
xylocaine spray	☐	
difflam	☐	
adcortyl	☐	
becotide	☐	
betnesol	☐	
prednisolone	☐	
antifungals	☐	
antivirals	☐	
dietary elimination	☐	
clinical trial (specify)	☐	

3. Review ☐ time ☐

Figure 2.2. Second page of the special form provided for first appointment of all patients with RAS. This form should be completed by the clinician.

Patients with iron deficiency were treated with ferrous sulphate, 600 mg. daily (200 mg eight hourly), for a period of three months.

Identified vitamin B_{12} deficient patients were also investigated for anti-parietal cell antibodies and anti-intrinsic factor antibodies. Investigation and replacement therapy for these patients were done by a haematologist or their general medical practitioner. In the case of folate deficiency, patients were also referred to a haematologist.

All patients with haematological deficiencies were reviewed after three months of replacement therapy and in most cases, patients reviewed in a further 3, 6 and 12 months to assess the results of replacement therapy on the severity of the aphthous ulcers. The criterion for the effect of replacement therapy was the patient's satisfaction. If patients reported a decrease in healing time and pain only, but the ulcers were still present as before, slight improvement was recorded as number one. Mild improvement was a decrease in the number of ulcers in addition to a reduction in pain and healing time (number two). Improvement of ulceration was when only one or two small ulcers with a short period of healing time appeared during the review period (number three). Patients who had no ulcers during follow-up were regarded as having had a remission due to replacement therapy (number four).

Venous blood was removed to find the effect of replacement therapy. In the case of persisting iron deficiency after treatment with Ferrous Sulfate tablets, patients were referred to a haematologist for further investigations.

Table 2.1. The normal range of routine Haematological elements tested.

	Minimum	Maximum
Ferritin (ng/ml)	20	330
Vitamin B_{12} (pg/ml)	175	800
Folate (ng/ml)	75	400
Haemoglubolin Male	12.5	18.0
(g/dl) Female	12.0	16.5
MCV (fl)	80	100

In addition, the sex and age of this group of patients was studied separately to compare them with the first group of 252 patients. Data from second group of 213 patients were not recorded on the Oral Ulceration Software, because at that period of time the software was not available.

To estimate the real difference in sex and age of the recurrent aphthous stomatitis patients compared with a control group, the whole patient population who attended the Oral Medicine Clinic in Glasgow were chosen as control group.

All patients received a topical medication for symptomatic relief. In severe cases, patients received systemic corticosteroids. These patients were reviewed on a regular basis. Topical medication was prescribed depending on severity, site and number of ulcers. For instance, in patients with major and long lasting ulcers, tetracycline mouthwash was the medication of choice. When the patient suffered minor ulcers on the tongue, soft palate or roof of the mouth, beclomethasone spray (BPC) or betamethasone mouthwash (BPC) was prescribed. For ulcers located in the buccal mucosa, buccal or labial sulcus, triamcinolone in dental paste (BPC) was the medication of choice. On the next appointment, if a medication was not effective, another one was prescribed. Combined drug therapy, such as tetracycline mouthwash and a topical corticisteroid were prescribed in some severe cases.

2.2.3. Allergy Investigation (Patch Testing)

Patients were tested to the items listed in table 2.2. All materials were applied under Finn chambers to the back or left arm for 48 hours. Then they were removed and after one hour examination was made for redness, blistering or weeping and patient was

asked whether there was any itching. These reactions were graded according to how severe they were and a further 48 hours later, another examination was made to see whether any reaction was still present. Itching and persistent reactions were taken to be positive and allergic. Other reactions might be considered to be irritant and non-specific.

In addition to the above testing technique, all the reagents were also applied to the inner aspect of the forearm under occlusion on Finn chambers. This time the tests were only left on for 20 minutes. A few minutes after removal, examination was made for a red or white, itching, or palpable area with a red flare around it (urticaria). Symptoms might be itchy, stinging or burning sometimes. The sites of the tests were marked and the patient was asked to observed this area during a period of 6 hours thereafter. Patients kept a note of the period of time which any red or itchy marks lasted and further similar reactions which were not evident immediately after removing the test. A positive reaction was considered to be those which gave visible sensations at that site experienced by the patient. The symptoms and visible changes required to be present for a period of two hours before the test was considered to be positive. The control group was tested to the same allergen materials except mercury and amalgam.

Table 2.2. The materials tested in RAS patients in Patch Testing.

Mercury
Amalgam
Nickel
Cobalt
Formaldehyde
Neomycin
Antimicrobial agents
Perfume
Domestic/Personal materials.
Benzoic acid
Sorbic acid
Sodium metabisulphate
Cinnamonaldehyde
Chocolate
Salicylic acid
Tartrazine
Glutamic acid
Butylated hydroxytoluene
Butylated hydroxyanisole

2.2.4. Statistics

The statistical significance of differences between clinical observations in patients with recurrent aphthous stomatitis and the total patient population who attended the Oral Medicine Clinic in the same period of time was analysed by non-parametric Chi-square tests using Minitab software (PC computer). For extracting the data from the Oral Ulceration Software, Access Software was applied and then the data was stored and analysed in Excel version five Software.

2.3. Results

The results of these observations are separately presented for different clinical features related to the disease.

2.3.1. Age

Data from first group of 252 patients indicated that there were 14 patients under the age of fifteen years, and 86 patients with recurrent aphthous stomatitis aged between sixteen and thirty years at the time of presentation. Also, 114 patients with recurrent aphthous stomatitis were referred for treatment between the age of thirty and fifty years. Only 38 patients were referred for treatment over the age of fifty years. (Table 2.3)

In the second group of 213 patients at presentation, 24 were under 15 years of age, 65 patients were between 16 and 29 years of age, 90 patients were in third and fourth decade of their lives. Only 34 patients were older than 50 years at presentation. A

similar pattern of age distribution therefore was observed in the second group of the patients. (Table 2.3)

The age distribution, in all patients who attended the Oral Medicine Clinic in the same period of time as the first group, showed more patients in the fourth decade of life, however from 8180 patients 3737 of them were over 50 years old.

In general, in both groups of patients with recurrent aphthous stomatitis, the prevalence of the disease was increased in the third and fourth decade of life (Figure 2.3) as compared with the whole patient population attending the Oral Medicine Clinic, whose numbers were increased in the fourth decade of life and over 50 years. There was no statistical significant difference in distribution of age at presentation between male and female patients.(Table 2.3)

2.3.2. Sex

From the total number of 252 patients in the first group 190 were females and 62 were males. (Table 2.3)

These patients were part of the 8180 patients, comprising 2519 males and 5661 females attended the Oral Medicine Clinic.

In second group of 213 patients with recurrent aphthous stomatitis, 68 males and 145 females underwent investigations and treatment for recurrent aphthous stomatitis. (Table 2.3)

Table 2.3. Age at presentation and sex distribution in two groups of patients with RAS.

	0-10	11-15	16-20	21-30	31-40	41-50	51-	total
1st. group	7	7	23	63	77	37	37	252
Male	3	2	7	14	18	9	9	62
Female	4	5	16	49	59	28	29	190
2nd. group	4	20	19	46	61	29	34	213
Male	1	9	6	10	23	11	8	68
Female	3	11	13	36	38	18	26	145
Total	11	27	42	109	138	66	71	465

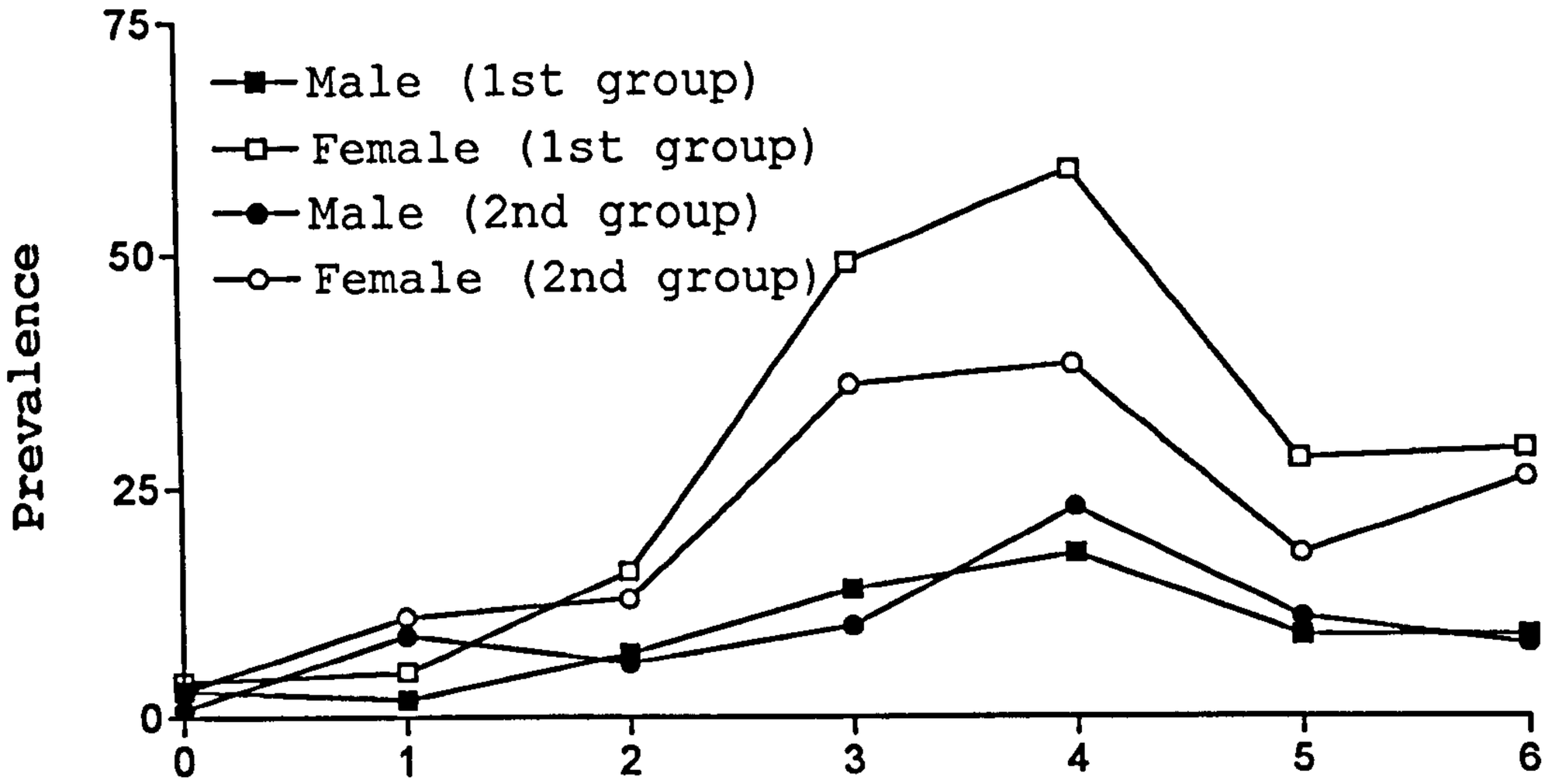

Figure 2.3. Age at presentation and sex distribution of first 252 and second 213 patients with RAS. The age and sex distribution is the same in both groups.

2.3.3. Diagnosis

Recurrent aphthous stomatitis was diagnosed clinically according to the definition of Lehner (1968) for major and minor aphthae and Cooke (1960) for herpetiform ulcers. In this thesis, from a total number of 252 patients, 241 patients were diagnosed as having minor aphthae, six patients had major aphthae and three had herpetiform ulcers (See section 1.2). In two other patients, ulcers were suspected to be related to denture trauma rather than recurrent aphthous stomatitis. (Table 2.4)

In some patients the diameter of ulcers was more than ten millimetres and healing time took more than four weeks. However no scar tissue was clinically observed after the healing process. These ulcers were categorized as minor recurrent aphthous ulcers.

2.3.4. Smoking

From a total number of 252 patients, 230 were non-smokers and 20 patients smoked less than 15 cigarettes a day. Only two patients were heavy smokers (30-40 cigarettes per day). Three patients strongly believed that their ulcers initially started when they stopped their smoking habit.

2.3.5. Haematological Deficiencies

All patients with recurrent aphthous stomatitis underwent haematological investigations. Venous blood was removed from all patients on the first visit for full blood count and assays of plasma ferritin, red cell folate and serum vitamin B_{12}.

Table 2.4. Distribution of sub-groups of RAS in first group of 252 patients

Mio RAS	Major RAS	Herpetiform RAS	Other	Total
241	6	3	2	252

In first group of 252 patients, 69 aphthous patients had iron deficiency (ferritin level < 20 ng/ml). (Table 2.5)

In the 69 patients with a low level of ferritin only 13 patients had overt deficiency with a low haemoglobin, 10 patients had a low MCV (Table 2.1) and three patients had anaemia with a low haemoglobin and a low MCV. Persistent iron deficiency was observed in seven patients who were referred to either their general practitioner or a haematologist for further investigations. (Table 2.5)

No folate deficiency was observed in this group of patients. (Table 2.5)

Fifteen patients were diagnosed as having vitamin B_{12} deficiency (vitamin B_{12} level < 175 pg/ml). These patients were investigated for the presence of anti-intrinsic factor antibodies and anti-parietal cell antibodies. Three patients had at least one of these antibodies present in their serum. Vitamin B_{12} deficiency with an abnormal blood film was observed in one patient with a low MCV, one patient with a low haemoglobulin. (Table 2.6)

All patients with vitamin B_{12} deficiency were referred to haematologist for further investigations. None of them were diagnosed to have pernicious anaemia.

In the group of 252 patients, venous blood of 66 individuals (17 males, 49 females) were tested for presence of IgA anti-gliadin antibody. In seven patients, IgA anti-gliadin antibodies were positive, however anti-endomysial antibody were negative indicating that these patients did not have coeliac disease. It has been reported that these patients however may develop gastrointestinal tract dysfunction in the future (Koninckx et al. 1984; O`mahony et al. 1990).

Table 2.5. Prevalence of haematological deficiencies in two groups of RAS patients and controls.

Deficiency	folate	B_{12}	ferritin	fol. & fer.	B_{12} & fer.	Abnormal FBC	Total
RAS*	0	13(5.2%)	67(26.6%)	0	2(0.8%)	22(8.7%)	252
RAS**	1(0.5%)	4(1.9%)	54(25.4%)	2(0.9%)	7(3.3%)	17(8.0%)	213
Controls	0	2(5%)	33(36.7%)	0	5(5.6%)	9(10%)	90

*First group and **Second group of RAS patients

Table 2.6. Patients with vitamin B_{12} deficiency and their full blood screen and auto-antibodies results.

Pt. Number	B_{12}	Hb	MCV	Intrinsic Factor antibodies	Parietal cells antibodies
1	↓*	↓	N	-	-
2	↓	N	↓	-	+
3	↓	N	N	+	+
4-15	↓	N	N	-	-
Total	15	1	1	1	2

*↓ low level, N normal level, - negative result.

The second group of 213 patients who attended the Oral Medicine Clinic during the period of April 1993 until April 1994 were investigated for the same haematological deficiencies.

In addition to this group with recurrent aphthous stomatitis, 90 patients with normal oral mucosa were also investigated as a control group.

Folate, vitamin B_{12} and ferritin deficiencies were identified in 68 patients (11 male, 57 females) with recurrent aphthous stomatitis (31.92%) and 40 (1 male, 39 females) patients with normal mucosa (44.44%). (Table 2.5, Figure 2.4)

Latent deficiencies were identified in 51 patients with recurrent aphthous stomatitis and 27 individuals in control group. In these patients haematological deficiencies could not be diagnosed with only a full blood count assay. (Table 2.7)

2.3.6. Allergy

The results of allergic reactions to relevant materials such as food additives were compared with controls (Table 2.8). In the present study, only food additives which are more related to oral ulceration were analysed in 285 patients.

Benzoic acid caused skin allergic reactions in 26 of 285 (9 per cent) recurrent aphthous stomatitis patients. Positive reactions to sorbic acid were observed in one of the 285 (0.3 per cent) of the recurrent aphthous stomatitis patients. Sodium metabisulphate and cinnamaldehyde caused allergic reactions in 10 (3.5 per cent) and seven (2.5 per cent) of 285 patients respectively. Also, allergic reactions to chocolate

Table 2.7. Prevalence of haematological deficiencies in the second group of 213 RAS patients

Deficiency	RAS patient	Controls
folate	1 (0)	0
B_{12}	4 (4)	2 (2)
ferritin	54 (39)	33 (23)
fol. & fer.	2 (1)	0
B_{12} & fer.	7 (7)	5 (2)
Abnormal FBC	17	13
TOTAL Pts.	213	90

*Latent deficiencies shown in bracelets.
** There is no significant difference between RAS and control patients.

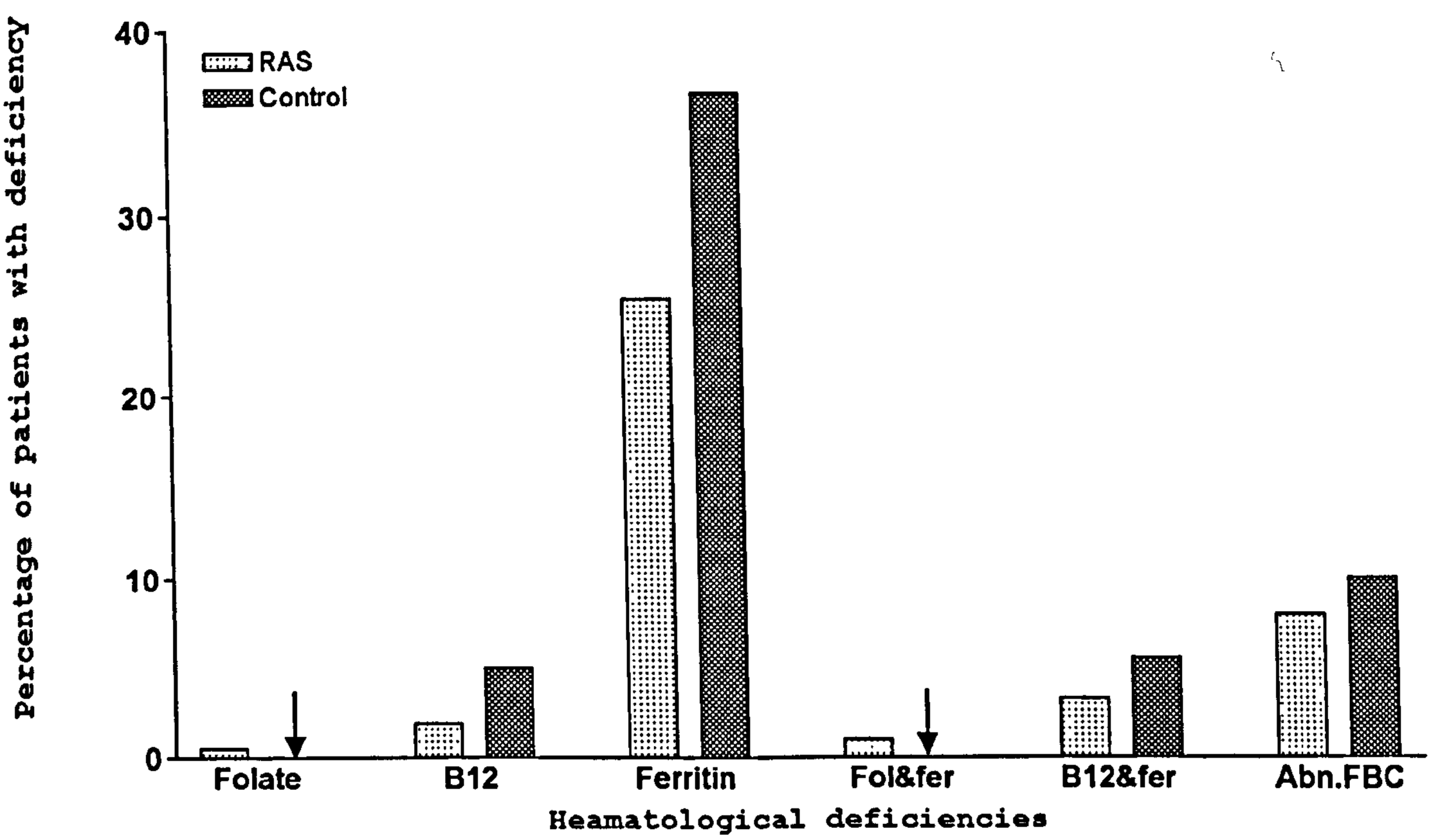

Figure 2.4. Haematological deficiencies in the second group of 213 RAS patients compared with the control group. Haematological deficiencies are not increased in RAS patients compared with controls.

↓ shows that there is no folic acid deficiency in the control group.

were identified in only one of the 247 (0.4 per cent) recurrent aphthous stomatitis patients tested for this material. (Table 2.8)

These results were not significantly different in the control group with the exception of allergy to benzoic acid which was significantly higher in the control group. Allergy to benzoic acid was observed in 28 of 100 control subjects. Sorbic acid, sodium metabisulphate, cinnamaldehyde and chocolate caused allergic reactions in three, six, seven and one of the 100 control subjects. (Table 2.8)

The length of time was not enough to do appropriate review and investigate the effect of withdrawing allergens from those patients with a positive allergic reaction. Therefore the role of allergy in the aetiology of recurrent aphthous stomatitis could not be entirely excluded.

2.3.7. Treatment

After primary investigation, patients with haematological deficiencies were treated to replace the deficient factors.

In iron deficient patients Ferrous Sulfate (600 mg. daily) for three months was prescribed. Patients with vitamin B_{12} deficiency were treated by either their general practitioner or a haematologist. In addition, all patients with milder ulcers, were symptomatically treated by topical medications such as triamcinolone in dental paste (BPC), beclomethasone spray (BPC), betamethasone mouthwash (BPC), chlorhexidine gluconate (BPC) mouthwash, benzydamine hydrochloride mouthwash

Table 2.8. Allergic reactions to some food additives in RAS and Controls.

Allergens	RAS	Controls	Stat. Difference*
Benzoic acid	26 (11%)	28 (%)	S
Sorbic acid	1 (0.4%)	3 (%)	NS
Sodium Metabisulphate	10 (4%)	6 (%)	NS
Cinnamaldehyde	7 (3%)	7 (%)	NS
Chocolate	1 (0.4%)	1 (%)	NS
Total	285	100	

* Chi-squre test and Fisher's exact test ($p<0.05$)

and spray, tetracycline hydrochloride (BPC) mouthwash or lignocaine hydrochloride (BPC). Those patients with severe ulceration were prescribed systemic corticosteroids. (Table 2.9) From a total of 252 patients, beclomethasone spray (BPC) was the most pleasant medication for recurrent aphthous stomatitis patients. This was because of ease of application of the drug especially in children and those with handicaps.

Beclomethasone spray (BPC) was prescribed in 45.6 per cent of the patients in the first group. Betamethasone mouthwash (BPC) (0.5 mg per-tablet) was used by 24.2 per cent of the patients. Benzydamine hydrochloride mouthwash, usually prescribed for very mild ulcers, was used by 22 per cent of the patients. Triamcinolone in dental paste (BPC) was given to 19.4 per cent of the patients. Tetracycline mouthwash was prescribed to 17 per cent of patients with more severe ulceration. Chlorhexidine gluconate (BPC) mouthwash was prescribed to 11.5 per cent of the patients for prophylaxis and treatment of secondary infection, usually combined with other topical medication. Lignocaine hydrochloride (BPC) spray was given to 2.8 per cent of the patients who did not find other medication effective for relief of severe pain from the ulcers. Systemic prednosolone was prescribed only for 2 per cent of the patients with very severe and constant ulceration. (Table 2.9)

In addition, 66 patients were treated in a clinical trial with 5 aminosalicylate (Mesalazine) cream, which will be explained in chapter three.

Table 2.9. Medications used for treatment of RAS patients in this study.

Medication	Patients (per cent)
Beclomethasone spray	45.6
Betamethasone M.W.	24.2
Benzydamine hydrochloride	21.8
Triamcinolone in dental paste	19.4
Tetracycline hydrochloride M.W.	17.0
Chlorhexidine gluconate M.W.	11.5
Lignocaine hydrochloride	2.8
Systemic prednisolone	2.0

In the second group of patients the effect of replacement therapy was assessed. This assessment was only applied in the second group who were studied from April 1993, while the length of the time was not enough for reviewing the first group of patients.

Twenty-five patients diagnosed with iron deficiency showed some degree of improvement in the severity of ulceration. Twelve patients had no response to replacement therapy.

In patients with vitamin B_{12} and folic acid deficiencies, seven patients reported some improvement in their ulceration. In 14 patients, in whom replacement therapy did not decrease the severity of ulceration, three had persistent haematological deficiencies and they were referred to the haematologist for further investigations. (Table 2.10)

2.4. Discussion

Recurrent aphthous stomatitis affects individuals of all ages. The prevalence of recurrent aphthous stomatitis in this study was increased in the third and fourth decades of life, however, previous reports, showed an increased prevalence of the disease in the second and third decades of life (Farmer, 1958; Lehner, 1968; Brody and Silverman, 1969; Donatsky, 1973; Scully and Porter, 1989). This might be due to a higher degree of contact with predisposing factors such as food additives in the adult population or higher numbers of adults in the fourth decade of life seeking treatment than a younger population.

Also it has been reported that the severity of ulcers in male subjects increases in the first and third decades of life whereas in females, ulcers are more severe in the second and third decades (Farmer, 1958). But in this study the severity of ulcers were

Table 2.10. Response to replacement therapy after 3-6 months review.

response	Iron therapy	vitamin B_{12} injection	folic acid therapy	other*
0	12	1	1	3
1	6	1	0	0
2	5	1	0	0
3	5	1	1	0
4	9	3	0	0

* patients in whom replacement therapy was not effective.

increased in the third and fourth decade of life in both sexes (Figure 2.5). This may be due to an increased number of all patients in the fourth decade who attended the clinic in the same period of time. However the pattern of age distribution was significantly different ($p<0.002$) between the first group of 252 patients and whole patients population. The number of patients aged over 50 years was significantly increased in the whole patient population compared with the first group of 252 aphthous patients.

Many investigators have claimed that recurrent aphthous stomatitis involves female subjects more than males (Brody and Silverman, 1969; Donatsky, 1973; Farmer, 1958; Sircus et al. 1957).

Compared with all patients who attended the Oral Medicine clinic, there was no significant difference between aphthous patients and total patients according to sex.

This result shows that in general female patients may attend the clinic more than males, or males do not pay enough attention to their disease unless it becomes very severe.

All together, this data can not demonstrate a real significance between male and female patients with respect to the prevalence of recurrent aphthous stomatitis.

The prevalence of minor recurrent aphthous stomatitis was higher than major and herpetiform ulceration. These results support the other reports about the prevalence of different types of recurrent aphthous stomatitis (Tyldesley, 1973; Stokes and Koprince, 1982; Bagan et al. 1991).

Figure 2.5. Sex distribution with respect to age in first group of 252 RAS patients. There is no significant difference between sex distribution in respect to the age.

Haematological deficiencies are not the main aetiologic factors in recurrent aphthous stomatitis but as many investigators reported (Walker, 1973; Wray et al. 1975; Wray et al. 1978; Hutcheon et al. 1978; Challacombe et al. 1983; Tyldesley, 1983; Porter et al. 1988; Miller et al. 1994) haematological deficiencies play a major part in the management of recurrent aphthous stomatitis. In both groups of patients with recurrent aphthous stomatitis almost the same results were obtained.

In first group of 252 patients 33.33 per cent of the patients had haematological deficiencies and in second group 31.91 per cent.

In second group of 213 patients, however, there was not any significant differences in haematological deficiencies compared with the control group, (Figure 2.4) but replacement therapy decreased the severity of ulceration in up to 41 per cent in patients. Complete remission was reported in 18.2 per cent of the patients with different haematological deficiencies.

In addition, only 5.16 per cent of the patients in the first group (252 patients) and 7.98 per cent of the patients in the second group (213 patients) had identified overt deficiencies with abnormal blood films. These results showed the importance of full haematological investigations including assays of ferritin, folic acid and vitamin B_{12}, in the management of recurrent aphthous stomatitis. The same conclusion was made from previous studies (Hutcheon et al. 1978; Wray et al. 1978; Tyldesley, 1983; Porter et al. 1988). Although in the study by Porter et al (1988) latent deficiency of iron was significantly higher in recurrent aphthous stomatitis patients compared with

controls. This difference was not found in the present study between the second group of patients and controls. (Table 2.7)

The incidence of folic acid deficiency has been dramatically decreased during the last decade, but the potential involvement of coeliac disease in recurrent aphthous stomatitis cannot be ignored. There was not any significant increase in the number of patients with positive IgA anti-gliadin antibodies and also patients with positive anti-gliadin antibodies were not diagnosed as having coeliac disease. Such a result may however lead the clinician to investigate the possiblity that patients may develop coeliac disease in future despite a normal folic acid level.

This study indicates not only that treatment of haematological deficiencies might reduce the severity of recurrent aphthous stomatitis, but also it may lead the clinician to detection of possible underlying systemic diseases.

Allergies may not play a major role in patients with recurrent aphthous stomatitis. However patients who have undergone dermatology allergic testing (Patch Testing) and allergies were identified, and who then avoided the allergens, showed some improvement in their ulceration. In the present study, there was no increase in the prevalence of allergy to food additives in recurrent aphthous stomatitis, and the difference between recurrent aphthous stomatitis and control subjects in the prevalence of allergic reactions to food additives was not significant. However the prevalence of allergic reactions to benzoic acid in control subjects was significantly higher than recurrent aphthous stomatitis patients. A high percentage of recurrent aphthous stomatitis patients were allergic to dental materials (67 per cent), but this

test was not applied in control subjects. Indeed, there were patients whose ulcers improved as the result of replacing dental restoration with mercury-free materials. A case of allergy to materials such as intrauterine contraceptive device (Saf-T-coil 32-s) and contact lenses (soflens) has been reported (Gordon and Gordon, 1974). However the allergen components of these materials was not mentioned. In a recent study, Patch Testing (standard European series) was undertaken in 21 recurrent aphthous stomatitis patients (Nolan et al. 1991a). From 21 patients 20 showed a positive reaction to a number of materials which clinically are considered to be relevant. As the result of dietary advice, 18 patients showed an improvement in the severity of the disease. Despite the absence of a control group, the authors concluded that food sensitivity and allergies to other materials could be considered as a possible aetiologic factor in recurrent aphthous stomatitis. (Nolan et al. 1991a) In a double-blind trial, 30 per cent of foods which caused histamine release (*in vitro*), were correlated to an increased incidence of oral lesions (Wray et al. 1982). However, in this study, dietary manipulation did not eliminate the ulceration in any patients. The increased serum antibodies to some food antigens such as cow's milk proteins and food antigens has been reported to be significantly different in recurrent aphthous stomatitis patients compared to a healthy population but this difference was not observed when compared to other ulcerative conditions (Taylor et al. 1964; Thomas et al. 1973). It is possible that, the local breakdown of oral mucosa allows increased absorption of immunogenic materials in foods which might increase the tendency to develop IgE antibodies to foodstuffs.

In the present study, the prevalence of allergy to the tested foodstuffs was not increased in recurrent aphthous stomatitis patients, but the effect of withdrawing allergen in patients with a positive allergic reaction has shown that allergy may play a minor role in the aetiology of recurrent aphthous stomatitis. Inadequate follow-up time was available to answer this question.

However most of the topical medications had some benefit for the patients, but in some severe cases systemic cortocosteroids were prescribed.

Treatment is a very important part of the management of patients with recurrent aphthous stomatitis, but without complete investigation and follow up of their current medications, needs cannot be fully satisfied.

Because there is no predictable cure for this disease, in any treatment plan, a safe, effective and pleasant medication seems to be desirable for symptomatic relief of ulceration.

In a recent report (Porter et al. 1993) of audit of information about patients with recurrent aphthous stomatitis, 20 per cent of the patients with abnormal haematological and serologic findings were not appropriately investigated or referred to a suitable medical specialist while in the present study, although a larger number of patients were studied, complete investigation and medical management were carried out in all patients with haematological deficiencies unless patients did not wish to continue the treatment process.

Chapter Three. The Effect of Topical 5 Amino-Salicylate

3.1. Introduction

5-aminosalicylic acid is a salicylate that is used for its local effects in the treatment of inflammatory bowel disease (Azad Khan et al. 1977; Insel, 1990). Generally, salicylates relieve those types of low intensity pain that arise from integumental structures rather than from viscera, especially headache, myalgia and arthralgia. They alleviate pain by virtue of peripheral action. Direct effects on the CNS also may be involved (Insel, 1990).

Salicylates also can influence the metabolism of connective tissue, and these effects may be involved in their anti-inflammatory action. For example, salicylates can affect the composition, biosynthesis or metabolism of connective tissue mucopolysaccharides in the ground substance that provide barriers to the spread of infection and inflammation (Insel, 1990).

Although the mechanism of action in 5 amino-salicylate is unknown and probably complex, it is likely to have a local action by analogy with ulcerative colitis (Azad Khan et al. 1977).

Mesalazine (5 amino-salicylate) has been of benefit in the treatment of inflammatory bowel disease for over 40 years, principally because of its local actions (Insel, 1990).

Collier et al (1992) investigated the effect of 5 amino-salicylate in 22 patients (age 18-75 years) with recurrent aphthous stomatitis. In this double-blind study, patients were assessed before treatment and after seven, 14 and 28 days of treatment. Pain and difficulty with eating were assessed using a 100 millimetres linear scale marked by the

subjects. The number and location of ulcers were recorded by the authors. The results showed 80 per cent reduction in pain in the treatment group compared with placebo by day seven, and by day 15, none of the ulcers in treatment group was painful while six out of eight patients in the placebo group were complaining of the pain (Collier et al. 1992). In this study 5 amino-salicylate significantly reduced the healing time, number of ulcers and difficulty in eating as well as pain compared with placebo (Collier et al. 1992).

However, Bagg et al. (1985) reported that 5 amino-salicylate was ineffective in treatment of recurrent aphthous stomatitis, compared with placebo. In this study, 25 patients with recurrent aphthous stomatitis entered a double-blind trial with crossover. Patients randomly used 5 amino-salicylate, 200 mg or an identical placebo (to be dissolved in 10 mls of water) as a mouthwash for six weeks. After a subsequent period of two weeks of no treatment, the alternative preparation was used for a final six weeks. Patients recorded the number of ulcers present, the number of new ulcers and a score for pain or discomfort on a visual analogue scale (Pimlott and Walker, 1983). At the end the ulcer incidence (mean of number of new ulcers) and ulcer prevalence and duration of lesions (sum of daily counts of ulcers present) and the period of remission (mean number of ulcer-free days) were measured. The analysis of obtained data did not show any statistically significant beneficial effect of the 5 amino-salicylate.

5 amino-salicylate has immunosuppressive properties (Insel, 1990). There is also evidence that autoimmmune processes may play a role in ulcerative colitis (Basu and

Chesner, 1990) and recurrent aphthous stomatitis (Wray, 1984). Therefore this clinical investigation was aimed at assessing the therapeutic effect of topical 5 amino-salicylate in recurrent aphthous stomatitis.

3.2. Materials and Methods

3.2.1. Subjects

From 252 patients with recurrent aphthous stomatitis (see section 2.2.1), 66 patients entered a clinical trial with more than three ulcers per-month. Selection was based solely on a history of recurrent aphthous stomatitis and availability and willingness to participate in the study. The age of the patients was between 17 and 57 years. Fifty two of the patients were female subjects and 14 males. Patients were investigated to exclude any haematological deficiency, renal and hepatic impairment. They were asked about any history of salicylate hypersensitivity and any regular anti-inflammatory analgesics, other analgesics, steroid therapy, anti-cancer or immunosuppressive therapy. Female patients were not pregnant or nursing mothers and all claimed to practice contraception.

During the treatment, patients received clinical advice on elimination of any possible environmental factors, such as trauma of the oral mucosa, possible allergic components and dental hygiene.

3.2.2. Ethical Approval

The study was approved by the local ethical committee, in Glasgow Dental Hospital and School.

Before the start of trial, all patients were fully informed about the clinical trial and possible side effect of medication and informed consent was obtained. They were all reassured that they could withdraw during the study for any reason without affecting their current treatment in the clinic. A direct phone number was given to all patients in case of any possible side effects arising or necessary information being required.

3.2.3. Experimental Design

The design was a randomised double-blind therapeutic trial of 5 amino-salicylate against placebo lasting four weeks in each limb of the study, with cross over after a two-week period of time as a wash-out stage. Pre-packaged randomised and coded supplies of 5 amino-salicylate cream and identically packaged placebo medications were supplied by Harefield Pharma Associates Limited. A code book identifying the numbered cream supplies was available after the trial was ended.

3.2.4. Subjects' Daily Records

All patients kept a careful daily record, noting the localization, number, size, pain and duration of ulcers during the trial. In a provided chart (Figure 3.1), patients recorded a number between zero to three (severity of ulcer) to illustrate the pain. To record the size, patients were given some figures in a form which indicated the size of ulcers from one to ten millimetres in diameter. In addition, a figure of an open mouth, helped the patients to locate the ulcers on the figure (Figure 3.2). These daily records of ulcers were collected in all three stages of the trial.

Patient Name:_____ Hospital Number._____ Patient (*trial identification*) number _____

	MONTH																										
	DAY																										
[illegible]	DATE																										
1	SIZE																										
1	PAIN																										
2	SIZE																										
2	PAIN																										
3	SIZE																										
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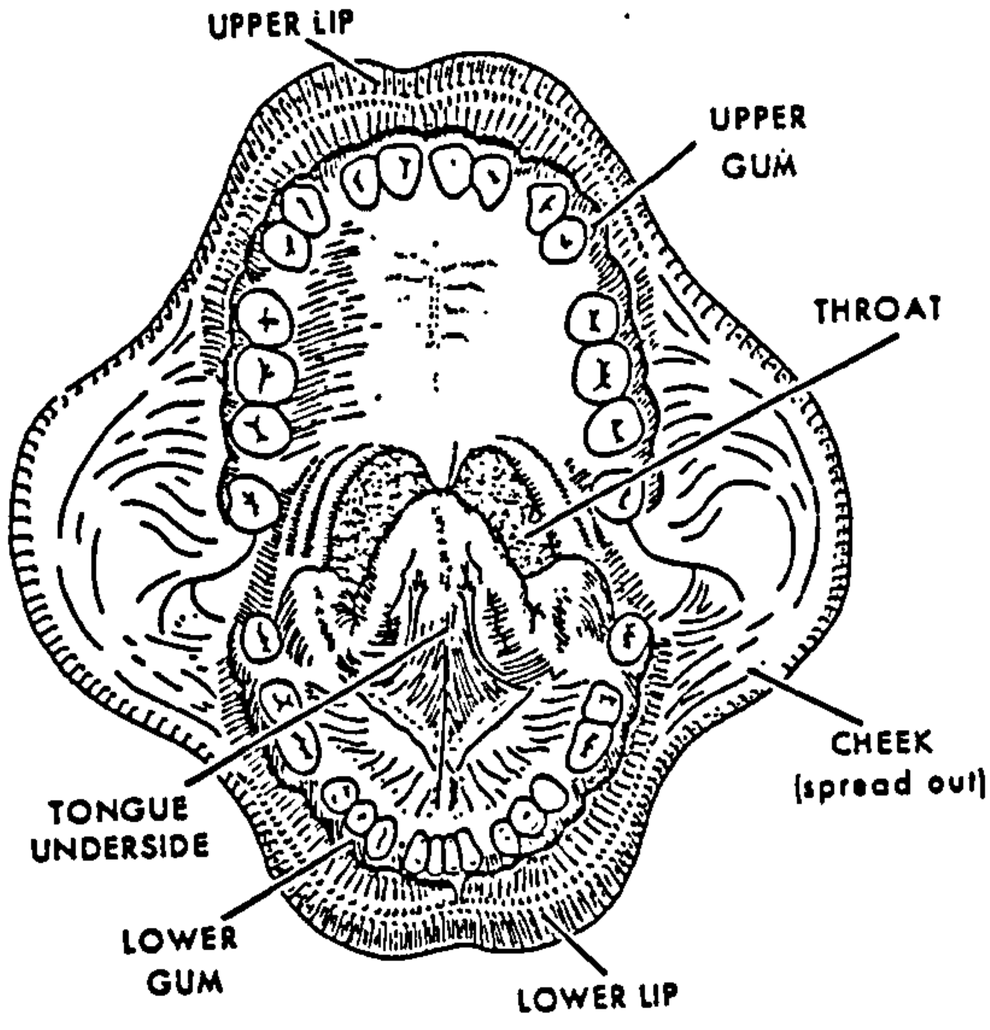
Figure 3.1: First page of diary card provided for RAS patients to record the number, size, pain and location of their ulcers. A diagram helps patients measure the size and pain of each ulcer. (see figure 3.2)



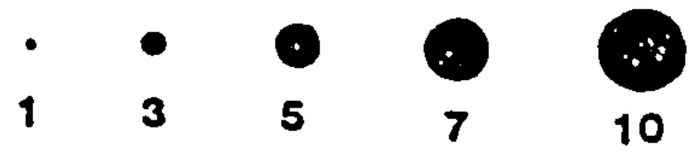
INSTRUCTIONS:

THIS SIDE

MARK & NUMBER EACH ULCER

OTHER SIDE

RECORD ULCER SIZE IN mm



RECORD PAIN

(no pain - 0, slight pain - 1, moderate pain - 2, severe pain - 3.)

Figure 3.2. Second page of diary card provided for RAS patients to record the number, size, pain and location of their ulcers.

3.2.5. Pre-Treatment Procedure

In this period of study, patients were not prescribed any topical medication or systemic analgesics. Patients were asked to write any topical medication or systemic analgesics which they may use during this period of the trial. In this first four weeks, 222 patients received a form to record their ulcers as has been explained in the previous section (section 3.2.4). Venous blood was removed for haematological investigation from all patients at their first attendence.

3.2.6. First Treatment Procedure

Patients who had any haematological deficiencies were excluded from the trial. Also, those who had less than three ulcers did not continue into the first treatment period. Sixty six patients received the randomized creams of whom 33 used the 5 amino-salicylic acid cream and 33 placebo. Patients were asked to apply the cream three times daily on the ulcers for a maximum period of four weeks. In addition to the cream, all patients were given a new form to record their ulcers during this period of the trial.

3.2.7. Wash Out Period

Between the first and second period of the trial, patients were not given any medication, to avoid any effect of the first treatment on the second part of treatment. This two weeks was called the wash-out period.

Patients returned the first cream and the ulcer diary. At this visit, an appointment was made for the second part of the treatment.

3.2.8. Second Treatment Procedure

Fifty two patients finished the first period of the trial and 47 of them agreed to continue with the second treatment. Patients received the second cream and a new ulcer diary form to record their ulcers as they did before. In this period of study, 24 patients received the 5 amino-salicylate and 23 the placebo.

At the end of this period, patients returned the second cream and ulcer diary.

3.2.9. Measurement

All indices which were recorded on the provided PC computer program for oral ulceration (see section 2.2.2) as follows:

a) The mean number of ulcers in the pre-treatment period and each period of the treatment.

b) The mean of size score in all three periods of the trial.

c) The mean of pain score in all three periods of the trial.

d) The mean of healing time in all three periods of treatment.

e) The mean score for efficiency of the cream on reduction of pain which patients reported after each period of treatment.

f) The mean score for efficiency of the cream on reduction of healing time which patients reported after each period of treatment.

3.2.10. Statistics and Data Analysis

Data were analysed using Minitab Software (PC computer). First, the statistical significance of observed data from each period of the trial was assessed by the non-parametric, Fridman test. This test was selected because of its power to show the statistical differences within one group of subjects.

Then, data from the first group of patients who applied the 5 amino-salicylate cream in first treatment was analysed with data from second group of patients who applied placebo in their first treatment, using the non-parametric Mann-Whitney U test. This test initially is helpful in analysing data in two sets of subjects.

3.3. Results

At the end of the trial the code of each package of the medication was opened.

Forty-two out of 66 patients completed the trial. Three patients used none of the creams because, they did not have any ulcers after entering the trial, four patients did not have any ulcers to continue in the second period of the trial. One patient became pregnant during the study, six patients could not use the cream because of the taste and colour of the cream and the rest of the patients who did not complete the study, failed to attend the clinic for their appointments as scheduled. (Table 3.1)

Three of the patients who did not have any ulcers after the first period of the treatment, just applied the placebo. In total, out of ten patients who failed to attend

the clinic, five received the active cream and five placebo. Of those six patients who did not like the cream, only two used the placebo and the other four patients were not happy with the active cream.

The first data obtained during active treatment was compared with the placebo, then data from the active treatment and placebo were compared with the pre-treatment data separately.

These results, did not show any significant difference between 5 amino-salicylate cream and placebo on their effect on healing time ($p=0.12$). Also 5 amino-salicylic acid cream was not statistically effective in decreasing the healing time compared with the pre-treatment period ($p=0.75$). The same result was obtained on the effects of placebo in decreasing the healing time compared with the pre-treatment period ($p=0.35$). (Table 3.2, Figure 3.3)

The topical active cream was not statistically effective in reduction of pain, compared with placebo ($p=1.0$) and the pre-treatment period ($p=0.06$). The placebo cream had the same lack of effect as the active cream compared with the pre-treatment period ($p=0.06$). (Table 3.2)

The results did not show any significant effect of the active cream on the number of ulcers compared with placebo ($p=0.38$) and the pre-treatment data ($p=0.4$). However placebo had a significant effect on the reduction of the number of ulcers compared with the pre-treatment period ($p=0.008$). (Table 3.2)

Table 3.1. Patients who did not complete the trial.

Cause	Number
Patients with no ulcers	3
Patients with no ulcers in the 2nd treatment	3
Failed to attended patients	10
Pregnant	1
Dislike of the cream	6
Total	23

The data obtained from patients after each treatment also did not show any significant differences in efficiency of active cream in healing time (p=0.84) and reduction of pain (p=0.31) compared with placebo. (Table 3.2)

Therefore, in this first analysis of the data, the results did not show any significant difference between the medication and placebo, and also no differences between each of the first and second treatments when compared with the pre-treatment period except the effect of placebo on decreasing the number of ulcers. (Figure 3.3)

To address the complexities in the first analysis, data obtained in each period of the trial were analysed without any knowledge about the codes of the creams. Each period of treatment was compared with all other periods. The results showed significant differences in decreasing the pain (p = 0.014) and significant differences (p = 0.016) in decreasing the number of ulcers between the data obtained in the pre-treatment period compared with the second treatment period regardless of whether it was active or placebo. There was no significant difference in reduction of size (p=0.53) and healing time (p=0.35) in this analysis. These results did not observe any difference in the first period of treatment compared with the pre-treatment period. The effect of the active cream on the size of the ulcer was not significant compared with the placebo (p=0.1) and the pre-treatment period (p=1.0). Neither was the effect of placebo compared with the pre-treatment period (p=0.2) (Table 3.2). However, from the pre-treatment period onwards an increased difference was observed in each subsequent period.

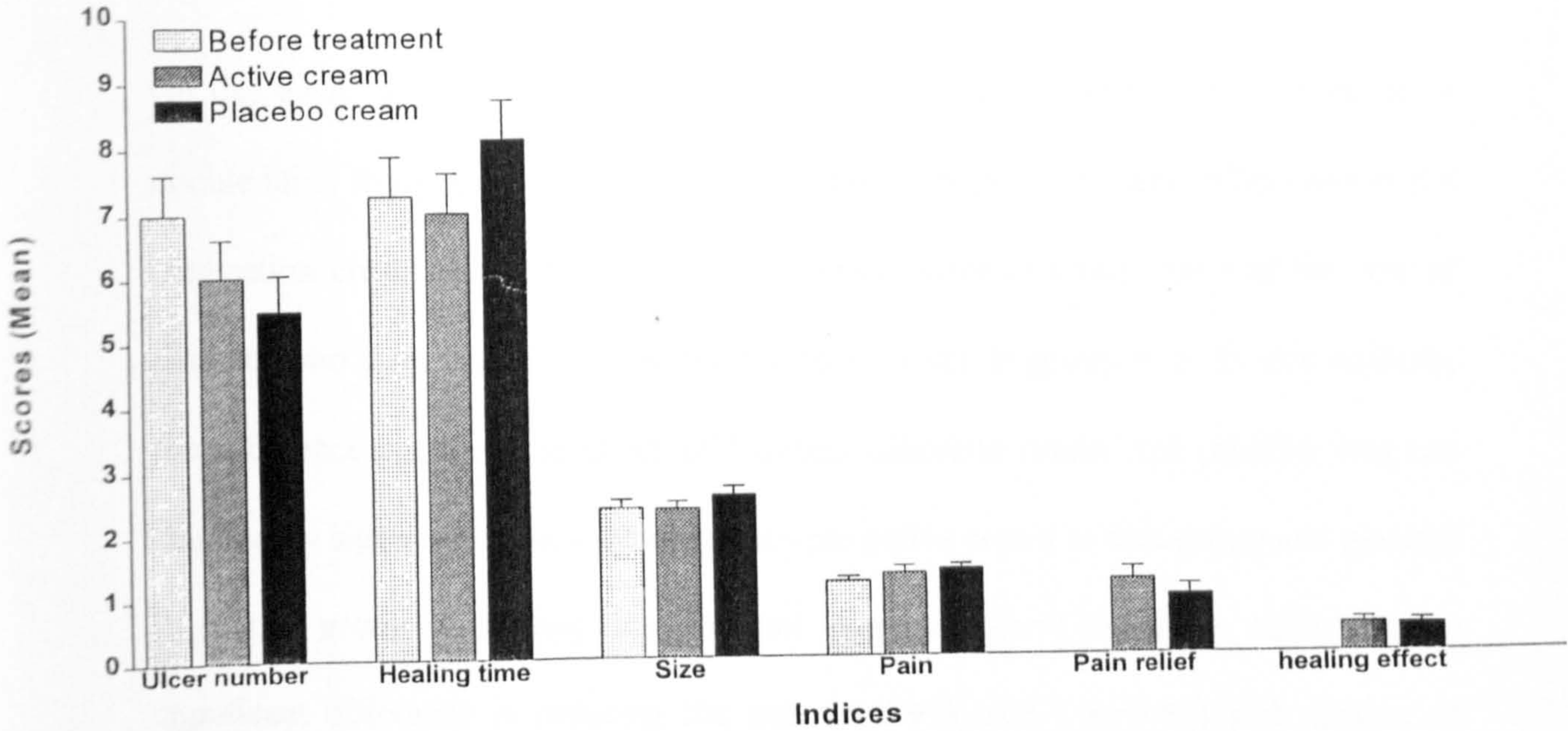


Figure 3.3. The effect of 5 amino-salicylate cream in aphthous ulcers compared with before treatment (no medication) and placebo in a double-blind cross-over trial. (n=42) There is no significant difference on aphthous lesions in patients before treatment and when patients were using placebo or active cream.

These differences were postulated to be as the result of clinical advice and maybe because of the effect of the base of the cream which covers the ulcers and reduced the pain and protected the ulcers from secondary infection. Therefore, it was concluded that a double blind cross over trial was not the appropriate way to study the effect of this topical medication in recurrent aphthous stomatitis.

On this basis, patients were thus divided in two groups to analyse the results as a double blind study. Patients were divided in two groups, 22 patients who applied the medication cream in the first period of treatment were in group one and the rest of patients who took the placebo in first treatment were in group two. In this analysis, the difference between the effect of 5-amino salicylate cream and placebo was not statistically significant. The difference between active cream in first group and placebo in second group on healing time was not significant ($p=0.73$). Also, there was no significant difference in reducing the pain ($p=0.99$), size ($p=0.85$) and number of ulcers ($p=0.90$) in two groups. (Table 3.3)

When comparison of the groups who took active or placebo cream in the second period were analysed there was also no significant difference in healing time ($p=0.06$), pain ($p=0.73$), size ($p=0.29$) and number of the ulcers between the two groups ($p = 0.28$). (Table 3.4, Figure 3.4)

Table 3-2. Summary of the data collected from diary cards.(Double blind cross over)

	Before treatment	Active cream	Placebo cream
Mean no. of ulcers	7.00±0.61**	6.02±0.59	5.48±0.57
Mean of healing time (day)	7.27±0.61	7.00±0.62	8.13±0.60
Mean of size score (mm)	2.38±0.13	2.37±0.11	2.58±0.14
Mean of pain score (0to 3 units)	1.19±0.07	1.31±0.11	1.37±0.07
Mean of the efficiency of the cream on pain (0 to 3 units)	-	1.19±0.18	0.93±0.17
Mean of the efficiency of the cream on healing time(0 to 1 units)	-	0.45±0.08	0.43±0.08

* Number of patients = 42

** Standard error

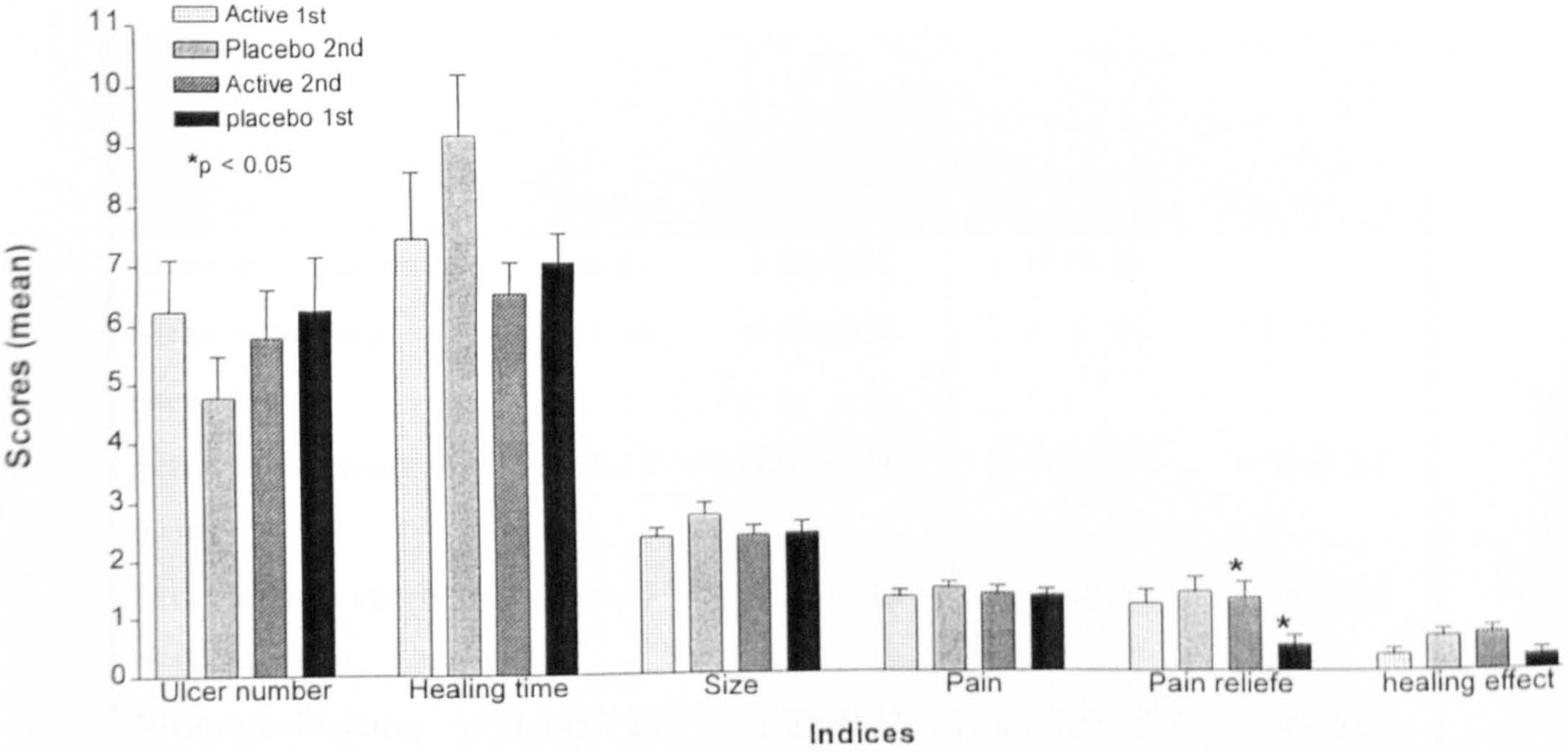


Figure 3.4. The effect of 5 ASA in aphthous lesions compared to placebo in two groups of patients. First those who used the active cream in first period of the trial and second those who used the active cream in the second period of the trial.

*shows a significant difference on relief of pain between patients who used the active cream in the second period of their treatment compared to placebo which has been used in the first period. This difference was reported by patients. There is no significant difference between the active cream and placebo in other aspects of the disease.

Table 3-3. Summary data collected from patients' diary form. (Double blind study)

	Active		Placebo	
	1st. treatment	2nd. Treatment	1st. treatment	2nd. treatment
Mean no. of ulcers	6.23±0.87	5.80±0.81	6.25±0.88	4.77±0.71
Mean of healing time (day)	7.45±1.10	6.51±0.50	7.01±0.51	9.15±1.01
Mean of size score (mm)	2.36±0.15	2.38±0.16	2.41±0.19	2.73±0.21
Mean of pain score (0 to 3 units)	1.28±0.11	1.34±0.12	1.31±0.10	1.44±0.10
Mean of efficiency of the cream on pain (0 to 3 units)	1.14±0.25	1.25±0.27	0.45±0.15	1.36±0.25
Mean of efficiency of the cream on healing time (0 to 1 units)	0.27±0.10	0.65±0.11	0.25±0.1	0.59±0.11

* In first active treatment and second placebo treatment number of patients=22, in second active treatment and first placebo treatment number of patients=20

However, the information obtained from patients on preference after each period of treatment in reduction of pain and healing time indicated a significant effect of 5 amino-salicylate cream in decreasing the pain (p = 0.04) but not a statistically significant effect on healing time (p=0.88), compared with the placebo in group of patients who used the active cream in second period of their treatment. (Table 3.3, Figure 3.4)

3.4. Discussion

Topical 5 amino-salicylic acid has been effective in the treatment of ulcerative colitis and Behçet's syndrome (Azad Khan et al. 1977; Ranzi et al. 1986). However this anti-inflammatory medication was not reported to be significantly effective in recurrent aphthous stomatitis when it was used as a mouthwash (Bagg, 1985), while its topical cream was effective in reduction of pain, number and duration of ulcers (Collier et al. 1992).

In this study, a statistically significant effect of the 5 amino-salicylate cream was not detected in patients' daily record of ulceration. However, patients could distinguish the medication from placebo and they reported a significant reduction in the pain of the ulcers as the result of the medication in second period in treatment. This effect, in the second period of treatment might be the effect of other factors such as avoiding the environmental predisposing factors, as well as medication. (Figure 3.4)

In this clinical trial of topical 5 amino-salicylate cream, the results suggest that in diseases like recurrent aphthous stomatitis, it may not be appropriate to use a cross-

over study. The reason for this is the process of the disease is not predictable in different individuals, and in the second period of treatment the severity of the condition may be totally different for reasons other than the effects of the medication. For instance, 5 amino-salicylate cream has been studied in another clinical trial, using a double-blind method (Collier et al. 1992). In that study, the significant effect of 5 amino-salicylic acid was reported to decrease the number, duration, pain and size of the ulcers (Collier et al. 1992). While in another double-blind cross-over study, there was no significant difference between 5 amino-salicylate and placebo on the oral ulceration (Bagg, 1985). Although, despite different clinical designs, fundamental differences between these two studies was in the method of applying the medication. When the medication was used as a mouth wash, (Bagg, 1985) the duration of activity of 5 amino-salicylate may be very short while in the format of the cream not only would this duration of activity increase but also, the cream covered the ulcers and separated them from any aggravating factors or infection and this would not only help the relief of pain but also decrease the healing time.

In the study by Collier et al (1992) subjects were volunteers who understood the aim of the study while in the present study, subjects were patients referred to the Oral Medicine Clinic for treatment of recurrent aphthous stomatitis. However patients were fully informed about the trial, but it was not possible to avoid giving clinical advice to exclude environmental aetiologic factors in some patients. As a result, the cross-over study was not successful in distinguishing any effects of 5 amino-salicylate.

There was no significant difference between the amount of the placebo and active cream, used by patients. The average weight of the cream, used by patients, was 4.64 g. for placebo and 4.19 g. for active medication. The maximum amount of cream was used by those patients with seven or more ulcers per month.

In this trial, no side effects were observed as result of using the 5 amino-salicylate.

However the present results do not strongly support the previous study on the effect of 5 amino-salicylate (Collier et al. 1992), but, the effect may be extended in the use of the cream for longer periods of time. Given this, 5 amino-salicylate cream can be beneficial for patients with moderate symptoms, but to assess the benefits of the cream in recurrent aphthous stomatitis, this medication should be compared with other current medications for treatment of recurrent aphthous stomatitis such as Adcortyl in Orabase.

Chapter Four. Search for Human Herpes Virus-6, Varicella Zoster, Cytomegalovirus and *Mycobacterium paratuberculosis* DNA

4.1. Introduction

Despite many investigations toward the aetiology of recurrent aphthous stomatitis, none of the aetiologic factors mentioned in the previous chapters, could explain the initiation or the cause of this disease. The majority of oral mucosal ulcerations, other than recurrent aphthous stomatitis, result from viral infections. (Gajdusek, 1977)

Immunological features have mainly referred to either autoimmunity or viral pathogenicity (Pedersen, 1993). The autoimmune hypothesis is based on a hypersensitivity reaction to human oral mucosal cells and cross-reactivity between streptococci and oral mucosal cells (Pedersen, 1993). However the clinical and laboratory investigations do not support an autoimmune theory. Autoimmune diseases are more prevalent in females and increase with advancing age, (Ollier and Symmons, 1992; Pedersen, 1993), whereas the prevalence of recurrent aphthous stomatitis is increased in the second and third decades of life, and the disease affects the two sexes almost equally (Pedersen, 1993). There is not strong evidence to establish an auto-antigen in patients with aphthous ulcers (Pedersen, 1993). Like recurrent aphthous stomatitis, viral infections are usually self-limiting (Jawetz et al. 1989). On the other hand, recurrent aphthous stomatitis, as mentioned under aetiologic factors, may have a genetic background, as does autoimmune disease (Ollier and Symmons, 1992). However, this similarity exists in other viral diseases such as recurrent herpes labialis (Russell and Schlaut, 1977). One of the early hypotheses for HLA disease associations is that the cell surface HLA molecule could act as a receptor for a specific virus and provide a means for it to infect the cell. Several examples have been

described in the mouse where H-2 molecules can specifically bind to viruses (Ollier and Symmons, 1992), although there is no evidence in man for HLA being a viral receptor. However, it is clear that cell surface markers in man, such as CD4, can act in this capacity e.g. as a receptor for human immunodeficiency virus (Ollier and Symmons, 1992). This hypothesis could explain the genetic and environmental component in the aetiology of many diseases such as recurrent aphthous stomatitis.

As viruses are the strongest infective candidates for a role in the production of autoimmune disease (Ollier and Symmons, 1992), the immune imbalances may be the result of viral involvement. Despite the inability to isolate viruses from recurrent aphthous stomatitis lesions (Hooks, 1978), a viral involvement cannot be excluded in the aetiology of recurrent aphthous stomatitis. Indeed, support for the involvement of herpesviruses in recurrent aphthous stomatitis has come from serological studies. Serum levels of total immunoglobulins in patients with recurrent aphthous stomatitis have been reported to be higher than normal values and this humoral abnormality was of a non-specific nature in recurrent aphthous stomatitis (Sun et al. 1986). Increased serum IgM antibody against varicella zoster virus (VZV) and human cytomegalovirus (HCMV) have been reported in patients with recurrent aphthous stomatitis, and serum IgG antibody against VZV and HCMV were also increased in patients, especially at the time of remission (Pedersen and Hornsleth, 1993). Antibody levels of serum anti-VZV IgM antibody were statistically significantly higher in the patients compared with the control group (Pedersen and Hornsleth, 1993). Although, there was no statistically significant difference in the level of serum anti-HCMV IgG antibody between patients in the active stage of ulceration and the control group, the serum

anti-HCMV IgG antibody concentrations in patients in the remission stage were statistically significantly higher than those of controls and those in patients in the active stage (Pedersen and Hornsleth, 1993), and a similar finding has been reported in another study (Sun et al. 1996). The level of serum IgG antibody against human herpesvirus-6 (HHV-6) was not different from the control group (Pedersen and Hornsleth, 1993).

Previous microbiological investigations were not successful in isolating any viruses in recurrent aphthous stomatitis. The polymerase chain reaction (PCR) however, allows for sensitive and specific detection of viral DNA or RNA.

Recently, the exquisitely sensitive PCR technique has been successfully used to detect herpes viral DNA in aphthous lesions. VZV DNA has been found in most tissue from recurrent aphthous stomatitis patients (Pedersen, 1993; Pedersen et al. 1993) and another study has identified HCMV DNA in 2 of 4 pre-ulcerative oral aphthae from Behçet's syndrome patients and in 3 of 9 pre- ulcerative recurrent aphthous stomatitis specimens but not in any of 5 control samples (Sun et al. 1996). HSV-1 DNA has been detected in the peripheral blood leucocytes of patients with Behçet's syndrome who demonstrated aphthous ulceration but not in oral ulcer biopsies from these patients (Studd et al. 1991). To date, there have been no other reports in which PCR has been used to identify other viruses in recurrent aphthous stomatitis. Previous studies have not detected HSV-1 DNA. As the result it did not seem to be necessary to repeat those investigations. However, the lack of information on detection of VZV,

HCMV and HHV-6 was encouragment to design an experiment to investigate the involvement of these herpesviruses in recurrent aphthous stomatitis.

Biopsy samples and blood were removed from 21 recurrent aphthous stomatitis patients in the early stage of ulceration. Serum samples were tested for detection of antibodies against herpesviruses tested in PCR which will be explained in detail in next chapter. The purpose of the study presented here was to investigate the possible presence of HHV-6, HCMV and VZV in aphthous lesions by a nested PCR method.

In most of the bacteriological studies L-form *Streptococcus sanguis*, Neisseria and staphylococcus were obtained from major and minor recurrent aphthous stomatitis, (Barile et al. 1963; Graykowski et al. 1964; Stanley et al. 1964) and the cross-reactivity between *Streptococcus sanguis* and human oral mucosa has been discussed as a pathologic factors in recurrent aphthous stomatitis (Donatsky, 1975). There has been no report to indicate the presence of *Mycobacterium para-tuberculosis*.

Recently, Hassan et al (1995) reported 73 percent homology between mycobacterial peptide 91-105 and human mitochondrial 116-130 peptide. It is also reported that four peptides from the 65 KD mycobacterias heat shock proteins and four peptides from the homologous sequence of the 60 KD human heat shock proteins can cause lymphoproliferative response in Behçet's syndrome (Fortune et al. 1996).

Heat shock proteins are very conserved and may cause cross-reactivity between microbial and cellular host materials (Hasan et al. 1995). However this raises the question that if molecular mimicry between microbial and human cellular heat shock proteins were damaging the immune response, it would be expected to involve other

parts of the human body. The author suggested that the involvement of oral mucosa is because of the high load of micro-organisms in oral cavity which increases this cross-reactivity and causes tissue damage (Hasan et al. 1995).

The presence of *Mycobacterium paratuberculosis* was also investigated as described later.

4.2. Polymerase Chain Reaction, Nested Polymerase Chain Reaction and Hot Start

The polymerase chain reaction (PCR) is an *in vitro* technique which allows the amplification of a specific deoxyribonucleic acid (DNA) region that lies between two regions of known DNA sequences (Newton and Graham, 1994). This method allows for sensitive and specific detection of trace copies of particular genetic sequences against a large background of irrelevant DNA. This makes PCR ideally suited for detecting viruses in human tissue.

Nested PCR primers are ones that are internal to the first primer pair. The larger fragment produced by the first round of PCR is used as the template for the second PCR. Nested PCR can also be performed with one of the first primer pair and a single nested primer. The sensitivity and specificity of both DNA and RNA amplification can be dramatically increased by using the nested PCR method. The specificity is particularly enhanced because this technique almost always eliminates any spurious non-specific amplification products (Newton and Graham, 1994).

Hot start is a modification of PCR which is a separation of one or more important components of the PCR by wax such that all reaction components are mixed after

denaturation of the template. It is more efficient to separate the reaction component physically with a material that can be used as a barrier but which will melt on raising the temperature, therefore causing mixing of all reaction components at the start of the PCR (Newton and Graham, 1994).

4.3. Materials and Methods

4.3.1. Materials

Consumables used during the course of the studies presented in this chapter were obtained from the sources listed in appendix II.

4.3.2. Subjects and Ethical Approval

Twenty-one patients referred to the Oral Medicine Clinic were informed and asked to undergo biopsy for research purposes. Informed consent was obtained from all patients . According to the classification of recurrent aphthous stomatitis (Lehner, 1968), all patients were diagnosed as having the minor type of the disease. The average age of the patients was 36 years (22-63 years) and there were six males and 15 females (Table 4.1).

In addition 20 patients diagnosed as having oral lichen planus, who underwent biopsy for their management in the Oral Medicine Clinic, were used as disease controls in this study. The mean age in this group was 45 years (24-71) and they were eight males and 12 females (Table 4.1).

Table 4.1. RAS and OLP patients and control subjects who underwent biopsy for PCR experiment.

	Age	Male	Female	Total
RAS patients	22-63	6	15	21
OLP patients	24-71	8	12	20
Controls	21-79	5	8	13

Healthy control subjects were those who attended Oral Surgery for dental extraction under surgical operation. A piece of mucosa flap removed for study purposes during operation. This group included 13 individuals with no history of oral ulceration. The consent was obtained from these patients by the surgeon in charge of the operation and all patients were fully informed about the purpose of the study. The mean age in this group was 43.2 years (21-79 years) and five males and eight females were in this group.(Table 4.1)

4.3.3. Collection of Samples

Biopsy specimens from patients with recurrent aphthous stomatitis were collected within 48 hours of ulceration, biopsy specimens of oral lichen planus and 13 normal oral mucosa samples were obtained as controls. The biopsy samples of aphthous patients were generally smaller than oral lichen planus and control specimens. Tissue biopsies were stored at -70°C prior to DNA extraction.

4.3.4. DNA Extraction

Frozen tissue samples were manually homogenised in 1 ml of TEN buffer (50 mM Tris-HCl, 100 mM EDTA, 150 mM NaCl) per 200 mg of tissue. To each 200 mg of homogenised tissue was added 250 µl 6M guanidinium isothiocyanate (in 0.1 M Tris HCl, pH 7.6) and this was incubated overnight at 37°C. The mixture was boiled for 20 minutes, an equal volume of molecular biology grade water was added, and then the sample was centrifuged at 12.500 RPM for one minute using an Eppendorf centrifuge (model 5415c). The supernatant was retained and the genomic DNA was

purified using the Wizard DNA Clean Up Kit in accordance with the manufacturer's instructions. For each extraction, tissue DNA was eluted in a final volume of 50 µl.

To confirm successful extraction of PCR-amplifiable DNA from tissues, PCR was carried out on each DNA sample with nested primers specific for the human ß-haemoglobin gene for which the method will be described.

4.3.5. Selection of Primers for Nested PCR

The nested primer pairs for use in PCR were those previously described for HHV-6, HCMV and VZV (Wakefield et al. 1992). The primer sequences, annealing temperatures used and expected size of amplification products are shown in Table 4.2. The nested primers for *Mycobacterium paratuberculosis* were those described in another investigation (Lisby et al. 1994). The primer sequences and expected size of amplification products are demonstrated in Table 4.3.

4.3.6. Nested Polymerase Chain Reaction Using Viral Primers

For nested PCR analysis, 5 µl of extracted DNA was added to 95 µl of 1 X PCR buffer (10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 2.0 U of Dynazyme I DNA polymerase (Flowgen Instruments Ltd, Lichfield, England), 0.2 mM dNTPs and 200 ng of each "outer" primer pair. Primers were separated from other components of the reaction mixture by a layer of wax (Dynawax; Flowgen), thus preventing the reaction from starting until melting of the wax upon commencement of thermal cycling. This 'hot start' PCR method improves the specificity and yield of reaction products. Amplification was carried out in an

OmniGene Thermal Cycler. After an initial denaturation step at 94°C for five minutes, 35 cycles of denaturation at 94°C for one minute, annealing of primers at 50-60°C for two minutes and primer extension at 72°C for one minute was carried out, followed by a final extension step at 72°C for 10 min. For the second round of PCR, 25 cycles of amplification were carried out using two µl of first round product as the source of template DNA and 200 ng of each "inner" primer pair. The annealing temperatures used for each set of "outer" and "inner" primers is indicated in Table 4.2.

4.3 7. Nested PCR Using *Mycobacterium paratuberculosis* Primers

A fragment of the IS900 multi-copy insertional gene element of *Mycobacterium paratuberculosis* (Lisby et al. 1994) was selected for this study and two sets of primers were collected (Table 4.3) for use in a nested PCR. One µL of extracted DNA was added to 99 µL of solution containing 50 mM KCl, 10 mM Tris-HCl (PH 8.3), 0.01 per cent (w/v) gelatin, 1.0 nM $MgCl_2$, 200 µM of each deoxynucleotide (dNTP, 2 units Ampli TAQ (Perkin Elmer Cetus) and 0.6 µM each of the outer primers (Table 4.3). PCR was performed for 25 cycles of denaturation at 94°C for 45 seconds, annealing of primers at 56°C for 90 seconds, and primer extension at 72°C for 45 seconds. For the second round of PCR, 5 µL of the first round products as a template was transferred to a new PCR reaction identical to the first one with the exception of using 0.4 µM each of the inner primers (Table 4.3). PCR for the second round was performed for 40 cycles using the same profile as in the first reaction.

Table 4.2. HHV-6, HCMV and VZV nested PCR primers used: sequences, annealing temperatures and PCR product sizes.

Virus and gene	Primer sequences (5'-3')	Nucleotide location	Annealing temperature (°C)	Product size (base pair)
HHV-6 (13R)	1. AAGCTTGCACAATGCCAAAAAACAG	17627-17603	60	223
	2. CTCGAGTATGCCGAGACCCCTAATC	17405-17429	60	
	3. TCCATTATTTGGCCGCATTCGT	17602-17580	60	173
	4. TGTTAGGATATACCGATGTGCGT	17430-17452	60	
HCMV (gB)	1. GAGGACAACGAAATCCTGTTGGGCA	1942-1966	58	150
	2. GTCGACGGTGGAGATACTGCTGAGG	2091-2067	58	
	3. ACCACCGCACTGAGGAATGTCAG	1967-1989	50	100
	4. TCAATCATGCGTTTGAAGAGGTA	2066-2044	50	
VZV (gene 29)	1. ACGGGTCTTGCCGGAGCTGGT	51067-51087	60	272
	2. AATGCCGTGACCACCAAGTATAAT	51338-51315	60	
	3. ACCTTAAAACTCACTACCAGT	51091-51111	50	208
	4. CTAATCCAAGGCGGGTGCAT	51298-51279	50	

Primers 1 and 2 are the first round "outer" set, primers 3 and 4 are the second round "inner" set.

Table adapted from reference (Wakefield et al. 1992).

Table 4.3. Mycobacterium paratuberculosis nested primer used sequences and expected product size.

Gene	Primer sequences (5`-3`)	Nucleotide location	Product size (base pair)
PTB1*	GCATGGTTATTAACGACG	290-310	437
PTB2*	CGAAAGTATTCCAGCAGC	710-720	437
PTB5**	CCGATCAGCCACCAGATCGG	660-670	289
PTB12**	GTTGATTGCGGCGGTGAC	360-380	289

* Outer primers
**Inner primers

4.3.8. Nested PCR Using Human β Haemoglobin Primers

For nested PCR for the detection of human β haemoglobin, the same PCR solution as the previous experiment was used. A 2.5 μL volume of a one μg/μL solution of each first round oligonucleotide was added to the solution in the first round as outer primers. In the second round, 2.5 μL of a 1 μg/μL solution of each of the second round oligonucleotide primers (inner primers) was used. A 2 μL volume of first round product was added to the solution as the template for second round. Amplification was carried out with initial denaturation step at 94 °C for five minutes, 35 cycles of denaturation at 94°C for one minute, annealing of primers at 64°C for two minutes and primer extension at 72°C for two minutes, followed by a final extension step at 72°C for 10 minutes.

For the second round of nested PCR, amplification was repeated for 35 cycles with the same time and temperature parameters as above except for a 55°C annealing temperature in the second step.

4.3.9. PCR Quality Control

In order to avoid contamination, strict procedures were employed when carrying out PCR. Separate rooms were used for sample preparation, setting up of PCR reactions and post-PCR analysis. Filter tips were used at all stages, except when adding samples to the reaction mixture when positive displacement tips were used. Positive and negative controls were used with each batch of samples being analysed. The positive control was 10 ng of plasmid DNA containing the cloned herpesviral gene against

which the primers were targeted instead of the sample. Positive control in nested PCR for *Mycobacterium paratuberculosis* was plasmid containing the cloned IS900 insertion element of paratuberculosis against which the primer for nested-PCR were targeted, whereas the negative control contained sterile water instead of the sample. For detection of human β haemoglobin the positive control was not employed.

4.3.10. Analysis of PCR Products

15 µl of each reaction product was electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 µg/ml), using a 100 base pair (bp) DNA ladder (Pharmacia Biotech, Milton Keynes, England) as a size marker, and visualized under UV illumination. PCR positivity for HHV-6, HCMV, VZV and *Mycobacterium paratuberculosis* was indicated by the appearance of DNA bands of 173- bp, 100- bp, 208-bp and 289- bp respectively. (Figure 4.1, 4.2, 4.3, 4.4)

4.3.11. Statistics

The statistical significance of observed differences was assessed by Fisher's exact probability test. A level of $p<0.05$ was chosen to reflect statistical significance.

4.4. Results

All tissue DNA samples demonstrated PCR positivity for the human ß-haemoglobin gene, which was indicated by the amplification of a 165-bp product (Figure 4.5). For each batch of samples being analysed by PCR, positive controls were always positive and negative controls always negative, thereby excluding the possibility of contamination and validating the results obtained.

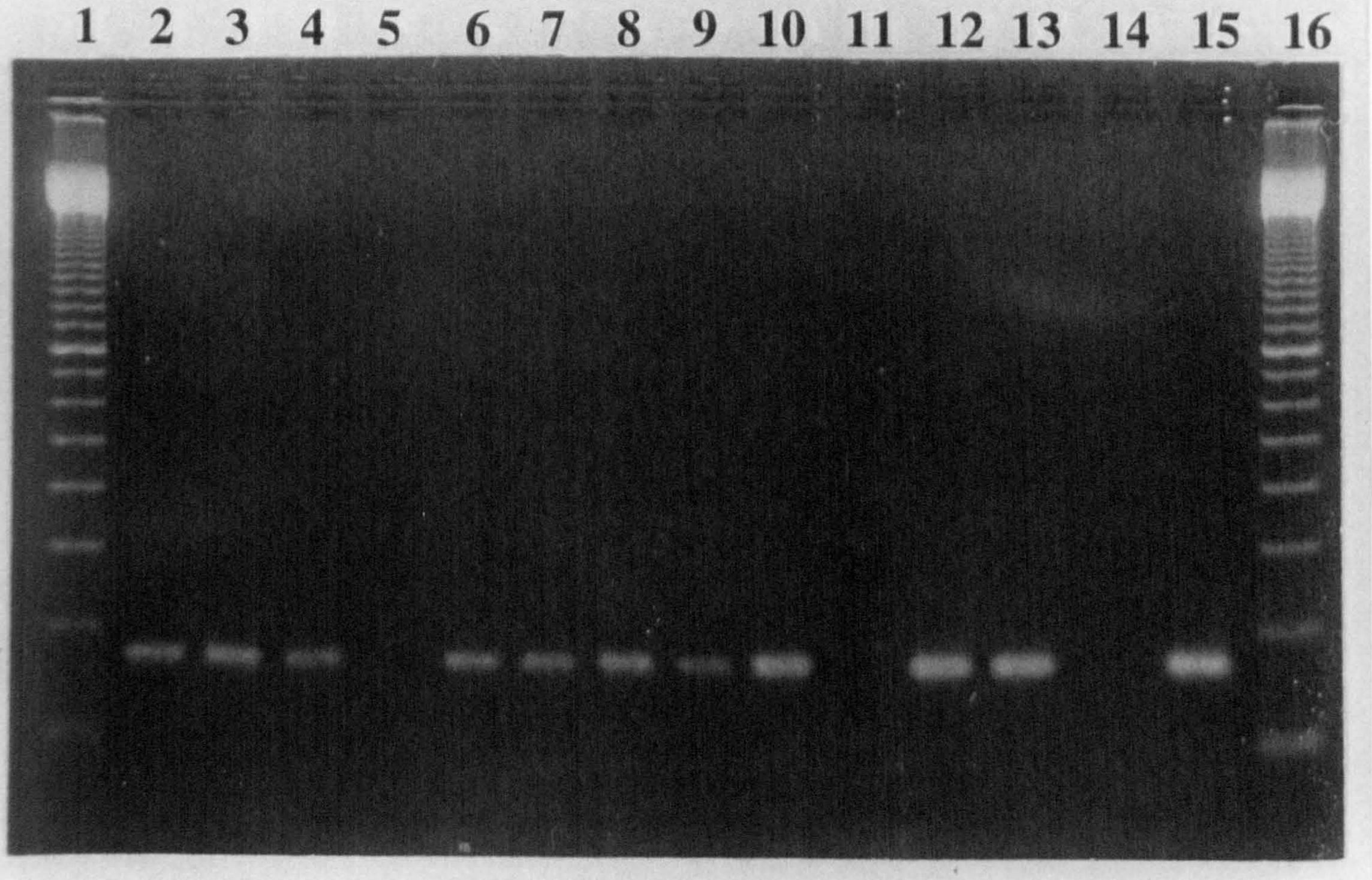


Figure 4.1. Two per cent agarose gel electrophoresis of selected products obtained following nested PCR with HHV-6 primers. 100-bp DNA ladder (lanes 1 and 16), RAS samples (lane 2 to 8), oral lichen planus samples (lanes 9 to 13), negative control (lane 14) and positive control (lane 15).

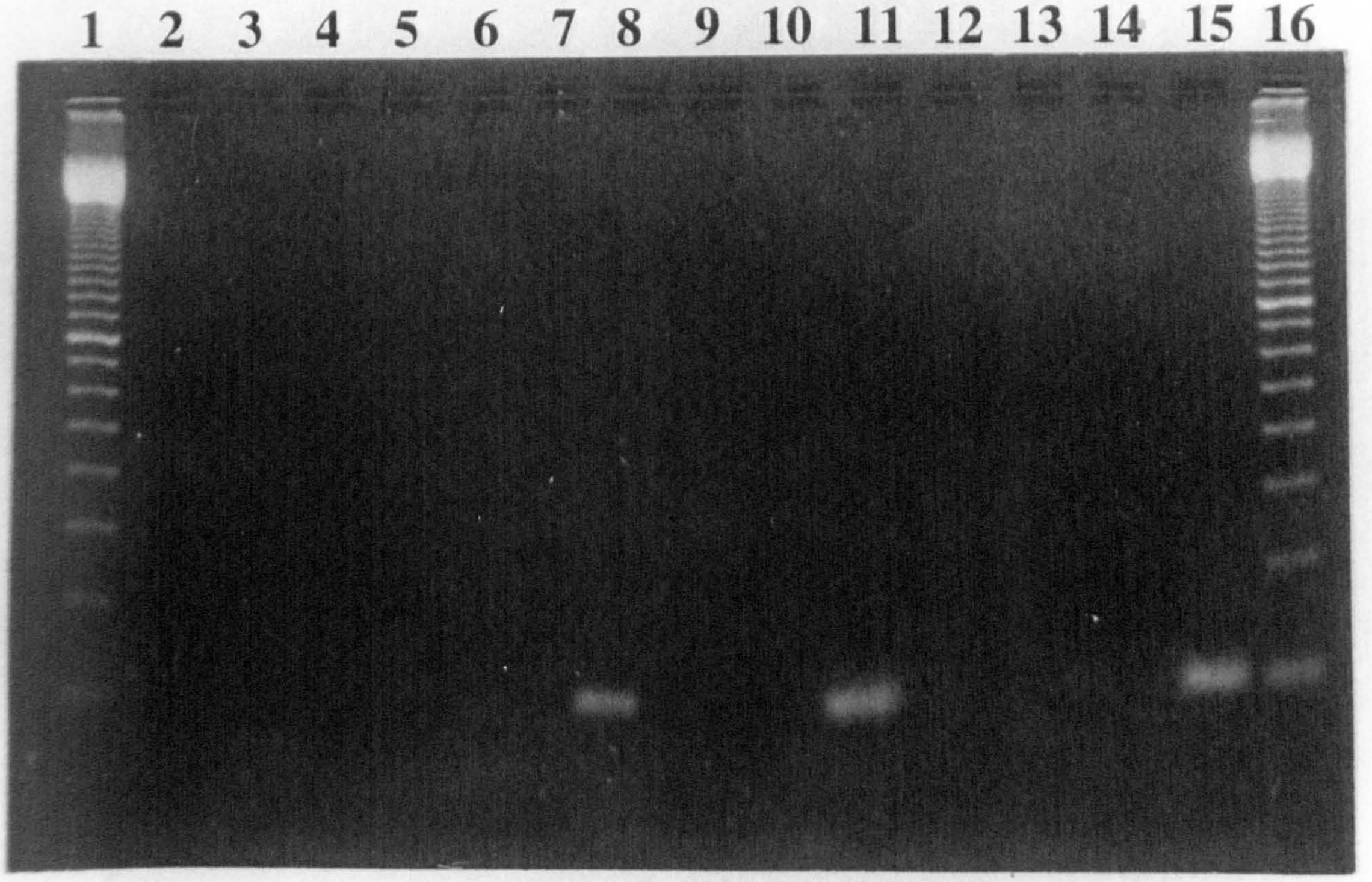


Figure 4.2. Two per cent agarose gel electrophoresis of selected products obtained following nested PCR with HCMV primers. 100-bp DNA ladder (lane 1 and 16), RAS samples (lane 2 to 7), oral lichen planus samples (lane 8 to 13), negative control (lane 14) and positive control (lane 15).

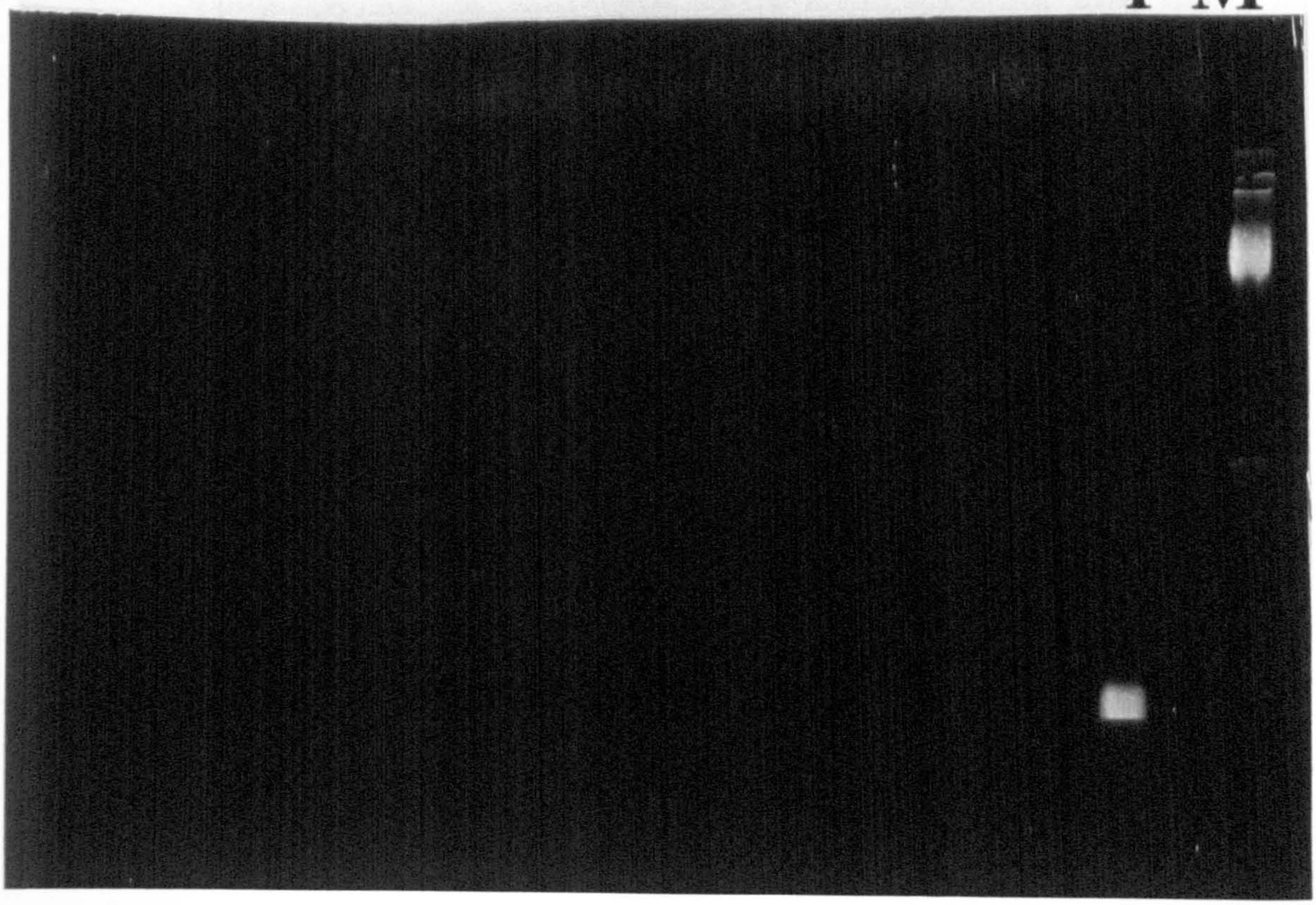


Figure 4.3. Two per cent agarose gel electrophoresis of selected products obtained following nested PCR with VZV primers. 100-bp DNA ladder (lane M), positive control (lane P) and the remaining lanes are negative control and biopsy samples which all show negative results.

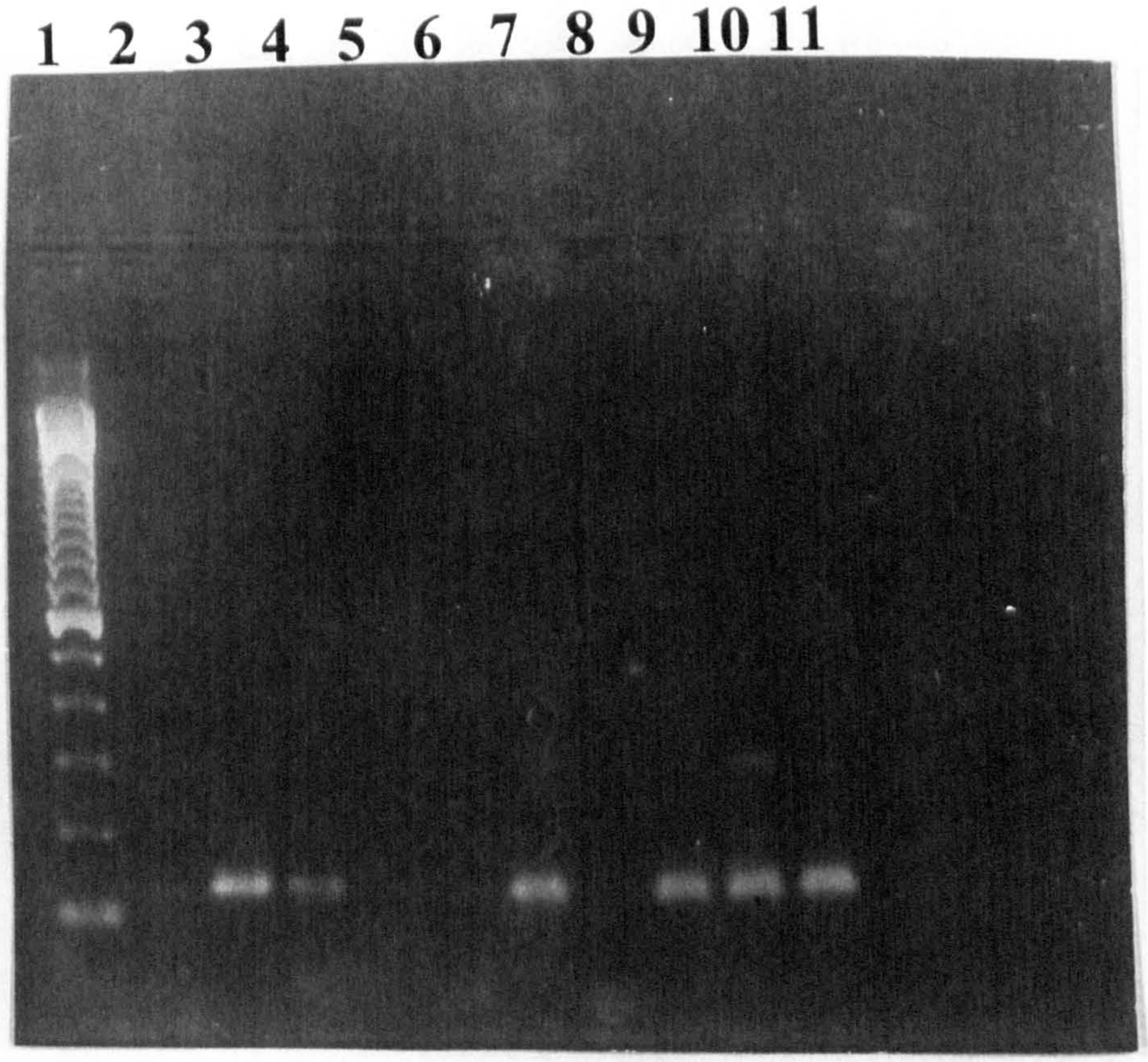


Figure 4.4. Two per cent agarose gel electrophoresis of selected products obtained following a nested PCR with *Mycobacterium paratuberculosis* primers. 100-bp DNA ladder (lane 1), RAS samples (lanes 2,3,4,7,9,10), oral lichen planus samples (lanes 5,6,8), positive control (lane 11) and negative control (lane 12).

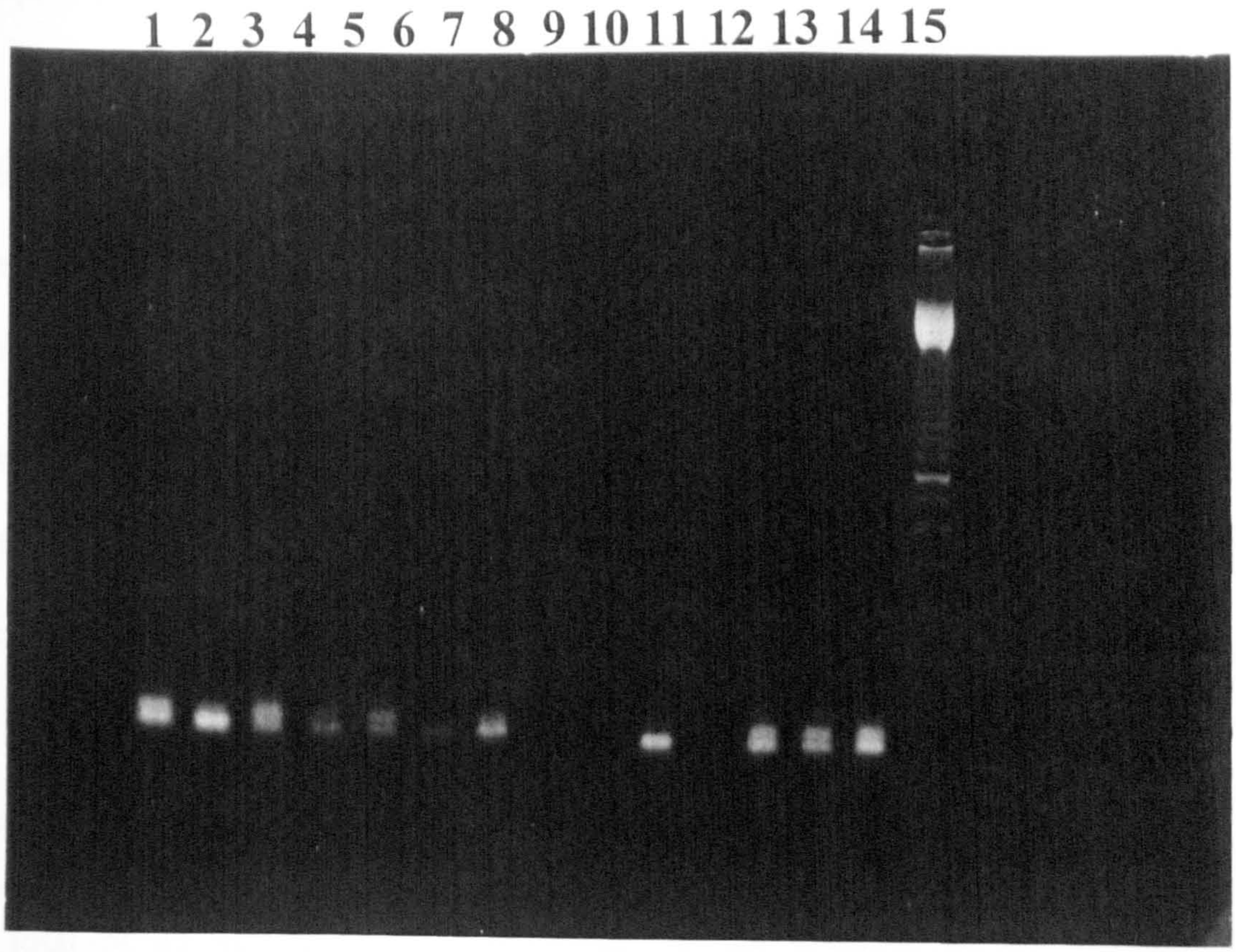


Figure 4.5. Two per cent agarose gel electrophoresis of selected products of a nested PCR with human β haemoglobin primers. Negative control (lane 1), DNA samples extracted from RAS, oral lichen planus and control biopsies (lane 2 to 8,11,13,14,15), empty lanes (lanes 9,10,12) and 100-bp DNA ladder (lane 15).

4.4.1. Human Herpes Virus 6, Human Cytomegalovirus and Varicella Zoster Virus

The PCR results obtained are summarized in Table 4.4. HHV-6 DNA was present in six samples from recurrent aphthous stomatitis patients and four samples from patients with oral lichen planus. HCMV DNA was detected in two oral lichen planus samples, whereas VZV DNA was not detected in any type of sample (Figure 4.3). None of the healthy controls were positive for either HHV-6, HCMV or VZV.

The difference of detection rate of HHV-6 DNA in recurrent aphthous stomatitis DNA compared with lichen planus biopsies was not significant ($p=0.77$) also there was no statistically significant difference in the presence of HHV-6 DNA in recurrent aphthous stomatitis patients compared with normal controls ($p=0.08$). (Figure 4.6)

4.4.2. *Mycobacterium paratuberculosis*

A total of 32 samples were investigated for the presence of *Mycobacterium paratuberculosis* DNA. *Mycobacterium paratuberculosis* DNA was present in four samples from a total of 15 patients with recurrent aphthous stomatitis. There were no DNA positive for this micro-organism in 13 samples of oral lichen planus patients and four normal biopsies. (Figure 4.4) There was no statistically significant difference between recurrent aphthous stomatitis patients and oral lichen planus group ($p=0.18$) and normal samples ($p=0.7$).

Table 4.4. Number of RAS, lichen planus and normal biopsies positive for HHV-6, HCMV and VZV DNA as determined by PCR. *N* denotes the number of biopsies analysed for each tissue type.

	HHV-6	HCMV	VZV
RAS (*N*=21)	6	0	0
lichen planus (*N*=20)	4	2	0
normal (*N*=13)	0	0	0

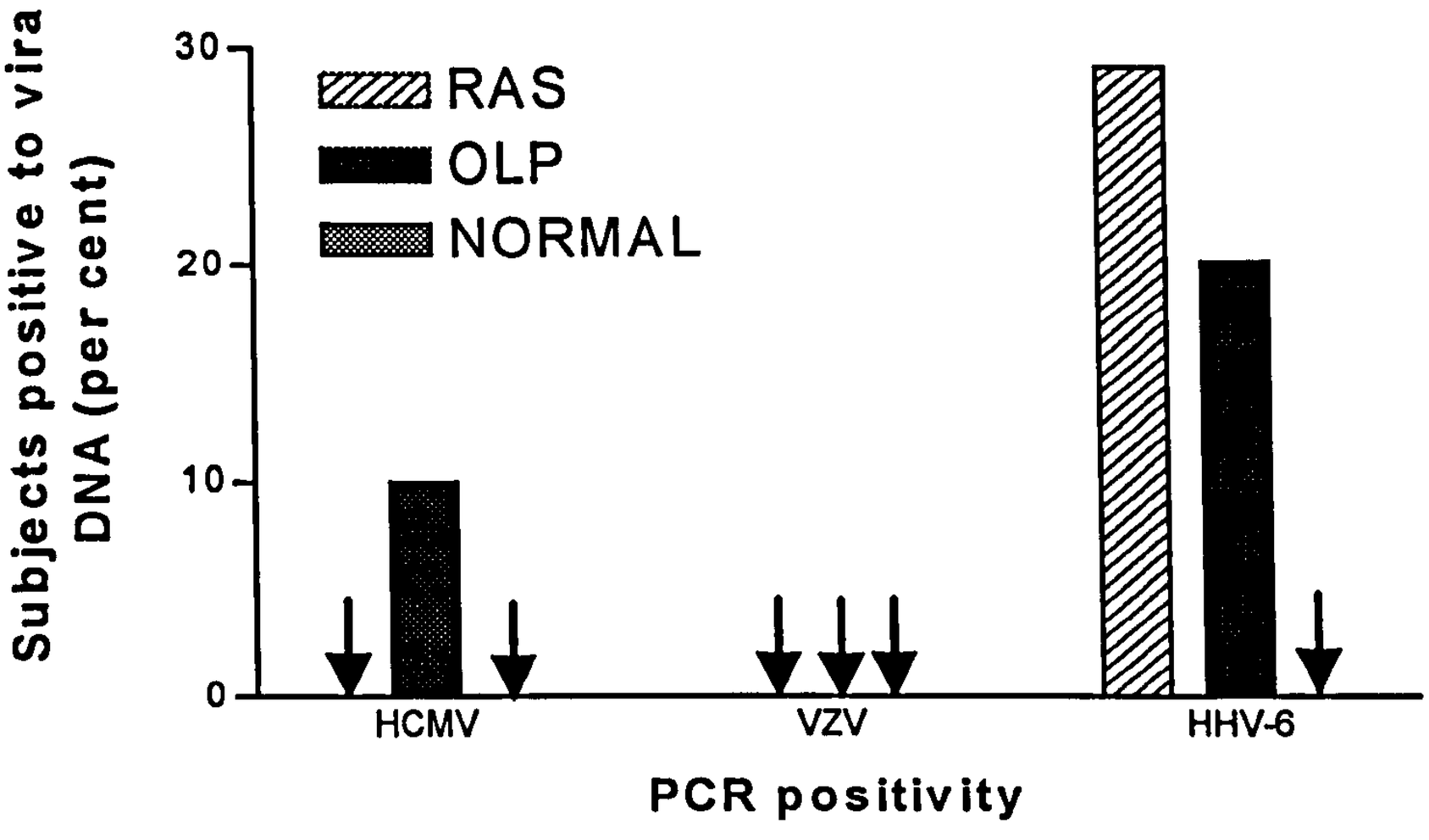


Figure 4.6. PCR positivity in RAS patients compared to oral lichen planus (OLP) patients and normal subjects to HCMV, VZV and HHV-6 DNA. No DNA of VZV and HCMV was detected in RAS lesions. VZV DNA was not found in any of the samples. HCMV DNA was found only in 10 per cent of samples obtained from oral lichen planus.

↓ Shows the PCR negative groups.

4.5. Discussion

The clinical signs and histological features of recurrent aphthous stomatitis and Behçet's syndrome have suggested a possible viral aetiology, although virus has never been successfully isolated (Hooks, 1978). The application of the PCR technique has revolutionized our approach for investigating possible viral involvement in recurrent aphthous stomatitis and other related conditions. In one study, PCR detected VZV DNA in all 20 recurrent aphthous stomatitis biopsies examined (Pedersen et al. 1993) but an unusual finding of that study was that VZV DNA was only amplifiable using one set of primers tested, which targeted the protein kinase 66 (PK66). Two other primer sets, one targeting another region of the VZV PK66 gene and the other targeting the VZV thymidine kinase gene, gave negative PCR results. Whilst the authors offered no explanation for this discrepancy, their results are clearly at odds with the findings of the present study, which was unable to identify VZV DNA in any of the samples examined, despite using two rounds of PCR with nested primers for increased sensitivity and specificity.

In another study it was reported that PCR detected HCMV DNA in three of nine (33 per cent) recurrent aphthous stomatitis biopsies (Sun et al. 1996) but not in any control samples. Using *in situ* hybridisation, HCMV DNA was found in three of 29 (10 per cent) oral mucosal ulcers (Leimol-Virtanen et al. 1995) but not in any recurrent aphthous stomatitis biopsies (Sun et al. 1996). In this study, it was not possible to demonstrate the presence of HCMV DNA in any recurrent aphthous stomatitis samples but the study demonstrated its presence in two oral lichen planus

samples. The combined evidence from these studies suggests that HCMV can be found in a small proportion of oral ulcers, and therefore cannot be entirely dismissed as an aetiological agent in some cases of recurrent aphthous stomatitis.

In the present study, HHV-6 DNA was detected in six of 21 (29 per cent) recurrent aphthous stomatitis biopsies. This is the first study to investigate the possible involvement of HHV-6 in recurrent aphthous stomatitis biopsy tissue by PCR. There is currently some controversy as to the true prevalence of HHV-6 in saliva of healthy donors. Using both isolation and PCR detection methods, some studies have reported a prevalence rate of approximately 90 per cent (Jarrett et al. 1990; Levy et al. 1990; Cone et al. 1993) whereas others have reported a much lower frequency of detection of between three and 7 per cent (Kido et al. 1990; Saito et al. 1991; Di Luca et al. 1995). Although it is difficult to fully explain the different detection rates of HHV-6 in the saliva of healthy persons in these studies, it is possible that the culture and PCR methods employed also detected HHV-7 or simply that the amount of saliva analysed in the various studies was variable, and if the viral load is low then small amounts of saliva would give negative results. In one particular study using PCR, HHV-6 was found in 63 per cent of salivary gland biopsies and 3 per cent of saliva samples from healthy persons, and in 17 per cent of saliva samples from patients with a history of recurrent aphthous stomatitis. However, HHV-7 was found at markedly higher frequencies, being detected in 75 per cent of salivary gland biopsies and 55 per cent of saliva samples from healthy persons, and in 66 per cent of saliva samples from recurrent aphthous stomatitis patients (Saito et al. 1991). PCR did not detect significant differences in the presence of HHV-6 and HHV-7 and their viral loads

between saliva from recurrent aphthous stomatitis patients and from healthy persons and the authors concluded that HHV-6 and HHV-7 probably do not play a role in the pathogenesis of recurrent aphthous stomatitis. Although we did not look at the saliva of recurrent aphthous stomatitis patients in our study for the presence of HHV-6, the low incidence found in some of the studies described above, taken together with the observation that all of the normal control tissues in our study, and indeed the vast majority of the recurrent aphthous stomatitis and lichen planus tissues analysed, were PCR-negative for HHV-6, suggests that salivary contamination of biopsy specimens is unlikely to be a problem.

In summary, the results of this study suggest that HCMV and VZV are not aetiological agents of recurrent aphthous stomatitis. Although there is not any identified clinical manifestation for HHV-6 in the oral cavity, recurrent aphthous stomatitis may be one of the features of HHV-6 reactivation, in some cases.

In the present study, four patients with recurrent aphthous stomatitis also demonstrated *Mycobacterium paratuberculosis* in their oral ulceration. Although there was no significant difference between recurrent aphthous stomatitis patients and controls, the presence of this micro-organism only in recurrent aphthous stomatitis samples, could not exclude mycobacteria from the aetiology of recurrent aphthous stomatitis. However the recurrent nature of the disease cannot be explained with the hypothesis of cross-reactivity of heat shock proteins of oral mucosa and mycobacterium.

Chapter Five. Antibodies to Human Herpes Virus-6, Cytomegalovirus and Varicella Zoster Virus

5.1. Introduction

Humoral immunity is a main system for protecting the host against infection by micro-organisms. Immunoglobulins as the important part of this system, are classified in to five major groups according to their size, charge, carbohydrate content and amino acid composition (IgG, M, A, E and D) (Ollier and Symmons, 1992). The major serum antibodies are of IgG class. IgM protects the host in the early stages of infection, while IgG replaces the IgM in later stages of infection and it can be detected very late after the infection has disappeared. IgM antibodies are the first to be detected after infection and the presence of anti-viral IgM is a sign of recent infection (Scully and Samaranayake, 1992). Secretory IgA is the principal and most important immunoglobulin in secretions and on mucosal surfaces including the oral mucosa (Scully and Samaranayake, 1992).

Specific antibodies play an important role in modulating viral infections. They may interact with the virion itself, and either neutralize the virus or destroy the virus-infected host cell by a number of mechanisms. Viruses may well however, be intracellular and thus inaccessible to antibody (Scully and Samaranayake, 1992).

In recurrent aphthous stomatitis, there have been no raised antibodies against herpes simplex virus using neutralization tests reported (Stark et al. 1954). Recently, raised specific human cytomegalovirus (HCMV) IgM was identified in recurrent aphthous stomatitis patients, especially in the remission period of the disease (Pedersen and Hornsleth, 1993; Sun et al. 1996). These results are in agreement with recent findings which show antibody production in cells infected with HCMV are too late to prevent

initiation of persistent infection (Mims and White, 1984). However, specific HCMV IgG + IgM was not different in recurrent aphthous stomatitis and control subjects (Pedersen and Hornsleth, 1993).

At least a four-fold increase in specific VZV IgM antibody was reported (Pedersen, 1989b), although, the antibody titre against VZV was less raised in a subsequent study (Pedersen and Hornsleth, 1993).

Specific HHV-6 IgG antibody was detected in most of the patients with recurrent aphthous stomatitis and controls (Pedersen and Hornsleth, 1993), but there has been no investigation in to specific HHV-6 IgM antibody in recurrent aphthous stomatitis to distinguish the stage of infection in this disease.

Recent investigation has shown the involvement of HCMV or VZV in the aetiology of recurrent aphthous stomatitis (Pedersen, 1989b; Pedersen and Hornsleth, 1993; Sun et al. 1996), however the previous section of this thesis could not support this hypothesis.

Viral involvement in the aetiology of recurrent aphthous stomatitis is one of the most plausible hypotheses, and clinical features and laboratory findings increase the possibility for herpesvirus involvement (see section 4.1). The aim of this part of the present study, subsequent to the molecular biological investigation, was to evaluate the involvement of human herpes virus-6, cytomegalovirus and varicella zoster virus in recurrent aphthous stomatitis by detection of specific immunoglobulins against these viruses.

5.2. Materials and Methods

5.2.1. Materials

Enzygnost, anti-HCMV IgG, anti-VZV IgG, anti-HCMV IgM, anti-VZV IgM ELISA kits and supplementary reagents for Enzygnost/TMB were obtained from Behring, Hoechst UK Ltd., 50 Salisbury Road, Hounslow, Middlesex, UK.

HHV-6 IgG ELISA (Abi), HHV-6 IgM IFA (Abi) were obtained from AutoGen Bioclear UK Ltd, Butts Farm, Potterne, Devizes, Wiltshire, UK.

5.2.2. Ethical Approval

The study was approved by the local ethics committee of Glasgow Dental Hospital and School. Consent was obtained for the collection of samples from all patients.

5.2.3. Subjects

a) Venous blood was obtained from 22 patients with recurrent aphthous stomatitis, who attended the Oral Medicine Clinic (within 48 hours of initiation of the ulcer episode). Twenty-one patients were those whose biopsy samples were used in PCR experiment explained in the previous chapter.

b) Venous blood samples were obtained as disease controls from 24 oral lichen planus patients, who had undergone biopsy and haematological investigation as part of their treatment. Twenty of these patients were those whose biopsies were tested in the PCR experiment described in previous chapter.

c) Fifteen patients were individuals without a history of recurrent aphthous stomatitis, who attended the oral surgery unit for removal of their wisdom teeth. This group

were also included as an additional control group. Also, 13 of these individuals were those whose biopsy specimens were used in PCR tests which previously have been described.

5.2.4. Collection of Blood

Ten mls of venous blood were collected from subjects using a plain 10 ml vacutainer tube. The blood was allowed to clot and subsequently centrifuged for 10 minutes at 1000 RPM using a MSE, super minor centrifuge. The serum was then separated and aliquoted to sterile microtubes. The sera were stored at -70°C.

5.2.5. Detection of IgG Antibody to HCMV

The ELISA kit, Enzygnost anti-HCMV IgG (Behring) was employed according to the manufacturer's instructions. The serum samples and reference were pre-diluted 1 to 20 by adding 0.02 ml of the serum to 0.4 ml of pre-coloured sample buffer POD (Tris buffer 0.3 mol/l which is pre-coloured 1 to 20 by blue colour provided by the supplementary kit) and mixed well. In to each reaction well, 0.20 ml of sample buffer POD (Tris buffer 0.3 mol/l) was introduced, then 0.020 ml of the pre-diluted reference P/N (human IgG specific for HCMV in Tris buffer solution of 20 mmol/l)) was pipetted in to first pair of reaction wells coated with antigen (derived from HCMV-infected cells) and control antigen (derived from non-infected cells) respectively. The same amount of pre-diluted test samples were pipetted into the next pairs of reaction wells respectively and finally into the last pair of the wells, in each set of the experiment, 0.020 ml reference P/N was introduced. Then plate was sealed and

incubated in a moist chamber for 60 minutes at 37°C. After incubation, the plate was washed in a plate washer for four times using the washing solution provided in the supplementary reagent kit (Behring). The amount of 0.10 ml of the diluted enzyme conjugate (Fab's component of rabbit antibody which has been conjugated with peroxidase) was added to each well and the plate was sealed and immediately incubated in a moist chamber for 60 minutes at 37°C. After this second incubation the plate was washed as before. After that, 0.10 ml of diluted substrate was added to each well and the plate incubated for 30 minutes at room temperature (18-25°C) protected from light. After this period, the enzyme reaction was stopped by adding 0.10 ml of stopping solution peroxidase to each well. The yellowish-green colour reaction was evaluated by using a microplate reader (Dynatech MR5000) at a wavelength of 450 nm.

5.2.6. Detection of IgG Antibody to VZV

The ELISA kit employed was Enzygnost anti-VZV IgG (Behring), used according to the manufacturer's instructions. Method for this test was exactly the same as to the one for anti-HCMV IgG which was explained in the previous section with the exception of the specific VZV reference P/N and the specific VZV antigen and control antigen coated plate were used in this experiment.

5.2.7. Detection of IgG Antibody to HHV-6

The ELISA kit, HHV-6 IgG antibody (Abi) was employed and run according to manufacturer's instructions. Positive and negative controls as well as samples were

diluted in a separate dilution plate by adding 0.010 ml of each of the controls in each of the wells (three wells for negative and two wells for positive controls) and also 0.010 ml of the test samples to the rest of the wells (two wells for each test sample). Then 0.20 ml of sample diluent was added to each well in addition to a blank well. By using a multichannel pipette, 0.10 ml of each of the diluted controls, samples and blank were transferred to the antigen-coated plate, after they were mixed properly. The coated-plate was sealed and incubated at 37°C for 30 minutes. In the next step, plate was washed three times by an automatic plate washer. Then 0.10 ml of pre-diluted horseradish peroxidase (HRP) conjugate was added in each well and incubated at 37°C for 30 minutes. Washing was repeated in the same manner as before and 0.10 ml of HRP substrate was introduced to each well. The plate was incubated at room temperature (20-25°C) for 30 minutes in the dark. The reaction was stopped by addition of 0.10 ml of stopping solution in to each well and the yellowish colour of reaction was measured within thirty minutes at wavelength of 450 nm. using a microplate reader (Dynatech 5000). The microplate reader was blanked on the reagent blank well. (Figure 5.1)

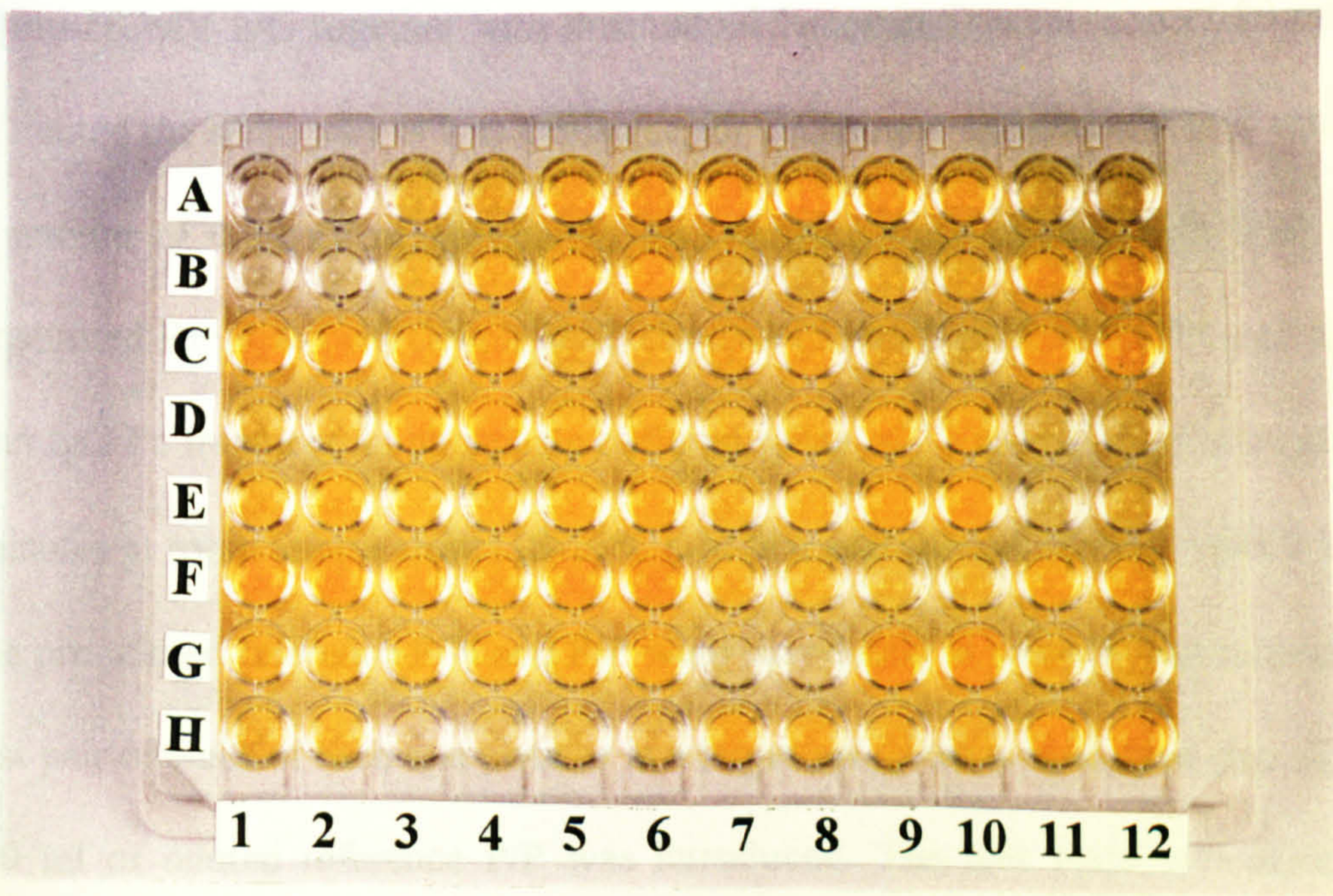


Figure 5.1. HHV-6 IgG antibody ELISA plate , coated with HHV-6 antigen, showing the yellowish colour change after addition of stopping solution. A1 is a blank well, A2, B1 and B2 are negative controls, C1, C2, H11 and H12 are positive controls. In each pair of wells (for instance D1 and D2), one sample test was incubated. All yellowish coloured wells show positive IgG antibodies against HHV-6 in sera of subjects. Un changed coloured wells (such as G7 and G8) show negative IgG antibody against HHV-6 in sera of subjects.

5.2.8. Detection of IgM Antibody to HCMV

The ELISA kit, Enzygnost anti-HCMV IgM (Behring) was employed according to the manufacturer's instructions. The serum samples and reference P/N (human monoclonal antibodies to HCMV in Tris buffer solution of 20 mmol/l) and reference P/P (anti-HCMV IgG together with rheumatoid factor in Tris solution of 20 mmol/l) were diluted the same way as was explained for Enzygnost anti-HCMV IgG antibody.

The amount of 0.20 ml of pre-diluted test samples were treated with 0.20 ml of reconstituted RF absorbent (lyophilized, consists of sheep antibodies directed against human IgG Fc fragment, in phosphate buffered saline of 10 mmol/l) and incubated for 15 minutes at room temperature (18-25°C). In first and second pairs of wells 0.150 ml of the pre-diluted reference P/N and P/P were introduced in to each well respectively. In last pair of wells of each test plate or in a larger set of tests, in the last pair of wells, 0.150 ml of diluted reference P/P was introduced. The test plate was sealed and incubated at 37°C in a moist chamber. After 60 minutes incubation, the plate was washed in a plate washer, four times using washing solution provided with the supplementary reagent kit (Behring). In next stage, test plate was incubated with 0.10 ml of diluted anti-human IgM/POD conjugate (Goat antibody conjugated with peroxidase in Tris buffer solution of 0.05 mol/l) in each well, for 60 minutes at 37°C in a moist chamber. The next step immediately started with washing the plate as before, then 0.10 ml of diluted substrate was introduced in to each well. The plate was sealed and incubated for another 30 minutes at room temperature protected from light. Finally the reaction was stopped by adding 0.10 ml of stopping solution POD

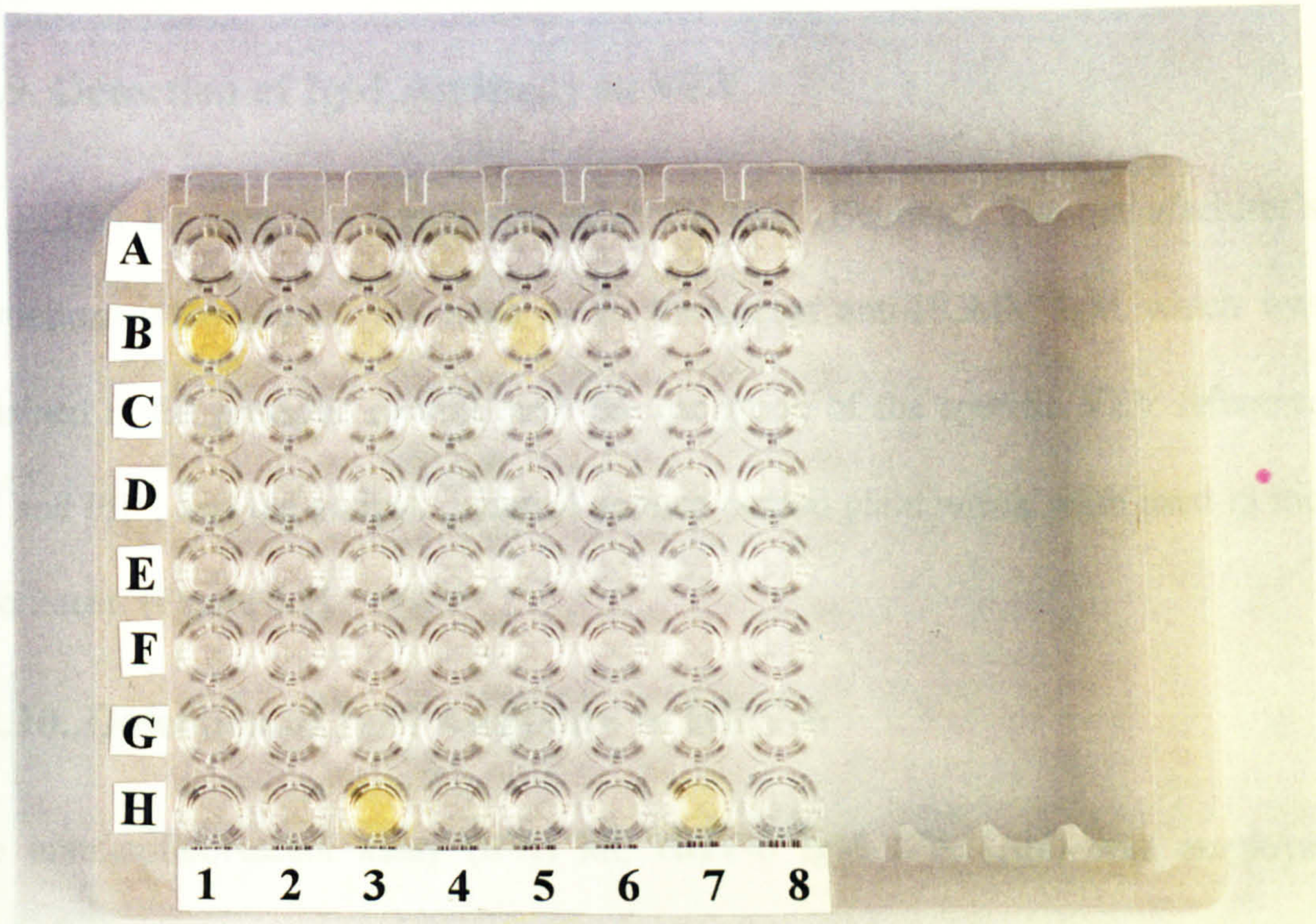


Figure 5.2. ELISA antigen-control antigen coated plate showing the Enzymatic colour change for specific IgM antibodies positivity in sera of subjects against HCMV (first two pairs of columns) and VZV (second two pairs of columns) after addition of the stopping solution. The odd-number columns contain test antigen and even-number contain control antigen. A1/2 were incubated with reference P/N, B1/2 and H3/4 were incubated with reference P/P of the specific HCMV IgM ELISA kit. B3/4 show the presence of IgM antibody in sera of a subject. A5/6 were incubated with reference P/N, B5/6 and H7/8 were incubated with reference P/P of the specific VZV IgM ELISA kit.

to each well. Photometric evaluation was carried out by using a microplate reader (Dynatech MR 5000) at wavelength 450 nm. (Figure 5.2)

5.2.9. Detection of IgM Antibody to VZV

The ELISA kit used was Enzygnost anti-VZV IgM (Behring). The manufacturer's instructions were exactly the same as to Enzygnost anti-HCMV IgM which was explained in the previous section, with the exception of the specific VZV reference P/N and P/P , and the antigen , control antigen coated plate which were used in this experiment. (Figure 5.2)

5.2.10. Detection of IgM Antibody to HHV-6

The immunofluorescent assay (IFA) kit, HHV-6 IgM IFA (Abi) was employed according to the manufacturer's instructions. Positive and negative controls as well as serum samples were diluted by IgG sorbant by adding 0.004 ml samples to two drops (approximately 0.070-0.080 ml) of IgG sorbant. After one to two minutes incubation at room temperature and centrifugation for 30 seconds in a table-top MSE-MLES centrifuge (≥1500 RPM), 0.015-0.020 ml (~1/2 drop) of the IgG depleted positive control and negative control were placed on wells number one and two respectively. On well number three, 0.020 ml of IgG sorbant was placed. IgG depleted and diluted patients sera were placed on the remaining wells. Slides were incubated in a moist chamber for three hours at 37°C. In the next step, slides were rinsed in a gentle stream of washing buffer (provided with the kit), then washing was continued for five to 10 minutes in a washing buffer using a staining dish while slowly stirring on a magnetic stir plate. Slides were dried with the provided blotter and approximately

0.020 ml (~1/2 drop) of FITC anti-human IgM conjugate was deposited on each well. After that, slides were incubated for 30 minutes in a moist chamber at 37°C. In the next stage slides were washed and dried as before and approximately 0.010 ml of mounting solution was placed on each well. The slides were then covered with a cover slip and their positivity was examined under a fluorescence microscope using 20× magnification. The slides were examined blind by two observers. Results were compared and in the case of disagreement, sera was retested.

5.2.11. Test Quality Control

Enzygnost anti-HCMV and anti-VZV IgG

According to manufacturer's instruction each pair of reference P/N wells must reach or exceed a value of 0.5 ΔA (ΔA is the difference in absorbance between antigen and control antigen wells). Anti-HCMV was considered negative when ΔA of the sample is less than 0.1 and positive when ΔA exceeds 0.2. The test samples with a ΔA value between 0.1 and 0.2 were retested and if the result still did not show the appropriete quality, it was classified as an equivocal result.

HHV-6 IgG antibody

To obtain the optical density of the samples, a simple calculation was done. To calculate the cut-off value, the average reading values of three negative was obtained and then the result was multiply by two. An optical density (OD) ratio for each sample was calculated by dividing the mean of the reading values of each sample by the cut-off value. If the OD ratio was equal to or less than 0.75, the result was considered as negative and if the OD ratio was equal or more than one, the result was considered as

positive. An OD ratio of between 0.76-0.99 was counted as an equivocal (borderline) result. Both of the positive control wells should have a reading of ≤1.0. All three negative control wells should have a reading of ≤0.125. If any of the negative control results deviate from the average of the three by greater than 50 per cent, the assay is invalid and should be repeated.

Enzygnost anti-HCMV and VZV IgM

Each pair of reference P/P must reach or exceed a minimum absorbance value of 0.2 ΔA. Also ΔA for the reference P/N must be within the negative result (ΔA<0.1). A positive result is when the ΔA value is more than 0.2. The samples with a ΔA value of between 0.1 and 0.2, must be retested and if still ΔA still does not reach the positive or negative values, the test result is classed as equivocal.

HHV-6 IgM IFA

The control IgM positive should show diffuse to restricted green staining of the infected cell. The uninfected cells should exhibit a red colour due to counter stain. The negative control serum should only show red coloured cells and the IgG sorbant control should only show red colour staining of cells demonstrating that FITC conjugate was not detected (Figure 5.3). The HHV-6 antibody positive test serum for IgM should show punctuate diffuse staining of the infected cells as observed in the control IgM positive serum (Figure 5.4).

During all experiments, test samples were coded and the diagnosis of the samples was not clear, until the end of the test.

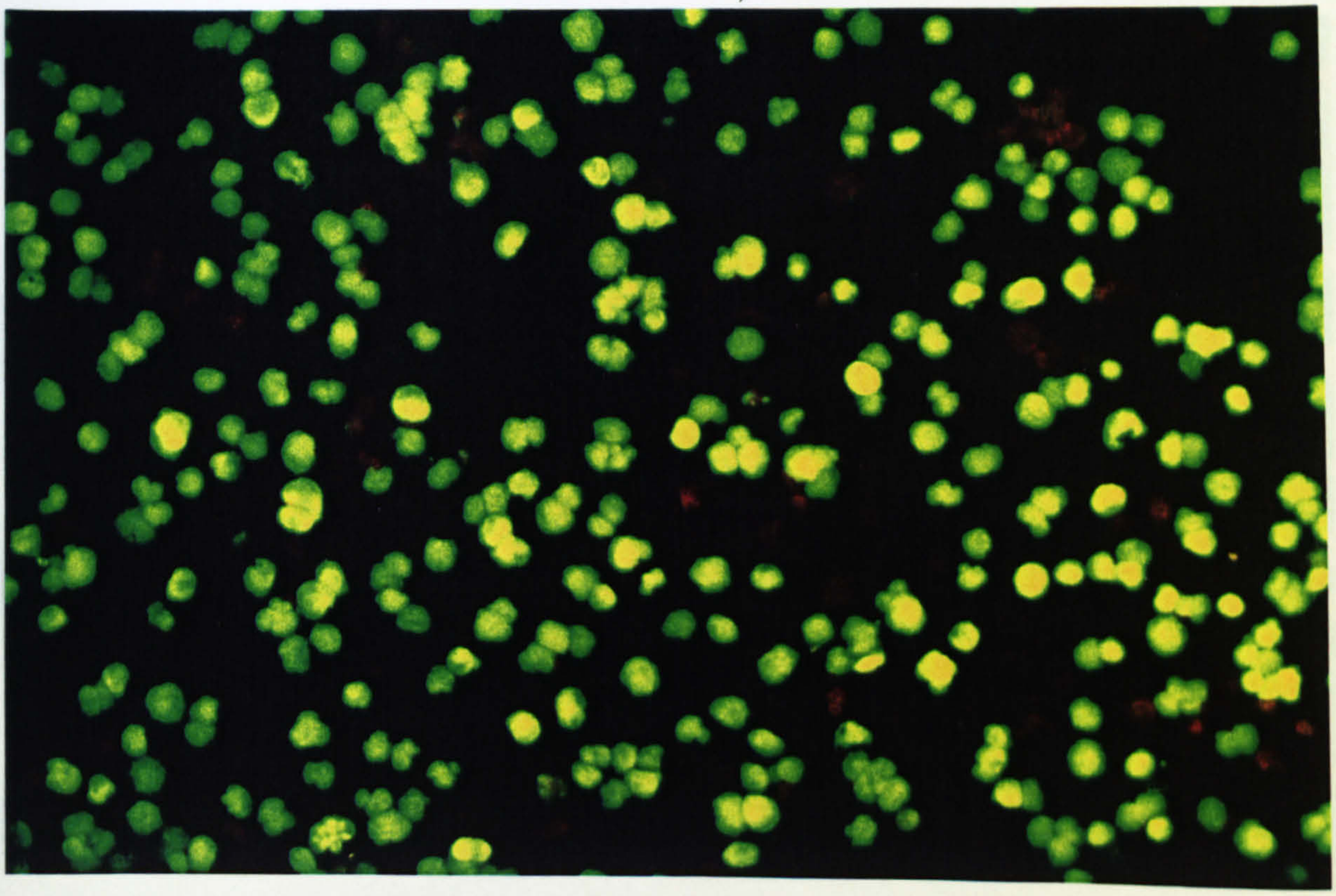


Figure 5.3. Positive staining of cells affected by addition of HHV-6 IgM antibody positive sera of a RAS patients. Bright green coloured cells show a positive reaction with IgM antibody against HHV-6. Positive control should also produce the same colour as the above picture.

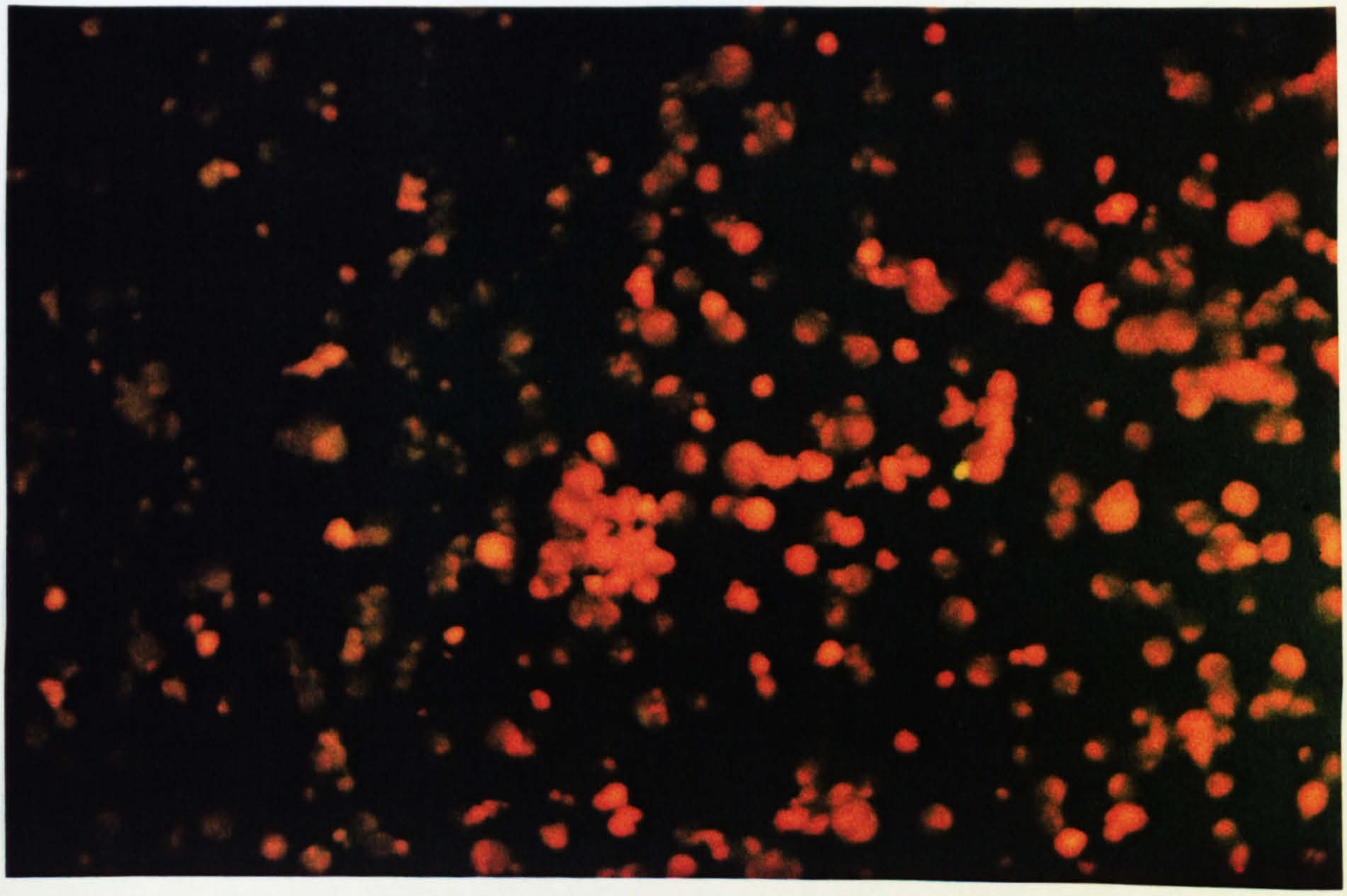


Figure 5.4. Negative staining of cells unaffected by addition of HHV-6 IgM antibody negative sera. Also, addition of IgG sorbant and negative control stain the same colour as negative results.

5.2.12. Statistics

The statistical significance of difference in data obtained from each test was assessed by non-parametric, Fisher's exact test or Chi-square test. A level of $p \leq 0.05$ was chosen as indicative of statistical significance.

5.3. Results

5.3.1. IgG Class Antibodies Against VZV, HCMV and HHV-6

For HCMV, the prevalence of IgG class antibody was less than the other two viruses. Eight sera (36 per cent) from recurrent aphthous stomatitis patients were positive. Also in 11 (46 per cent) oral lichen planus patients and seven (47 per cent) healthy individuals IgG antibody against HCMV was detected (Table 5.1). By applying a chi-square test the difference in specific HCMV IgG, between recurrent aphthous stomatitis patients and the two other groups, was not significant ($df=1$, $p>0.05$) (Figure 5.5).

All patients with oral lichen planus and all in the control group were sero-positive to specific VZV IgG class antibody. From 22 patients with recurrent aphthous stomatitis only in two of them was specific VZV IgG class antibody not detected and the rest of the patients (91 per cent) were sero-positive against VZV IgG class antibody. (Table 5.1) There was no statistically significant difference in specific IgG class antibody against VZV, between recurrent aphthous stomatitis patients and oral lichen planus ($p=0.45$). Also no significant difference was observed between recurrent aphthous stomatitis patients and controls ($p=0.69$). (Figure 5.5)

Specific IgG class antibody to HHV-6 was detected in all patients suffering from recurrent aphthous stomatitis and all healthy individuals in the control group. Also, in the oral lichen planus group 22 patients (92 per cent) were sero-positive for specific IgG class antibody against HHV-6 (Table 5.1). The differences in specific HHV-6 IgG class antibody between recurrent aphthous stomatitis patients and oral lichen planus ($p=0.53$) and also with healthy individuals ($p>1.00$) were not significant. (Figure 5.5)

5.3.2. IgM Class Antibodies Against VZV, HCMV and HHV-6

Specific HCMV IgM class antibody was detected in one (five per cent) recurrent aphthous stomatitis patient, two (eight per cent) oral lichen planus patients and two (13 per cent) controls. (Table 5.1) Specific HCMV IgM class antibody also was not statistically different between recurrent aphthous stomatitis patients and oral lichen planus patients ($p>1.00$) and healthy individuals ($p=0.66$). (Figure 5.5)

Specific VZV IgM class antibody was negative in all patients and controls. (Table 5.1) Specific HHV-6 IgM was strongly positive in 21 (95 per cent) recurrent aphthous stomatitis patients, 17 (71 per cent) lichen planus patients and eight (53 per cent) controls. (Table 5.1) Although the difference in specific HHV-6 IgM class antibody between recurrent aphthous stomatitis patients and oral lichen planus was not significant ($p=0.29$), there was a statistically significant difference between the prevalence of specific HHV-6 IgM antibody in recurrent aphthous stomatitis patients and the control group ($p=0.0076$). (Figure 5.5)

Table 5-1. Specific antibodies to HCMV, VZV and HHV-6.

	HCMV		VZV		HHV-6		Total
	IgG	IgM	IgG	IgM	IgG	IgM	patients
RAS	8*	1	20	0	22	21	22
OLP	12	2	24	0	22	17	24
Normal	8	2	15	0	15	8	15
Difference	NS**	NS	NS	NS	NS	S	

* Number of patients with positive specific antibodies to HCMV, VZV and HHV-6.

** Fisher's exact test, $p<0.05$

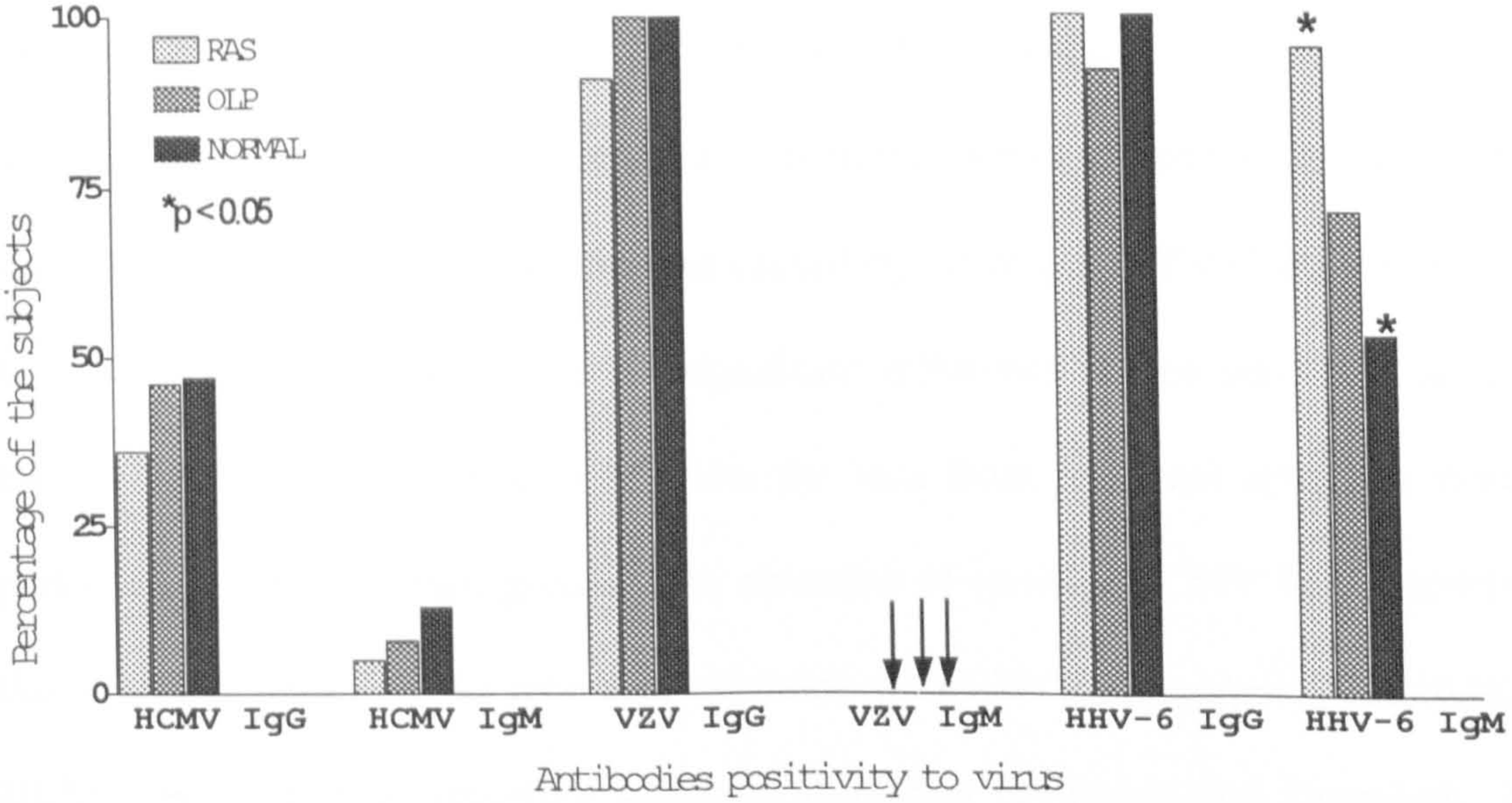


Figure 5.5. Specific antibodies against HCMV, VZV and HHV-6 in sera of RAS patients compared to oral lichen planus (OLP) and normal subjects. The antibodies against HCMV and VZV was not significantly different between RAS patients and the two other groups.

* shows that there was a significant difference between IgM antibodies against HHV-6 in sera from RAS patients and normal individuals.

↓ shows sera negative for specific IgM antibodies against VZV.

5.4. Discussion

Routine serological investigation is not of diagnostic value in the diagnosis of herpesvirus infection, however, the detection of specific IgM antibody or an increase of specific IgG antibody in relation to disease activity, is usually indicative of an infection or reactivation of the virus (Pedersen, 1993). The nature and extent of humoral immune responses to recurrent herpetic lesions may be different in different patients. IgM appears in some patients but is not a consistent marker in recurrent genital, mucosal or ocular herpes simplex virus infection or other diseases caused by HCMV or VZV (Lopez et al. 1993). In the present study, there was no significant difference in the detection of specific HCMV IgG or IgM antibodies between the sera from recurrent aphthous stomatitis patients and the other two groups. The elevation of specific HCMV IgM, especially in the remission period of the recurrent aphthous stomatitis may suggest a reactivation of HCMV as a cause of recurrent aphthous stomatitis (Pedersen and Hornsleth, 1993). However, this elevation was not statistically significant in another group of recurrent aphthous stomatitis patients compared with controls in the same study (Pedersen and Hornsleth, 1993). A study on anti-HCMV IgG antibody showed increased levels of specific HCMV IgG antibody in the sera of recurrent aphthous stomatitis patients compared with control group (Sun et al. 1996).

The ELISA kit employed in this study was a diagnostic kit, routinely used to measure the level or positivity of sera against VZV. Almost all sera were sero-positive to specific IgG antibody which is the reaction of the immune system after primary infection or reactivation of the virus or the effect of vaccination. Specific VZV IgM was not detected in any of sera obtained from recurrent aphthous stomatitis, lichen planus or healthy

individuals indicating that there has been no history of VZV infection in the previous two or three years. (Figure 5.5)

In studies by Pedersen (1989b) and Pedersen and Hornsleth (1993), increased antibody against VZV, especially VZV IgM has been reported in patients with recurrent aphthous stomatitis in contrast to controls. In data presented in this thesis, no significant difference was observed in specific VZV IgG antibody in sera from recurrent aphthous stomatitis patients compared to the other two groups. Two to three days after a varicella infection, anti-VZV of the IgG, IgM and IgA classes are detectable in serum (Zoulek, 1985). Anti-VZV IgM and IgA will disappear after one or two years, while anti-VZV IgG persists for many years (Zoulek, 1985). The present results show that probably, no infection or reactivation of the virus had occurred during at least the last two or three years, while all serum samples from recurrent aphthous stomatitis patients were collected in the active stage of the disease. In addition, in previous studies (Pedersen, 1989b; Pedersen and Hornsleth, 1993), a history of infection or reactivation of virus had not been considered.

In the present study, almost in all patients and controls specific HHV-6 IgG antibody was detectable and no significant difference between the groups was observed. In contrast, the number of sero-positive samples to specific HHV-6 IgM antibody, which appear in first step of infection and reactivation of the virus, was significantly higher in recurrent aphthous stomatitis patients compared with healthy individuals ($p=0.0076$) (Figure 5.5). Specific HHV-6 IgG was detected in 34 per cent of sera from healthy donors, while none of these samples were positive for specific HHV-6 IgM (Cuende et al. 1994). This result indicates a previous infection by HHV-6 but not recent infection or reactivation of virus (Cuende et al. 1994). In addition, 67 per cent of recurrent aphthous stomatitis patients

and 94 per cent of controls have been reported to be IgG antibody sero-positive against HHV-6 (Pedersen and Hornsleth, 1993). There has been no other investigation to show IgM sero-positivity against HHV-6 in recurrent aphthous stomatitis.

In the immunoflourescence assay, non-specific positive reactions can occur in samples from patients with auto-immune disease which show antinuclear antibody reactivity (Manufacturer's instructions). Hence the aetiology of recurrent aphthous stomatitis remains unclear, though viral or/and auto-immunity may be involved. The results obtained from the specific HHV-6 IgM by immunoflourescence assay support the above hypothesis. Since there is more clinical and laboratory evidence to support viral involvement (Section 4.1), HHV-6 is more likely to be considered as a possible cause of the disease. These results were supported by the PCR findings in the previous chapter.

In conclusion, the involvement of VZV and HCMV was not supported in this study while the antibodies against HHV-6 showed that this virus might be a cause of recurrent aphthous stomatitis by inducing autoimmunity or causing immunity imbalance.

Chapter Six. General Discussion

6.1. Introduction

There is an enormous variation in the presenting signs and symptoms among patients with recurrent aphthous stomatitis. Some individuals are quite incapacitated by painful, severe oral ulcers, while others have such mild involvement that they fail to seek treatment for the disorder. A number of general considerations, regarding the problems associated with investigating patients with recurrent aphthous stomatitis must be taken into account. These will be discussed in the first instance.

Despite the broad spectrum of disease severity seen among patients with oral ulceration, there is no doubt that recurrent aphthous stomatitis exists as a distinct clinical entity. There are no laboratory methods to identify the disease and diagnosis is based on the clinical features and exclusion of other diseases with similar oral manifestations. It is therefore difficult to determine whether the syndrome is one condition which is clinically modified by a variety of influences or a group of clinically similar conditions with distinct aetiologies. Certainly the clinical syndrome of recurrent aphthous stomatitis maybe divided into the major and minor forms and herpetiform ulceration. Attempts to further classify subgroups of patients with nutritional deficiencies as having clinically distinguishable "pseudo-aphthae" (Challacombe et al. 1977a) have not gained support. The relationship of recurrent aphthous stomatitis to Behçet's syndrome is unclear, although an aetiological link has again been hypothesised.

The recent investigations, on cross-reactivity between human and microbial heat shock proteins show that recurrent aphthous stomatitis and Behçet's syndrome have different patterns in cross-reactivity to heat shock proteins. For instance, in Behçet's syndrome,

this cross-reactivity is more significant between four peptides of 65 KD human heat shock proteins, whereas in recurrent aphthous stomatitis only peptide 91-105 derived from 65 KD mycobacterial heat shock protein which has not any significant cross-reactivity in Behçet's syndrome, caused a significant lymphoproliferative response as the result of homology with 60 KD mitochondrial 116-130 peptide. (Hasan et al. 1995)

The ulceration seen in recurrent aphthous stomatitis is recognised as a distinct clinical entity on the basis of the shared end-organ effect of the disease, which is recurrent, idiopathic and mucosal ulceration. There is a diversity of host or environmental factors which may initiate the mucosal destruction and cause the disease. The point at which these should be regarded as distinct or one disease process, however is not sufficiently understood. In this thesis, therefore, minor ulcers have been studied as in *in vitro* experiments. The association of recurrent aphthous stomatitis with Behçet's syndrome is unclear and patients with the latter condition of Behçet's syndrome were excluded from experiments.

The observation that some women have recurrent aphthae which are related to menstruation, but that the disease can progress independently of such host factors in the male population, lends credence to the idea that hormonal and other host factors such as haematological deficiencies or psychological factors may not be of central importance to the pathogenesis. However environmental factors may have a uniform effect if host susceptibility could be controlled. As cited in the introduction to this thesis, replacement of haematological deficiencies is therapeutic although a universal effect can not be demonstrated.

Furthermore, recurrent aphthous stomatitis is almost unique in that the disease is characterised by rapid spontaneous regression and healing of oral lesions indicating a transient defect of host resistance to mucosal ulceration. In addition, this makes assessment of response to any treatment difficult.

The stage of the disease at which the patient is studied is also important. A reproducible pattern of recurrence is not evident among the patients. Indeed, the majority of individuals can experience developing and healing lesions simultaneously.

Although, the cause of recurrent aphthous stomatitis is not demonstrable yet, generalised abnormalities have been shown. Circulating levels of immunoglobulins or immune complexes may be altered, but this may be an epiphenomenon giving little information on the aetiology of the ulceration. Other factors, however, such as cell surface antigens are not tissue-specific and may reveal a genetic susceptibility to the disease regardless of the organ of origin. Assessment of the haematological status of an individual is carried out by examination of the peripheral blood, the assumption that circulating levels of these elements reflect tissue concentrations being well accepted.

As reviewed in chapter one, there is a wealth of evidence to suggest that immunological alterations are present in patients with recurrent aphthous stomatitis. Therefore, it seems likely that activation or alteration of the effector limb of the immune system is important in the end stages of the pathogenesis of recurrent aphthous stomatitis.

In addition to the problems of selecting patients for study, there is also the difficulty that the patient population is probably pre-selected on the basis of their attitudes to medical

care. This can affect the results obtained from prevalence of different sex and ages in patient population.

This thesis has been an attempt to explore the relevance of several host and environmental factors in the pathogenesis of recurrent aphthous stomatitis with particular emphasis on the viral aetiology. Also, an attempt has been made to find a clinically safe and effective medication to relieve the symptoms of the disease. A number of both clinical and laboratory studies were conducted on over 465 patients with the history of recurrent aphthous stomatitis. This chapter was aimed at further discussion of those findings which were presented and discussed in chapters two to five.

6.2. Clinical and Epidemiological Findings

Epidemiological data amassed from patients reveal similarities in the clinical presentation between the two groups of 252 and 213 patients with recurrent aphthous stomatitis. The prevalence of the disease in the present study (Chapter 2) was increased in the third and fourth decades of life (Figure 2.3). However in the review of the literature (chapter one) this prevalence has been reported to increase in the second and third decade of life. Such dissimilarities may be due to differences in patient populations, or due to diverse host or environmental factors. Despite many reports indicating an increased susceptibility of females to recurrent aphthous stomatitis compared to males (Sircus et al. 1957; Farmer, 1958; Brody and Silverman, 1969; Donatsky, 1973), such a difference was not observed in the present study. The number of female patients in general who attended the Oral Medicine Clinic for any treatment is higher than males. As a result the sex difference between male and female patients with recurrent aphthous stomatitis might be a reflection of this general trend rather than a female preponderance in aphthae sufferers.

The prevalence of the subgroup of minor recurrent aphthous stomatitis was higher than other reports which reviewed in chapter one.

Almost all recurrent aphthous stomatitis patients were non-smokers and not smokeless tobacco users. This result shows that nicotine and cigarettes may have some preventative or therapeutic effect in recurrent aphthous stomatitis. A preliminary trial on recurrent aphthous stomatitis patients with nicorette chewing tablets has supported this hypothesis (Bittoun, 1991). Nicotine might have an anti-oestrogen effect (Baron, 1996). Also cigarette smoking has been reported to affect several elements of immune function, including those of T-cell function and antibody responses (Baron, 1996). Despite all the investigations on the effect of smoking on the immune system, little has been discovered about the preventative or therapeutic effects of smoking in diseases like recurrent aphthous stomatitis.

Haematological screening was carried out on the first group of 252 patients and the second group of 213 patients. In the first group 33.33 per cent and in the second group 31.91 per cent of the patients had some haematological deficiencies (Table 2.6). This prevalence of haematological deficiencies has increased in the Glasgow population since 1975 (Wray et al. 1975; Miller et al. 1994). This increase may be due to the change of diet or the higher number of females in the study populations. Also, different criteria applied in the study may have caused these changes. It should also be considered that folic acid deficiency has almost disappeared in recurrent aphthous stomatitis patients in recent investigations (Miller et al. 1994). Indeed, many of the deficiencies were themselves latent, that is, they had produced no recognisable abnormalities in the peripheral blood screening alone. Therefore, a full blood count would be insufficient to

detect an underlying abnormality in these patients. In the first group of 252 patients, 69 had a low level of ferritin (Table 2.6). Overt deficiency with a low level of haemoglobin, MCV or both of them was observed in 26 patients. The remaining 43 patients had latent iron deficiency. Deficiency of vitamin B_{12} has not been generally recognised as a cause of recurrent aphthae, though isolated cases have been reported (Hjorting-Hansen and Bertram, 1968; Walker, 1973; Porter et al. 1988; Palopoli and Waxman, 1990). In the first group of 252 patients, vitamin B_{12} deficiency was diagnosed in 15 subjects. Three of these patients had at least one of the anti-parietal cell or anti-intrinsic factor antibodies present in their serum (Table 2.7). Patients with vitamin B_{12} deficiency with no obvious clinical explanation were referred to a haematologist for more investigations and further management. In these 15 patients with vitamin B_{12} deficiency, 13 had latent vitamin B_{12} deficiency which by full blood count alone would not be identified. Folic acid deficiency as a cause of recurrent aphthous stomatitis is also not generally accepted, though several cases of folic acid deficiency with oral ulceration have been reported (Hutcheon et al. 1978; Tyldesley, 1981). No patient with folic acid deficiency was diagnosed in this group. These results supported the notion that with the change of diet to consuming more green vegetable, folic acid deficiency would almost disappear in the population (Miller et al. 1994). To investigate the effect of these changes on prevalence of coeliac disease in patients with aphthae, venous blood of 66 patients in the first group were tested to detect IgA anti-gliadin antibodies in recurrent aphthous stomatitis patients. In seven patients, IgA anti-gliadin antibody was present, but anti-endomysial antibody, which is a diagnostic test for coeliac disease, was negative in all cases. The presence of anti-gliadin antibody in the sera from some of the recurrent aphthous stomatitis patients

with a normal diet could be explained as latent coeliac disease (O`mahony et al. 1990). Also significant raised levels of IgG antibody to gliadin in the sera from patients with ulcerative colitis and Crohn's disease has been reported (Williams et al. 1996).

In the second group with 213 patients with a history of recurrent aphthous stomatitis who attended the Oral Medicine Clinic in the period of April 1993 to April 1994, haematological deficiencies had almost the same pattern as first group (Table 2.5). In addition, 90 patients with normal oral mucosa were also investigated as a control group. Folic acid, vitamin B_{12} and ferritin deficiencies were diagnosed in 68 (32 per cent) of recurrent aphthous stomatitis patients and 40 (44 per cent) of controls. The increased prevalence of haematological deficiencies in the control group compared to the recurrent aphthous stomatitis group was perhaps due to the high number of females (83 per cent): female patients in the recurrent aphthous stomatitis group represented 68 per cent. However, there was no significant difference between recurrent aphthous stomatitis and control subjects in the prevalence of haematological deficiencies. These data show that haematological deficiencies is not a prevalent factor in causing recurrent aphthae. After the haematological deficiencies were diagnosed, patients underwent replacement therapy. During the follow-up period, 41 per cent of the patients had some improvement in their ulceration.

The precise role of iron, vitamin B_{12} or folic acid deficiencies in the pathogenesis of recurrent aphthous stomatitis remained speculative, although malnutrition can interfere with any of the mechanisms that act as barriers to the progress of micro-organisms throughout the body. If viruses were a cause of recurrent aphthous stomatitis, nutritional deficiencies could depress the cell mediated immune response and thus cause an increase

in the susceptibility to certain viral infections or reactivation (Mims and White, 1984). For instance, conjunctivitis can progress to cause severe eye damage and blindness, especially if there is associated vitamin A deficiency (Mims and White, 1984). The small ulcers of the mouth that constitute Koplik's spots in normally nourished children, can enlarge to form massive ulcers or necrosis of the mouth (cancrum oris) (Mims and White, 1984).

There have been a few experimental investigations of the impact of malnutrition on viral infections. In one experiment on mice which from the age of three weeks had received only one-quarter of the food intake necessary for normal growth, they developed marked atrophy of the lymphoid tissues and were then much more susceptible than normal mice to coxackievirus B3 infection. These observations showed that an inadequate host immune response to infection was caused by malnutrition (Mims and White, 1984). Also, defects of cell mediated immunity have been reported in iron deficient patients (Joynson et al. 1972). The oral ulceration which occurs with folic acid antagonist drugs such as methotrexate is also well recognised and responds to topical folinic acid. Dreizen et al (1970) induced oral ulceration in marmosets by feeding them a diet free of folic acid. However, in the present study, replacement of iron, folic acid or vitamin B_{12} did not completely resolve the ulceration in most of the patients.

In the present study, the prevalence of allergic reactions to food additives were not different between recurrent aphthous stomatitis and control subjects. Thus allergy seems to play a minor role in those recurrent aphthous stomatitis patients with a positive allergic reaction. In a study by Wray et al (1982), 60 recurrent aphthous stomatitis patients were screened clinically for a history of allergies and their leucocytes tested *in*

vitro for histamine release to food and respiratory allergens. In this group of patients, 18 had a history of clinical allergy to environmental antigens and this was confirmed by a histamine release assay. In 18 patients, an ability of foods to induce ulceration was found. However in eight of the patients this was correlated with the histamine release assay (Wray et al. 1982). Also dietary manipulation could not eliminate the ulceration in any of the patients (Wray et al. 1982). As a result, it was concluded that, although foods can cause oral lesions, probably through an IgE-mediated mechanism, there may be other contributing factors and patients with *in vitro* reactivity to foods who did not develop oral lesions, may lack other factors or may have protective mechanisms such as blocking antibodies (Wray et al. 1982).

Despite reports supporting a possible relationship between food sensitivity and recurrent aphthous stomatitis (Wright et al. 1986; Nolan et al. 1991a), in the present study, such relationship seems only a minor factor in causing aphthous lesions.

The oral mucosa is influenced directly by oestrogen in a fashion similar to vaginal epithelium (Ferguson, 1975). Oestrogen and progesterone also affect vascular permeability and the inflammatory response (Lindhe et al. 1968; Lindhe and Branemark, 1968). Sex hormones can influence the immune system and cause a higher frequency of autoimmunity in females (MacPhail et al. 1993). Oestrogen can increase antibody production by inhibiting T-suppresser (CD8+ cells) activity, while progesterone increases T-suppresser function (MacPhail et al. 1993). T-suppresser cells play an important role in the regulation of the immune response to antigens, by turning off the positive effector T cells after they have completed their function and are no longer needed (Mims and White, 1984). In some viral infections, T-suppresser cells increase due to the infection

(such as herpesvirus infection) (Roizman et al. 1993), and the combined effect of lack of oestrogen and a viral infection, may lead to damage of the host cells and induce an autoimmune-like disease. However, if the virus is present in the oral cavity, according to this hypothesis, it might induce oral epithelial damage and cause diseases such as recurrent aphthous stomatitis. Cyclical changes of the sex hormones and latency of some viruses in the oral cavity such as herpesvirus may explain the recurrent nature of the disease. Since, recurrent aphthous stomatitis is not related to the menstrual cycle in all female patients and the sex difference is not significant in recurrent aphthous stomatitis patients, this hypothesis cannot be accepted in all cases.

The results presented in this thesis have a bearing on the management of patients with recurrent aphthous stomatitis. Thus 41 per cent of patients with haematological deficiencies responded to replacement therapy. This therapeutic effect indicates the relevance of these blood disorders regardless of their aetiology. As discussed previously, it was not possible to distinguish those individuals with deficiencies without resort to haematological investigation.

The majority of patients will not adequately benefit from preventive measures such as replacement of iron, vitamin B_{12} or folic acid. These patients must unfortunately rely on local symptomatic therapy. In the observations described in this thesis, patients were maintained on topical corticosteroids (paste, mouthwash or spray) and this regime was supplemented with local anaesthetics and antiseptic mouthwashes (Table 2.9). Only a minority of patients were ultimately given intermittent systemic corticosteroids because of the severity of the disease. As no patients are cured by symptomatic therapy, this group continues to illustrate the symptoms of the disease until spontaneous remission or

improvement occurs. Patients who have undergone an induced or spontaneous remission are presumably still predisposed to oral ulceration which could recur under appropriate environmental or endogenous conditions. This is consistent with the findings previously discussed, that the incidence of recurrent aphthous stomatitis was increased in individuals with positive family history (Miller et al. 1980). In addition there are several reports indicating an association of recurrent aphthous stomatitis with the HLA type A_2, B_{12} and DR_7 (Challacombe et al. 1977b; Lehner et al. 1979a; Gallina et al. 1985). This could not be confirmed by another study (Dolby et al. 1977). The HLA histocompatibility locus can be associated with different diseases such as autoimmune diseases or disease with unknown aetiology. However until now, no certain role of HLA has been identified in the development of autoimmunity and related diseases (Ollier and Symmons, 1992). One of the hypotheses for HLA disease associations is that the cell surface HLA molecule could act as a receptor for a specific virus and provide a way for it to infect the cell. It makes individuals carrying one particular gene susceptible to disease. Since the association of the HLA histocompatibility locus with viral disease has been reported (Russell and Schlaut, 1977), diseases with autoimmune or unknown aetiology origin such as recurrent aphthous stomatitis which are related to HLA antigens may have a viral cause. Thus, although a role for genetically determined cell-surface antigens in the immunopathogenesis of recurrent aphthous stomatitis has been suggested, the mechanisms involved still remain undefined.

6.3. Effect of 5 Amino-Salicylate

A randomised double-blind cross-over study was conducted into the effect of 5 amino-salicylate as part of the investigation of patients seen in the Oral Medicine Clinic.

Previous reports had indicated that topical 5 amino-salicylate caused an improvement in selected patients with recurrent aphthous stomatitis (Collier et al. 1992) and one patient with Behçet's syndrome (Ranzi et al. 1986). However this effect was not shown by another study (Bagg, 1985). In the present study no significant effect was seen by analysing the data obtained from patients' diary cards (Figure 3.3). However a general improvement was noticed during the course of the treatment regardless of the effect of the active or placebo cream. As a result, the data was studied by dividing the patients in two groups. This time, the study was analysed as a double-blind trial. Although, there was no significant different between the two groups with respect to the patients' diary card, the effect of 5 amino-salicylate cream was seen in the patients' self reports (Figure 3.4). This time a higher number of patients preferred the active cream to placebo, because of the positive effect of the 5 amino-salicylate in decreasing the pain compared with placebo. Regardless the results show that 5 amino-salicylate has little effect on relief of the symptoms in recurrent aphthous stomatitis. They may prove however that a double-blind trial with cross-over may not be the appropriate way to investigate the effect of drugs in recurrent aphthous stomatitis. The reason is that the process of the disease is not predictable in different individuals and in the second period of the trial the severity of the condition may be totally different for reasons other than the effects of the medication. This trial also highlights, the problems encountered in trying to objectively assess patient responses. These results indicate that, although patient and investigator assessment of the number and size of ulcers correlates well (Graykowski and Kingman, 1978), it may be necessary to assess patients subjectively for these parameters during the trial. Such a protocol would eliminate patient subjectively and allow easier collection of

data. Pain must remain a subjective symptom and further, the observation that many of the patients noticed subjective pain reduction on any medication emphasises the limits of this parameter in open trials.

6.3. *In Vitro* Investigations

Since it has been accepted that viral or microbial involvement as a first event may cause autoimmune-like disease, the hypothesis of involvement of autoimmunity in several diseases can be no longer accepted (Ollier and Symmons, 1992). As viruses are the strongest candidates for inducing autoimmune diseases (Ollier and Symmons, 1992), the immune changes in recurrent aphthous stomatitis patients reviewed previously, could be a secondary phenomenon to a viral involvement in recurrent aphthous stomatitis.

Autoimmune diseases are more prevalent in females and increase with advancing age (Ollier and Symmons, 1992; Pedersen, 1993), whereas the prevalence of recurrent aphthous stomatitis is increased in the third decade of life and the first appearance of recurrent aphthous stomatitis is mainly in the first and second decade of life (Farmer, 1958; Brody and Silverman, 1969; Donatsky, 1973). Observations in this thesis also show that the disease is more predominant in the fourth decade of life and there is no significant difference between male and female in involvement of the disease. The clinical features of recurrent aphthous stomatitis also, are very similar to those seen in latent viral infections such as herpes simplex virus and for both diseases, recurrent herpes labialis and recurrent aphthous stomatitis, an HLA background has been postulated (Challacombe et al. 1977b; Russell and Schlaut, 1977; Lehner et al. 1979a).

By immunofluorescent and immunohistochemistry methods, as has been reviewed before, the level of serum IgG and IgM was significantly increased in recurrent aphthous stomatitis patients compared with controls (Sun et al. 1986). The level of IgG subclasses has been reported to be normal in most patients. However 8.4 per cent of recurrent aphthous stomatitis patients had marginally raised IgG1 levels and 1.4 per cent a significantly low level of IgG3 (Porter et al. 1992). Most of the antiviral IgG is of the IgG3 subclass, while IgG1 responses are also particularly raised in herpesvirus infections (Scully and Samaranayake, 1992). Marginal raised IgG1 levels in recurrent aphthous stomatitis patients also might be due to a herpesvirus infection.

It has been reported that in recurrent aphthous stomatitis lesions, the percentage of T cells with CD8+ receptors (T suppressor) was increased and as a result the CD4+/CD8+ cell ratio is decreased (Kayavis et al. 1987; Savage et al. 1988; Pedersen et al. 1989). Also, in the peripheral blood of recurrent aphthous stomatitis patients, the number of T-helper/inducer cells (Cdw29) was increased and the number of T-suppressor/inducer cells (CD45R) decreased during both remission and the early stage of ulceration (Savage et al. 1988; Landesberg et al. 1990). The CD4/CD8+ ratio usually increases in autoimmune diseases such as multiple sclerosis, autoimmune hemolytic anaemia and erythema nodosum (Bach and Bach, 1981). This increase seems to be the effect of decrease in CD8+ T-lymphocytes (Raeman et al. 1981). T-lymphocyte counts in patients with an early stage of HIV infection showed a strong relationship between an increased number of CD8+ cells and development of intra-oral lesions (Melnic et al. 1991). Changes in CD4+ cells were not found to be related to the presence of oral lesions (Melnic et al.

1991). Also, in HCMV infection, the ratio of circulating CD4+/CD8+ T-lymphocytes is decreased due to a marked increase in the CD8+ T-cell subset (Roizman et al. 1993).

The comparison between the CD4+/CD8+ cells ratio and changes in T-lymphocyte subclasses explained above, show that changes in T-cell subclasses in recurrent aphthous stomatitis are more similar to those in viral diseases than autoimmune diseases.

The virus-carrier lymphocyte was identified in the sera from patients using an immunofluorescent-antibody test (Nasz et al. 1971). In this study antigens of adenovirus type I and herpesvirus caused blastogenic transformation in a significant proportion of lymphocytes from the blood of aphthous patients (Nasz et al. 1971).

Antibody levels against herpes simplex virus are not raised in sera from recurrent aphthous stomatitis patients, also such patients do not have positive skin tests to herpes virus (Stark et al. 1954; Sircus et al. 1957; Sallay et al. 1973). Herpes simplex virus has not been isolated from any lesion of recurrent aphthous stomatitis (Sallay et al. 1973). In more recent studies, part of the genum of herpes simplex virus was identified in peripheral blood mononuclear cells of recurrent aphthous stomatitis patients by applying PCR and *in situ* hybridisation (Eglin et al. 1982; Studd et al. 1991). These data did not encourage another investigation to find involvement of herpes simplex virus in recurrent aphthous stomatitis.

In the present study, there were no significant differences in levels of IgG antibodies against HCMV, VZV and HHV-6. The specific VZV IgG antibody was positive in all patients and controls. Also specific HHV-6 IgG was positive in almost all patients and controls. IgG against HCMV was also positive in 36 per cent of recurrent aphthous

stomatitis, 50 per cent of oral lichen planus patients and 53 per cent of controls. Whereas specific IgM antibody was negative against VZV, IgM antibody against HCMV was not significantly different between patients and controls. But, on the other hand, the prevalence of positivity of IgM antibody against HHV-6 was significantly different between sera from recurrent aphthous stomatitis patients and control subjects without a history of recurrent aphthous stomatitis. These findings were similar to those from the PCR investigations. Biopsy samples used in the PCR tests were obtained from 54 patients whose blood was removed for serological investigations. Biopsy and blood samples were obtained at the same visit from patients and controls.

In the PCR investigations VZV DNA was not detected in any of the biopsy samples. HCMV DNA was identified only in two biopsy samples (10 per cent) of oral lichen planus patients and in none of the recurrent aphthous stomatitis or control subjects. However, HHV-6 DNA was found in four biopsies (20 per cent) of oral lichen planus and six samples (29 per cent) of recurrent aphthous stomatitis patients. In none of the controls was HHV-6 DNA detected. Although the difference in detection of HHV-6 DNA between recurrent aphthous stomatitis and non-aphthous control subjects was not statistically different ($p=0.08$). This difference could show possible involvement of HHV-6 in recurrent aphthous stomatitis. This hypothesis was supported by data obtained from the immunofluorescent assay, showing a significant difference in IgM antibody against HHV-6 between recurrent aphthous stomatitis and control subjects ($p=0.0076$). However it should be considered that the number of control subjects in the serological study was higher than in the PCR investigation. The difference in the PCR result might

have been increased if a higher number of biopsy samples were obtained from non-aphthous control subjects.

Increased specific HCMV IgG was identified in recurrent aphthous stomatitis patients compared with controls especially in the remission period of the disease (Pedersen and Hornsleth, 1993; Sun et al. 1996). Also by applying the PCR method, HCMV DNA was recovered in oral lesion specimens of two out of four patients with Behçet's syndrome and three out of nine patients with recurrent aphthous stomatitis (Sun et al. 1996). Using *in situ* hybridisation, HCMV DNA was found in three of 29 oral mucosal ulcers but not in any recurrent aphthous stomatitis biopsies (Sun et al. 1996). Our study did not find any significant difference in positivity to antibodies against HCMV. Also no HCMV DNA was identified in recurrent aphthous stomatitis samples. However in two of the 20 biopsies from oral lichen planus subjects, HCMV was identified. This supports the previous findings by *in situ* hybridisation (Leimol-Virtanen et al. 1995).

The increased antibody levels against VZV, especially IgM has also been reported in recurrent aphthous stomatitis patients compared with control subjects (Pedersen, 1989b; Pedersen and Hornsleth, 1993). In addition, VZV DNA has been detected in all biopsies from 22 recurrent aphthous stomatitis patients (Pedersen et al. 1993), but an unusual finding of this study was that VZV DNA was only amplifiable using one set of primers tested, which targeted the protein kinase 66 (PK66). Two other primer sets, one targeting another region of the VZV PK66 gene and the other targeting the VZV thymidine kinase gene, gave negative PCR results. Whilst the authors offered no explanation for this discrepancy, their results are not supported with the findings of the present study, in which using two rounds of PCR with nested primers it was not possible

to identify VZV DNA in any of the samples which were examined. These findings by PCR support the presented serological features where no specific IgM antibody against VZV was found in any of the serum samples. The serological findings indicate that there has been no infection or reactivation of the VZV in any of the subjects whose blood was tested.

In the literature pertaining to recurrent aphthous stomatitis, there is only one investigation on antibodies against HHV-6 (Pedersen and Hornsleth, 1993). In this study IgG antibody was tested and there was no significant difference in antibody levels in recurrent aphthous stomatitis patients compared with controls (Pedersen and Hornsleth, 1993). In the present study, in 96.7 per cent of all serum samples, specific HHV-6 IgG antibody was detected. There was no significant difference between recurrent aphthous stomatitis patients and controls. These findings support previous results on the high prevalence of specific antibodies against HHV-6 in different populations (Knowles and Gardner, 1988; Briggs et al. 1988; Levy et al. 1990; Pedersen and Hornsleth, 1993). In this thesis, PCR detected HHV-6 DNA in six of 21 (29 per cent) recurrent aphthous stomatitis biopsies. Also specific HHV-6 IgM was found in 21 of 22 (95.5 per cent) sera from recurrent aphthous stomatitis patients. This finding was significantly different from control subjects. This is the first study to investigate the possible involvement of HHV-6 in recurrent aphthous stomatitis by applying PCR to aphthous lesions and sera from recurrent aphthous stomatitis patients to detect HHV-6 DNA and specific HHV-6 IgM.

All patients in whom HHV-6 DNA was present in their aphthous lesions, specific HHV-6 IgM was also detected in their sera. However in the other 15 patients with positive IgM antibody, PCR did not detect any HHV-6 DNA in their aphthous ulcers. The presence of

HHV-6 DNA has been identified in peripheral blood monocytes and saliva samples of healthy individuals (Jarrett et al. 1990; Kido et al. 1990; Cone et al. 1993; Cuende et al. 1994), although IgM antibody against HHV-6 was not present in any of the subjects (Cuende et al. 1994). In this study, HHV-6 DNA was detected in peripheral monocytes of eight of 20 individuals when one microgram of DNA was amplified, but when the amount of DNA was increased to five micro-gram, in 18 of 20 individuals HHV-6 DNA was present (Cuende et al. 1994). The authors suggested that HHV-6 is present in a high proportion of the healthy population but in minimal amounts (Cuende et al. 1994). Detectable HHV-6 DNA in some individuals seronegative to the virus also has been reported (Cone et al. 1993).

In the present study, saliva samples and peripheral blood cells were not tested to find the presence of the virus in recurrent aphthous stomatitis and control subjects. It is possible that the higher number of recurrent aphthous stomatitis patients with positive IgM against HHV-6 is either due to the presence of the viral DNA in saliva or peripheral blood monocytes in larger amount than healthy individuals as the result of reactivation of the virus or alternatively PCR could not detect viral DNA in some of the biopsy samples because of the small amount of DNA extracted from biopsies. The biopsy samples from recurrent aphthous stomatitis patients were generally smaller than those from lichen planus and normal individuals. In one study, HHV-6 was found in 63 per cent of salivary gland biopsies, three per cent of saliva samples from healthy individuals and in 17 per cent of saliva samples from patients with a history of recurrent aphthous stomatitis (Di Luca et al. 1995). In this study PCR did not detect significant differences in the presence of HHV-6 and its viral load between saliva from recurrent aphthous stomatitis patients

and from healthy subjects. However, it is not clear that saliva samples were collected during the active ulceration or the remission period. If the samples were collected during the remission period of the disease, then it cannot be concluded that reactivation of the virus does not play a role in the aetiology of recurrent aphthous stomatitis.

The question of contamination of samples during biopsy causing false positive results in the PCR findings has been discussed in section 4.5. In addition, the significant difference in IgM antibody against HHV-6 between recurrent aphthous stomatitis and normal subjects support the PCR results.

The reported benefit of antiviral drugs in recurrent aphthous stomatitis could also support the involvement of viruses in aetiology of this disease. Although the effect of oral aciclovir has been denied in one report (Wormser et al. 1988), a high dose of oral aciclovir was found to be effective in other clinical trials (Pedersen, 1992; Ozturkcan et al. 1996). Although in these reports (Pedersen, 1992; Ozturkcan et al. 1996), the effect of high dose aciclovir was assumed to support the recent findings of involvement of VZV in recurrent aphthous stomatitis (Pedersen, 1989b; Pedersen et al. 1993; Pedersen and Hornsleth, 1993), the data presented in this thesis are not in agreement with those studies. The homology between HHV-6 and HCMV (Wahren and Linde, 1991; Chou and Marousek, 1992) suggests that this virus is likely to be inhibited by ganciclovir and foscarnet (Burns and Sandford, 1990; MacPhail et al. 1993). But the high dose of 25 to 50 μM aciclocir (*in vitro*) was found to cause four and eight fold reductions in DNA synthesis respectively (Burns and Sandford, 1990). Given this, the effect of high dose aciclovir in improving the symptoms of recurrent aphthous stomatitis may indicate the

involvement of a virus less sensitive to aciclocir such as HHV-6 in recurrent aphthous stomatitis.

The isolation of HHV-6 has been successful from CD4+ CD8- and CD3+ CD4+ cells which suggests that cells with the CD4+ surface marker are involved in the primary infection (Wahren and Linde, 1991). Most cells infected *in vitro* carry the CD4 cell surface antigens, which have been suggested as receptors not only for HIV but also for HHV-6 (Wahren and Linde, 1991). The highest HHV-6 antibody titres have been found in patients with Epstein Barr virus (EBV) and HCMV infections (Wahren and Linde, 1991). It is likely that a previous HHV-6 infection becomes reactivated when a primary infection with another herpesvirus takes place (Wahren and Linde, 1991). It is also possible that HHV-6 causes reactivation of other herpesviruses such as HCMV and EBV which also reside in blood cells (Wahren and Linde, 1991). Therefore, the high titre of specific HHV-6 antibodies in other diseases with a different pathogenesis than HHV-6, make it difficult to evaluate the involvement of HHV-6 by detecting its specific antibodies titres. Although our findings in the serological investigations of HHV-6 are supported by the PCR results, the possible involvement of other herpesviruses which were not investigated is not excluded.

In conclusion, the results of laboratory investigation presented in this thesis, suggest that HCMV and VZV are not aetiological agents of recurrent aphthous stomatitis. Although there is no established clinical manifestation for HHV-6 in the oral cavity, recurrent aphthous stomatitis may be one of the features of HHV-6 reactivation.

The possible involvement of bacteria in the aetiology of recurrent aphthous stomatitis for a long time has been an important issue. In most of the bacteriological studies an L-form

of *Streptococcus sanguis* , Neisseria and staphylococci were obtained from major and minor recurrent aphthous stomatitis (Barile et al. 1963; Graykowski et al. 1964; Stanley et al. 1964), and cross-reactivity between *Streptococcus sanguis* and human oral mucosa has been discussed as a pathologic factor in recurrent aphthous stomatitis (Donatsky, 1975). There have been no reports to show the presence of *Mycobacterium paratuberculosis*. In the present study, PCR detected *Mycobacterium paratuberculosis* DNA in four recurrent aphthous stomatitis tissues (19 per cent) but in none of the other samples. Recently, Hassan et al (1995) reported 73 per cent homology between mycobacterial peptide 91-105 and human mitochondrial 116-130 peptide. Heat shock proteins are very conserved and may cause cross-reactivity between microbial and cellular host materials (Hasan et al. 1995). The involvement of oral mucosa in this cross-reactivity procedure, might be because of the high load of micro-organisms in the oral cavity (Hasan et al. 1995).

Indeed a rabbit antiserum against a 65 KD heat shock protein of *Mycobacterium tuberculosis* reveals a corresponding 65 KD band with all six *Streptococcus sanguis* strains examined and *Streptococcus pyogenes* but not with *Streptococcus salivarius* (Lehner et al. 1991). Significant anti-65 KD heat shock protein and anti-*Streptococcus sanguis* ST3 antibodies were found in the sera from patients with rheumatoid arthritis and recurrent aphthous stomatitis but the anti-*Streptococcus sanguis* KTH-1, KTH-2 and KTH-3 antibodies were found only in Behçet's syndrome. Since the mycobacterias recognise only 30 per cent of the entire length of the 65 KD heat shock protein, the streptococcal antigens may share some of the remaining 70 per cent of the 65 KD protein and other heat shock proteins. (Lehner et al. 1991) These findings raised the possibility

of cross-reactivity between mycobacterial and streptococcal antigens and also the possible cross-reactivity between these speices with oral mucosa, causing diseases like recurrent aphthous stomatitis.

Although in the present results, there was no significant differences between recurrent aphthous stomatitis patients and controls with respect to the presence of *Mycobacterium paratuberculosis* the presence of this micro-organism only in recurrent aphthous stomatitis samples, could raise the possibility of involvement of this bacterium in the aetiology of recurrent aphthous stomatitis. More investigation is necessary to explain the recurrent mode of the disease and possible involvement of microbial heat shock proteins in recurrent aphthous stomatitis.

6.4. Conclusion

Review of the above discussion could be concluded as follow:

All patients should be screened haematologically for haematological deficiencies. Blood tests should include individual assays for iron, whole blood folate and vitamin B_{12}, in addition to standard haematological examination of the peripheral blood. Dietary manipulation should be considered in those patients with suspected allergic reactions to foods. Patch testing is the test of choice to identify allergic patients.

The symptoms of recurrent aphthous stomatitis in the majority of patients could be improved by local symptomatic therapy in the form of antiseptics, local anaesthetics and topical corticosteroids. A small number of patients require on intermittent systemic corticosteroids.

A symptomatic effect of 5 amino-salicylate on decreasing the pain was observed in some patients, but this effect was not very obvious compared to placebo. Also, this study showed that a cross-over trial is not the study of choice for investigating the effect of drugs in recurrent aphthous stomatitis.

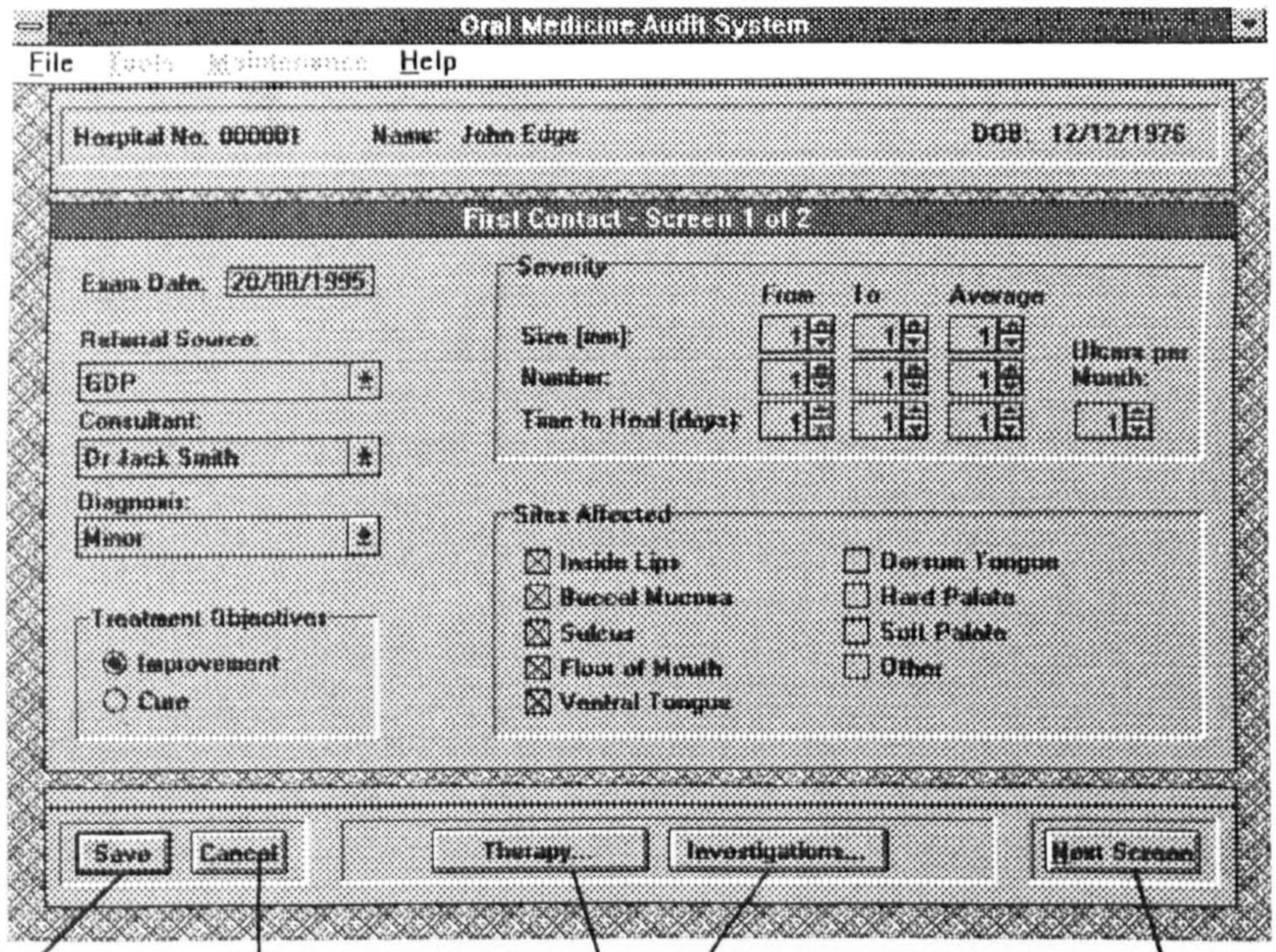
In the review of the literature, it seems that the central defect, present in patients with aphthae, is immunological. But the main cause of these immunological changes is not yet clear. A viral cause is the most plausible factor in causing all immunological imbalance in recurrent aphthous stomatitis. However a virus has not been identified.

From the herpesvirus group, HHV-6 is most likely to be a cause of ulceration in this disease. This involvement has been supported by the findings of PCR and the serological studies on specific IgG and IgM antibodies against this virus. A difference of $p=0.08$ was observed in the positivity of HHV-6 by PCR in aphthous lesions compared with normal samples. This difference was increased to $p=0.0079$ by IFA in specific HHV-6 IgM antibodies when patients' sera were tested compared with the control group. Such differences were not detected with HCMV and VZV.

Although DNA of *Mycobacterium paratuberculosis* was detected in four of 15 samples of aphthous lesions and none was found in controls, an involvement of this bacteria could not be supported only by these findings and further investigation, especially in relation to human heat shock proteins is recommended.

Appendix I. The computer software provided for Oral Medicine Audit system, specific to oral ulceration.

First Contact Screen- First of Two



The **Save Button** will save the details entered into the screen to the Database.

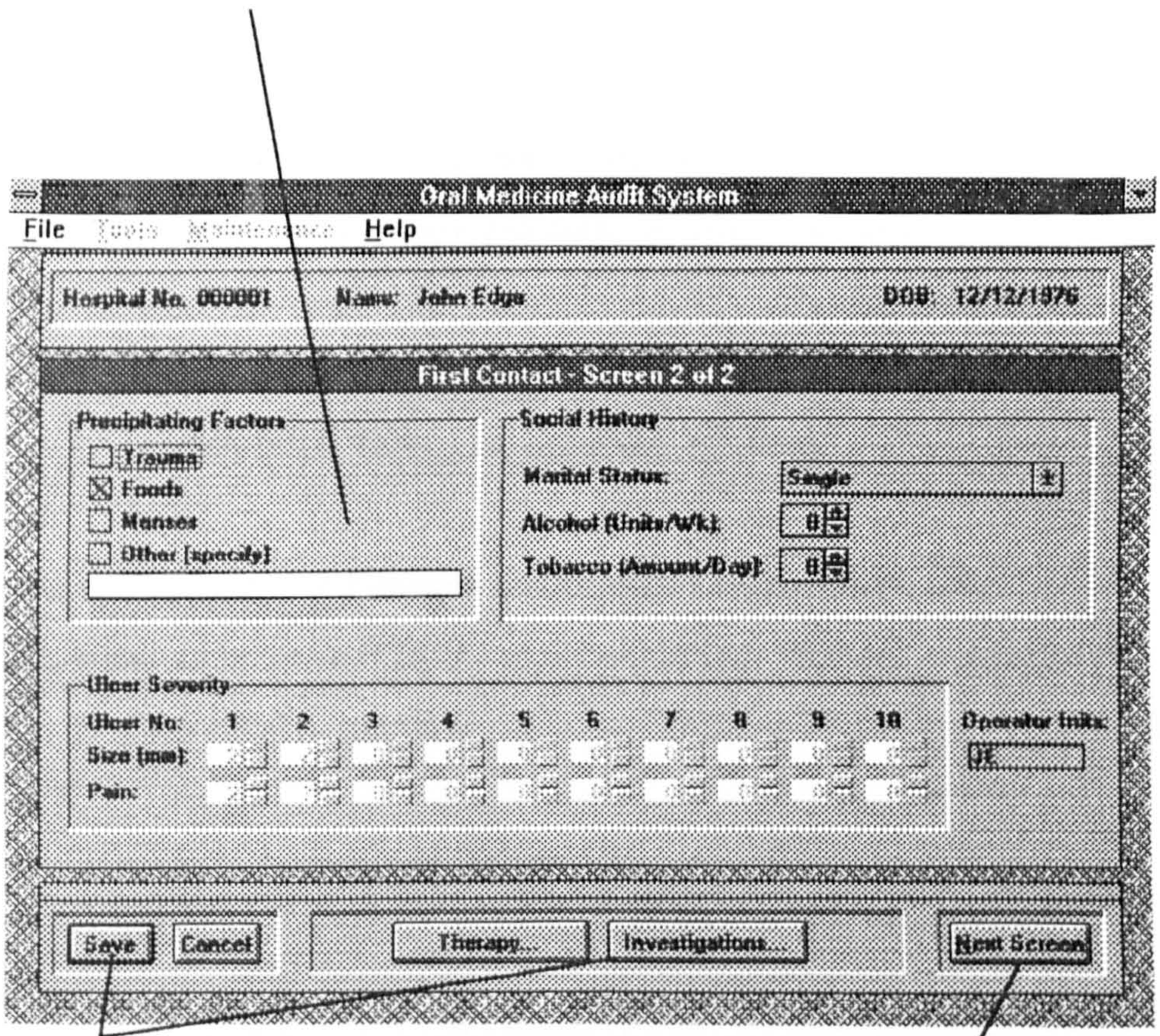
The **Cancel Button** does NOT save the data entered in these screens.

Next Screen displays the second of two screens for inputting data relating to the First Visit.

Therapy and Investigation Buttons lead to a series of screens allowing entry of data with respect to these areas

First Contact- Second of Two.

Detail of screen different to First screen.



The Buttons at the bottom of the screen do not change between the First and Second of the **First Contact** screen. The functionality of these buttons is exactly the same as on the first screen described in page 3-9

Flips back to the first of the screens of data.

Appendix II. Materials used in PCR and their sources.

DNA

Oligonucleotide primers for human herpes virus 6, human cytomegalovirus, human varicella zoster virus, *Mycobacterium paratuberculosis* and human β haemoglobin primers: Cruaclen Ltd, Glasgow, United Kingdom.

DNA from HHV-6, HCMV and VZV (as positive control): Gift from Mr. Duncan Clark, Royal Free Hospital, School of Medicine, London.

IS900 DNA from *Mycobacterium paratuberculosis* (as positive control): Gift from Professor John Hermon-Taylor, St. George's Hospital, Medical School, London.

Enzyme

Dynazyme I: Flowgen Instruments Ltd, Lichfield, England.

Chemicals

PCR buffer (Dynazyme ™): Flowgen Instruments Ltd, Lichfield, UK.

Trise base, ethidium bromide, EDTA, sodium chloride: SIGMA, Dourest, UK.

Guanidine isothiocyanate solution, agarose: GIBCO-BRL, life technologies LTD, Paisley, UK.

Dynawax: Flowgen Instruments Ltd, Kent, UK.

dNTP:

sd H_2O:

Miscellaneous Items

Polaroid film type 667: SIGMA, Dorset, UK.

Omni Gene Thermal Cycler: Hybaid Ltd, Teddington, UK.

DNA Purification System

Wizard DNA Clean up kit:

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