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RECURRENT APHTHOUS ULCERATION

Immuno-pathological aspects

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Academic dissertation

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Yliopistopaino

This work is dedicated to those who
suffer or might suffer from
recurrent aphthous ulceration

CONTENTS

LIST OF ABBREVIATIONS

ABC	avidin-biotin-peroxidase complex
"$ T cell	alpha/beta T cell
ADCC	antibody-dependent cellular cytotoxicity
AEC	3-amino-9- ethylcarbazole
AECA	anti-endothelial cell autoantibodies
BD	Behçet's disease
CD	cluster of differentiation
CD4+	helper-inducer T lymphocyte
CD8+	suppressor-cytotoxic T lymphocyte
CMV	cytomegalovirus
DAB	3,3-diaminobenzidine tetrahydrochloride
EBV	Epstein-Barr virus
FGF-7	fibroblast growth factor-7
(* T cell	gamma/delta T cell
G-CSF	granulocyte-colony stimulating factor
HCl	hydrochloric acid
HHV-6	herpes hominis virus-6
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
Hsp	heat shock protein
HSV	herpes simplex virus
HuRAU	herpetiform ulcer RAU
ICAM-1	intercellular adhesion molecule-1
IgG	immunoglobulin G (also IgM, IgA, IgD, IgE)
IL-10	interleukin 10
IFN-(	interferon-gamma
kDa	kilodalton
LFA-3	lymphocyte function-antigen-3
MaRAU	major RAU
MC	mast cell
MHC	major histocompatibility complex
MiRAU	minor RAU
MMPs	matrix metalloproteinases
NK-cell	natural killer cell
NO	nitric oxide
PAP	peroxidase-antiperoxidase
PCR	polymerase chain reaction
RAU	recurrent aphthous ulcer
RNA	ribonucleic acid
TBS	tris-HCl buffered saline
TCR	T-cell receptor
TNF-"	tumor necrosis factor-"
TU	traumatic ulcer
VZV	varicella-zoster virus

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications, referred to in the text by their Roman numerals (I-V).

I. Natah SS, Häyrinen-Immonen R, Hietanen J, Malmström M, Konttinen YT. FactorXIIIa-positive dendrocytes are increased in number and size in recurrent aphthous ulcers (RAU). J Oral Pathol Med 26: 408-13, 1997.

II. Natah SS, Häyrinen-Immonen R, Hietanen J, Malmström M, Konttinen YT. Quantitative assessment of mast cells in recurrent aphthous ulcers (RAU). J Oral Pathol Med 27: 124-9, 1998.

III. Natah SS, Häyrinen-Immonen R, Hietanen J, Malmström M, Konttinen YT. Immunolocalization of tumor necrosis factor-" expressing cells in recurrent aphthous ulcer lesions (RAU). J Oral Pathol Med 29: 19-25, 2000.

IV. Natah SS, Hietanen J, Häyrinen-Immonen R, Malmström M, Konttinen YT. Expression of cell proliferation-associated nuclear antigen (Ki-67) in recurrent aphthous ulcers. Oral Med Pathol 3: 29-34, 1998.

V. Natah SS, Häyrinen-Immonen R, Patinen P, Hietanen J, Malmström M, Savilahti E, Konttinen YT. Increased density of lymphocytes bearing (/* T-cell receptors in recurrent aphthous ulceration (RAU). Int J Oral Maxillofac Surg 29: 375-80, 2000.

INTRODUCTION

"As it takes two to make a quarrel, so it takes two to make a disease, the microbe and its host"
Louis Pasteur, French chemist

Recurrent aphthous ulcer (RAU) seems to be as old as humanity itself. The Father of Medicine, *Hippocrates* (460 to 370 BC) is credited with the first use of the term "aphthai" in relation to focal painful inflammation of the oral mucosa, although valid clinical description of RAU only appeared in 1898 in a paper published by Mikulicz and Kümmel (Mikulicz von and Kümmel 1898, Sircus et al. 1957). RAU is one of the most common and poorly understood mucosal disorders. It is found in men and women of all ages, races, and geographic regions (Embil et al. 1975). It occurs more frequently in times of stress (Sibley 1899, Andrews and Hall 1990), and it is estimated that at least 1 in 5 individuals is afflicted with RAU (Axéll and Henricsson 1985a). Much progress has been made over the last four decades on the epidemiology, clinical description, predisposing factors, and symptomatic treatment of RAU. Considerable research attention has been devoted to elucidating the etiology of RAU. Local and systemic conditions, genetic, immunologic, and microbial factors all may play a role in the pathogenesis of RAU. However, to date, no principal cause has been discovered (Ship 1996, Porter et al. 1998).

Since the etiology is unknown, the diagnosis is entirely based on history and clinical criteria and no laboratory procedures exist to confirm the diagnosis (Ship 1996). There is no curative therapy to prevent the recurrence of ulcers, and all available treatment modalities can only reduce the frequency or severity of the lesions. Although RAU may be a marker of an underlying systemic illness such as coeliac disease (Meini et al. 1993), or may be present as one of the features of Behçet's disease (International Study Group for Behcet's Disease

1990), in most cases no additional body systems are affected, and patients remain otherwise fit and well.

The aetiopathogenesis of RAU is not fully understood. Different etiologies and different mechanisms might be operative in the aetiopathogenesis of aphthous ulceration, however, pain, recurrence, self-limitation of the condition, and destruction of the epithelium seem to be the ultimate outcomes. For better understanding of RAU, it is important to study the inflammatory cytokines network and the cells involved in the initiation and progression of inflammation. This information should provide clues to the cause(s) of RAU and may lead to the development of effective and rational treatment for the control of this condition.

In this work, the findings of an expansion of the dendritic cell system, high density and hyperactive mast cells, prominent expression of pro-inflammatory cytokine tumor necrosis factor-" (TNF-"), marked cell proliferation *in situ*, and high counts of intraepithelial (/* T-lymphocytes in RAU lesions are not the end of the RAU story, but the beginning of an exciting new chapter in our attempts to understand the etiopathogenesis of this fascinating, periodical and painful- and still enigmatic- condition.

REVIEW OF THE LITERATURE

"Let's make use of our knowledge today,
because tomorrow it may be too late"
Prof. Federico Mayor, The former Director General of UNESCO

Definition of RAU

An inflammatory condition of unknown etiology characterized by painful, recurrent (single or multiple) ulcerations of the oral mucosa (Graykowski et al. 1966).

Description and clinical forms of RAU

RAU has three different variants – minor aphthous ulcers, major aphthous ulcers, and herpetiform ulcers, according to the classification of Stanley (1972).

1) Minor RAU (MiRAU) is the common variety, affecting about 80% of RAU patients (Porter et al.1998), and is characterized by painful round or oval shallow ulcers, regular in outline, and usually less than 10 mm in diameter, with a gray-white pseudomembrane surrounded by a thin erythematous halo. MiRAU usually occurs on non-keratinized labial, buccal mucosa and the floor of the mouth, but is uncommon on the keratinized gingiva, palate, or dorsum of the tongue. MiRAU is the most common form of childhood RAU (Field et al. 1992). These lesions recur at varying frequencies (from every few years to almost constantly) and heal within 10-14 days without scarring (Porter et al. 1998).

2) Major RAU (MaRAU), also known as *periadenitis mucosa necrotica recurrens* occurs in approximately 10% of RAU patients (Rennie et al. 1985). These lesions are similar in appearance to minor RAU, but they are larger than 10 mm in diameter, single or multiple and

very painful. MaRAU has a predilection for the lips, soft palate, and fauces, but can affect any site (Scully and Porter 1989). The ulcers of MaRAU persist for up to 6 weeks or more and often heal with scarring. MaRAU usually has its onset after puberty (Scully and Porter 1989).

3) The third and least common variety of RAU is **herpetiform (HuRAU)**. The name is derived from the supposed resemblance to the intraoral lesions of primary herpes simplex virus (HSV) infection, but HSV cannot be isolated from HuRAU lesions or from any other forms of RAU (Macphail et al. 1991). This form is characterized by multiple recurrent crops of small, painful ulcers that are widely spread and may be distributed throughout the oral cavity. As many as 100 ulcers may be present at a given time, each measuring 2-3 mm in diameter, although they tend to fuse, producing large irregular ulcers. They usually heal without scar formation, the healing time of an individual lesion being 7 to 10 days. HuRAU occurs more often in women and has a later age of onset than other types of RAU (Lehner 1977, Scully and Porter 1989, Porter and Scully 1991).

Recurrence is the hallmark of RAU, and one variant of the disease is generally present in patients, but two forms may coexist, or a change in clinical expression may be seen with time (VanHale et al. 1981).

Epidemiology of RAU

It has been estimated that 20% of the general population will suffer from RAU at some time in their lives (Sircus et al. 1957; Axéll and Henricsson 1985a). In childhood, RAU is the most common form of oral ulceration (Field et al. 1992). It seems to be more common in children and adults of higher, rather than lower, socio-economic status (Ship 1966, Crivelli et al. 1988).

In cross sectional study RAU lesions were found in about 2% of Swedish adults (Axéll and Henricsson 1985a). RAU prevalence varies from 5 to 66% of the population depending on the group studied (Fahmy 1976; Miller et al. 1977a). RAU seems to be infrequent in Bedouin Arabs (Fahmy 1976) and is more common in Western countries (Embil et al. 1975). The peak age of onset is the second decade (Sircus et al. 1957, Lehner 1968), and a high prevalence and severity of disease has been found in students with a high socio-economic background (Ship 1966, Ship 1972, Miller et al. 1977a).

Factors predisposing to RAU

Age and sex

The prevalence of RAU detected during oral examination (average time point prevalence) was found to be about 1% in children of developed countries (Kleinman et al. 1994), but 40% of children (aged 15 years or less) may have a history of RAU, with ulceration beginning before 5 years of age and the frequency of affected patients rising with age (Miller et al. 1980, Peretz 1994). In the adult population, the first ulceration appears before the age of 30 in 60-85% of patients (Rennie et al. 1985). A slight predominance was found for females (Axéll and Henricsson 1985a), and there may also be a female predisposition in affected children (Field et al. 1992). A decreased prevalence has been noted in males, though not females, over the age of 50 in the Scottish population (Sircus et al. 1957) whereas Axéll (1976) found a decrease in prevalence with age in both sexes in the Swedish population.

Family and heredity

In some individuals, RAU may have a familial basis. Possibly more than 40% of

patients may have a familial history of RAU (Sircus et al. 1957). Patients with a positive family history of RAU develop oral ulcers at an earlier age and have more severe symptoms than individuals with no family history of RAU (Ship 1965, Miller et al. 1977a, Miller et al. 1980). The probability of a sibling developing RAU is influenced by the parents' RAU status (Ship 1972) with increased risk in children of two affected parents (67-90%), as well as there being a high correlation of RAU in identical twins (Miller et al. 1977b). Nevertheless, there is a clear variability in host susceptibility, which can be explained by a polygenic inheritance, with the penetrance being dependent on environmental factors (Ship 1965, 1972).

Genetic factors have been implicated by numerous studies on the association of RAU and the genetically determined human leukocyte antigen (HLA) subtypes. An increase in the frequency of HLA2 (Challacombe et al. 1977), B12 (Lehner et al. 1982, Malmström et al. 1983), B51 and Cw7 in Jewish patients (Shohat-Zabarski et al. 1992), DR2 (Lehner et al. 1982, Özbakir et al. 1987), DR4 in Turkish RAU patients (Özbakir et al. 1987), DR5 and A28 in Greeks (Albanidou-Farmaki et al. 1988), DR7 and MT3 (Gallina et al. 1985) in Sicilians and DRw9 in Chinese patients (Sun et al. 1991a) has been noted in patients with RAU. There may be a negative association with HLA-B5 in Sicilians (Gallina et al. 1985) and DR4 in the Greek population (Albanidou-Farmaki et al. 1988). Many studies have reported a variety of associations or absence of associations (Platz et al. 1976, Dolby et al. 1977, Gallina et al. 1985, Özbakir et al. 1987) between RAU and a particular HLA antigen. This could be explained by the variable ethnic backgrounds of studied patients, or more likely the multiple etiologic basis for RAU. The above mentioned literature, however, suggests that RAU, at least in certain persons, has a genetic basis.

RAU and hormonal changes

It appears from different conflicting studies (Ship et al. 1961, Dolby 1968, Segal et al. 1974, Boggess et al. 1990, McCartan and Sullivan 1992) that a minor subset of women with RAU have cyclical oral ulceration related to the onset of menstruation or the luteal phase of menstrual period. A complete remission during pregnancy have been reported (Sircus et al. 1957, Vincent and Lilly 1992) but with exacerbations occurring in the puerperium (Dolby 1968). Sircus and co-workers (1957) had reported that almost no men developed RAU after the age of 50, whereas 10% of women had their first episode between 50-59. However, the association between RAU and menopause (McCartan and Sullivan 1992) has not been established.

Food hypersensitivity

Some studies correlate the onset of ulcers with exposure to certain foods, such as cow's milk (Thomas et al. 1973), gluten (Wray 1981a, O'Farrelly et al. 1991), chocolate, nuts (Wray et al. 1982b), cheese (Hay and Reade 1984), azo dyes, flavoring agents and preservatives (Wright et al. 1986, Nolan et al. 1991b) but Eversole and co-workers (1982) did not find any significant association of RAU with 3 specific food items (tomatoes, strawberries and walnuts). Some studies have noted an increased prevalence of atopy among RAU patients (Tuft and Ettleson 1956, Wilson 1980), whereas Wray and co-workers (1982b) found no significant difference in the incidence of atopy in RAU patients compared with normal population.

Drugs

Drugs such as non-steroidal anti-inflammatory drugs (NSAIDS) (the proprionic acid and

phenylacetic acid, diclofenac) rarely give rise to oral ulcers similar to those of RAU, along with genital ulceration (Healy and Thornhill 1995) or only oral ulcers in case of piroxicam (Siegel and Balciunas 1991). However, such type of ulcers usually occur as an adverse side effect and disappear with discontinued usage of the drug. A recent French study (Boulinguez et al. 2000) has found that typical clinical description of aphthous ulcer and/or clinical presentation suggesting the diagnosis of aphthous ulcers were noted for 8 drugs (Table 1), along with another group of 20 drugs where the diagnosis of aphthous ulcers remained to be confirmed.

Table 1. List of drugs for which a complete clinical description of RAU or indicative photograph was available.

Drug-induced RAU	Reference
Captopril	Corone et al. 1987
Gold salts	Kuffer et al. 1976
Nicorandil	Shotts et al. 1999
Niflumic acid	Kuffer et al. 1976
Phenindione	Kuffer et al. 1976
Phenobarbital	Kennet 1968
Piroxicam	Siegel and Balciunas 1991
Sodium hypochloride	Menni et al. 1988

Hematinic deficiencies

Hematinic deficiencies have been found in about (20%) of patients with RAU (Field et al. 1987). In several studies, deficiencies of iron, vitamin B12, and folate have been reported (Wray et al. 1975, Ferguson et al. 1976, 1980, Tyldesley 1981, Challacombe et al. 1983, Porter et al. 1986), although in many cases the deficiencies were marginally low (Field et al. 1987).

However, Olson and colleagues (Olson et al. 1982) found that vitamin B12, folate and iron deficiencies were not significantly different between the patients with RAU and controls. Another pilot study on 22 HIV-infected patients with RAU suggested that vitamin B12 or folate deficiencies were not risk factors for HIV-associated RAU (MacPhail and Greenspan 1997). Low serum ferritin levels were found in 8-12% of patients with RAU, compared with 3-5% in controls, and the level did not differ in different subtypes of RAU (Challacombe et al. 1983). However, in the majority of cases, there was no identifiable underlying cause for those RAU patients who had a ferritin deficiency (Porter et al. 1988). In a Scottish study, Nolan and co-workers (1991a) found that 28.2% of patients with RAU had deficiencies of vitamins B1, B2, and/or B6. They showed also that patients who have both RAU and a vitamin B deficiency could benefit from vitamin replacement therapy. It appears from the above mentioned literature that the wide variations in the findings may be due to differences in genetic background and dietary habits of examined patients, or the multi-factorial etiology of RAU.

Zinc deficiency

The improvement of RAU with zinc sulphate supplementation were described in an open trial (Merchant et al. 1977) and in a case report (Endre 1991) of aphthous ulcers with zinc deficiency and immunodeficiency, but such improvement could not be confirmed in later studies (Merchant et al. 1981, Wray 1982a). In a Chinese study (Pang 1992) , the level of serum zinc of 75 cases of RAU was found to be on a lower level within normal range, and serum copper was also normal. So far no information exists on the association of RAU and other trace elements.

Environmental factors

Stress

Earlier studies have documented an association between RAU and a variety of psychological factors including anxiety, repressed hostility, as well as job related and other stressors (Sircus et al. 1957, Ship et al. 1960, 1967, Miller et al. 1977a). Conversely, other studies have failed to reveal any association between anxiety (Heft and Wray 1982), depression (Ferguson et al. 1984), psychological life stress (Pedersen 1989) and recurrences of RAU. A more recent study, in which the relaxation/imagery treatment program was used (Andrews and Hall 1990) found a significant decrease in the frequency of ulcer recurrence for all treated subjects. Although the majority of studies have been unable to validate the concept that stress plays an important role in the development of RAU, the literature continues to report that stress may play a role in precipitating RAU.

Local trauma

A subset of patients with RAU are predisposed to develop aphthae at sites of trauma (Ross et al. 1958, Wray et al. 1981b). Why local trauma such as anaesthetic injections, toothbrushing, and dental treatment (Kvam et al. 1987) would trigger aphthous ulceration in these patients is still unknown.

Tobacco

Several reports document the negative association between smoking and the occurrence of RAU (Shapiro et al. 1970, Axéll and Henricsson 1985b). Such a negative

association has also been documented with use of smokeless tobacco (chewing tobacco and snuff) (Grady et al. 1992), as well as in patients who are smokers and seropositive for HIV (Greenspan et al. 1992). Paradoxically, the majority of patients with RAU are nonsmokers (Rennie et al. 1985), for instance, in a more recent study (Tuzun et al. 2000) only 9% of RAU patients were found to be active smokers compared with 25% among the control subjects.

Nicotine has been found to be beneficial in RAU (Bittoun 1991) and in inflammatory bowel disease (Lashner et al. 1990), and its effects may result from influences on nerve function, although these agents may also exert direct anti-inflammatory effects. However, the mechanism by which cigarette smoking protects against RAU is still unknown.

Infectious factors

Bacterial agents

In 1963, Barile and co-workers (1963) isolated *S. oralis* (previously known as *S. Sanguis* 2A) from an aphthous ulcer lesion. Other subsequent studies (Donatsky 1975, Donatsky 1976 a,b) have found raised levels of antibodies against certain oral streptococcal strains in patients with RAU when compared with controls. Cross-reaction of antibacterial antibodies with oral mucosa has been postulated as an immunopathogenic mechanism in RAU (Donatsky 1975). Later studies, however, have not confirmed these observations (Hoover et al. 1984, Greenspan et al. 1985, Riggio et al. 2000). In a serological test, *Helicobacter pylori* does not appear to be of etiologic significance in the development of RAU (Porter et al. 1997). Another study had detected *H. pylori* DNA in swabs from 23 of 32 RAU lesions using polymerase chain reaction (PCR) assay (Birek et al. 1999), but in a more recent study, the culture of *H. pylori* from 12 RAU lesions were found to be negative (Shimoyama et al. 2000).

Suggesting that *H. pylori* might not have a direct association with RAU.

Viral agents

A possible viral cause for RAU has been suggested by several researchers. Sallay and co-workers (1973) isolated adenoviruses from oral aphthae, but there was no antibody response to adenovirus in RAU. Adenoviruses are ubiquitous organisms and these results need confirmation. Studd et al. (1991) detected HSV-1 DNA in only 2 of 11 biopsies of oral aphthae from RAU patients. Other studies failed to detect HSV antigen in the biopsies (Poulter and Lehner 1989), and HSV cannot be cultured from the RAU lesions (Donatsky et al. 1977, Macphail et al. 1991). Antivirals such as acyclovir, highly effective against HSV, appear to have only equivocal clinical effect on RAU (Wormser et al. 1988, Pedersen 1992). Patients with RAU were found to have higher titers of IgM against varicella-zoster virus (VZV) and cytomegalovirus (CMV) than control subjects (Pedersen and Hornsleth 1993a). Further studies have detected VZV-like DNA (Pedersen et al. 1993b), CMV-DNA (Sun et al. 1996), Epstein-Barr virus (EBV-DNA) (Sun et al. 1998) in some oral ulcer biopsy specimens from some RAU and/or Behçet's disease (BD) patients. However, VZV could not be cultivated from any of the oral ulcer biopsies and VZV antigen was not detected in any of the smears. A further study using PCR, has detected herpes hominis virus-6 (HHV-6- DNA) in six of 21 RAU lesions (6/21), whereas VZV-DNA and CMV-DNA were not detected in any RAU samples (Ghodratnama et al. 1997). The detection of human herpesvirus DNA from the oral mucosa and peripheral blood mononuclear cells of patients with RAU appears to represent normal viral shedding rather than a direct causal mechanism in this disorder (Brice et al. 2000).

Overall, the evidence for involvement of viruses such as HSV, VZV, CMV, EBV and HHV-6 in RAU is conflicting. It is possible that RAU is a non-specific response with multiple etiologies and represents the final common pathway of mucosal inflammation or, alternatively, the dormant herpesviruses might be reactivated by the immuno-dysregulation known to be associated with RAU.

Heat shock proteins

Cross-reactivity between mycobacterial 65-kDa heat shock protein (Hsp) and *Streptococcus sanguis* has been demonstrated, and significantly elevated levels of serum antibodies to recombinant 65-KDa mycobacterial Hsp were observed in RAU (Lehner et al. 1991). The lymphocytes of RAU patients have a significantly increased lymphoproliferative response to peptide epitope 91-105 of the 65-kDa mycobacterial Hsp in the ulcerative stage as opposed to the period of remission (Hasan et al. 1995). There is some cross-reactivity between the microbial 65-kDa Hsp and the 60-kDa human mitochondrial Hsp. Thus, RAU may be a T-cell-mediated response to antigens of *S. sanguis* that cross-react with the mitochondrial Hsp and induce oral mucosal damage (Hasan et al. 1995). Conversely, other studies (Van Eden et al. 1998) have suggested that immediate upregulation of Hsps in any cell type, everywhere in the body, as a consequence of stress may trigger T cells with a regulatory phenotype. This would provide the immune system with an immunoregulatory mechanism which acts to monitor and control dangerous or potentially deleterious inflammatory responses. However, whether Hsps in RAU are protective or destructive or have a dual role is still unclear.

Serology of RAU

Increases in serum IgA, IgG, IgD and IgE have been reported in some groups of RAU patients (Scully et al. 1983, Lehner 1969b, Ben-Aryeh et al. 1976), whereas in other groups of RAU patients IgG, IgM and IgA were found to be normal or reduced (Malmström et al. 1983, Bagg et al. 1987). A previous study by Porter *et al.* (1992) in a group of 71 RAU patients showed no significant changes in serum levels of IgG_1, IgG_2, IgG_3 or IgG_4, but a more recent study found low serum levels of IgG_2 during the quiescent period of the disease (Vicente et al. 1996). The presence of raised levels of anti-endothelial cell auto-antibodies (AECA) lends support to the hypothesis that a vasculitic process may underlie some cases of RAU (Healy et al. 1996). Circulating immune complexes were found to be present in some patients (Burton-Kee et al. 1981, Lehner et al. 1982). However, complexes have not reliably been demonstrated in MiRAU (Bagg et al. 1987). Serum levels of C9 (Lehner and Adinolfi 1980) and $2 microglobulin (Scully 1982b) have been reported to be raised in some patients, and may represent a non-specific acute phase response (Rennie et al. 1985).

IMPORTANT SYSTEMIC DISEASES ASSOCIATED WITH RAU

Coeliac disease

Coeliac disease is characterized by inflammatory changes in the small intestinal mucosa induced by a component of the gluten protein of wheat. Recent studies by Lähteenoja et al. (2000a,b) have shown inflammatory changes with increased lamina propria and intraepithelial helper-inducer T lymphocyte (CD4+) and suppressor-cytotoxic T lymphocyte (CD8+) cells in the oral mucosa of coeliac disease patients after a local challenge with gliadin. The prevalence of patients with coeliac disease who have concurrent

RAU ranges from 10% to 18% (Tyldesley 1981, Ferguson et al. 1980, Majorana et al. 1992, Meini et al. 1993), with an increase in frequency of HLA-DRw10 and DQw1 in coeliac disease associated with RAU (Majorana et al. 1992, Meini et al. 1993). However, the aphthae usually disappear with appropriate management of the coeliac disease (Ferguson et al. 1980). On the other hand, it has been found that about 5% of RAU patients suffer from coeliac disease (Ferguson et al. 1976, Veloso and Saleiro 1987). Such RAU patients may have particularly IgA-class reticulin and/or gliadin antibodies (Ferguson et al. 1980, Merchant et al. 1986).

Patients who have RAU with no detectable clinical or histological evidence of coeliac disease on jejunal biopsy may respond to gluten withdrawal (Wray 1981a, Wright et al. 1986).

In contrast, another study failed to demonstrate any benefit from gluten withdrawal in aphthous patients, suggesting that the improvements have been due to a placebo effect (Hunter et al. 1993). So far no studies have used the new diagnostic markers of coeliac disease such as anti-tissue transglutaminase and anti-endomysial antibodies for screening patients with RAU.

Behçet's disease

BD is a multisystem disorder that affects predominantly young men of Mediterranean, Middle Eastern and Japanese descent. Classically, it features a triad of MiRAU, genital ulcers and ocular lesions (Shimizu et al. 1979). In 1990 (International Study Group for Behcet's Disease 1990), the criteria for the diagnosis of BD were redefined to include the presence of oral aphthous ulcers plus any two of the following: genital ulcers, typical defined eye lesions (such as uveitis, hypopyon and iridocyclitis), typical defined skin lesions and a positive pathergy (cutaneous puncture hyperreactivity) test. Aphthous ulcers are present in 99% of

patients with BD and are the first symptoms to appear in 67% of patients (Lehner 1977). The three types of RAU, minor, major, and herpetiform are also found in BD and there are no features which differentiate the oral ulcers in BD from those of RAU (Lehner 1978). Although the oral ulcerations in BD are both clinically and histologically identical to those seen in RAU, the exact relationship of these diseases is still unknown.

Cases of complex aphthosis or bipolar aphthosis (present with oral and genital aphthae, but no systemic signs or symptoms) may represent an atypical form of BD (Jorizzo et al. 1985), and follow-up of such patients may eventually disclose more complete expression of BD (Jorizzo et al. 1985). A high frequency of RAU was found among relatives of patients with BD (Arber et al. 1991). Furthermore, RAU has some, but not all, of the immunological abnormalities that arise in BD. In this respect, it has been suggested that RAU and BD might be different degrees of the same disease spectrum (Lehner and Batchelor 1979). However, RAU is usually confined to the oral mucosa in otherwise healthy individuals while in BD it affects the oro-genital mucosa. The cause of this extension of localization to non-oral location is unclear.

HIV-associated RAU

Severe episodes of RAU have been observed in patients infected with HIV. The ulcers are of the minor, major and herpetiform types and are often located on the soft palate, tonsils or tongue, where they hinder eating and speaking. Macphail et al. (1991) showed that 66% of HIV patients affected by RAU had the usually uncommon herpetiform or major types and that patients with MaRAU were significantly more immuno-suppressed than those with MiRAU or HuRAU in that they had fewer CD4 and CD8 lymphocytes. The role played by the

marked neutropenia seen in most of the HIV patients with MaRAU is unclear, but the healing of the ulcers without resolution of the neutropenia argues for the ulcers being MaRAU rather than neutropenic ulcers. About half (44%) of the patients denied or could not recall having had RAU during their childhood, which was presumably before they became infected with HIV. The rest (56%) gave a definite history of childhood RAU and described the ulcers as MiRAU (Macphail et al. 1991). Patients with HIV infection have an overall prevalence rate of recurrent aphthae ranging from 1% to 4% (Phelan et al. 1991, Muzyka and Glick 1994).

Although the lesions are mainly oral, HIV-associated aphthae have been reported in the esophagus and more distal gastrointestinal tract (Bach et al. 1990). HIV-associated RAU lesions tended to be more severe and longer lasting, and may cause debilitating pain with associated alteration of important oral functions such as speaking, chewing and swallowing which ultimately lead to malnutrition and weight loss, compromise a patient's ability to take medications and seriously interfere with the patients' quality of life (Muzyka and Glick 1994). As progress in the treatment of HIV disease results in more patients living longer in a state of significant immuno-suppression, managing severe RAU may become an increasing challenge (Macphail et al. 1991).

Although it has not yet been definitely accepted that RAU-like lesions found in association with HIV infection are indeed RAU, they meet the diagnostic criteria for RAU, they respond to treatment like RAU, and therefore, until proven otherwise, they must be considered RAU (MacPhail et al. 1992). Although HIV DNA has been identified in buccal mucosal scrapings from apparently healthy mucosa of (18/45) HIV-seropositive subjects (Qureshi et al. 1997), to my knowledge no studies have demonstrated the presence of HIV in oral ulcers.

It is unknown whether such lesions represent a localized auto-immune reaction, developing in response to an undefined antigen which triggers a normal immunologic response or represent an overactive HIV in the mucosa of a T cell deficient host.

Important effector cells participating in the inflammatory events of RAU

Neutrophils

Although the chemotactic function of neutrophils is normal in RAU (Abdulla and Lehner 1979, Dagalis et al. 1987), their marked concentration at the ulcer area in the ulcerative phase of the lesion suggests that may they play an active role in the pathogenesis and/or healing of RAU. Indeed, the production of oxygen radicals by neutrophils in RAU was found to be similar to controls (Wray and Charon 1991), and their phagocytic function does not seem to be defective (Ueta et al. 1993). Oral aphthae are a prominent feature of cyclic neutropenia (Scully et al. 1982a), and major aphthae in HIV-infected patients have been associated with a depressed absolute neutrophil count (Macphail et al. 1991). The rapid healing of aphthae on a regimen of granulocyte-colony stimulating factor (G-CSF) (Manders et al. 1995) and the clinical response to similar regimens in patients with cyclic neutropenia (Fink-Puches et al. 1996) suggest that neutrophils are important in the healing of recurrent aphthae.

On the other hand, human neutrophil-type matrix metalloproteinase-8 (MMP-8) was found intracellularly in the ulcer area, and extracellularly in the area of basement membrane lateral to the ulcer (Häyrinen-Immonen et al. 1993) suggesting that neutrophils containing MMP-8 are likely to be involved in the tissue destruction seen in aphthae.

However, the exact role of the neutrophils in the pathogenesis or healing of recurrent aphthae is still not known and remains to be identified.

Macrophages

In spite of the fact that macrophages are likely to participate in every stage of the inflammatory process, they have not yet been adequately studied to definitively establish their exact role in RAU pathogenesis. Griffin (1982) has found that macrophages were seen in the lamina propria but not in the epithelium of RAU lesions, and form a large proportion of the infiltrate in the early phase of the ulcer (VanHale and Rogers 1984). Another histopathologic study of RAU (Schroeder et al. 1984) has found the presence of numerous macrophages loaded with phagolysosomes containing debris of neutrophilic granulocytes, implying that macrophages mainly function to clear the tissue of neutrophil remnants.

The results of Häyrinen-Immonen et al. (1991) indicated that CD11b and nonspecific esterase-positive mature tissue macrophages formed about 14% of all inflammatory cells in RAU lesions, with increased distribution around the periphery of the lymphoid cell infiltrates.

Mast cells

Mast cells (MCs) that have the ability to provide numerous mediators (Brody and Metcalfe 1998) have long been regarded as potentially important cells in the inflammatory events of RAU. In a histopathological study using Alcian blue/Safranin staining of 15 MiRAU, Dolby and Allison (1969b) found that the MC count in the first 2 days did not differ from the normal buccal mucosa. While there was approximately 50% reduction in the MC count in lesions of more than 48 hours duration. In contrast to findings in Dolby and Allison's study, increased numbers of MCs were noted by Lehner (1969a) in all three types of RAU (minor, major, and herpetiform) and in oral aphthae associated with BD, particularly with toluidine blue stain. Such increase in MC numbers was also found in skin lesions and oral aphthae in

patients with active BD (Lichtig et al. 1980). The above mentioned results seem suggestive of an active role of MCs in RAU pathogenesis.

Factor XIIIa+ dendrocytes as a member of the subepithelial immune system

Factor XIIIa-positive dendrocytes are normal residents of the dermis and subepithelial connective tissues. They share a number of molecules, such as CD11b, CD14, CD18 and CD36 with monocyte-macrophages (Caux et al. 1996). Their morphology and expression of HLA-DR suggest that they are capable of processing and presenting antigens (Headington 1986, Drijkoningen et al. 1987, Cerio et al. 1989, Weber-Matthiesen and Sterry 1990). Negative reactivity with antibodies recognizing CD1a and the absence of Birbeck granules and ATPase activity distinguish the FXIIIa dendrocytes from Langerhans' cells (Sontheimer et al. 1989, Headington and Cerio 1990, Moschella and Cropley 1990, Nestle et al. 1993a). Cytoplasmic vacuoles containing hemosiderin and melanin implicate the FXIIIa+ dendrocyte as a phagocytic cell (Headington and Cerio 1990, Altman et al. 1992). Furthermore, it has been shown that isolated FXIIIa+ dermal dendrocytes were potent stimulators of resting T-cells (Nestle et al. 1993a, b). There has been much speculation about the role of FXIIIa+ dendrocytes in various pathological conditions based on their increased number in such diverse diseases as psoriasis (Nickoloff and Griffiths 1990), lichen planus (Regezi et al. 1994), atopic dermatitis (Headington 1986), and Kaposi's sarcoma (Nickoloff and Griffiths 1989). FXIIIa+ dendrocytes bear high amounts of major histocompatibility complex (MHC) class II molecules on their surface, and are very potent antigen presenting cells *in vitro*.

A subpopulation of these cells acquires certain ultrastructural features of Langerhans cells *in vitro* such as Birbeck granule and CD1a (Nestle and Nickoloff 1995a). These cells may

be precursors of epithelial Langerhans cells and may play a role in submucosal immune responses. Given their prevalence in subepithelial connective tissue, and their *in vitro* functional capacity, it is appropriate to conclude that FXIIIa+ dendrocytes are indeed important members of the subepithelial immune system, and may have a role in stimulating mucosal immunity via presenting antigens locally to infiltrating T-cells (Nestle and Nickoloff 1995b, Thomas 1996, Yoo et al. 1998).

Gamma/delta T-lymphocytes

Two classes of T cell antigen receptors (TCRs), the "$ TCR and the (* TCR, have been identified. (/* T-cells share many cell surface proteins with "/$ T-cells and are able to secrete lymphokines and express cytolytic activity in response to antigenic stimulation (Munk et al. 1990). Moreover, (/* T-cells and natural killer (NK)-cells share similar responses upon activation (Haas et al. 1993). However, differences between (/* T-cells and "/$ T-cells are numerous. First, (/* TCRs are more closely related to immunoglobulins (Igs) than to "/$ TCRs (Rock et al. 1994). Thus, (/* TCRs bind antigens in a different manner than "/$ TCRs. The second difference relates to their activation kinetics. A consistent feature of (/* T-cell responses is a localized, rapid and transient release of bioactive polypeptides such as interferon-gamma (IFN-() (Ferrick et al. 1995) following activation. This response can precede "/$ T-cell activation by several hours to days. Third, (/* T-cells have the ability to recognize non-peptidic molecules commonly associated with micro-organisms and stressed cells (Boismenu and Havran 1997). In general, recognition of these antigens by (/* cells involves the antigen receptor but does not require antigen processing and presenting cells or MHC gene products.

Although in rodents (/* cells are preferentially localized in epithelial tissues such as skin, intestine, and lung (De Libero 1997), they are rare in the normal human oral epithelia (Pepin et al. 1993, Patinen et al. 1997). However, increased (/* T-cell numbers have been found to be associated with a variety of infectious and auto-immune conditions such as coeliac disease, multiple sclerosis and rheumatoid arthritis (Haas et al. 1993; Hayday and Geng 1997). Although (/* T-cell population constitutes only about 5% of circulating T cells, they are much more common in the peripheral blood of patients affected by RAU or Behçet's disease, especially during the active phase of the disease (Suzuki et al. 1992, Pedersen and Ryder 1994). Interestingly, Hasan and colleagues (1995) showed that (/* T-cells from patients with RAU have a specific, proliferative response to heat shock protein peptides.

Cytokines, such as IL-7 released within the epithelial microenvironment, may play a role in proliferation and differentiation of (/* T-cells in epithelia (Fujihashi et al. 1996a). On the other hand, the epithelial-cell-specific fibroblast growth factor (FGF)-7 produced by (/* T-cells may play a role in healing epithelia damaged by infection or inflammation, by prompting cell growth and hence reinstalling tissue integrity (Boismenu and Havran 1994). However, the role of (/* T-cells in mucosal immunity might not be restricted to nursing the epithelial injury.

A previous study using a murine model (Jones-Carson et al. 1995) showed that (/* T-cells lining the orogastric tract can stimulate the production of nitric oxide (NO) by neighboring epithelial cells. This finding may be important because human oral epithelial cells might also be stimulated by neighbouring (/*-T-cells-derived IFN-((Freysdottir et al. 1999) to produce NO via upregulation of inducible NO synthase (iNOS) expression.

As a gaseous free radical, NO could serve as a physiological cytoprotective agent for the mucosa. However, large amounts of NO alone, or after reaction with superoxide released

from activated phagocytes (Beckman et al. 1990, Kimura et al. 1998), could lead to epithelial autotoxicity (Flak and Goldman 1996) and possibly ulceration.

TNF-" and recurrent aphthous ulceration

The possible relevance of TNF-" to the pathogenesis of RAU stemmed from observations that thalidomide, which reduces the activity of TNF-" by accelerating the degradation of its messenger RNA (Moreira et al. 1993), and pentoxifylline, which inhibits TNF-" production (Zabel et al. 1993), were found to be effective in the treatment of RAU in HIV-infected patients and in otherwise healthy persons with RAU (Revuz et al. 1990, Thompson 1995, Pizarro et al. 1995). An enhanced release of TNF-" by peripheral blood monocytes of patients with RAU has also been demonstrated (Taylor et al. 1992).

Furthermore, a recent study has shown low resting levels of interleukin-10 (IL-10) mRNA in non-lesional mucosa of RAU patients and high levels of the pro-inflammatory cytokines, IL-2, IFN-(, and TNF-" mRNAs in lesional and nonlesional mucosa of patients with RAU compared with controls (Buno et al. 1998). However, the contribution of TNF-" to RAU pathogenesis is not fully understood at present.

HISTOPATHOLOGY OF RECURRENT APHTHOUS ULCER

I. The ulcer area

Superficial tissue necrosis with fibrinopurulent exudate consisting of clotted fibrin, numerous red blood cells forming hemorrhagic foci, neutrophils and cellular debris covers the necrotic area. The epithelium is infiltrated with variable numbers of intraepithelial lymphocytes and some neutrophils (Stanley 1972). Neutrophils predominate in the immediate

ulcerated area, although peripheral areas surrounding the ulcer remain mononuclear in nature (Lehner 1969a; Mills et al. 1980; Schroeder et al. 1983; Häyrinen-Immonen et al. 1991).

II. The area lateral to the ulcer

Defined as the epithelium covered area from the edge of the ulcer and sideway to the periphery of the biopsy. An intense leukocytic infiltration with predominance of lymphocytes in non-ulcer regions, where they outnumbered neutrophils (Mills et al. 1980; Häyrinen-Immonen et al. 1991). Monocyt/macrophages are also numerous in the tissue adjacent and lateral to the ulcer. The density of MCs is increased in the lamina propria (Lehner 1969a, Schroeder et al. 1984). The lymphocytes in RAU lesion are primarily T cells, and only 5-12% of all cells in the lesion are B cells (Häyrinen-Immonen et al. 1991). A small proportion of plasma cells and eosinophils can be found and more often in the older lesion (Lehner 1969a). Dilatation of blood vessels is a constant and prominent feature of RAU lesions as are foci of perivascular mononuclear cell infiltrates (Lehner 1969a; Schroeder et al. 1984).

IMMUNOHISTOPATHOLOGY OF RAU

Immunological aberrations involving both cell-mediated and humoral immunity have been reported in previous studies of RAU (Porter et al. 1998). Both class I and II MHC antigens were found to be expressed on the epithelial basal cells in preulcerative RAU lesions and more diffusely within the epithelium at the ulcer stage, consistent with active cell-mediated inflammation (Savage et al. 1986, Poulter and Lehner 1989). *In vitro* studies have shown that peripheral lymphocytes from patients suffering from RAU were found to be directly cytotoxic against oral epithelial cells (Dolby 1969a, Rogers et al. 1976), which, however,

has not been confirmed by others (Peavy et al. 1982, Gadol et al. 1985, Burnett and Wray 1985). RAU patients have significantly increased antibody-dependent cellular cytotoxic (ADCC) activity in the early stage of the disease (Greenspan et al. 1981).

Immunofluorescence studies demonstrated deposits of IgG, IgM, IgA, and C3 in and along mucosal blood vessels and in the cytoplasm of stratum spinosum cells in aphthous ulcers lesions from patients with RAU and Behçet's disease (Lehner 1969a, VanHale et al. 1981, Malmström et al. 1983). Previous studies on peripheral NK-cells in patients with RAU have been contradictory as their percentages have been reported to be either increased (Greenspan et al. 1982) or similar to that of controls (Savage et al. 1988, Pedersen and Pedersen 1993c). Furthermore, Thomas et al. (1990) found that depletion of CD-16 positive cells (NK-cells) produced no change in cytotoxicity towards the oral epithelial target cells. Another report demonstrated that among patients with major RAU, NK cell activity is increased when active oral lesions are seen, depressed during periods of resolution and normal in patients in remission (Sun et al. 1991b).

Formation of perivascular lymphocyte infiltrates are probably in part mediated by endothelial intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-antigen-3 (LFA-3)-binding to their counterpart ligands lymphocyte function-antigen-1 (LFA-1) and CD-2 on lymphocytes, respectively (Häyrinen-Immonen et al. 1992b, Verdickt et al. 1992). ICAM-1 is expressed on the epithelium and submucosal capillaries and venules, suggesting that it may support T-cell adhesion and control the trafficking of leukocytes into the submucosa and epithelium (Savage et al. 1986, Häyrinen-Immonen et al. 1992b, Eversole 1994, Healy and Thornhill 1999), while LFA-3 and its counterpart ligand CD-2 are likely to be involved in T-cell activation in RAU (Häyrinen-Immonen et al. 1992b). Increased numbers of CD1+ Langerhans

cells were found in the epithelium and lamina propria in BD and RAU (Poulter and Lehner 1989, Häyrinen-Immonen 1992a).

It is thus evident that there is no unifying theory of the immunopathogenesis of RAU.

AIMS OF THE STUDY

"It is not the answer that enlightens but the question"
Eugène Ionesco, Romanian-born French playwright

1) To look for morphologic evidence of the involvement of factor-XIIIa+ dendrocytes in the pathogenesis of RAU by assessing their frequency and spatial distribution in RAU compared to traumatically induced oral ulcers.

2) By using specific immunohistochemical markers for MCs, we aimed to re-study the density, distribution and degranulation activity of MCs in RAU and traumatic oral ulcers.

3) To assess the cellular localization and extent of expression of the key pro-inflammatory cytokine TNF-" in RAU lesions and traumatic oral ulcers.

4) To investigate whether the cell proliferation activity in RAU lesions differs from that of the traumatic oral lesions, and to clarify whether the infiltrating inflammatory cells proliferate locally by using the proliferation marker Ki-67.

5) To examine whether the density of lymphocytes bearing (/* T-cell receptors is increased locally in RAU lesions compared to clinically normal appearing oral mucosa from the same patients, and to correlate this density of lesional (/* T-cells to the duration of RAU lesions.

PATIENTS AND METHODS

"What we observe is not nature itself,
but nature exposed to our method of questioning"
Werner Heisenberg, German physicist and Nobel Prize winner

Patients and diagnostic criteria

The studied biopsy specimens consisted of four sets. The first set was comprised of 29 RAU lesions taken from 24 patients (14 women and 10 men, mean age=35 years, range=18-54, Table 2) with longstanding RAU of the minor type (according to the classification by Stanley, 1972). The second set is ulcerative lesions control consisted of 14 experimentally-induced oral traumatic ulcers (TU) were taken from 14 healthy volunteers (women=7, men=7, mean age=36 years, range 21-50) with no history of any mucosal disease (studies I, III, IV). The third set contained 10 specimens (used only in study V) from the non-lesional mucosa at the corresponding sites opposite to the RAU lesions, obtained from the same RAU patients included in study (V) to demonstrate eventual differences between RAU lesions and non-lesional mucosa. The fourth set, was clinically healthy oral mucosa (healthy control) from 16 healthy individuals with a negative history of RAU (women=10, men=6, mean age=35 years, range 15-58).

This work was carried out from 1995 to 2001 and the study protocol was approved by the human research Ethics Committee of the Institute of Dentistry, Faculty of Medicine, University of Helsinki, Finland, 1995. The informed consent was obtained from each participant according to the Declaration of Helsinki (4th amendment, 1989).

A tobacco-smoking history was obtained from sixteen RAU patients included in the study, only one had smoked tobacco in the past and the others had never smoked tobacco. Of the thirty healthy individuals (16 control and 14 TU subjects) recruited to this study, only

two men currently smoked tobacco and four had stopped smoking. All the healthy control subjects were in good general health.

All the patients included in this study had been investigated at the outpatient clinic of the Department of Oral Medicine, Institute of Dentistry, Helsinki, Finland, during previous episodes of ulceration. The results of full blood examination, serum vitamin B12 and folate were within normal limits. None of the patients or controls had a history of any gastro-intestinal signs or symptoms other than associated with minor illness. None of the patients had a history of HIV infection. One of the patients had suffered from occasional genital ulcers in association with oral aphthae, but she had no systemic signs or symptoms of BD. Apart from the RAU complaint, all the patients were otherwise clinically healthy. All RAU lesions studied were 2-10 days old, MiRAU, and painful. The histological picture was that of a nonspecific ulcer.

During the study more than one sample of RAU lesions were obtained from some RAU patients to match the location and duration of the TU lesions (Table 2).

Table 2. Distribution of the patients with RAU according to study, age, gender, duration of RAU, age of the ulcer, and site of biopsy.

Patient	Study	Age (yrs)	Sex	Duration of RAU (yrs)	Age of RAU (days)	Site of RAU biopsy
1	II	37	F	22	10	Buccal
2	II, III, V	34	F	20	6	Labial
3	II, III V	26 27	F	13 14	2 2	Labial Buccal
4	II	29	F	15	7	Labial
5	I, II, IV	36	F	16	3	Labial
6	II, III, V	25	M	10	3-4	Buccal
7	I, II, III, IV, V	45	F	30	3	Buccal
8	I, II, IV, V	35	M	25	2	Buccal
9	III V	28 30	F	12 14	4 5	Buccal Labial
10	II, III, IV	40	F	33	2	Buccal
11	I, II	51	F	34	3	Labial
12	I, II, III, IV	33	F	19	3	Buccal
13	II	21	F	10	-	Labial
14	I III	22	M	12	1 3	Buccal Buccal
15	II, III V	46 49	M	32 35	5 4	Buccal Labial
16	II V	22	M	10	- 7	Buccal Buccal
17	IV, V	37	F	23	3	Buccal
18	III	53	F	43	-	Labial
19	I, II, III, IV	29	M	11	2-3	Buccal
20	IV	40	F	20	3	Buccal
21	I, III	54	M	37	3	Labial
22	IV	18	M	8	3	Buccal
23	IV	45	M	22	2-3	Buccal
24	V	21	M	12	3	Buccal

The inclusion criteria for the RAU patients selected in this study were as follows

1) The patients should have classical minor RAU: history of recurrent painful ulcers on non-keratinized mucosa, clinical appearance of RAU and histopathological findings of RAU.
2) Severe RAU condition: defined as at least one episode per month on average.

RAU Patients were excluded from the study if they had

1) a history or manifestation of any systemic illness or immunodeficiency state.
2) chronic or acute infection such as recent viral illness.
3) recurrent intraoral herpes simplex virus lesions or other oral mucous membrane diseases.
4) been taking any medication that might interfere with the study parameters (such as steroids or immunosuppressive drugs.
5) treated their current ulcer with any form of systemic or topical medication.

Inclusion/exclusion criteria for the healthy control subjects selected for this study

1) good general condition with absence of any health problem
2) comparable in age to RAU patients
3) negative history of RAU condition
4) lack of acute infection
5) negative history of medication or trauma-induced ulcers

Due to absence of a definitive etiology or diagnostic test for RAU, the identification of RAU in a clinical practice usually relies on combinations of a history, clinical features and histopathology. I propose in this thesis a set for diagnostic criteria for RAU which were meant to distinguish RAU from other diseases, and to be practical, all were based on working knowledge of aphthous ulcers and clinical experience. They were not tested for sensitivity and specificity and further studies are required for the widespread use of these criteria. Further refinement of the diagnostic criteria described here will depend on properly conducted studies to validate them.

The diagnosis of primary RAU minor (idiopathic) or secondary RAU minor (that occur in association with systemic diseases) can be made if the condition fulfills the four major criteria (which are necessary to establish the diagnosis of RAU) plus at least one of the minor (supportive) criteria.

Table 3. The major criteria for recognising and diagnosing the condition of RAU minor.

Major criteria	Description
1. External appearance	Single or multiple round-oval shaped ulcers, never preceded by vesicles. The ulcers are shallow and have regular margins, yellow-gray base surrounded by thin erythematous halos, variable in size, but less than 1 cm in diameter.
2. Recurrence	At least three attaks of RAU within the past 3 years and do not recur at the same focal site.
3. Mechanical hyperalgesia	The lesion is painful and the pain is exaggerated by moving the area affected by the ulcer.
4. Self-limitation of the condition	The ulcer heals spontaneously without seqeulae, either with or without treatment.

Table 4. The minor criteria for recognising and diagnosing RAU minor.

Minor criteria	Description
1. Family history of RAU	A positive family history of RAU is present.
2. Age of onset	The first RAU attack started before the age of 40.
3. Location of ulcers	Occur on non-keratinized oral mucosa.
4. Duration of the lesion	Each bout of ulceration lasts from a few days to two weeks.
5. Pattern of recurrence	Irregular
6. Histological examination	Shows non-specific inflammation.
7. Presence of precipitation factor	The attacks are triggered by hormonal changes, exposure to certain food or drug, intercurrent infections, stress and local trauma.
8. Presence of hematinic deficiencies	Laboratory investigations reveal an accompanying hematinic deficiency. Particularly, ferritin, folate, iron, vitamin B and zinc.
9. Negative association with smoking	RAU patient is a non-smoker or develops the ulcer after stopping smoking.
10. Therapeutic trial with gluco-corticosteroids	Positive response to treatment with local or systemic steroids.

Sample collection, processing and storage of control and RAU specimens

Control specimens of clinically healthy oral mucosa were obtained from buccal mucosa (twelve cases) or labial mucosa (four cases). All TU lesions were obtained from the buccal mucosa. RAU lesions were obtained from the buccal and/or labial mucosa. All biopsies were performed under local anaesthesia (20mg/ml Xylocain + 12.5 ：g/ml adrenalin, Astra Co., Södertälje, Sweden). The anesthetic solution was injected deep into the tissue in the area from where the specimens were taken. Ulcer specimens were obtained as excisional biopsies, and for control purposes, specimens from clinically healthy oral mucosa were obtained from healthy individuals by the same technique. Those samples (studies I-IV), which were fixed in 10% buffered neutral formalin, were embedded in paraffin and cut at 6 ：m thickness. Biopsy specimens of study V were divided into two pieces. One-half of each specimen was fixed in buffered neutral formalin and embedded in paraffin for routine histopathological examination using hematoxylin and eosin stain. The second half of each specimen was snap frozen in liquid nitrogen, then embedded in Tissue-Tek OCT compound (Lab-Tek Products, Elkhart, IN, U.S.A.) and stored at -70№C until used for further studies.

Experimental induction of traumatic ulcers (TU)

TU was induced experimentally in each normal volunteer by removing an ellipse of normal buccal mucosa under local anaesthesia. No sutures were placed, the wound measure 0.5×1 cm, being left open for 24-72 h, after which time the induced lesion had the appearance of traumatic ulcer. At this stage the ulcer was removed under local anaesthesia.

In study (IV), due to rapid resolution of TU lesions, 48 and 72 h were chosen as time points following induction of the trauma for study and comparison with aphthae of similar

lesional duration. All biopsies were taken in the morning (9 am-12 noon) to avoid the diurnal variation in the proliferation activity of the epithelium (Kellett et al.1989). For publications (I-IV), all TU samples were processed as formalin-fixed specimens.

Histologic staining

All samples used in the present study were histologically examined using hematoxylin-eosin staining. All aphthae samples were in ulcerative stage and showed histological signs of inflammatory cell infiltration.

Paraffin-embedded tissue sections were deparaffinized three times in xylene, rehydrated in graded ethanol from 100% to 70%, then stained with Delafield's hematoxylin for 10 min. Sections were rinsed in distilled water, immersed in acidic ethanol (250 ml of 70% ethanol + 2.5 ml of 37% HCl for 20 sec) and washed in running water for 10 min. The sections were incubated in Eosin BA for 5 min. Finally, rinsed in a graded ethanol series from 70% to 100%, cleared in xylene and mounted in Diatex (Becker Industrifärg AB, Märsta, Sweden).

Antigen-retrieval methods

The antigen epitopes hidden by aldehyde cross-links were disclosed either by proteolytic digestion with pepsin enzyme (studies I-III) or by heating tissue sections in slightly acidic citrate buffer solution using microwave oven (study IV) (Boon 1994; Mighell et al. 1998).

A) Pepsin treatment

Tissue sections were incubated in 0.4% pepsin (40 mg pepsin in 10 ml distilled water) plus 100 ：l 1N HCl (0.01N HCl) for 30 min at +37<C. Then washed three times with TBS.

B) Microwave treatment

Sections were cut onto slides coated with a strong adhesive (3-aminopropyl-triethoxysilane). Tissue sections were deparaffinized, rehydrated and placed in a plastic box filled with 10 mM tri-sodium citrate buffer, pH 6.0, and heated in a conventional microwave oven for 10 min at 650W to unmask the antigen. While undergoing microwave processing, slides were always covered with a buffer. After heating, slides were permitted to cool down to room temperature over a period of 30-60 min.

Table 5. Primary antibodies used in the studies

Antigen	Antibody	Source	subtype	Concentration/dilution	manufacturer
Factor-XIIIa	Polyclonal	Rabbit	IgG	100U/ml; 1:900	Calbiochem, USA
Mast cell tryptase	AA1 Monoclonal	Mouse	IgG_1	105 mg/L; 1:50	Dako, Denmark
IgE	specific for Epsilon-Chains	Rabbit	IgG	7.7 g/L; 1:800	Dako, Denmark
TNF-"	Monoclonal	Mouse	IgG_1	200 :g/ml; 1:50	Santa Cruz, USA
Ki-67	Polyclonal	Rabbit	IgG	0.25 g/L; 1:500	Dako, Denmark
T-cell receptor (*	TCR *1 Monoclonal	Mouse	IgG_1	100 :g/ml; 1:100	T-cell Sciences, USA
T-cell receptor "$	$F1 Monoclonal	Mouse	IgG_1	100 :g/ml; 1:100	T-cell Sciences, USA
T-cell receptor CD3	Leu4 Monoclonal	Mouse	IgG_1	50 :g/ml; 1:400	Beckton-Dickinson, USA

Immunohistochemistry protocol

Avidin-biotin-peroxidase complex (ABC) staining

The expression of antigen was evaluated by immuno-histochemical staining using the avidin-biotin-peroxidase complex (ABC) method (Vectastain Elite-ABC-peroxidase, Vector Lab).

Paraffin-embedded tissue sections were deparaffinized three times in xylene, rehydrated in ethanol (absolute ethanol 5 min, 94% v/v ethanol 5 min, 70% v/v ethanol 5 min) and rinsed in tap water. All sections were washed in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl (TBS). Disclosing of antigen epitopes were done either by pepsin treatment (studies I-III) or by microwave treatment (study IV). The intrinsic peroxidase activity was abolished by pretreating tissue sections in 0.3% H_2O_2 in methanol for 30 min. Non-specific binding sites were blocked by incubation in normal goat serum (studies I and IV) and normal horse serum (for studies III and V) (1:50, Vector Laboratories, Burlingame, CA, USA) for 20 min. The sections were then incubated with the following primary antibodies: rabbit antiserum against FXIII-a (1:900), monoclonal mouse anti-human TNF-" IgG_1 (1:50), rabbit anti-human Ki-67 IgG (1:500), mouse anti-human TCR *1 IgG_1 (1:100), mouse anti-human "/$ TCR IgG_1 (1:100), and mouse anti-human CD3 TCR IgG_1 (1:400) overnight at +4^{N}C. Bound antibodies were labeled with biotinylated goat anti-rabbit IgG (dilution 1:250-300, Vector Laboratory, Burlingame, CA, USA) or biotinylated horse anti-mouse IgG (dilution 1:50, Vector Lab) for 30 min followed by incubation in avidin-biotinylated peroxidase complex for 30 min. Finally the peroxidase-binding sites were visualized by 3,3'-diaminobenzidine tetra hydrochloride (DAB) (Sigma Chemical Co., St. Louis, MO, USA), 40 mg/150 mL TBS and 0.006% H_2O_2, treatment for 3 min (studies I, III, V) or revealed with a combination of 200:l H_2O_2 and 3-amino-9- ethylcarbazole (AEC 40mg + 12 ml dimethyl formamide) for 20 min

(study IV). After immersion in DAB solution, the specimens were counterstained in Mayer's hematoxylin (Mayer, Diagnostica Merck, Darmstadt, Germany) for 45 sec, dehydrated through graded alcohol series, cleared in xylene and mounted in Diatex (Becker Industrifärg AB, Märsta, Sweden) or in aqueous mounting medium (glycerin-gelatin solution, Dako, study IV). Between each step, the slides were washed three times in TBS. All incubations were performed at +22°C if not otherwise indicated.

For frozen tissue specimens (study V) 6 µm thick cryostat sections mounted on gelatin coated slides were air dried for 30 min, and fixed in acetone for 20 min at +4°C, then in chloroform for 20 min at 22°C. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in absolute methanol for 30 minutes at 22°C. Then the sections were processed for immuno-histochemical ABC staining as described above.

Peroxidase-anti-peroxidase (PAP) complex staining

Paraffin embedded sections were deparaffinized three times in xylene, rehydrated in ethanol (absolute ethanol 5 min, 94% v/v ethanol 5 min, 70% v/v ethanol 5 min) and rinsed in tap water. All sections were washed in TBS. After disclosing of epitopes with pepsin, the intrinsic peroxidase activity was abolished by H_2O_2-methanol pretreatment. Mast cell tryptase and IgE were demonstrated with the unlabelled antibody enzyme method PAP (peroxidase-anti-peroxidase). The tissue sections were treated sequentially with: 1) normal rabbit serum (for MC staining) or normal goat serum (for anti-IgE staining) (dilution 1:66 in TBS) for 20 min. 2) the primary monoclonal mouse anti-human mast cell tryptase or the primary rabbit anti-human IgE overnight at +4°C. 3) rabbit anti-mouse or goat anti-rabbit immunoglobulins for 30 min. 4) incubation with the appropriate PAP (PAP mouse for MC tryptase staining and PAP rabbit for IgE staining, (1:100; Dakopatts A/S, Glostrup, Denmark). The specimens were

washed with TBS three times for 5 min between each step. Finally the peroxidase-binding sites were visualized by DAB and H_2O_2 treatment for 3 min. The specimens were counterstained in Mayer's hematoxylin for 45 sec, dehydrated through graded ethanol series, cleared in xylene and mounted in Diatex.

Specificity

The specificity of the reaction was tested by omission of the primary antibodies from the staining sequence. In addition, normal goat, rabbit, or mouse serum, was used as appropriate instead of the primary antibodies as an additional negative staining control (studies I; II, IV). For studies III and V, an isotype specific IgG_1 antibody raised against *Aspergillus niger* glucose oxidase (monoclonal mouse anti-*aspergillus niger* IgG_1), an enzyme not present or inducible in mammalian tissues, was used at the same concentration as, and instead of, the primary antibody as a negative staining control.

ABC staining was not used in study II because of the possibility of false positive staining of MCs (Hsu and Raine 1984). No positive sample controls were used in this study.

Assessment and quantification of immuno-histochemical staining

In studies I-III the number of positively stained cells was calculated by means of the video image digital analysis system (VIDAS, Kontron, Münich, Germany) linked to a low-light charge screen coupled with a CCTV camera (HV/720K, Hitachi, Denshi, Osaka, Japan) mounted on an Olympus BH-2 light microscope. The VIDAS system consisted of semi-automatic Kontron image analysis and processing systems (Kontron Bildanalysis, Eching, Germany) equipped with a VIDAS 2.1 programme (Kontron). After the adjustment of illumination and focus, the images were recorded in digitized form in a computer. After the

parameters were set for the detection of positive cells, they were fixed and used during the whole analysis procedure. The evaluation of the staining in studies (IV) and (V) was undertaken using an Olympus BH 2 (Olympus Japan Corp., Ltd., Tokyo, Japan) light microscope.

The number of positive cells was calculated in three serial sections in all study samples (except in study III, where only one section from each sample was counted) to get an estimate for a population mean. Using the formula:

$$t = \frac{\bar{x} - \mu}{SEM}$$

it was calculated that the mean of five high-power fields is not significantly ($p>0.05$) different from the population mean (t refers to t-statistics, x=the sample mean, μ=the population mean, SEM=standard error of the mean). Results are expressed as the number of positive cells per one mm^2 of tissue (studies I, II, V) or averaged in each specified area and expressed as count of cells/0.2 mm^2 (studies III and IV).

The calculations were performed under 400× magnification and were done in the surface epithelium at the edge of the ulcers and sideways, and in the subepithelial lamina propria (defined as that immediately beneath the basal cell layer of the epithelium, to a constant depth of 0.22 mm) in areas beneath and lateral to the ulcer, as well as from the sites of mononuclear inflammatory cell infiltrations, and deeper connective tissue areas (I-V). The overall positive cell counts were determined by counting 14 sequential high power fields (field area 0.0704 mm^2), outlining an area of connective tissue that extended 1 mm horizontally (starting just beneath the epithelium from the edge of the ulcer and laterally to the periphery of the biopsy) and vertically to a depth of 1 mm.

Statistical analysis

The results are given as mean and standard deviation to describe the dispersion of the data. The means of the three groups were compared by using the Kruskal-Wallis rank test, and the comparisons between the pairs of means by using the Mann-Whitney U test, with a downward adjustment of the " level to compensate for multiple comparisons. Spearman's rank correlation was used to measure the correlation between MC density and duration of the lesions (study II), and linear correlation was calculated between the densities of intraepithelial (/* T-cells and the ages of RAU lesions (study V). A *P* value of 0.01 or less was considered statistically significant. BMDP-PC, version 7.01 (BMDP statistical software, Cork, Ireland) was used for all calculations.

RESULTS

“A ship in port is safe, but that is not what ships are for,
sail out to sea and do new things"
Admiral Grace Hopper, computer pioneer

Factor XIIIa+ dendrocytes in RAU (I)

In healthy controls, FXIIIa+ dendrocytes were mainly found in the lamina propria and just beneath the basal epithelial cell layer and most of them had slender or spindle shapes. Although the distribution and shape of FXIIIa+ cells in TU samples were similar to those seen in healthy controls, many FXIIIa+ round-shaped dendrocytes were seen within the inflammatory infiltrates of TUs.

In aphthae, FXIIIa+ cells were found in lamina propria, within and lateral to the mononuclear inflammatory cell infiltrates and in perivascular areas. The cells had a spindle, round or dendrite-like appearance. FXIIIa+ cells localized within the mononuclear cell infiltrates were 80% more numerous in aphthae than in TU lesions (199±67 vs 110±31 cells/mm^2, P<0.001). Dendrocytes in RAU were larger in size (up to 2 times as large as in controls) and had more prominent dendritic processes (Fig. I) than those seen in TUs or in healthy controls.

Overall, FXIIIa dendrocytes were 50% more numerous in RAU than in TU or healthy controls (Fig. II).

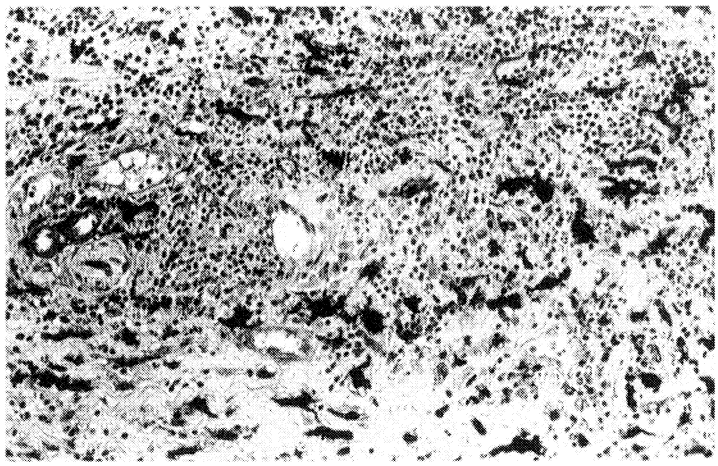

Fig. I. *Immunohistochemical staining of RAU lesion revealing FXIIIa+ dendrocytes within and at the periphery of the mononuclear inflammatory infiltrate of RAU. Note that cells are large with prominent dendritic processes, x160.*

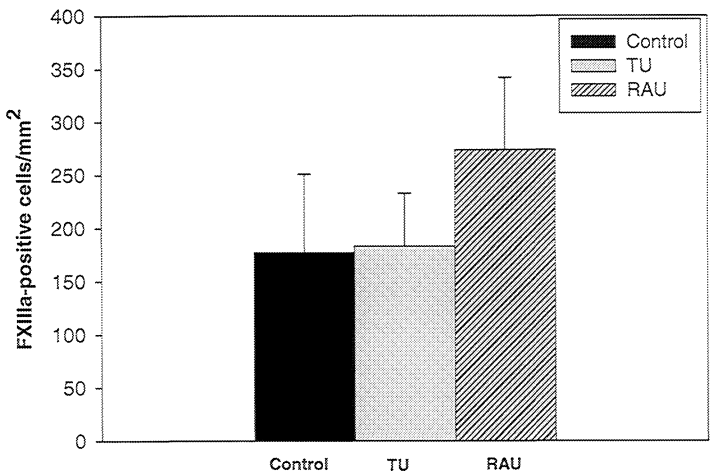

Fig. II. *A diagram showing that the number of FXIIIa-positive cells is 50% higher in RAU than in TU or healthy control mucosa.*

Mast cells in RAU (II)

Tryptase staining

MC tryptase staining was either strong and confined to cytoplasm or was weaker and diffuse, suggestive of degranulation.

Few or no MCs were seen at the base of the aphthous ulcer or close to the ulcer region. In 3 of 15 RAU lesions, MCs were seen to infiltrate the epithelium and were localized in the epithelial basal layer (Paper II: Fig. 2a). Many of these cells were degranulated and surrounded by extracellular tryptase staining (paper II: Fig. 2b). Whereas in the TUs and healthy control sections MCs were absent in the epithelial compartment.

MCs in the lamina propria were 30% more numerous in RAU than in TU lesions. Numerous tryptase positive MCs were intermixed with the other infiltrating mononuclear cells. The inflammatory infiltrates of the RAU lesions showed 1.6 times more MCs than inflammatory cell infiltrates in TUs (118±31 vs 75±18 cells/mm^2, P<0.001). Moreover, many MCs at these sites were degranulated, as evidenced by diffuse extracellular tryptase staining (halos of tryptase) around the MCs (paper II: Fig. 3), in contrast to only a few such cells in the inflammatory cell infiltrates in TUs.

In aphthae, MCs were seen in connective tissue, particularly at the interface between inflammatory cell infiltrates and connective tissue, where they often associated with sites of connective tissue that appear disrupted (paper II: Fig. 4). The MC count in connective tissue in RAU, lateral to the inflammatory infiltrates, was 70% to 80% greater than the count in the connective tissue in TUs or controls. No difference was seen in the densities of MCs between TUs and controls and no correlations could be found between the MC density and duration of RAU or TU lesions. Overall, MCs were 63% more numerous in RAU lesions than in control (Fig. III).

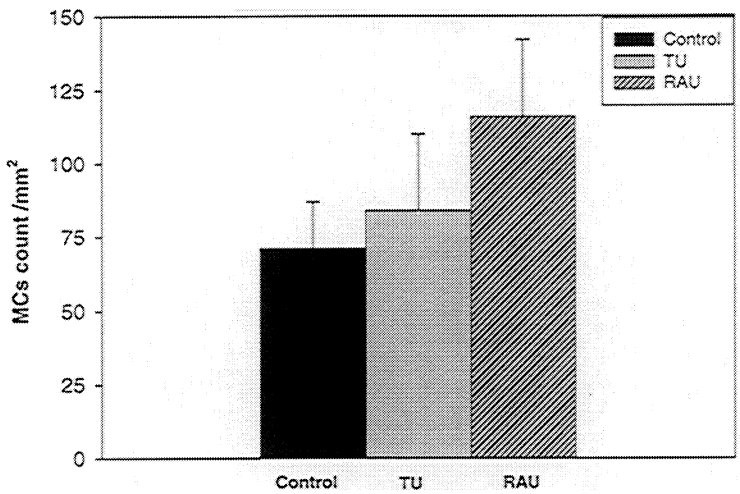

Fig. III. *MCs are 63% more numerous in RAU than in control mucosa.*

IgE staining

IgE was seen as ring-shaped membranous staining. In all sections studied, IgE-positive cells showed a similar frequency and distribution to tryptase-positive MCs. The number of IgE-positive cells was significantly higher in the RAU lesions than in the TU or control sections (108±24 vs 70±12 vs 74±13 cells/mm^2, P<0.001).

TNF-α (III)

TNF-α immunoreactivity was present in four cell types, namely macrophage-like cells, MCs, lymphocytes and endothelial cells. Staining was mostly cytoplasmic, with marked perinuclear accentuation, whereas in MCs it seemed to be confined to the cytoplasmic granules. The intensity of the TNF-α staining appeared more greater in the macrophage-like

cells than in the other TNF-α immunoreactive cell types. No TNF-α immunoreactivity was found in neutrophils or in epithelial cells in RAU or TU.

TNF-α staining in RAU was mostly seen in macrophages and lymphocytes within the inflammatory infiltrates as well as in MCs in perivascular areas (paper III: Fig. 1). Approximately 32% to 60% of the mononuclear cells in aphthae were found to be TNF-α immunoreactive (paper III: Fig. 2).

MCs within the inflammatory cell infiltrates or those at the peri-inflammatory infiltrates in connective tissue showed weak staining and fewer positive cells. Moderate staining of vascular endothelial cells was seen at the edge of the lesion and occasionally in deep connective tissue areas. The counts of TNF-α-positive cells in the lamina propria and deeper connective tissue areas in RAU were significantly higher than those seen in the corresponding sites of TU (paper III: table 1; $P<0.001$). Overall, TNF-α-positive cells were 3 to 4-fold higher in RAU than in TU (Fig. IV).

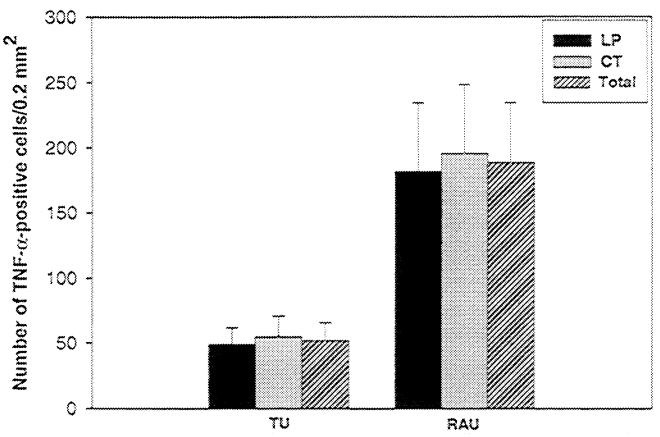

Fig. IV. *The number of TNF-α-positive cells is 3 to 4-fold higher in RAU than in TU.*

Expression of nuclear antigen Ki-67 in RAU (IV)

1) Epithelial compartment

The staining pattern of proliferation-associated antigen Ki-67 was strong and always confined to the nuclei. Ki-67 positivity was mainly seen in the basal and suprabasal epithelial cells. The epithelium surrounding RAU and TU showed an increase in the number of Ki-67 positive cells. There were few proliferating cells in the migrating epithelium at the edges of the ulcer with absence of epithelial outgrowth in 6 out of 10 RAU. In the basal layer of both RAU and TUs, Ki-67+ proliferating cells were more numerous among the cells in the deepest epithelial rete ridges than among the cells overlying the connective tissue papillae.

Epithelium in RAU samples showed an increase in intraepithelial lymphocytes compared with TU and some of the intraepithelial lymphocytes displayed Ki-67. Such cells were located in the interepithelial cell spaces, as single cells or, more rarely, as very small clusters. However, such cells were relatively few and could easily be distinguished from Ki-67 positive epithelial cells, which had intercellular bridges.

The Ki-67 positive cell count was 30% greater in the epithelium of RAU than in the epithelium of TU (Fig. V).

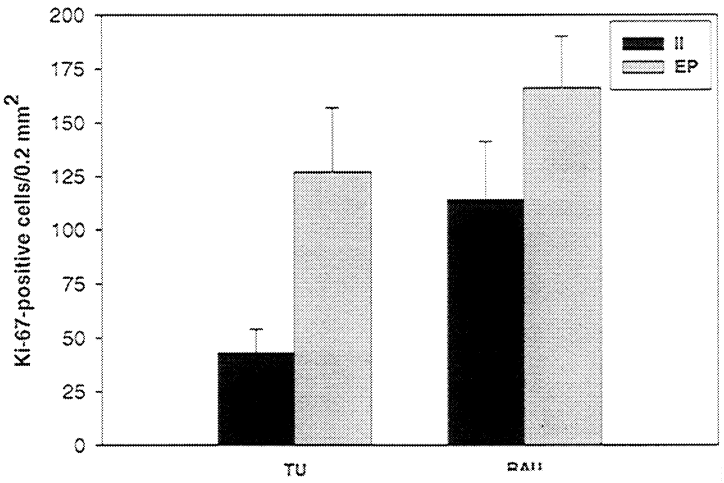

Fig. V. *The number of Ki-67+ cells in epithelium (EP) is 30% higher in RAU than in TU. Note also that the number of Ki-67+ cells in inflammatory infiltrates (II) is 3-fold higher in RAU than in TU lesions.*

2) Connective tissue compartment

Ki-67 positive mononuclear inflammatory cells were in RAU found mostly at the interface between the neutrophil-rich areas and the mononuclear inflammatory cell-rich areas (Fig. VI). In both RAU and TU, locally proliferating cells were mainly lymphocytes, followed by macrophage-like cells, and also a few fibroblast-like cells were Ki-67 positive.

The connective tissue of both types of ulcers contained many foci of dense perivascular mononuclear inflammatory cell infiltrates, with numerous actively dividing Ki-67+ cells. 9% of the mononuclear cells were Ki-67 positive in RAU, with only 3% being Ki-67 positive in TU ($P<0.001$).

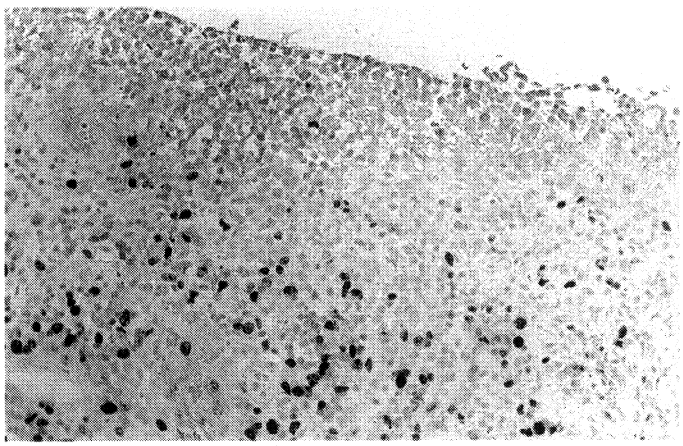

Fig. VI. *An aphthous ulcer lesion showing numerous Ki-67+ cells at the interface between the neutrophil-rich area and the mononuclear inflammatory cell infiltrate. (ABC staining, x250).*

CD3, α/β and γ/δ T-cell lymphocytes in RAU lesions (V)

1) Lymphocyte populations in the connective tissue area

CD3 and TCR staining was seen as ring-shaped membranous staining of lymphocytes. The density of CD3 positive cells in the inflammatory cell infiltrates in the subepithelial compartment in RAU was about ninefold higher than in the non-lesional mucosa of RAU patients. In all samples T-cells expressing the α/β TCR formed the dominant subset of CD3 positive T-cells.

γ/δ T-cells were rare or absent in the non-lesional mucosa and in the mucosa of healthy controls. By contrast, γ/δ T-cells were more numerous in all RAU lesions, especially within the inflammatory cell infiltrates and at the perivascular locations. The γ/δ T-cell count was high in connective tissue of RAU (200±126 cells/mm^2) compared to controls (4±4 cells/mm^2;

P<0.0001) or to non-lesional mucosa from RAU patients (5±7). Moreover, the mean percentage of (/*+ T-cells among total CD3+ lymphocytes was increased in the connective tissue area from 4% and 5% in controls and non-lesional mucosa, respectively, to 19% in RAU.

2) Lymphocyte populations in the epithelium

In epithelium, CD3+ lymphocytes were approximately 3-fold more frequent in RAU samples than in controls. Both "/$ and (/* T-cells were scattered as single cells between basal and suprabasal epithelial cells. The count of "/$ T-cells was high in the RAU epithelium (118±33 cells/mm^2) compared to non-lesional mucosa (72±24 cells/mm^2; *P*<0.001) or to healthy controls (63±27 cells/mm^2; *P*<0.001).

Intraepithelial (/*+ T-cells were seen in 5 of the 10 non-lesional mucosa, and in 4 of the 10 controls, whereas in aphthae they were seen in all 10 RAU samples. A wide variation was observed in (/* T-cell counts in the epithelium of RAU, as they localized focally, homing some epithelial ridges more than others. The highest densities of (/* T-cells were usually seen in the lower epithelial layers and in the basement membrane zone (Fig. VII). The number of (/* T-cells was high in the epithelium of RAU (70±34 cells/mm^2) compared with the epithelium of non-lesional mucosa (2.8±06 cells/mm^2; *P*<0.0001) or epithelium of healthy controls (1.2±1.5 cells/mm^2; *P*<0.0001). In the epithelial area the proportion of (/* T-cells of CD3 positive cells was 2% and 6% in controls and non-lesional mucosa, respectively, and 36% in RAU (*P*<0.0001; Fig. VIII). No significant differences were found in (/* T-cell counts between the non-lesional mucosa and healthy controls, and no correlation was found between the densities of intraepithelial (/* T-cells and the duration of RAU lesions.

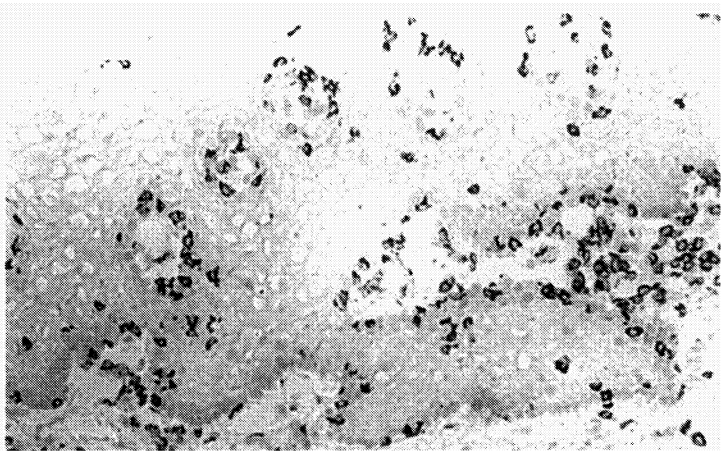

Fig. VII. *Numerous γ/δ T-cells are found in RAU lesion, particularly in the lower epithelial layers and in the basement membrane zone, x250.*

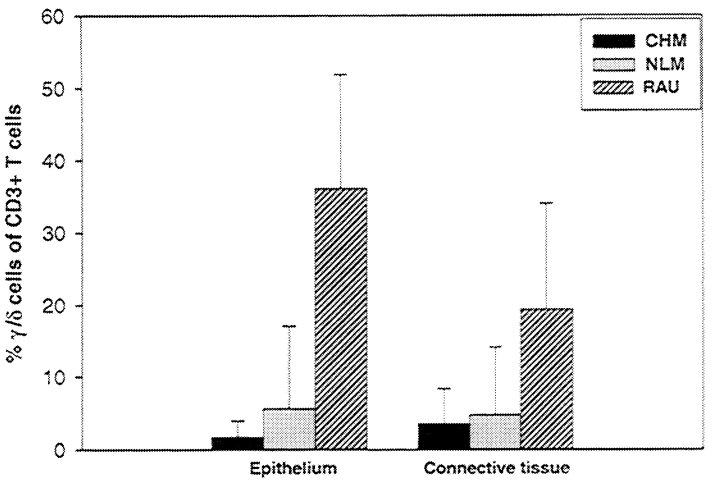

Fig. VIII. *Percentage of γ/δ T-cell counts expressed as a proportion of CD3+ T-cells in clinically healthy mucosa (CHM), non-lesional mucosa (NLM) and RAU.*

DISCUSSION

"The probability of success is difficult to estimate, but if we never search, the chance of success is zero"
Astrophysicist Giuseppe Conconi concludes about searching of extraterrestrial intelligence

Diagnosis

Although the criteria for diagnosing RAU were applied to all RAU patients recruited in this study, the use of these criteria for diagnosing RAU has some limitations. First, there is no known laboratory procedure available to establish the definite diagnosis, and histopathological examination of the biopsies do not provide such a definitive diagnosis. Second, detailed virological investigations of lesional tissue or serum are usually not warranted, except to exclude atypical herpetic infection (Porter et al. 1998).

However, the high reproducibility and uniformity of the clinical picture and course in all our patients suggests that all the RAU samples that were included in this study represent recurrent aphthous ulcers. Furthermore, all patients with features suggesting systemic disease, immunodeficiency state or neutropenic ulcers were excluded.

In spite of the fact that RAU is a common condition, its histopathology and time course have been rather infrequently studied. For instance, studies on RAU lesions at the pre-ulcerative stage are few in number and include very few subjects, probably because biopsy specimens of true pre-ulcerative stage of RAU are extremely difficult to obtain (Stenman and Heyden 1980). Therefore, in this study we dealt only with biopsies obtained from ulcerative and painful lesions, 2-10 days after their development.

The selection of a severe form of RAU conditions (defined as one attack of RAU per month or more for a period of at least one year) in this study was done to maximize the

possibility of observing significant changes that may occur at the cellular or subcellular level. Although the patient group in each individual study was small, there were no remarkable differences in the histopathologic features of RAU. This would suggest that the findings in this study may be generally applicable.

Staining and evaluation methods

Most of the biopsy specimens in this study were stained by the ABC staining method introduced by Hsu and et al. (1981). The strong affinity of avidin for biotin gives this method high sensitivity and usually gives excellent results on fixed, paraffin-embedded specimens.

The peroxidase-anti-peroxidase (PAP) method has been used in study II instead of the ABC method to avoid possible interference of tryptase staining with non-immunochemical staining of MCs as a result of endogenous avidin-binding activity of MC granules (Hsu and Raine 1984). To reduce the problem of non-specific background staining, we used mostly monoclonal antibodies for their ability to bind specifically to one epitope on the immunogen, and separate incubations with blocking normal sera prior to application of the primary antibodies were used to inhibit non-specific immunoglobulin and other protein binding sites.

In this study, a 10% neutral buffered formalin solution was used as a fixative because it is well tolerated by the tissues, has good penetration, and gives clear morphological details (Leong 1993). Furthermore, it is suitable for identification of cytoplasmic and surface membrane antigens (Farmilo and Stead 1989). Acetone was used in (study V) because it produces good preservation of surface membrane antigens in cryostat sections (Farmilo and Stead 1989). To achieve maximal staining sensitivity, the signal-to-noise ratio was increased by suppressing the endogenous peroxidase activity with methanol-H_2O_2 treatment in addition

to the normal serum blocking step (Petrusz 1983). The semi-automatic VIDAS image analyzer version 2.1 employed in this study has the advantages of accuracy, repeatability, and ease of use.

Control specimens

Both of the control groups and RAU patients in this study were comparable in age factor but were not well matched in gender factor as the controls and RAU groups were not equal in sample size and the the number of female participants in this study were slightly more than the males. A third set of control samples were obtained only for study V by taking specimens from the normal appearing mucosa of RAU patients, at sites contralateral to RAU lesions. These control groups were considered as very appropriate for detection of any specific cellular changes (concerning the topics dealt with in this study) that have occurred at inflammatory sites of RAU. Labial and/or buccal areas were selected as sampling sites for clinically healthy control mucosa to match the same location areas of RAU lesions. Clinically normal oral mucosa consisted of stratified squamous epithelium supported by fibrous connective tissue. There was a small number of scattered chronic inflammatory cells in the connective tissue areas. This could be explained by the fact that oral mucosa (due to its anatomical state and function) is especially exposed to stimuli of physical, chemical and microbiological origin. These noxious effects may increase the inflammatory cells pool within the mucosal compartment.

Buccal mucosa was chosen as a site for induction of TUs to match the location of RAU lesions, and for convenience, i.e. easy accessability and less painful than other areas. We chose only those volunteers with a negative history of RAU for induction of TU lesions

because a local trauma may trigger RAU in people susceptible to aphthous ulcerations but not in healthy controls (Wray et al. 1981b). The histology of TU differs from that of RAU in the less marked mononuclear and perivascular mononuclear inflammatory cell infiltration, a more pronounced neutrophil leukocyte and fewer MCs. The control tissue samples were fixed and processed identically with the diseased RAU samples to avoid artefactual differences.

Factor XIIIa+ dendrocytes (I)

Factor XIIIa is expressed by subepithelial dendrocytes. Factor XIIIa as a marker has the advantage of staining subepithelial dendrocytes but not epithelial Langerhans-type dendritic cells. Langerhans cell-independent pathway is another pathway for handling the antigens that are formed and/or enter via the subepithelial compartment (Streilein 1989, Yardy Tse and Cooper 1990, Van Wilsem et al. 1994). It is conceivable that Langerhans cell-independent pathway may play an important role in RAU inflammation because the starting focus of RAU pathology lies in the subepithelial compartment with early accumulation of mononuclear lymphoid cells (Stanley 1972).

FXIIIa + dendrocytes seem to be involved in the antigen handling in the connective tissue, because they are much more frequent than Langerhans' cells and because they are capable of presenting antigen *in vitro* and possibly *in vivo* (Yardy Tse and Cooper 1990). Moreover, the antigen-presenting potency of FXIIIa+ cells has been found to equal that of dendritic cells isolated from epidermis or blood (Nestle et al. 1994, Nestle and Nickoloff 1995b).

In this study we found that FXIIIa+ cells may acquire a spindle-shaped, rounded or dendritic appearance, which may reflect differences in maturation development or cellular and functional diversity of cells that express FXIIIa (Cerio et al. 1989, Ádany et al. 1988, Penneys

1990). Interestingly, the morphology of the FXIIIa+ cell population in RAU and TU lesions had changed markedly towards dendrite-like (and, to a minor extent, round) at the expense of the spindle-shaped cells. And many of these cells appeared to have enlarged (up to two times as large as that of control) and revealed long processes.

A constant finding in the present study was the appearance of numerous FXIIIa+ dendrocytes interspersed among lymphocyte infiltrates and in close proximity to many blood vessels. This finding contradicts partially previous results from other group (Regezi et al. 1993) showing enlarged factor XIIIa+ dendrocytes but without increase in the the density of such cells in RAU in HIV-positive patients. This discrepancy could perhaps be explained by the differences in the subjects of study, as they have used only HIV-positive patients for study and they did not use mucosa from healthy control for comparison.

We found that FXIIIa+ dendrocytes were in close contact with the infiltrating lymphocytes. Such observations suggest that FXIIIa+ dendrocytes may serve as antigen-presenting cells at the connective tissue compartment to infiltrating T lymphocytes after the emigration of these T cells from the blood vessels into the site of inflammation (Cerio et al. 1989, Sontheimer et al. 1989, Lappin et al. 1996). This view might be further supported by our previous finding in study (IV) that at least part of the infiltrating lymphocytes were replicating locally in the perivascular areas in RAU lesions.

Moreover, it has been found that MCs are intimately associated with dermal dendrocytes (Yoo et al. 1998) and that FXIIIa expression by dermal dendrocytes increases when MCs degranulate and release TNF-" (Sueki et al. 1993). These data, in addition to our findings on the functional activity status of MCs in RAU (study II), suggest a close relationship between these cells at an anatomical and functional level. Furthermore, the XIIIa+

dendrocytes accumulated also in the connective tissue, lateral to inflammatory infiltrates of RAU, which may suggest a regulatory function in this site since these cells are also believed to have a role in extracellular matrix deposition and regulation of connective tissue remodeling and repair that may accompany the inflammation process (Hermanns-Le et al. 1999, Monteiro et al. 1999).

Overall, the results demonstrated that FXIIIa+ dendrocytes were increased in number and size in RAU, found in abundance in lamina propria, within the inflammatory cell infiltrates and in deeper connective tissue, particularly in association with blood vessels. They may have a prominent role as antigen presenting cells in the immuno-surveillance system of the submucosa, and/or are involved in tissue repair and extracellular matrix deposition.

Mast cells (II)

MCs are effector cells of inflammation and immunity (Galli et al. 1999). Their specific locations within tissues, immediately beneath and, in some cases, within epithelial surfaces as well as near blood vessels, nerves and glands and their ability to respond quickly to changes in the immune status of their microenvironments (Wedemeyer et al. 2000), led us to propose a potential role for MCs in RAU pathogenesis.

There are remarkable similarities between the triggering factors for MC degranulation such as drugs (Bosso et al. 1991, Horinouchi et al. 1993), hormones (Tsakalos et al. 1993), infections (Sugiyama 1977), stress (Gui 1998; Alexacos et al. 1999; Wilson and Baldwin 1999) and trauma (Elsayed and Dyson 1993) and those factors precipitating RAU such as stress, food, drugs, hormonal changes, trauma and infections (Porter et al. 1998). Previous studies on the frequency of MCs in RAU lesions have yielded conflicting results, as being either increased

(Lehner 1969a), unchanged, or decreased (Dolby and Allison 1969b, Müller and Lehner 1982) in RAU lesion.

Tryptase and chymase, are the two major MC proteinases. “Connective tissue” MC population expresses both tryptase and chymase (MC_{TC}), while “mucosal” MCs express tryptase but no chymase (MC_T). However, the oral mucosa contains a mixed population, with a predominance of "connective tissue" phenotype (Walsh et al. 1995). In this study, tryptase, which present almost exclusively in the granules of human MCs and expressed by both subtypes of MCs of the oral mucosa is employed as a specific marker for MCs.

It is possible that some MCs have totally released their granular contents leading to the formation of “phantom MCs”, and this could lead to an erroneously "low number" of MCs. I therefore used surface bound IgE as a second marker, because it is not affected by excessive degranulation of MCs.

The results obtained with the anti-IgE staining were similar to those found by tryptase staining, suggesting that tryptase is a very sensitive marker for MCs.

In normal oral mucosa and in the majority of RAU samples, MCs were not found in epithelium. It was an interesting finding that some MCs were localized intraepithelially, close to the basal cell layer in 3 of 15 RAU lesions. MCs can infiltrate the epithelium in certain circumstances, especially in parasitic and allergic inflammations (Fokkens et al. 1992). The presence of MC in the epithelium of only 3 RAU lesions may have a number of interpretations. One attractive possibility is the heterogeneity of RAU with respect to etiology. RAU may represent a single condition with multiple causes and the etiology of these 3 RAU lesions could be due to allergic cause.

Localization of MCs near, or even in, the epithelium may represent an interaction

between active degranulated MCs and epithelium of RAU. However, the absence of MCs in the area close to the ulcer suggests that ulceration probably was not dependent upon direct MC involvement. An indirect role in the early events cannot be ruled out, in view of our observation of increased MC density and activity at the basement membrane zone, lateral to the aphthous ulcers. The degranulation of MCs in close proximity to the basement membrane results in the release of tryptase and chymase, which can promote both the inflammatory reaction and the cleavage and activation of enzymes that are known to promote degradation of the basement membrane component (Gruber et al. 1990a, Lohi et al. 1992, Häyrinen-Immonen et al. 1993). They can therefore participate in the formation of the ulcer. Conversely, the mere presence of MCs and their degranulation activity at the basement membrane zone does not necessarily mean that they play only a destructive role. MCs might be involved in the repair process. For instance, MC tryptase was found to be a mitogen for epithelial repair and to facilitate recruitment of granulocytes (Cairns and Walls 1996). In this respect, higher proliferation activity was found in the RAU epithelium than in TU epithelium of similar lesional duration (study IV). Furthermore, Thompson and associates (1991) have provided evidence that certain murine MC lines are capable of laminin synthesis. Thus MCs in regions lateral to aphthous ulcers may contribute to increased epithelial mitosis and/or formation of the basement membrane. The possibility that MCs were able to perform different or even opposite functions during the course of RAU inflammation or at the different microenviromental areas in RAU is not surprising, as MCs can respond to antigens, pathogens or their products by undergoing alterations in phenotype, function, survival, and as some of these changes can be reversible (Galli et al. 1999).

Although this study showed that the majority of the MCs in RAU were apparently

degranulated, it was difficult to ascertain whether a partial degranulation of MCs in the tissue was due to an inflammatory process or due to sampling and processing procedures. However, the presence of a “halo” of tryptase around a proportion of MCs in the tissue sections is a reliable indication of MC degranulation (i.e. the released granules lost their round shape and appeared as diffuse staining around the cell indicating that dissolution of the contents of such granules have occurred in a time preceded the sampling and processing procedures). For this reason, this paper focuses mainly on those observations where halos of tryptase around some MCs were encountered.

The significant increase in MC numbers and degranulation within the RAU mononuclear cell infiltrates, compared with those seen in TU inflammatory cell infiltrates, suggests that such an increase is not a response to ulceration in general but is more specific to aphthae.

The functional significance of MC accumulation in the connective tissue along the borders of the inflammatory infiltrates, especially at the interfaces between the inflammatory infiltrates and connective tissue, is unclear. MC degranulation at these interfaces may contribute to localized degradation of extracellular matrix, both by the stimulation of matrix-degrading enzymes, metalloproteinases (MMPs) production by resident connective tissue cells (Yoffe et al. 1984, Yoffe et al. 1985, Häyrinen-immonen et al. 1993, Kanbe et al. 1999), and by the subsequent activation of released inactive precursors (proMMPs) by the MC proteinases (Gruber et al. 1990b, Lees et al. 1994, Suzuki et al. 1995). Recently, it was also found that chymase is able to cleave and activate procollagenase directly (Saarinen et al. 1994), and can function as an interleukin-1$ convertase (Mizutani et al. 1991). Thus, both tryptase and chymase of MCs in RAU may initiate a sequence of events leading to degradation of the extracellular matrix, which is an essential step for inflammatory cell movement and migration

through extracellular tissues.

Although the majority of RAU lesions were early, i.e. 2-3 days old, the results show that the age of RAU and TU has no appreciable effect on MC density in such lesions. Our data showed that MCs are 63% more numerous in RAU than in clinically healthy mucosa, which is less than that reported by Lehner (1969a), who described a 100% rise in the number of MCs in minor aphthous ulcers. However, it is in conflict with the results of Dolby & Allison (1969b), who reported no difference in the number of MCs between controls and early (1-2 days old) RAU and also described a reduction of MC numbers in lesions of more than 2 days duration. The most likely explanation for this discrepancy is the use of different stains like toluidine blue or alcian blue/safranin to identify and localize MCs in the two previous studies. However, the markers and methods employed in the present study were more sensitive and allow all MCs in the tissues to be detected, regardless of their phenotype and state of degranulation.

Our results, however, provide no clues as to the reasons for increased MCs in RAU lesions. Finally, our results emphasize the importance of tryptase enzyme as a marker for the localization and activation of MCs. The significant increase in number and degranulation activity of MCs in aphthae suggests an active role for MCs in the pathogenesis of RAU.

TNF-" (III)

Although a previous study showed mRNA for TNF-" in inflamed RAU mucosal tissue, little is known about its cellular distribution at sites of RAU inflammation. To address this point immunohistochemistry was used to determine the cellular localization of TNF-" in RAU lesions compared with TU lesions.

Our results showed that there was no TNF" immunoreactivity in the epithelial cells. This was unexpected because epithelial cells participate in immune regulation and in the maintenance of mucosal integrity by generating a range of biologically active mediators. However, this does not mean that these cells are not producing and secreting this cytokine. It is possible that such cell types may contain insufficient TNF-" to be demonstrated by our immuno-histochemistry, or that the epithelium of RAU is not an important source of this cytokine. However, we cannot rule out the possibility that epithelium may act as a target for TNF-" that was produced by infiltrating inflammatory cells. TNF" has been found to possess *in vitro* cytotoxic activity against a variety of tumor cells (Williamson et al. 1983, Deem et al. 1991) and to a lesser extent against some normal cell lines depending on the concentration of TNF-" and on the simultaneous presence of other cytokines (Taverne et al. 1987, Wu et al. 1996). Although the hallmark of RAU is the destruction of epithelium leading to ulceration, the relative importance of the role of cytotoxicity versus cytokine release in mediating epithelial cell death and aphthous ulceration is still unknown.

Although it has been reported that neutrophils can produce TNF-" (Dubravec et al. 1990), the immunohistochemical results did not show TNF-" immunoreactivity in neutrophils in either TU or RAU lesions. It may be that neutrophils do not produce TNF-" as a response to surgically induced trauma or to the antigenic stimulation that is supposed to trigger aphthous ulceration. However, TNF-" produced by other cells may act on neutrophils to promote their adherence to endothelial cells and migration to the inflammatory areas. TNF-" may also enhance phagocytosis, respiratory burst, reactive oxygen generation and degranulation of the neutrophils (Gamble et al. 1985, Shalaby et al. 1985, Klebanoff et al. 1986, Tsujimoto et al. 1986).

Because of the possibilty of slight nonspecific background staining it is difficult to make comment on TNF-" release into the extracellular compartment of RAU and TU lesions.

The present study shows that the expression of TNF-" is marked in macrophages and occurs mainly in mononuclear inflammatory cell infiltrates. TNF-" is secreted by activated monocyte/macrophages and many other cells in response to bacterial toxins, inflammatory products, and other stimuli (Vasalli 1992). But the precise factor(s) which activate macrophages to produce TNF-" in the RAU lesions remain(s) uncertain.

Our findings show that numerous lymphocytes in mononuclear inflammatory cell infiltrates in RAU biopsies were positive for TNF-". This means that these cells are activated, and express TNF-" in RAU lesions (Kinkhabwala et al. 1990, Deem et al. 1991).

The finding of TNF-" localized to MCs adds to the previous studies demonstrating TNF-" production by human MCs (Ohkawara et al. 1992, Walsh et al. 1991, Walsh et al. 1995). It is worthwhile to mention that only MCs are known to store preformed TNF-" in significant quantities and to release it upon immunologic stimulation (Gordon and Galli 1990). TNF-" derived from MCs and other adjacent mononuclear inflammatory cells seems to be a particularly important candidate for initiating the increased expression of cell adhesion molecules like ICAM-1 that has been described in blood vessels close to the inflammatory infiltrates and lateral to the aphthous ulcer sites (Häyrinen-Immonen et al. 1992b). MCs within mononuclear inflammatory cell infiltrates showed weaker TNF-" immunoreactivity than those MCs in deeper connective tissue. This weak TNF-" immunoreactivity in MCs might be due to ongoing production and release of this cytokine. This is also in line with the previous findings that showed prominent activation and degranulation of MCs in RAU inflammation (study II).

Moderate immunoreactivity for TNF-" was observed in some vascular endothelial cells at the lamina propria lateral to the ulcer and in the blood vessels of deep connective tissue. This suggests that the production of TNF-" by RAU vascular endothelium is minimal and the major sources of TNF-" that act on vascular endothelium in RAU are the adjacent mononuclear inflammatory cells.

The difference in TNF-" immunoreactivity between RAU and TU controls appears to relate to an increased expression of this cytokine in inflammatory cells in RAU. However, the detection of TNF-" containing cells in TU lesions is not unexpected, since numerous infiltrating inflammatory cells are usually present within the inflammatory infiltrates in TUs, and local expression of inflammatory mediators is an essential component of the normal healing process.

In view of the high density of TNF-" immunoreactive cells in RAU lesions and the well characterized effects of this cytokine, it is probable that TNF-" makes a significant contribution to inflammatory events in RAU lesions.

Cell proliferation in RAU lesions (IV)

As a result of mediators released in the vicinity of postcapillary venules, leukocyte adhesion molecules are induced on endothelial cells. The circulating lymphocytes then roll, adhere and transmigrate into inflammation areas via local blood vessels. Once in the perivascular compartment, extravasated lymphocytes may produce a variety of pro-inflammatory molecules, and the immune response is amplified by local T cell proliferation stimulated by interaction between these cells and antigen-presenting cells in the connective tissue compartment.

In this study we raised the issue of the relatively long persistence of RAU lesions and whether the cell proliferation in RAU lesions differs from that of the TU lesions.

Ki-67 used in this study is a good marker of cell-cycling, has a short half-life (90 min), and the nuclei can be scored as either positive or negative for Ki-67 immunoreactivity, (Bruno and Darzynkiewicz 1992, Duchrow et al. 1994).

The absence of epithelial outgrowth in 6 out of 10 RAU might be due to ongoing epithelial destruction or inability of the cells at the edge of the ulcer to switch from the stationary to locomoting epithelial phenotype. For instance, Richards *et al.* (1996) found disruption in the deposition of laminin-5 and an apparent lack of fibronectin (which provides the provisional matrix for subsequent cell migration) at the edges of some of the RAU lesions (Grinnell et al. 1981).

The low mitotic activity in the epithelial outgrowth of both RAU and TU suggests that the advancing epithelial tongue, which extended over the ulcer margins, involves migratory, nondividing cells and only to a lesser extent proliferating cells.

Our results with the Ki-67 proliferation marker were mostly in line with the previous reports showing that DNA synthesizing cells in the oral mucosa are mainly localized at the lowermost part of the epithelial rete ridges, with only few DNA synthesizing cells overlying the connective tissue papillae (Hume 1989).

Few intraepithelial lymphocytes had displayed Ki-67 positivity, but their distinguished appearance from epithelial cells made it possible to demonstrate that most of the proliferating cells in RAU are epithelial cells.

Our finding of increased mitotic activity in RAU epithelium and the clinical observation that RAU lesions last longer than TU (Stanley 1972, Richards et al. 1996) seem paradoxical.

However, epithelial cell proliferation is not the sole determinant of re-epithelialization (Garlick et al. 1996). It seems likely that the epithelial cell migration is delayed in RAU lesion when compared with TU, as suggested by the absence of epithelial outgrowth in 6 of 10 RAU samples. The absence or inappropriate expression of keratinocyte integrins or their extracellular ligands as a result of inflammation may explain such an apparent paradox (Richards *et al.* 1996).

This immunostaining study gives no definite clues as to the type of inflammatory cells involved in proliferation, but it confirms that there are proliferating cells in the inflammatory infiltrates. Our results showed an inter-individual variation in the proportion of proliferating cells in the inflammatory cell infiltrates in RAU (9±4% of inflammatory cells were proliferating), which may reflect the dynamic nature of cell activation in RAU, the interpatient variations and the possible variation in the concentration and presentation of the antigens, i.e. it may imply that not all RAU lesions contain the same amount of triggering antigen(s).

Although the majority of infiltrating inflammatory cells in TU lesions were not proliferating, the presence of some cycling lymphocytes in TU infiltrates could represent physiological processes that act to stimulate epithelial repair and protect against infection. In this respect Reusch and colleagues (1991) found that lymphocytes infiltrate the epidermis in sterile wound healing, which suggests that in the absence of foreign antigenic stimulation, the immune system can be activated by traumatic injury. The antigen presenting cell system has been suggested to be activated by microenvironmental tissue damage (Ibrahim et al. 1995) which leads to immune stimulation (Evavold et al. 1993) and subsequently to activation and proliferation of the infiltrating lymphocytes.

In situ T cell proliferation has been shown to be a feature of several diseases believed

to be immunologically mediated (Wood et al. 1986, Séron et al. 1989, Morganroth et al. 1991), which suggests that it is a mechanism which operates in different inflammatory lesions and is not restricted to RAU inflammation.

It has been previously reported that 45% of all lymphoid cells in RAU lesions show signs of previous antigenous contact (Häyrinen-Immonen 1991). Antigen-experienced cells can be activated by a broader range of antigen presenting cells, respond faster and more effectively upon new encounter with the antigen (even outside the context of the lymphoid tissues) than naive cells (Byrne et al. 1988). They may represent the cellular basis for the high proliferative response at sites of RAU lesions.

In conclusion, our results demonstrate that increased proliferative activity is present in RAU epithelium and that the longer persistence of RAU lesions (compared to TU) cannot be explained by a lack of new epithelial cell production. Furthermore, mononuclear cell replication *in situ* represents one mechanism for the expansion of the inflammatory cell infiltrates at the sites of RAU and TU lesions.

Intraepithelial lymphocytes (V)

Previous studies have found a high percentage and activity of (/* T cells in peripheral blood of patients with RAU and Behçet's disease (Suzuki et al. 1992, Pedersen and Ryder 1994, Freysdottir et al. 1999). Such findings imply a possible role for (/* T cells in the immunopathogenesis of RAU.

The early changes in RAU lesions are characterized by the early influx of lymphocytes and neutrophils (Savage et al. 1985) into the inflammatory RAU site via blood vessels in lamina propria. Initially, there is subepithelial aggregation and proliferation of lymphocytes *in*

situ and, subsequently, active intraepithelial migration.

The sequence of cellular inflammatory events in RAU inflammation culminates in a marked migration of lymphocytes into the epithelia. It seemed therefore important to study the proportion of (/* T-lymphocytes among the lymphocytes in the epithelium and in the subepithelial compartment of RAU lesions. Furthermore, there have been, to my knowledge, no reports concerning the occurrence of (/* T-cells in RAU lesions. In the present study, the distribution and frequency of (/* T-cells in RAU lesions were analyzed immunohistochemically.

The present data showed that the epithelium of RAU lesions contains three times as many CD3 positive lymphocytes as the epithelium of controls. These findings seem to agree with most former pathological studies that have found frequent infiltration of lymphocytes to the lower epithelial layers in RAU lesions (Lehner 1969a). In the present study, most of the CD3 lymphocytes in all samples expressed the "/$ TCR. Although we found that the absolute count of "$-TCR positive cells was increased in RAU lesions, their relative proportion was reduced compared with the mucosa of controls, apparently because of the relative increase of (/* T-cells.

Lymphocytes expressing (/* TCRs were found to comprise a small proportion (-2% of CD3+ T-cells) in the healthy control mucosa, a value similar to that previously reported (Pepin et al. 1993). In contrast to the results obtained in mice (Itohara et al. 1990), (/* T-cells were uncommon in the epithelium and in the connective tissue of normal human oral mucosa.

Previous work has shown an increased proportion of (/* T-cells in the peripheral blood of patients with RAU during both the inactive and active phase (Pedersen and Ryder 1994). Furthermore, Suzuki et al. (1992) have shown that the concentration of circulating (/*+ T-

cells was higher in patients suffering from Behçet's disease with mucocutaneous RAU compared to patients without mucocutaneous lesions. In the present retrospective study we were unable to determine if the increased percentage of (/* cells in RAU lesions simply reflected their high numbers in the peripheral blood.

Our results demonstrate a high density of (/* T-cells in inflammatory lesions in RAU, whereas, in non-lesional mucosa from opposite sites of RAU, (/* T-cells were rarely seen in the epithelium or in the connective tissue areas.

The requirements for (/* T-cell activation are less stringent than those for "/$ T-cell activation. For instance, (/* T-cells can recognize antigens directly, without the need for specialized antigen-presenting cells (Schild et al. 1994). Furthermore, (/* T-cells can also react to non-peptide antigens such as a lipid fraction of *M. tuberculosis* (Tsuyuguchi et al. 1991) and various phosphorylated metabolites (Porcelli et al. 1996). Such features of (/* T-cells would probably enable them to respond quickly to a variety of antigens before the "/$ TCR lymphocyte population begins to expand (De Libero 1997).

The bowel affected by coeliac disease contains higher than normal numbers of (/* T-cells in either active or "silent" periods of the disease (when the pathology is mildest). Furthermore, the density of such cells is higher in the epithelium than in the lamina propria (Kutlu et al. 1993). Such an expansion of (/* TCR positive intraepithelial lymphocytes might be driven by an intestinal allergy to cereal proteins associated with autoimmunity directed against tissue transglutaminase. In aphthae, the high density of (/* T-cells was present exclusively in RAU lesions and in both the epithelium and the subepithelial connective tissue. Thus, it is possible that some of the (/* T-cells were responding in an antigen-specific fashion and that antigenic stimuli inductive for (/* T-cells were expressed only during the

development and/or progression of RAU.

The exact biological function of (/* T-cells in the epithelium of RAU lesions remains unclear. However, Boismenu & Havran (1994) have shown that activated (/* T-cells produce the epithelial cell-specific fibroblast growth factor-7 (FGF-7). The effects of FGF-7 on the proliferation and differentiation of epithelial cells may help to reduce mucosal damage following tissue injury (Zeeh et al. 1996). This is in line with the findings of study (IV) on the expression of the cell proliferation-associated nuclear antigen (Ki-67) in RAU lesions that showed higher proliferation activity in the epithelium of RAU compared with that in the epithelium of controls.

Many reports have shown that different sub-populations of (/* T-cells have different functions and different patterns of cytokine secretion. For instance, Ferrick et al. (1995) found that (/* T-cells can produce either T-helper type-1 (Th1) or Th2 cytokines depending on the pathogen they are exposed to. However, the role of (/* T-cells may not be limited to merely enhancing epithelial proliferation or modulating immune responses of "/$ T cells (Fujihashi et al. 1996b). For instance, other investigators (Hamzaoui et al. 1994, Hasan et al. 1996) have suggested that (/* T-cells may play a role in immunological damage in RAU. The "Janus-like" coexistence of the cytotoxic and protective potential of (/* T-cells might enable them to play a dual function in the pathogenesis of RAU, thereby acting as immunoregulators to maintain the homeostasis and functional integrity of the epithelial barrier.

It should be pointed out that there was a wide variation in the density of (/* T-cells between RAU samples in this study. Similar wide inter-individual variations were also noted in the peripheral blood of Behçet's disease (Suzuki et al. 1992) and RAU patients (Pedersen and Ryder 1994). The reasons for these variations are currently unknown.

Although our results provide no answers regarding the function of (/* T-cells in RAU lesions, they showed that (/* T-cells are increased in RAU lesions and such an increase was concomitant with the RAU inflammatory process and did not extend to mucosal areas other than the inflammation sites.

SUMMARY & CONCLUSIONS

“Creativity is the ability to find the alternatives”
Edward De Bono, a contemporary thinker

For better understanding of RAU, it will be necessary to study the immunocompetent cells and the key pro-inflammatory cytokine TNF-" that are likely to have a role in aphthous ulcer pathogenesis. The purpose of these studies was to investigate the distributions and frequencies of the factor XIIIa-positive dendrocytes, mast cells, CD3+ lymphocytes and its subtype "$ and (* T-cells, as well as to evaluate the expressions of the pro-inflammatory cytokine TNF-" and the proliferation-associated nuclear antigen Ki-67 in RAU lesions compared with clinically healthy oral mucosa and/or with induced traumatic oral ulcers. Samples were incubated with the relevant antibodies (polyclonal rabbit anti-human factor XIIIa, monoclonal antibody specific for MC tryptase (AA1), polyclonal anti-IgE antibody, monoclonal antibodies specific for CD3, "/$ TCR and (/* TCR, monoclonal anti-human TNF-" antibody, rabbit anti-human Ki-67 nuclear antigen) in avidin-biotin-peroxidase complex (ABC) staining or with peroxidase-anti-peroxidase (PAP) complex staining. Results were quantified by means of VIDAs image analyser or conventional microscopy.

Most of the FXIIIa-immunoreactive cells in TUs and normal mucosa were spindle-shaped, whereas a relatively large, dendritic-like cell type was predominant in RAU lesions. FXIIIa+ dendrocytes were increased in number, and apparently also in size, in RAU lesions (274±68 cells/mm^2) as compared to controls (177±74 cells/mm^2, P<0.01), and to TU lesions (183±50 cells/mm^2, P<0.01). MCs were absent in the epithelium except in 3 out of 15 RAU lesions. MC activation/degranulation as judged by diffuse extracellular tryptase staining, was a common feature within RAU-inflammatory infiltrates and at RAU-inflammatory infiltrates-

connective tissue interfaces, which were often associated with connective tissue disruption. MCs were significantly increased in aphthae (116±26 cells/mm^2) compared with TU lesions (72±11 cells/mm^2, *P*<0.001) and healthy controls (71±16 cells/mm^2, *P*<0.001).

The number of lymphocytes expressing (/* TCRs was very low in non-lesional mucosa or in clinically healthy mucosa. By contrast, (/* T-cells were numerous and observed in all RAU lesions especially intraepithelially, within the inflammatory infiltrates and at perivascular locations. In epithelial areas, the average percentage of (/* T-cells among total CD3+ lymphocytes was increased from 2% and 6% in controls and non-lesional mucosa, respectively, to 36% in RAU. TNF-" immunoreactivity was seen in monocyte/macrophages, lymphocytes, MCs and vascular endothelial cells. TNF-"-containing cells were more numerous in aphthae (188±46 cells/0.2 mm^2) compared with controls (52±14 cells/0.2 mm^2, *P*<0.001). The epithelium of RAU contained 30% more proliferating cells than the epithelium of TU and about 9±4% of the infiltrating inflammatory cells in RAU lesions were Ki-67+ proliferating cells, compared to only 3±1% in TU inflammatory infiltrates.

In conclusion, the results of the studies show significant increase in density and obvious changes in the morphology and size of FXIIIa+ dendrocytes in RAU lesions. The increased number of MCs in RAU lesions, and the local MCs show signs of activation and degranulation suggesting an active involvement of this cell type in RAU pathogenesis. High expression of TNF-" in RAU lesions was seen in mononuclear inflammatory cells, MCs and vascular endothelial cells. This may reflect an active role for TNF-" in the recruitment and activation of leukocytes and MCs that are found in RAU. The expression of cell proliferation-associated nuclear antigen Ki-67 is increased in RAU compared with TU lesions.

The findings also indicate that local replication of the infiltrating mononuclear inflammatory cells represents another mechanism that may contribute to the formation of the inflammatory cell infiltrates at the inflammatorty sites of both RAU and TU. As in the peripheral blood of patients with RAU and Behçet's disease, (/* T-lymphocytes are also increased locally at the sites of RAU lesions suggesting that (/*T-lymphocytes may have a pathogenic and/or regulatory role in aphthous ulceration.

Finally, the progress of RAU research in recent years has been remarkable. However, many essential questions still remain: What is the etiology of RAU? Does RAU have a single etiology or multiple etiologies? Is the association of RAU with coeliac disease, HIV infection, and Behcet’s disease a meaningful guide towards elucidation of the etiology of RAU, or does it only represent a similarity between RAU lesions and similar ulcers in these other diseases? Future research may hold the answers to one or more of these questions.

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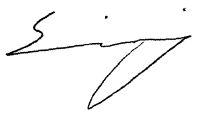

Sirajedin S. Natah

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Figure legends

Fig. I. Immunohistochemical staining of RAU lesion revealing FXIIIa+ dendrocytes within and at the periphery of the mononuclear inflammatory infiltrate of RAU. Note that cells are large with prominent dendritic processes, x160.

Fig. II. A diagram showing that the number of FXIIIa-positive cells is 50% higher in RAU than in TU or healthy control mucosa.

Fig. III. MCs are 63% more numerous in RAU than in control mucosa.

Fig. IV. The number of TNF-"-positive cells is 3 to 4-fold higher in RAU than in TU.

Fig. V. Tthe number of Ki-67+ cells in epithelium (EP) is 30% higher in RAU than in TU. Note also that the number of Ki-67+ cells in inflammatory infiltrates (II) is 3-fold higher in RAU than in TU lesions.

Fig. VI. An aphthous ulcer lesion showing numerous Ki-67+ cells at the interface between the neutrophil-rich area and the mononuclear inflammatory cell infiltrate. (ABC staining, x250).

Fig. VII. Numerous (/* T-cells are found in RAU lesion, particularly in the lower epithelial layers and in the basement membrane zone, x250.

Fig. VIII. Percentage of (/* T-cell counts expressed as a proportion of CD3+ T-cells in clinically healthy mucosa (CHM), non-lesional mucosa (NLM) and RAU.