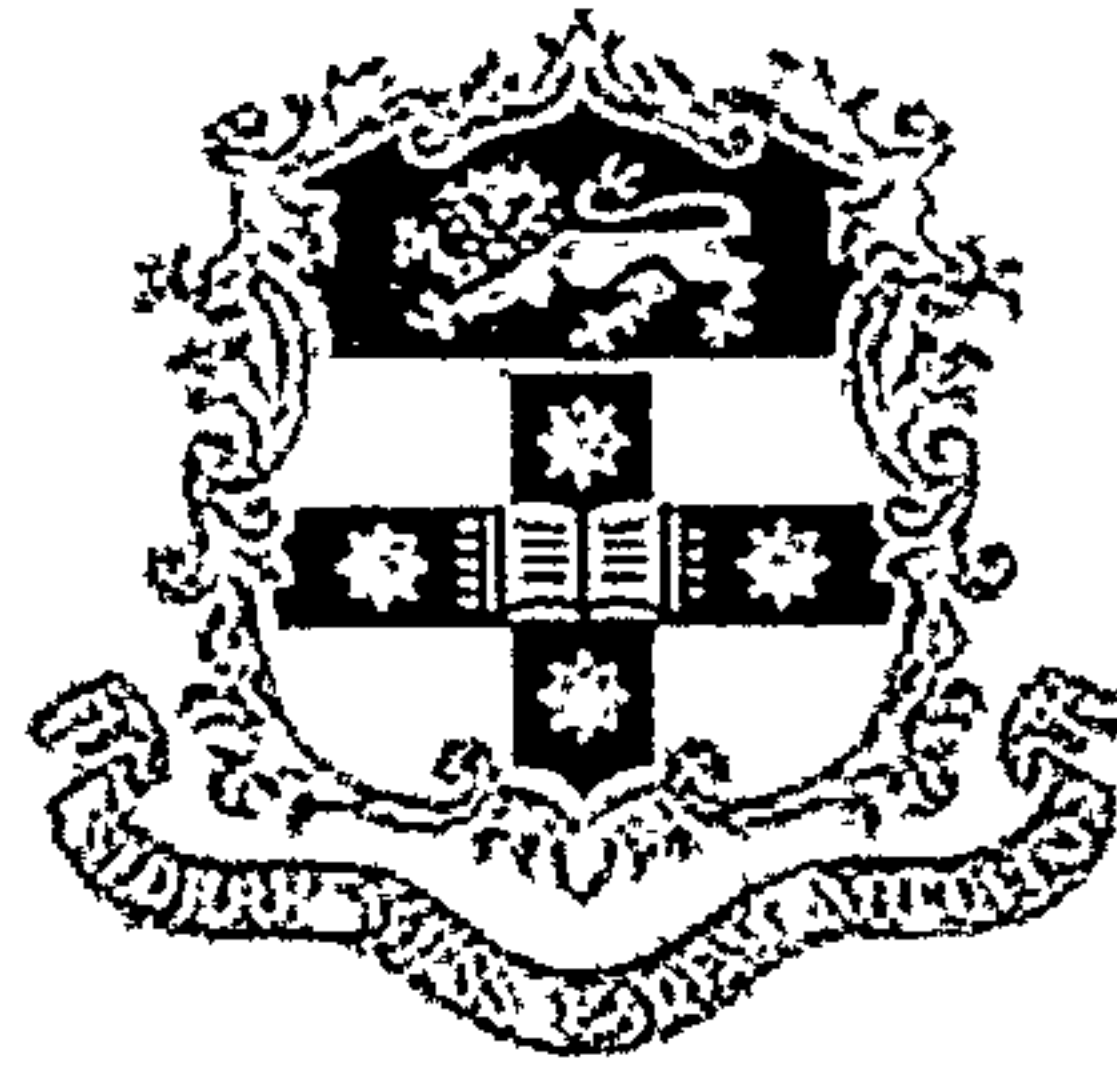

Faculty of Dentistry
The University of Sydney



**AUTOIMMUNITY IN CHRONIC
PERIODONTITIS**

A thesis submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy

Ping Ye

June 2003

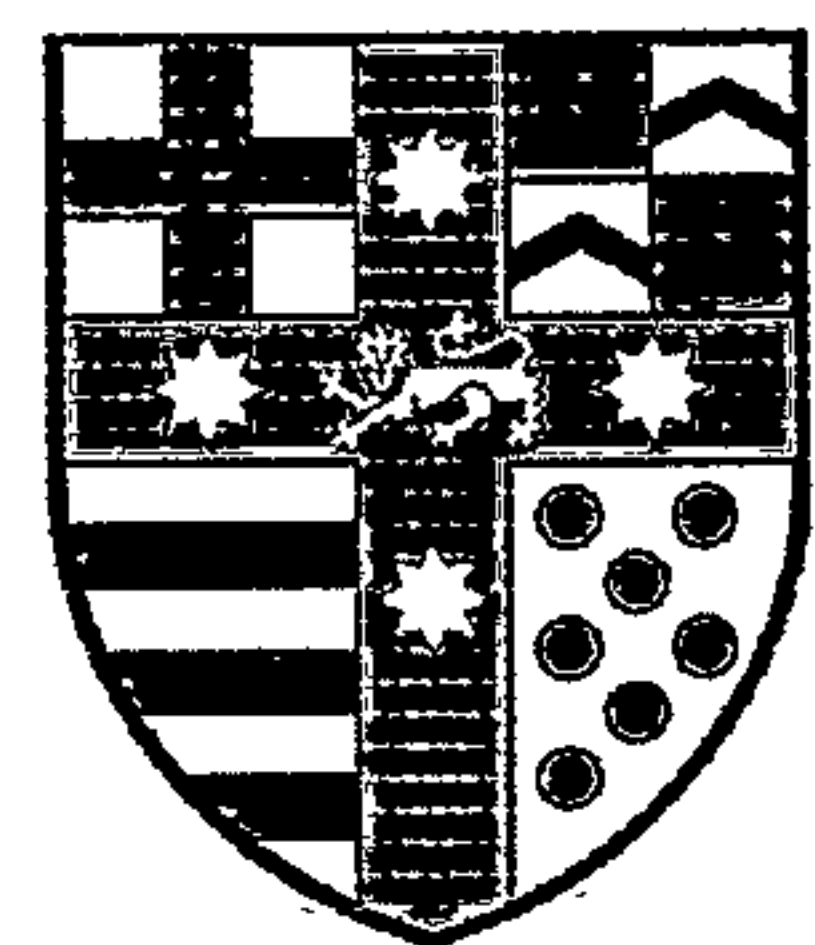


Institute of Dental Research

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FOREWORD

Structure of the Thesis

- **Chapter 1:** A brief review of the structure and function of the immune system is followed by an analysis of autoimmunity.

Subsequent experimental studies are presented in a self-contained paper format with linkage to preceding and following chapters. Some repetition of methods has been preserved where this facilitates analysis of the material presented.

Studies of tissue pathology

- **Chapter 2:** Structural proteins, E-cadherin, involucrin, and the gap junction connexins are considered to be important markers of tissue integrity, differentiation and communication in gingival epithelia.
- **Chapter 3:** Expression patterns of TGF- β s and their receptors in inflamed gingival epithelia are analysed with particular emphasis on the immuno-modulatory role of these growth factors.

Auto-reactive antibodies

- **Chapter 4:** The relationship of antibodies to epithelial components to disease indices.
- **Chapter 5:** Identification of a cluster of bacteria with potential to induce auto-reactive antibodies.
- **Chapter 6:** Identification of keratinocyte auto-antigens.

General discussion

- **Chapter 7:** A summary of thesis studies and the development of plans for future research in related areas.

- **Bibliography**
- **Appendices**

SUMMARY

Profound perturbation of epithelial structure is a characteristic feature of the immunopathological response to bacterial antigens considered to be central in the pathogenesis of the destructive lesion of periodontitis. The pathological basis for the disturbance of epithelial structure is not understood. It was demonstrated that the structural integrity and functional differentiation of the lining epithelium is compromised in relation to inflammatory changes associated with destructive periodontitis. In the pathological lining epithelium of the periodontal pocket there was a marked reduction of epithelial cadherin important in intercellular adhesion, of involucrin, a marker of terminal differentiation, and of the gap junction connexins that form intercellular communication channels. These changes were associated with alterations of filamentous actin expression, collectively indicating profound perturbation of epithelial structure. The data reported support the concept that the ability of the pathological lining epithelium to function as an effective barrier against the ingress of microbial products into the tissues is severely compromised (Ye *et al.*, 2000). In addition, a recent study (Ye *et al.*, 2003) by Western analysis of serum IgG from all 22 patients with chronic periodontitis tested indicated recognition of multiple epithelial components in individual patterns. In contrast, subjects with a healthy periodontium displayed only trace recognition of epithelial antigens. Levels of epithelial-reactive antibodies were significantly correlated with attachment loss as an indication of disease activity. To investigate a possible relationship between the bacterial flora adjacent to diseased sites and the presence of epithelial-reactive antibodies, subgingival plaque samples were taken from deep periodontal pockets and cultured anaerobically. Gram positive bacteria containing antigens potentially cross-reactive with epithelial cells were reproducibly isolated by probing membrane colony lifts with affinity-isolated (epithelial-specific) antibodies. The bacteria were identified as streptococci (*S. mitis*, *S. constellatus* and two *S. intermedius* strains) and *Actinomyces* (*A. georgiae*, and *A. sp. oral clone*) by 16S rDNA sequence homology. Recognition by affinity-isolated antibodies of antigens from the captured organisms was confirmed by Western analysis. Conversely, absorption of affinity-isolated antibodies with the bacterial species specifically reduced subsequent recognition of epithelial antigens. To identify the auto-antigens, a human keratinocyte cDNA expression library in Lambda phage was probed using pooled sera. Groups of

responders were detected for CD24 (a recently described adhesion molecule also known as P-selectin ligand), antioxidant protein 2 (a newly recognised member of the thiol-dependent anti-oxidant proteins), lactate dehydrogenase A, the transcription factor NFAT5, and for three genes encoding novel proteins. Six identified bacteria, especially *S. intermedius* were demonstrated to absorb antibodies reacting with identified auto-antigens in patterns varying between individuals.

This evidence indicated that during the course of periodontitis, subjects develop increased levels of antibodies to common oral bacteria amongst which are included tissue cross-reactive antigens. Periodontitis could therefore present a risk for the subsequent initiation or exacerbation of a broad spectrum of disease processes including autoimmune, inflammatory, proliferative and degenerative disorders.

DECLARATION OF AUTHENTICITY

The work described in this thesis was performed at the Institute of Dental Research, full time during the period from February 2000 to March 2003. All experiments were carried out by myself except where explicit reference to the work of others is given in the text or acknowledgments.

This thesis contains no material that has been accepted for the award of any other degree in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made.



Ping Ye

June 2003

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Lastly, I would like to thank the Department of Education, Training and Youth Affairs of Australia for the award of an International Scholarship that has given me the opportunity to perform Ph D studies.

PUBLICATIONS ARISING FROM THIS THESIS

Ye P, Chapple CC, Kumar RK, Hunter N. Expression patterns of E-cadherin, involucrin, and connexin gap junction proteins in the lining epithelia of inflamed gingiva. *Journal of Pathology* 2000; **192**: 58-66.

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Ye P, Harty DWS, Nadkarni MA, DeCarlo AA, Hunter N. Autoantibodies to epithelial components in periodontitis. 2001; reported in the *11th International Congress of Immunology, Stockholm, Sweden*.

Ye P, Harty DWS, Nadkarni MA, DeCarlo AA, Hunter N. Identification of auto-antigens in chronic periodontitis by antibody screening of a human keratinocyte cDNA library (in preparation).

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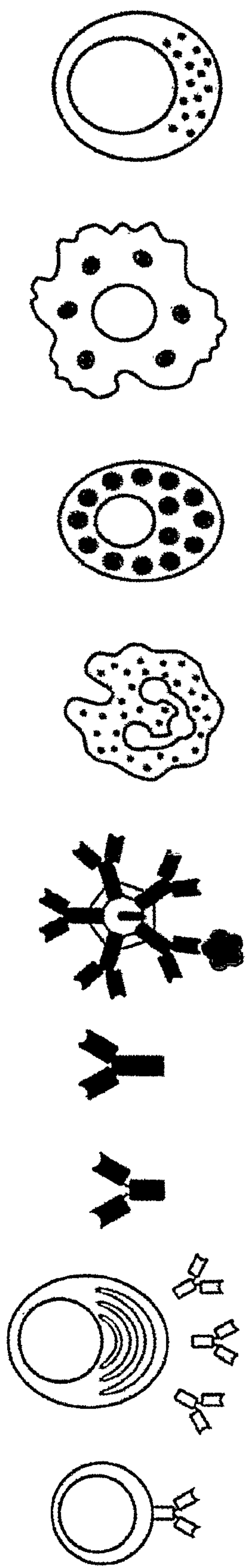
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CHAPTER 1

Literature

Review

AUTOIMMUNITY IN CHRONIC PERIODONTITIS

CHAPTER 1: AUTOIMMUNITY IN CHRONIC PERIODONTITIS

1.1 INTRODUCTION

Destructive periodontitis is characterised by the failure of the epithelial attachment to the tooth, resulting in a hyperplastic, poorly structured pocket epithelium associated with connective tissue changes including destruction of the specialised collagen fibre networks, manifest and characteristic vascular responses and perturbation of the local and systemic immune response. These changes are related to the action of microbial products, the low redox potential of the pocket creating an environment suitable for the abundant growth of anaerobic bacteria. The pathogenic processes of periodontal disease result in host responses to microbially-induced epithelial perturbation, tissue break down and local or systemic immune reaction. Although some microorganisms, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Bacteroides forsythus*, *Haemophilus actinomycetemcomitans* and *Treponema denticola*, have been considered as potential periodontal pathogens (Zambon, 1996), it has become apparent that pathogens are necessary but not sufficient for disease activity to occur (Socransky & Haffajee, 1992). An intensive research effort continues to study the bacteria that are responsible for periodontitis. However, the distribution of periodontal pathogenic bacteria is far wider than the distribution of periodontitis (Clarke & Hirsch, 1995). In many individuals the composition of the dental plaque flora is not substantially different in periodontitis versus gingivitis or health (Salvi *et al.*, 1997), indicating that the association between bacteria and periodontitis is insufficient to explain disease progression.

An autoimmune response in chronic periodontitis was suggested three decades ago by Brandtzaeg and Kraus (1965) but not proven. Since that time many investigators have raised the possibility of autoimmunity in periodontal disease (Gemmell *et al.*, 2002). Serum antibodies reactive with self-antigens have been reported in periodontitis patients (Govze & Herzberg, 1993; Anusaksathien *et al.*, 1992b; Anusaksathien & Dolby, 1991; Ranney, 1991; Jonsson *et al.*, 1991; Hirsch *et al.*, 1988). One mechanism for the generation of these antibodies is the existence of shared antigenic determinants in exogenous agents such as the oral flora (Ye *et al.*, 2003).

Possible mechanisms of autoimmunity in periodontitis:

1. Genetic predisposing, especially MHC genes.
2. Infectious agents, such as bacteria containing superantigens, which could elicit

antibodies cross-reactive with self-antigens.

3. Loss of self-tolerance.
4. Other factors may influence the development of autoimmunity in periodontitis, including aging, chronic stress, hormones, pregnancy and low birth weight, and other autoimmune diseases.

This chapter is divided into two sections. The first section presents an overview of immune system function in mucosa. The second section addresses possible autoimmune mechanisms related to the pathogenesis of periodontitis.

1.2 AUTOIMMUNE RESPONSES TO CHRONIC PERIODONTITIS

1.2.1 What are autoimmune diseases?

The immune system is a complicated network of cells and cell components (molecules) that normally work to defend the body and eliminate infections. Autoimmune disease occurs when a specific adaptive immune response is mounted against self, targeting the cells, tissues, and organs of a person's own body. There are many different autoimmune diseases, and they can each affect the body in different ways. For example, the autoimmune reaction is directed against the brain in multiple sclerosis and the gut in Crohn's disease (Hume & Radford-Smith, 2002). In other autoimmune diseases such as systemic lupus erythematosus (SLE), affected tissues and organs may vary among individuals with the same disease (Wolman *et al.*, 1988). One person with SLE may have affected skin and joints whereas another may have affected skin, kidney, and lungs. Ultimately, damage to certain tissues by the immune system may be permanent, as with destruction of insulin-producing cells of the pancreas in Type 1 diabetes mellitus (Joy *et al.*, 2002). Many of the autoimmune diseases are rare. As a group, however, autoimmune diseases afflict millions of people.

1.2.2 The epidemiology of periodontitis

Destructive periodontal disease remains amongst the most common infections of mankind. These insidious disease processes progressively lead to loss of function and culminate in the destruction of the dentition. About 35% of adults in the United States have periodontitis, of these, about 13% of adults over 30 years of age have a moderate or severe form of periodontitis; 22% have a mild form of the disease (Albandar *et al.*, 1999), and 10–15% of US adults are 'refractory' to therapy for chronic periodontitis

(Levine *et al.*, 2002).

However, morbidity rates for destructive periodontitis remain very high in developing nations particular in rural areas where logistical and financial constraints demand a different approach to the treatment and prevention of periodontal diseases. Thus national data for rural southern Thailand (Baelum *et al.*, 2003), obtained from 30-39- and 50-59-year-old rural Thais, indicated that the oral hygiene was poor with abundant amounts of both plaque and calculus. Gingival bleeding was essentially ubiquitous. The prevalence of attachment loss $>$ or $=$ 4 mm was 92% among 30-39-year-olds and 100% among 50-59-year-olds. The average percentage of sites affected in the two age groups was 23.9% and 63.9%. Pockets $>$ or $=$ 4 mm were seen in 84% of the 30-39-year-olds and in 93% of the 50-59-year-olds. These data may be indicative of the burden of periodontal disease in Asian populations.

Early-onset periodontitis is infrequent in all populations. Adult periodontitis is rather prevalent; however, advanced disease affects limited fractions of the population (probably less than 10 to 15%) (Papapanou, 1996). Mild periodontitis (PD) and moderate to severe PD infection were estimated to affect 22% and 13% of adults, respectively in US and Sweden (Albandar *et al.*, 1999; Norderyd & Hugoson, 1998). Destructive periodontitis is consistently more prevalent in males than females and more prevalent in African Americans and Mexican Americans than in whites (Borrell *et al.*, 2002; Albandar *et al.*, 1999).

1.2.3 How does the immune system work in periodontitis?

The immune system defends the body from attack by invaders recognized as foreign. It is an extraordinarily complex system that relies on an elaborate and dynamic communications network.

1.2.3.1 T and B cells

Two major classes of lymphocytes are T cells and B cells. T cells are critical immune system cells that help to destroy infected cells and coordinate the overall immune response.

During the course of migration through the thymic cortex immature thymocytes become clonally distinct on the basis of the structure of the T cell antigen receptor, thereby generating an enormously diverse repertoire for the recognition of antigens. This occurs by a process of random somatic translocation in genes contributing receptor

domains. Thymocytes are then positively or negatively selected on the basis of the capacity of the T cell antigen receptor to bind major histocompatibility antigens (MHC) expressed by thymic epithelial cells. Those thymocytes that bind receive thymic hormones released by the thymic epithelial cells, the so-called 'nurse cell' function. Current immune doctrine indicates that those thymocytes that bind with high affinity to self-antigens expressed in combination with MHC are killed by an active process of negative selection (Klein & Kyewski, 2000; Viret *et al.*, 2001; Clayton *et al.*, 1997). It is curious that this death signal in development is interpreted as a proliferative signal in the peripheral immune system. One possible explanation is that in the mature immune system co-ligation of MHC by CD4 expressed by the T cell, counteracts the apoptotic signal through the T cell receptor. The process of selection is not 'fail-safe' with evidence that small numbers of auto-reactive 'forbidden clones' thymocytes do escape as mature T cells into the peripheral environment (Lo *et al.*, 1997; Radvanyi *et al.*, 1993).

A key finding was that T cells recognize altered self-antigen expressed in combination with MHC. The importance of MHC structure in the control of the immune response is highlighted by the designation as immune-associated (Ia) genes. The selected evolutionary strategy of the acquired immune system is the presence of multiple alleles for genes encoding MHC i.e. an inherited basis for polymorphism. Variation in MHCII α and β chains will correspondingly determine the binding affinity for peptides of the antigen binding region contributed to by each polypeptide of MHC (Viret & Janeway, 2003; Stefanova *et al.*, 2002). Accordingly, individual variation in MHC platform will determine capacity to present particular antigens. In turn this will determine susceptibility to autoimmune diseases. In practice, the restricted expression of MHCII has the implication that auto-antigens would not normally be detected by auto-reactive T cells. This restriction breaks down, particularly in sites of chronic immunopathology such as periodontitis, where the release of interferon- γ from activated T cells powerfully induces MHCII expression by a wide spectrum of tissue cells (Yun *et al.*, 1999).

In birds, antibody producing lymphocytes undergo maturation in the gut-associated Bursa of Fabricius; hence the designation B cells. In mammals these cells have undergone maturation and selection in the bone marrow. Clonal diversity in B cells is generated by somatic mutation of genes encoding variable domains of immunoglobulins. Accordingly the structure and binding specificity of the

immunoglobulin receptor defines the clonal characteristics of B cells (Cooper, 2002). It is considered that selection of B cells is less stringent than for T cells, in part because B cells almost universally require T cell help to become activated, that is auto-reactive B cells normally would not pose a serious threat.

1.2.3.2 Macrophages and neutrophils

Macrophages and neutrophils are also important in autoimmune disease. Macrophages and neutrophils can destroy foreign antigens by making toxic molecules such as reactive oxygen intermediates. For example, in rheumatoid arthritis, reactive oxygen intermediate molecules and other toxic molecules are overproduced by macrophages and neutrophils invading the joints. The toxic molecules contribute to inflammatory damage.

1.2.3.3 MHC and co-stimulatory molecules

For a T cell to respond to a foreign antigen presented by MHC, another molecule on the antigen-presenting cell must send a second signal to the T cell. A corresponding molecule on the surface of T cells recognizes the second signal. These two secondary molecules of the antigen-presenting cell and the T cell are called co-stimulatory molecules. There are several different sets of co-stimulatory molecules that can participate in the interaction of an antigen-presenting cell with a T cell. Once the MHC and the T-cell receptor and the co-stimulatory molecules interact, there are several possible paths that the T cell may take. These include cell activation, tolerance, or death. The subsequent steps depend in part on which co-stimulatory molecules interact and how well they interact. For instance, Apolipoprotein E (ApoE) is synthesized by a variety of cells including macrophages. These cells activate T lymphocytes by antigen presentation, while the T-cell cytokine, interferon-gamma, inhibits macrophage ApoE expression. ApoE inhibits T-cell proliferation in culture. The ApoE-deficient (E0) mouse has enabled evaluation of the immunological role of ApoE by analysis of T-cell responses to an exogenous antigen (ovalbumin) and polyclonal mitogen (concanavalin A) in E0 and ApoE^{+/+} mice. Macrophages of E0 mice stimulated T-cell activation more effectively as antigen-presenting cells than macrophages from ApoE^{+/+} mice. Both proliferation and interferon-gamma secretion were enhanced in T cells activated in the context of antigen-presenting cells from E0 mice. Since macrophage-T-cell interactions depend on interactions between cell

surface molecules, the expression of such molecules was assessed after *in vivo* stimulation with interferon-gamma. This treatment caused an increased expression of the co-stimulatory surface proteins CD40 and CD80, and also of the major histocompatibility complex class II molecules I-Ab on macrophages of E0 mice compared with ApoE^{+/+}. The data suggest that ApoE inhibits T-cell activation by reducing the density of immune stimulatory proteins on antigen-presenting cells (Tenger & Zhou, 2003).

In relation to the lesion of chronic periodontitis, dendritic cells were demonstrated to be abundant in the inflammatory infiltrate; they were MHC-II positive, admixed with CD4-positive and CD8-positive T lymphocytes, and, they expressed CD54, CD80, and CD86. Dendritic cells often contacted lymphocytes and were also located within plasma cell aggregates (Mahanonda *et al.*, 2002). Within the lesion both CD86 and CD83 expression was upregulated on B cells indicating the potential of these cells to function as antigen presenting cells (APCs) in the regulation and maintenance of local T cell reactivity (Mahanonda *et al.*, 2002).

Few studies have been reported the function of macrophages in periodontal disease. However, while *H. actinomycetemcomitans* and *P. gingivalis* have been shown to activate monocytes and macrophages and stimulate the secretion of pro-inflammatory and tissue-destructive mediators (Zadeh *et al.*, 1999), a recent report has shown that there is no increase in macrophage numbers and little evidence of macrophage activation in advanced periodontitis compared with minimally inflamed tissues (Chapple *et al.*, 1998). During the initial stages of periodontal disease, macrophages may induce cytokine secretion and adhesion molecule expression by gingival keratinocytes.

1.2.3.4 Cytokines and chemokines

One way T cells can respond after the interaction of MHC and the T-cell receptor, and the interaction of co-stimulatory molecules, is to secrete cytokines and chemokines. Cytokines are polypeptides that may cause surrounding immune system cells to become activated, proliferate, or die. They also influence non-immune system tissues.

Chemokines are small peptides that function to attract cells of the immune system. Overproduction of chemokines contributes to the invasion and inflammation of the target organ, which occurs in autoimmune diseases. For example, overproduction of chemokines in the joints of subjects with rheumatoid arthritis may result in invasion of

the joint space by destructive immune system cells such as macrophages, neutrophils, and T cells (Buckley, 2003). More recent research has reported that macrophage chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1 α , MIP-1 β , interferon-gamma (IFN- γ)-inducible protein-10 (IP-10) and the regulated on activation of normal T cell expressed and secreted protein (RANTES), as well as the chemokine receptors CXCR3 and CCR5, were present in inflamed human gingival tissues (Kabashima *et al.*, 2002; Gemmell *et al.*, 2001).

Epithelial cell cytokines

Cytokines were initially thought to be produced solely by cells of the immune system; however, it is now established that many non-immune cells also produce cytokines. The mucosal compartment contains a variety of cells, including macrophages, epithelial cells, intraepithelial lymphocytes, mast cells, fibroblasts and dendritic cells, which are now known to secrete cytokines.

Gingival epithelial cells play an important role as the first barrier against the offending bacterial agents in the periodontal pocket. Oral gingival epithelial cells, produce a number of cytokines including interleukin-1 beta (IL-1 β), tumour necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Yamamoto *et al.*, 1994), IL-8 and transforming growth factor-beta (TGF- β) (Fitzgerald & Kreutzer, 1995; Seymour & Gemmell, 2001; Okada & Murakami, 1998). IL-6 is an important cytokine in the inflammatory response (reviewed by Akira *et al.*, 1993). Several studies indicate that IL-6 levels in gingival crevicular fluid increase during active periodontal disease (Geivelis *et al.*, 1993; Reinhardt *et al.*, 1993; Lee *et al.*, 1995), and gingival keratinocytes isolated from chronically inflamed gingiva produce IL-6 (Yamamoto *et al.*, 1994).

Studies described in this thesis determined by immunohistochemistry the expression patterns of the potent transforming growth factor family (TGF- β s) and their receptors I, II and III in the lining epithelia of inflamed gingiva. TGF- β 2, - β 3 and TGF- β RII expression was increased in the external oral epithelium of advanced periodontitis compared with the gingival attachment of minimally inflamed gingiva. The number of detectable TGF- β s and their receptors varied between patients (Ye *et al.*, 2003).

Epithelial cytokine responses to bacteria were first studied using epithelial cell lines from the human urinary tract. *Escherichia coli* was shown to stimulate kidney and bladder cell lines to secrete IL-6, and the highest IL-6 levels were produced after

exposure to uropathogenic *E. coli* strains (Hedges *et al.*, 1992). Thus, the ability to produce cytokines in response to microorganisms appears to be a general feature of epithelial cells.

Moreover, during periodontal disease, increased levels of inflammatory cytokines such as IL-1 and TNF- α (Heasman *et al.*, 1993) may induce over-expression of adhesion receptors by gingival keratinocytes. Clinical studies show that IL-8 levels in gingival crevicular fluid (GCF) (Mathur *et al.*, 1996) and gingival tissues increase significantly in diseased sites and are related to PMN influx (Tonetti *et al.*, 1994).

Further, T-helper (Th) cells can be divided into two functional subsets (Th1 and Th2). Th1 and Th2 subsets can be distinguished by their respective cytokine profiles. Th1 cells produce IL-2, interferon (IFN)- γ , and TNF- β , and are implicated in cell-mediated immune responses involving T-cell proliferation and macrophage activation. Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, thus favouring immune responses involving antibody production. Th1 and Th2 cells probably derive from common precursor cells which have an intermediary cytokine profile, and are referred to as Th0 cells. Differentiation of Th0 cells into Th1 or Th2 cells is influenced by factors related to the nature of the early immune response (Romagnani, 1992; O'Garra & Murphy, 1994).

As for skin keratinocytes, IFN- γ has been shown to upregulate HLA-DR expression by oral keratinocytes (Mutlu *et al.*, 1991), suggesting that this cytokine could play a role in regulating gingival keratinocyte interactions with T-cells. Several studies show that HLA-DR expression, which is minimal or absent in healthy tissues, is upregulated in inflamed tissues (Crawford, 1992; Nunes *et al.*, 1994). The observation that HLA-DR expression by junctional epithelium during inflammation appears less uniform than for ICAM-I suggests that HLA-DR may be down-regulated by a lack of responsiveness of the gingival keratinocytes to IFN- γ . However, a wider distribution of HLA-DR expression by gingival keratinocytes has been observed in some rapidly progressing lesions and may be indicative of a greater inflammatory response favouring disease progression, as shown in patients with Down's syndrome (Schoel *et al.*, 1995).

Epithelial cells in mucosal cytokine networks

Exogenous cytokines are known to have effects on epithelial cells; for example, interferon γ (IFN- γ) upregulates the expression of major histocompatibility complex (MHC) class I and II antigens, and intercellular adhesion molecule 1 (ICAM-1) by

human oral, intestinal and kidney epithelial cells (Brandtzaeg, 2001; Kvale *et al.*, 1992; van Dorp *et al.*, 1993). The expression of these markers has led to the proposal that epithelial cells can act as antigen-presenting cells. Epithelial cell involvement in mucosal cytokine networks was first postulated when IL-1 and TNF were shown to stimulate epithelial IL-8 secretion (Standiford *et al.*, 1990). It was suggested at the time that stimulated macrophages could be a source of inflammatory cytokines during mucosal infections.

Conclusions

(1) The epithelium plays an active role in mucosal immune responses. (2) Microbial activation of epithelial cells is one important regulatory pathway for the activation or suppression of mucosal inflammation and immunity, and is an important component in the pathogenesis of mucosal infections. (3) Cytokines are essential in controlling a variety of immune and innate mucosal functions and the role of epithelial cells as a source of these cytokines cannot be ignored in studying the mechanisms of defence by mucosal tissues against potential pathogens.

1.2.3.5 Antibodies

Some individuals develop antibodies reactive with many subgingival species, while other subjects show much less reactivity. For example, anti-*P. gingivalis* IgG can be detected in subjects with various forms of periodontitis (Unsal *et al.*, 1996; Kojima *et al.*, 1997; Whitney *et al.*, 1992). However, refractory subjects exhibited high levels of serum antibody to a wide range of subgingival species, with particularly high levels of serum antibody to *Streptococcus constellatus* (Colombo *et al.*, 1998b).

One study of antibodies to *P. gingivalis* in a group of subjects without periodontitis and without *P. gingivalis* infection showed that titres of IgG2 anti-*P. gingivalis* antibodies were significantly higher than those of the group of individuals who did have *P. gingivalis* in their plaque samples, with and without periodontitis (Pietrzak *et al.*, 1998). This suggests that IgG2 antibodies may have resulted in clearance of the organism.

Studies in this thesis (see Chapter 4), found significant negative correlation between anti-*P. gingivalis* IgG levels and attachment loss.

However, many reports have indicated no major differences in serum IgG levels reactive with subgingival plaque related to health, gingivitis and periodontitis (Tanner

et al., 2000). Susceptible subjects may be characterized by the production of non-protective antibodies (Gemmell *et al.*, 2002).

1.2.3.6 Autoantibodies

In some autoimmune diseases, B cells produce antibodies reactive with self antigens. Occasionally, these autoantibodies either interfere with the normal function of the tissues or initiate destruction of the tissues. In the skin disease pemphigus vulgaris autoantibodies break down epithelial integrity, resulting in skin blisters.

A number of mechanisms could account for the generation of autoreactive antibodies in association with periodontitis. The proportion of so-called autoreactive CD5B cells (CD5+CD19 positive) in peripheral blood lymphocytes was six fold higher in adult patients with advanced and generalized chronic periodontitis and four times higher in children with localized aggressive periodontitis than control subjects with healthy periodontia. Some 40-50% of the B cells in the peripheral blood of the periodontitis-susceptible individuals expressed markers for autoreactive features while less than 15% of the circulating B cells in the periodontally healthy subjects exhibited such markers. The periodontitis lesion in adult periodontitis patients contained a substantial number of B cells of which 30% demonstrated autoreactive markers (Berglundh *et al.*, 2002).

Cells producing autoantibodies are known to be present in chronically inflamed periodontal tissues. In sites of chronic inflammation, polyclonal B cell activators (PBA) are known to exhibit adjuvant activity when combined with foreign antigens. The adjuvant effect of microbial PBA may be important in anti-collagen antibody production and thus the localization of PBA in periodontal pockets may explain why anti-collagen antibody-forming cells (AFC) are restricted to chronically inflamed periodontal tissues (Hahn *et al.*, 1997).

In addition, a marked difference in the number and distribution of anti-neutrophil cytoplasmic antibodies (ANCA) with respect to periodontitis between RA and SLE was found. Hyper-responsiveness of B cells and polyclonal B activation in response to periodontopathic bacteria in SLE could account for the high titres of ANCA and the close association observed between those autoantibodies and periodontitis in SLE (Novo *et al.*, 1999). This suggested that a high incidence of (ANCA) frequently co-exists in SLE and periodontitis (Novo *et al.*, 1997).

1.2.3.7 Circulating immune complexes (CIC)

When many antibodies are bound to antigens in the bloodstream, they form a large lattice network called an immune complex. Immune complexes are harmful when they accumulate and initiate inflammation. For example, in diseases such as systemic lupus erythematosus (SLE), chronic IgG antibody production directed at self-antigens causes immune complex deposition in the tissues, especially small blood vessels.

Immune complexes (ICs) in gingival crevicular fluid (GCF) could be potentially useful to ascertain the risk of periodontal disease activity at specific oral sites. This may help to assess the usefulness of IC levels in GCF samples as possible diagnostic indicators of periodontal disease activity (Turner *et al.*, 1992). In localised juvenile periodontitis, high levels of circulating immune complexes (CIC) were noted. In addition, the levels of CIC were found to be significantly higher in diabetic patients with periodontitis compared to non-diabetics (Anil *et al.*, 1990).

1.2.3.8 Other important proteins in gingival epithelium

In a previous study (Ye *et al.*, 2000), it was demonstrated that the reactive pocket epithelium, which forms the critical interface with the pathogenic microbial plaque, displayed evidence of lack of structure. Thus E-cadherin, essential in intercellular adhesion and structure in a multi-layered epithelium as well as connexin gap junction proteins essential in intercellular communication, were markedly down-regulated. Further, there was scant expression of involucrin in the superficial layers indicating a lack of terminal differentiation. Also filamentous actin critical for cell structural integrity was poorly organised. The lining epithelium was also demonstrated to show intense expression of class II MHC. It is reported that antibodies against adhesion molecules can fundamentally disrupt epithelial structure and thereby destroy the essential barrier function of this tissue.

1.2.4 Summary

In summary, to define a disease as autoimmune, the tissue damage must be shown to be caused by an immune response to self antigens. Autoimmune diseases can be caused by autoantibodies or by autoimmune T cells, and tissue damage may result from direct attack on the cells bearing the antigen, from immune complex formation, or from local inflammation. Autoimmune diseases characterised by antibodies that bind to cellular receptors, causing either excess activity or inhibition of receptor function, fall into a

special class. T cells may be involved directly in inflammation or cellular destruction, and they are also required to sustain autoantibody responses.

1.3 POSSIBILITY OF AUTOIMMUNE MECHANISMS IN PERIODONTITIS

1.3.1 Genetic factors

Genetic factors can affect an individual's immune system and the responses to foreign antigens in several ways. Genes determine the conformation of MHC molecules that individuals express. In fact, some MHC genes are associated with autoimmune diseases. However, genes are not the only factors involved in determining an individual's susceptibility to autoimmune disease. For example, some individuals who carry disease-associated MHC molecules on their cells will not develop an autoimmune disease.

Certain diseases such as psoriasis can occur among several members of the same family. This suggests that a specific gene or set of genes predisposes a family member to psoriasis. Current research indicates for psoriasis, a pattern of polygenetic or multifactorial inheritance in the Chinese Han population (Zhang *et al.*, 2002). In addition, individual family members with autoimmune diseases may inherit and share a set of genes that confer susceptibility (Prahalad *et al.*, 2002); although they may develop different autoimmune diseases. For example, one first cousin may have lupus, another may have dermatomyositis, another rheumatoid arthritis.

Evidence for a genetic predisposition to periodontitis comes from four areas of research: 1) the study of inherited diseases and syndromes; 2) family studies; 3) twin studies; and 4) population studies (Hodge & Michalowicz, 2001). There are several features of the host's innate and acquired immune systems that have some heritable element and may contribute to genetic susceptibility to periodontitis. These include epithelial and fibroblast defects (Aldred & Bartold, 1998; Hassell *et al.*, 1997). A number of lines of evidence demonstrate that inherited traits such as some isoforms of IL-1 (Tai *et al.*, 2002; Kornman & di Giovine, 1998) and CD14 receptor (Holla *et al.*, 2002) influence the progression of chronic periodontitis (Michalowicz *et al.*, 1994).

So-called genotype-positive individuals have a 20 times increased risk of developing severe periodontitis after the age of 40 than those individuals who are genotype-negative (Kornman *et al.*, 1997). Genotypes linked with expression of high levels of IL-1 have been examined in a group of periodontitis patients, with 7/22 individuals being positive for this genotype (Engelbreton *et al.*, 1999). There was also a

trend toward higher IL-1 β levels in gingival biopsies of the positive group. Recently, the frequency of IL-1 β genotypes, including allele 2 of the IL-1 β +3953 restriction fragment length bi-allelic polymorphism, was shown to be significantly increased in patients with severe adult periodontitis compared with individuals with early and moderate disease (Gore *et al.*, 1998).

Bacterial transmission within families is insufficient on its own to account for the clustering seen in early-onset periodontitis (van der Velden *et al.*, 1993). It is possible that commensal bacteria acting in an individual with a defective immune response may be sufficient to cause tissue damage (Hart, 1996). Data from a twins study suggested that from 38% to 82% of the population variance for periodontal disease indices could be attributed to genetic factors (Michalowicz *et al.*, 1991).

1.3.2 Autoimmunity could be triggered by plaque bacteria in a variety of ways

Human autoimmune diseases often appear gradually, making it difficult to find out how the process is initiated. Nevertheless, there is a strong suspicion that infections can trigger autoimmune disease in genetically susceptible individuals.

1.3.2.1 Subgingival biofilms

Subgingival plaque is a microbial biofilm. Biofilms are defined as “matrix-enclosed bacterial populations adherent to each other and /or to surfaces or interfaces” (Costerton *et al.*, 1994). Although biofilms have been described for a number of years, more recent details concerning their molecular organization, physiochemical properties and growth characteristics have inspired investigators to consider biofilms as ecological communities as a whole (Costerton *et al.*, 1995). A recent review has summarized dental biofilms (Socransky and Haffajee, 2002). Subgingival biofilms constitute the basis of a substantial and continuing bacterial load. Socransky *et al.*, (1998) analysed 13,261 plaque samples, and divided the component organisms into 5 major complexes. 1) One complex consisted of the tightly related group: *Bacteroides forsythus*, *Porphyromonas gingivalis* and *Treponema denticola*. 2) The 2nd complex consisted of a tightly related core group including *Fusobacterium nucleatum/periodonticum*, *Prevotella intermedia*, *Prevotella nigrescens* and *Peptostreptococcus micros*. Species associated with this group included: *Eubacterium nodatum*, *Campylobacter rectus*, *Campylobacter showae*, *Streptococcus constellatus* and *Compylobacter gracilis*. 3) The 3rd complex consisted of *Streptococcus sanguis*, *S. oralis*, *S. mitis*, *S. gordonii* and *S.*

intermedius. 4) The 4th complex was comprised of three *Capnocytophaga* species, *Campylobacter concisus*, *Eikenella corrodens* and *Haemophilus actinomycetemcomitans* serotype a. 5) The 5th complex consisted of *Veillonella parvula* and *Actinomyces odontolyticus*. *A. actinomycetemcomitans* serotype b, *Selenomonas noxia* and *Actinomyces naeslundii* genospecies 2 (*A. viscosus*).

Periodontal disease is caused by bacteria found in dental plaque, and about ten species, mainly Gram-negative rods, have been identified as putative pathogens in periodontal disease. The Gram-negative bacteria *H. actinomycetemcomitans*, *P. gingivalis*, and *B. forsythus* are the most commonly associated with periodontitis (Zambon, 1996; Haffajee & Socransky, 1994). Accordingly, the pathogenic plaque is considered as a complex interactive microbial community.

In an investigation of the distribution of *H. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* in an Australian population, a total 6030 subgingival plaque samples were collected from 504 subjects. *H. actinomycetemcomitans* was detected in 22.8% of subjects, followed by *P. gingivalis* and *P. intermedia* in 14.7% and 9.5% of subjects respectively (Hamlet *et al.*, 2001). Known putative periodontal pathogens such as *P. gingivalis*, *B. forsythus*, and *T. denticola* were identified from multiple subjects, but typically as minor components of the plaque as seen in cultivable studies (Paster *et al.*, 2001). Most of the other predominant species were detected in both subjects with disease and those with healthy gingiva (Paster *et al.*, 2001). It has become apparent that pathogens are necessary but not sufficient for disease activity to occur (Haffajee & Socransky, 1992).

Certain bacteria, especially species of *Actinomyces* and streptococci, occur in human dental plaque and may contribute to various disease states including gingivitis, root surface caries and periodontitis. Therefore, *Streptococcus* spp. and *Actinomyces* spp have received more attention recently (Ochiai *et al.*, 1993).

Members of the genus *Actinomyces* are Gram-positive, generally facultatively anaerobic rods commonly isolated from the human mouth (Tanner *et al.*, 1994). In particular, species/genospecies 1 and 2, *A. odontolyticus*, *A. meyeri* and *A. georgiae*, have been recognized from human oral samples (Sato *et al.*, 1998). *Actinomyces* comprise a major segment of the indigenous human oral microbiota and are among the most predominant microorganisms in both supragingival and subgingival plaque (Ximeaez *et al.*, 1999).

As dental plaque matures, a microbial succession occurs in which *Streptococcus* spp. dominate in the early stages followed by an increase in *Actinomyces* spp. and other related Gram-positive bacteria. Some *Actinomyces* are pathogenic and induce chronic inflammatory lesions in man and animals (Ochiai *et al.*, 1993).

It is well known that *Actinomyces* can co-aggregate with Gram-positive bacteria such as *S. mitis* that contribute to the accumulation of dental plaque on tooth surfaces. In recent years, many investigators have demonstrated that Gram-negative bacteria that co-aggregate with *Actinomyces* spp. in the gingival crevice appear to be major pathogenic species in periodontitis (Ochiai *et al.*, 1993).

Recent studies reported that of the predominant species, *S. constellatus* was most associated with disease sites. Also *Actinomyces* were commonly detected in healthy and diseased sites (Paster *et al.*, 2001). *Actinomyces* spp., *Streptococcus* spp. and *Eubacterium* spp., were detected at different levels in adult periodontitis, localized juvenile periodontitis and rapidly progressive periodontitis subjects (Nonnenmacher, 2001).

Intraperitoneal immunization of Balb/c mice with subgingival plaque from patients with advanced periodontitis induced IgM antibodies reactive with antigens from a variety of oral bacteria including *Streptococcus mitis* and *Actinomyces georgiae* (Gmur *et al.*, 1999).

The subgingival microflora of refractory disease can be predominantly Gram-positive with elevated levels of *Streptococcus intermedius* (Magnusson *et al.*, 1996) and *Streptococcus constellatus* (Colombo *et al.*, 1998a).

Streptococci of the *milleri* group (SMG, see Chapter 4) consisting of *S. intermedius*, *S. constellatus* and *S. anginosus* are part of the normal flora of human mucous membranes. These streptococci have also been reported to be significant pathogens. They have also been repeatedly associated with serious suppurative infections. Evidence for the pathogenicity of the *Streptococcus milleri* group is scattered and mainly circumstantial. Further investigation is required to establish the pathogenicity and pathogenic mechanisms of these organisms (Gossling, 1988).

1.3.2.2 Molecular mimicry

Structural similarities between external antigens (bacteria, viruses, food antigens, etc) and self-components are believed to be one of the possible causes of autoimmunity (Tontsch *et al.*, 2000). It has also been suggested that autoimmunity may be initiated by

a mechanism known as molecular mimicry, in which antibodies or activated T cells generated in the response to an infectious agent, recognise self-antigens.

Microbial antigens can elicit antibody responses that react not only with the pathogen but also with host antigens that are similar in structure. For example, this type of response occurs after infection with some *Streptococcus* species that elicit antibodies that cross-react with kidney (Kraus *et al.*, 1990) or heart (Tontsch *et al.*, 2000) to produce disease. However, such responses are usually transient and do not lead to sustained autoantibody production since the helper T cells are specific for the microbe and not for self proteins. Host proteins that complex with bacteria can induce a similar transient response; in this case, the antibody response is not cross-reactive, but the bacterium is acting as a carrier, allowing B cells that express an autoreactive receptor to receive inappropriate T cell help. These and some other mechanisms that could allow an infectious agent to break tolerance are summarized in this chapter (1.3.2.3, below).

In addition, bacterial and viral infections may trigger autoimmune disease in genetically susceptible individuals by exposing autoreactive T cells to cross-reactive peptides (Wucherpfennig & Strominger, 1995). Similarly, enhanced lymphocyte stimulation by microbial substances may result in polyclonal activation of the lymphocytes, which then provides sufficient help to activate anergic autoreactive lymphocytes.

Acute rheumatic fever and rheumatic heart disease have been under investigation for decades and the range of cross-reactive tissue antigens for streptococcal-induced antibodies identified in rheumatic heart disease is still expanding. Cross-reactivity to antigens of group A streptococci has been proposed as the mechanism leading to cardiac damage in acute rheumatic fever and rheumatic heart disease. The mimicry of host antigens by infectious agents such as *Streptococcus pyogenes* may induce or uphold a cross-reactive autoimmune response to myocardial antigens (Fagan *et al.*, 2001; Tontsch *et al.*, 2000).

Various models suggest that for acute rheumatic fever, cross-reactive antibodies may either damage heart tissue directly or induce an imbalance in the immunological response in susceptible individuals, eventually leading to an autoimmune mechanism which is not yet fully understood. One study demonstrated that streptococcal M protein can induce an autoimmune cell-mediated immune attack on the heart valve in a Lewis rat model. The data support the hypothesis that a bacterial antigen can break immune tolerance *in vivo*, an important concept in autoimmunity (Quinn *et al.*, 2001).

Rheumatoid arthritis is a well-documented autoimmune disease. Rheumatoid factor (RF) had previously been reported in the serum of patients with periodontitis suggesting that those antibodies could be elicited by micro-organisms in the subgingival plaque. Further, *Capnocytophaga gingivalis* and *F. nucleatum* express surface antigenic epitopes that are antigenically related to determinants on IgG that may induce cross-reactive IgM-RF (The & Ebersole, 1996).

Other bacteria, such as *Helicobacter pylori* or *Helicobacter mustelae* induce autoantibodies that cross-react with human gastric mucosa (Croinin *et al.*, 1998, Negrini *et al.*, 1996; 1991; Appelmelk *et al.*, 1996) with implications for the pathogenesis of chronic atrophic gastritis. A relationship between periodontal disease and *H. pylori* infection has been identified in adults in the United States. Periodontal pockets with a depth of 5 mm or more were associated with increased odds of *H. pylori* seropositivity after adjustment for socio-demographic factors (Dye *et al.*, 2002).

Molecular mimicry as a mechanism for autoimmune disease is summarised in Table 1.1.

1.3.2.3 Loss of self-tolerance

One possible mechanism for the loss of tolerance to self-antigens is that the infectious agents induce co-stimulatory activity on cells expressing low levels of peptides, thus enhancing activation of autoreactive T cells.

Mechanisms maintaining tolerance against self-components, namely self-tolerance, are divided into central and peripheral tolerance (van Parijs & Abbas, 1998; Weintraub & Goodnow, 1998). In central tolerance, B lymphocytes with high avidity reactivity with a complex of MHC and self-peptide are deleted or rendered unresponsive during their ontogeny in primary lymphoid organs. However, it is known that such elimination is incomplete and potentially harmful auto-reactive lymphocytes are seeded into peripheral lymphoid organs. Regulatory mechanisms to maintain peripheral tolerance are therefore important in keeping mature autoreactive lymphocytes under control. Mechanism of peripheral tolerance includes cell death, anergy and possibly, suppression of autoreactivity by regulatory lymphocytes. Autoimmune diseases arise from failure to maintain such self-tolerance. Breakdown of peripheral tolerance likely involves presentation of auto-antigens to T-cells by dendritic cells and other antigen presenting cells (Ring & Lakkis, 1999; Banchereau *et al.*, 2000).

Table 1.1 Molecular mimicry mechanism in autoimmune diseases

Exogenous agents (Bacteria, Viruses, Food antigens, etc.)	Antibodies	Cross-reactive antigens	Autoimmune diseases	References
<i>Helicobacter pylori</i>	IgG	gastric antigens	Chronic atrophic gastritis	Negrini <i>et al.</i> , 1996; 1991
<i>Helicobacter mustelae</i>	IgG	gastric antigens	Ferret gastritis	Croinin <i>et al.</i> , 1998
<i>H. pylori</i> LPS	mAb	Lewis blood group antigens (Lewis x, y and H type I)	Type B gastritis	Appelmelk <i>et al.</i> , 1996
Group A streptococci (<i>S. pyogenes</i>)	IgG	myocardial antigens (myosin, creatine kinase, two mitochondrial proteins and stress proteins)	Acute rheumatic fever (ARF) Rheumatic heart disease (RHD)	Tontsch <i>et al.</i> , 2000
	mAB	Streptococcal M protein(Lewis rat)	Rheumatic heart disease (RHD)	Quinn <i>et al.</i> , 2001
subgingival plaque (<i>C. gingivalis</i> , <i>F. nucleatum</i>)	IgG	Rheumatoid factor (RF)	Rheumatoid arthritis	The & Ebersole, 1996
subgingival plaque (<i>Streptococcus</i> spp., <i>Actinomyces</i> spp.)	IgG	oral epithelial components (30 – 50 kD etc.)	Periodontitis*	Ye <i>et al.</i> , 2003
Reovirus type III	IgG	Cogan peptide	Cogan's syndrome	Lunardi <i>et al.</i> , 2002
Gliadin	mAb	enterocyte antigen (28kD, 62kD and 66kD)	Celiac Disease	Tuckova <i>et al.</i> , 1995

*The contribution of autoimmunity to periodontal disease is speculative.

It is believed that processes leading to self-tolerance are operative in both B and T cells although those for B cells are less clear (van Parijs & Abbas, 1998; Weintraub & Goodnow, 1998; Akkaraju *et al.*, 1997). Since production of antibodies by B cells generally requires T cell help, breakdown of self-tolerance in both B and T cell compartments is expected to take place. Alternatively, it is possible that ablation of self-reactive B cell tolerance is sufficient to trigger autoimmunity. However, experimental data supporting this notion have rarely been obtained.

Pemphigus vulgaris (PV), is a life-threatening autoimmune disease targeting skin and mucous membranes (Amagai, 1995; 1996). It is well established that in PV IgG antibodies against desmoglein 3 (Dsg3), a cadherin type intercellular adhesion molecule expressed in stratified squamous epithelia, play a pathogenic role causing blisters in the skin and mucous membranes. (Amagai *et al.*, 1991; 1994; Amagai, 1999).

Production of sufficient amounts of pathogenic anti-Dsg3 IgG causes the PV phenotype as a result of disruption of cell-cell adhesion of keratinocytes especially in oral mucous membranes (Amagai *et al.*, 2000). These findings indicate that loss of tolerance to Dsg3 in T cells as well as B cells is required for production of pathogenic anti-Dsg3 IgG antibodies and development of the PV phenotype.

Multiple factors triggering breakdown of tolerance include genetic background (Vyse & Todd, 1996; Kouskoff *et al.*, 2000). Because specific IgG antibody production is generally a consequence of cooperation between B and T cells, breakdown of self-tolerance in both B and T cell compartments seems important for autoantibody production. Thus, it is not surprising that breakdown of tolerance for both B and T cells is required for triggering PV phenotype. On the other hand, it has also been proposed that self-reactive B cells are widely distributed and breakdown of T cell tolerance is sufficient to trigger autoimmunity.

1.3.2.4 Superantigens

A novel class of microbial-derived T cell mitogens, referred to as superantigens, has recently been described. Superantigens are unique in that they induce a broad-spectrum activation and expansion of specific subsets of T cells in an antigen-independent manner, thereby causing immune dysfunction. Subsets of superantigen-expanded T cells can be identified with reagents that discriminate among different families of the variable domains of the T cell antigen receptor beta-chain (V beta). Since superantigens

expand one or a few of these T cell antigen receptor V beta families, T cell subsets that have been expanded by superantigens have restricted expression of one or a few V beta types (Zadeh & Kreutzer, 1996).

Superantigens are comprised of two groups of potent immunomodulatory molecules (Scherer *et al.*, 1993): 1) soluble exotoxins derived from Gram-positive bacteria, such as staphylococci and streptococci (Marrack & Kappler, 1990; Tomai *et al.*, 1991); and 2) viral superantigens such as those encoded by endogenous mouse mammary tumour viruses (Webb & Gascoigne, 1991). Superantigens play an important role in auto-immune disease by activating anergic T cells which then react with self-antigens (Kotzin *et al.*, 1993); they are also responsible for acute reactions such as toxic-shock syndrome and food poisoning (Ulrich *et al.*, 1995). Interestingly, superantigens may also induce T cell death and/ or anergy, since administration of staphylococcal exenterotoxin B leads to expansion of T-cell clones followed by Fas-mediated apoptosis and/or anergy (MacDonald *et al.*, 1991).

It is known that keratinocytes can be induced to express MHC class II (HLA-DR) molecules, and as superantigen presentation to T cells does not require antigen processing, it is possible that these cells may act as “non-professional” APCs. Studies *in vitro* have shown that human keratinocytes can provide the co-stimulatory signals necessary to induce superantigen-driven T cell proliferation, and that this function is largely inhibited by anti-ICAM-I monoclonal antibody, suggesting an important role for ICAM-I in the process (Nickoloff *et al.*, 1993; Strange *et al.*, 1994).

Oral streptococci which colonize tooth surfaces and gingival tissue in high numbers, play a role in the initiation of periodontal disease prior to the subgingival colonization by Gram-negative anaerobic species more commonly associated with periodontal disease. Historically, streptococci were among the first groups of micro-organisms to be associated with periodontal disease, because they were regularly isolated from subgingival plaque samples (Socransky & Haffajee, 1994). More recent evidence suggests that certain species of streptococci may be involved in disease progression, while others are regarded as forming part of a beneficial bacterial flora associated with quiescent or healthy sites (Socransky & Haffajee, 1992). Elevated counts of *Streptococcus intermedius*, a beta-haemolytic species, have been observed in sites exhibiting recent disease progression (Dzink *et al.*, 1988) and in refractory periodontitis (Walker *et al.*, 1993).

Recent evidence points to the possible participation of streptococcal

superantigens in the early stages of periodontal disease. In particular, it has been shown that a superantigenic fraction isolated from an *S. mitis* strain can selectively activate V β 2 and V β 5. T cells may contribute to the initiation of periodontal disease by exerting cytotoxic activities against epithelial cells (Matsushita *et al.*, 1995). Some bacterial antigens such as *S. aureus* and *P. intermedia* enterotoxins can selectively stimulate T cells that express specific V beta genes of the T cell antigen receptor (TCR) (Mathur *et al.*, 1995).

Since viridans streptococci, including *S. mitis*, form part of the resident oral flora (Frandsen *et al.*, 1991), it is tempting to speculate that the onset of gingival inflammation, the first stage in periodontal disease, might be related to the presence of virulent clonal types within the species (Hohwy & Killian, 1995), which may activate particular T cell populations. This concept is supported by the observation that similar T-cells, bearing V β 25 and V β 5. TCRs can be activated by superantigens derived from a particular *S. mitis* strain, and *S. pyogenes*, which is a common pharyngeal and dermal pathogen (Abe *et al.*, 1991; Matshushita *et al.*, 1995). These observations, together with the observed HLA-DR expression by gingival epithelial cells, suggest that these cells are capable of presenting bacterial superantigens to T cells. However, the question of whether activated T cells then cross-react with gingival epithelial cell components, as suggested for psoriasis, has not yet been addressed.

One study investigated the presence of putative superantigen-stimulated T cells in periodontitis sites, utilizing a panel of monoclonal antibodies to T cell antigen receptor V beta families. In most periodontitis patients examined, patterns of V beta expression among T cells were characteristic of superantigen stimulation, i.e., there was an elevation in the proportion of one or a few V beta families. Specifically, analysis revealed that T cell subsets expressing V beta 5a and V beta 5b, V beta 6, V beta 8 and V beta 12 were each elevated by greater than 2 standard deviations. The finding suggested that a large proportion of T cells in periodontitis sites have been stimulated and expanded by superantigens, presumably produced by periodontitis-associated bacteria (Zadeh & Kreutze, 1996). However, extracts of periodontopathic bacteria (*H. actinomycescomitans*, *P. gingivalis*, *P. intermedia* and *Prevotella nigrescens*) did not stimulate murine T cells in a manner consistent with superantigenic activation (Petit & Stashenko, 1996).

1.3.3 Other factors may influence the development of autoimmune diseases

Other less understood influences affecting the immune system and the course of autoimmune diseases: personal factors include aging, chronic stress, hormones, pregnancy and low birth weight. In addition, periodontitis also associates with other autoimmune diseases.

1.3.3.1 Aging:

There seems to be little doubt that the prevalence of periodontal disease increases with age (Albandar *et al.*, 1999). One might argue that some loss of alveolar bone and periodontal attachment is actually part of the normal physiology of aging and not a consequence of infectious pathology.

1.3.3.2 Chronic (Psychosocial) stress

The impact of stress on the immune system is well established, and a possible influence on a chronic inflammatory disease like periodontitis appears probable. The host defence mechanisms within the gingival/periodontal tissues seem to be responsible for most of the tissue damage and for the outcome and progression of the disease. Many researchers have reported an increase in stress is associated with the development of progressive periodontitis (Pistorius *et al.*, 2002; Genco *et al.*, 1998; 1999; Monteiro *et al.*, 1996 Clarke *et al.*, 1995; Breivik *et al.*, 1996). Chronic stress has been related to levels of the putative bacterial pathogens *B. forsythus* and *P. gingivalis* (Genco *et al.*, 1999). However, a direct association between periodontitis and stress remains to be proven, which is partly due to lack of an adequate animal model and the difficulty in quantifying amount and the time of stress and the detrimental effects of inadequate coping with stress (Hildebrand *et al.*, 2000).

Depressed immune responsiveness as a result of physical or mental stress has been postulated to be one of several factors involved in the aetiology of destructive periodontal disease. The stress-released hormones present in the gingival crevicular fluid may provide a source of nutrients that promote subgingival growth of periodontal pathogens. Microorganisms possess the ability to recognize hormones within the host and utilize them to adapt to their surroundings. Noradrenaline and adrenaline, which are released during human stress responses, may act as environmental cues to alter the growth of individual organisms within subgingival biofilms. Roberts *et al.*, (2002) reported that the most pronounced positive growth effects of noradrenaline were

demonstrated in *Actinomyces naeslundii* (+ 49.4%) and *Actinomyces gerencsariae* (+ 57.2%). The composition of the subgingival biofilm in response to stress-induced changes in local catecholamine levels may play a significant role in the aetiology and pathogenesis of periodontal disease.

1.3.3.3 Hormone changes and pregnancy

Women in the postmenopausal phase of life (47–55 age) have increased oral disease, including periodontitis (Friedlander, 2002).

A number of researchers have examined the association of hormone changes, systemic health and socio-cultural characteristics on periodontitis during pregnancy. Their reports indicate the relationship of clinical indices to demographic and socio-cultural variables (Yalcin *et al.*, 2002; Machuca *et al.*, 1999).

Changes in hormone levels, such as those that occur during puberty, pregnancy, menstruation and menopause, as well as those that occur with the use of hormonal supplements, have long been associated with the development of gingivitis. Further, bacterial anaerobes have been found to change in abundance during the normal hormonal cycle. In periodontitis, the inflammatory response results in ulceration of the gingiva and the subsequent entry of bacterial cells, bacterial products, peptidoglycan fragments and hydrolytic enzymes into the systemic circulation. The result is a systemic response of increased cytokines and other biological mediators, as well as increased levels of serum antibodies (Krejci & Bissada, 2002).

1.3.3.4 Low birth weight

Some researchers have found that pregnant women with periodontitis were 7.5 times more likely to have a pre-term low-birth-weight infant (PLBW) than were control subjects (Krejci & Bissada, 2002; Offenbacher *et al.*, 1998). In addition, microbial data indicate that four organisms associated with mature plaque and progressing periodontitis: *B. forsythus*, *P. gingivalis*, *H. actinomycescomitans*, and *T. denticola*, were detected at high levels in PLBW mothers (Offenbacher *et al.*, 1998).

1.3.3.5 Association with other autoimmune diseases

Diabetes

The epidemiology of autoimmune diseases was recently reviewed by Cooper and

Stroehla (2003). Many studies have reported significant positive associations between diabetes and severe periodontal disease (Firatli, 1997; Hugoson *et al.*, 1989; Papapanou 1996; 1999; Taylor *et al.*, 1996). Further, diabetes has been identified as one of the major risk factors for periodontal disease (Grossi *et al.*, 1994). The incidence of periodontitis is higher in teenagers and adults with insulin-dependent diabetes mellitus (IDDM) (Seppala *et al.*, 1997; Leo *et al.*, 1993). IDDM is an autoimmune disease and has been shown to have a clear basis of genetic susceptibility (Bach, 1994).

Type I, or insulin-dependent diabetes mellitus (IDDM), commonly develops before the age of 30 years; non-insulin-dependent diabetes, also referred to as Type II diabetes, is more prevalent and usually begins after the age of 40 years. Insulin-dependent diabetes mellitus has been associated with certain genetic factors and immune impairment that result in an increased susceptibility to infection, such that the risk of oral infections, including periodontitis, is high in adult populations of juvenile-onset insulin-dependent diabetes mellitus patients, especially those with elevated blood glucose levels (Mrena *et al.*, 2003; Gustafsson *et al.*, 2003; Rosenthal *et al.*, 1988; Blackwell *et al.*, 1989; Morinushi *et al.*, 1989). Type II diabetics seemed to have the same risk factors associated with the condition (Sobngwi *et al.*, 2003), including an increased risk for destructive periodontitis (Beck, 1990).

No study to date has reported the induction of periodontal disease in the diabetic individual without the local factors of plaque and calculus. Thus diabetes is a predisposing or modifying factor that can accelerate periodontal breakdown, which is initiated by aetiological microbial agents (Takahashi *et al.*, 2001).

Study of periodontal disease in pregnancy complicated by type 1 diabetes mellitus demonstrates that periodontal inflammation and destruction increases in pregnant diabetics as compared to non-diabetic pregnant patients (Guthmiller *et al.*, 2001).

Refractory periodontitis patients with IDDM have elevated serum glutamic acid decarboxylase (GAD) autoantibody levels immuno-reactive with the oral bacterium *P. gingivalis* (Sims *et al.*, 2001). In addition, there was a significantly higher level of circulating immune complexes (CIC) in the sera of patients with localised juvenile periodontitis (Anil *et al.*, 1990).

One study reported that cellular, vascular, and connective tissue changes in periodontitis indicative of increased catabolism rather than anabolism, are associated with poorly controlled long-term insulin-dependent diabetes (Seppala *et al.*, 1997).

In view of the results of the studies discussed above, it is reasonable to conclude that both Type I and Type II diabetes mellitus significantly enhance susceptibility to severe periodontitis. Increased susceptibility to periodontal diseases has been considered to be associated with impaired polymorphonuclear leukocyte functions (Noack *et al.*, 2000). Several studies in diabetic animals and humans have reported defects in neutrophil chemotaxis, adherence, phagocytosis and bacterial killing suggesting that these dysfunctions may lead to impaired host resistance to bacterial infections (Cutler *et al.*, 1991; Watanabe, 1990; Canturk *et al.*, 1998; Repine *et al.*, 1980).

The key pathogenic mechanism in IDDM is considered to be a T-cell mediated autoimmune response against the insulin-producing islet cells of the pancreas. The clinical onset of diabetes is often preceded by the appearance of serum autoantibodies to islet cells (ICA Ab), insulin (IA-2 Ab), and/or glutamic acid decarboxylase (GAD65) (Lernmark, 1994). The detection of GAD Ab in serum has been proven to be a good predictor of subjects who will later develop IDDM (Lernmark, 1994; Akamine *et al.*, 1997). GAD Ab has also been detected in some subjects who have been previously classified as having non-insulin-dependent diabetes mellitus (NIDDM), suggesting a possible role for a similar form of autoimmunity in NIDDM (Littorin *et al.*, 1999). Cell-mediated autoimmune responses to antigens shared by host tissues and periodontal bacteria have also been implicated in periodontal disease (Anusaksathien & Dolby, 1991; Anusaksathien *et al.*, 1992a; 1992b; Loesche & Lopatin, 1998; Petit *et al.*, 1999).

Human leukocyte antigen (HLA) association is apparent in both periodontal disease and IDDM. Carriage of both HLA DQ2 and DQ8 on chromosome 6 appears to be necessary, but not sufficient, to predict the onset of IDDM (Lernmak 1994) whereas HLA DQB1 and specific subtypes of HLA DR4 have been linked with increased risk for early onset periodontal disease (Ohshima *et al.*, 1996; Bonfil *et al.*, 1999). Further, cytotoxic lymphocyte antigen (CTLA) association has been reported for both IDDM (Nistico *et al.*, 1996) and periodontal disease (Aoyagi *et al.*, 2000). CTLA-4 genotypes have been related to cytokine responses that promote pathogenic autoimmune reactions that have been linked with T cell activation by *P. gingivalis* (Aoyagi *et al.*, 2000). CTLA-4 is a strong candidate gene for T cell-mediated autoimmune disease because it encodes a T cell receptor that mediates T cell apoptosis and is a vital negative regulator of T cell activation (Nistico *et al.*, 1996). The islet tyrosine phosphatase-like protein IA-2/ICA5 12bdc is a major auto-antigen in type 1 diabetes (IDDM) (Farilla *et al.*,

2002).

Recent study of periodontal treatment outcome in insulin-dependent (type 1) diabetes mellitus patients with severe periodontitis (IDDM/periodontitis) resulted in two key findings: 1. serum glutamic acid decarboxylase autoantibody (GAD65 Ab) levels were significantly associated with periodontal pocket depth change (PDC) and 2. serum IgG titres to *P. gingivalis* cells were positively associated with GAD65 Ab level in seropositive (GAD65 Ab +) patients (Sims *et al.*, 2002).

Down's syndrome

Individuals with Down's syndrome have a higher incidence of periodontitis (Hodge & Michalowicz, 2001). The majority of cases of Down's syndrome are trisomic for the whole of chromosome 21. This chromosome contains genes encoding a number of structural proteins (Sofaer, 1990). Patients with Down's syndrome also suffer from frequent upper respiratory tract infections, indicating an immune defect in resistance to bacterial infection (Hassell & Harris, 1995).

Rheumatoid arthritis

Individuals with moderate to severe periodontal disease are at higher risk of suffering from rheumatoid arthritis (Mercado *et al.*, 2000). Further, rheumatoid factor from periodontitis patients cross-reacts with epitopes on oral bacteria (The & Ebersole, 1996). Periodontitis differs from other chronic inflammatory diseases, such as arthritis, in that arthritis is characteristically a T-cell-dominated lesion instead of the plasma cell dominance seen in periodontitis. This is undoubtedly due to the omnipresent challenge presented by the subgingival microbiota, which provides antigenic drive for localized antibody production. There is a correlation between interleukin-6 levels in gingival crevicular fluid and periodontal status in patients with rheumatoid arthritis and adult periodontitis (Bozkurt *et al.*, 2000).

1.4 DIAGNOSIS OF PERIODONTITIS

This was reviewed by Kinane (2000). Periodontitis affects a subset of the population and current thinking is that progression of periodontal disease may be either continuous or cyclical (burst hypothesis). These features make screening and diagnostic tools desirable in the management of this disease. Although many potential markers exist, several difficulties hamper the ability to define diagnostic tests with proven utility. The

'gold standard' for active periodontal disease is not available and inflammation due to gingivally confined lesions (gingivitis) and periodontal inflammation which results in attachment loss is a potential confounder of any test based on assessing the host response elements of the disease. The current absence of proof for the progression of periodontal disease i.e., whether or not the burst hypothesis is correct, is a further problem.

In terms of screening tests for diseases such as the early-onset forms of periodontitis, the development of diagnostic tools involving genetic polymorphisms, specific genes, microbial species-specific DNA probe tests (van Arsdell *et al.*, 1996), enzyme analysis (Loesche, 1992), systemic antibodies or leukocyte cell surface markers of the patients, may become a clinical reality in time. One could envisage chair side tests using blood from thumb pricks being capable of determining a young individual's risk of developing disease at a later age, and thus the need for a timely prevention programme. Whether periodontal diagnostic tests will be developed that will indicate to the clinician the need for various treatment regimes other than root planing, is still an open question. For example, in the future, a test of specific antibody levels in patients prior to treatment might indicate their likely response to therapy and thus the need for additional therapeutic agents such as antibiotics. Periodontal diagnostic tests are still at an early stage of development and much work remains to be performed to fully validate their utility such that they become an important and cost effective aspect of clinical treatment planning, screening or patient monitoring.

1.5 IMMUNE THERAPY IN PERIODONTITIS

Active and passive immunization approaches have been developed for immunotherapy of these diseases. For example, immunization with the RgpA-Kgp proteinase-adhesin complexes of *P. gingivalis* protects against periodontal bone loss in the rat periodontitis model (Rajapakse *et al.*, 2002).

Recent advances in mucosal immunology and the introduction of novel strategies for inducing mucosal immune responses now raise the possibility that effective and safe vaccines can be constructed. In this regard, some successful results have been reported in animal experimental models. Further, there are attempts to construct single-chain variable fragments (ScFv) capable of neutralizing virulence factors (Abiko, 2000).

In profound periodontitis, however, hyper-responsiveness and hypersensitivity

of the immune system are counterproductive because of the destruction of the affected periodontal connective tissues. Intercellular adhesion molecule type 1 (ICAM-1) plays a key role in the onset and manifestation of inflammatory responses. Thus, inhibition of ICAM-1 expression could be of therapeutic relevance for the treatment of destructive periodontitis. Here, antisense oligonucleotides (AS-ON) directed against ICAM-1 suppress protein expression and mRNA levels specifically and effectively in primary human endothelial cells of different tissue origin. Moreover, down-regulation of ICAM-1 expression is also observed in AS-ON-transfected inflamed gingival tissue. Antisense-mediated inhibition of ICAM-1 expression has therefore been proposed as a therapeutic strategy against inflammation of human periodontal tissue (Nedbal *et al.*, 2002).

Antibacterial peptides, such as defensins and LL-37, are natural bactericidal components similar in potency to classic antibiotics. These peptides are produced at mucosal linings including the oral cavity (Putsep *et al.*, 2002).

A current goal in caring for patients with autoimmune diseases is to find treatments that produce remissions with fewer side effects. Much research is focused on developing therapies that target various steps in the immune response. New approaches such as therapeutic antibodies against specific T cell molecules may produce fewer long-term side effects than the chemotherapies that now are routinely used.



CHAPTER 2

**EXPRESSION PATTERNS
OF E-CADHERIN,
INVOLUCRIN
AND CONNEXIN GAP
JUNCTION PROTEINS IN
THE LINING EPITHELIA
OF INFLAMED GINGIVA**

CHAPTER 2: EXPRESSION PATTERNS OF E-CADHERIN, INVOLUCRIN AND CONNEXIN GAP JUNCTION PROTEINS IN THE LINING EPITHELIA OF INFLAMED GINGIVA

2.1 ABSTRACT

The structural integrity and functional differentiation of the lining epithelium was studied in relation to inflammatory changes associated with destructive periodontitis. In the different regions of lining epithelia from clinically healthy gingiva and periodontitis, the expression patterns of E-cadherin, which is critical in intercellular adhesion, of proteins associated with gap junction communication channels and of involucrin, which is a key marker of differentiation in stratified epithelia were compared. Filamentous actin (F-actin), which is important in cell structural integrity, attachment and migration was also examined. Semiquantitative immunohistochemical analysis revealed that in both clinically healthy gingiva and lesions of advanced periodontitis, expression patterns of E-cadherin, involucrin and connexins 26 and 43 were similar, with statistically significant reduction in staining intensity from the external oral epithelium through the gingival sulcus to the junctional epithelium or pocket epithelium respectively. Furthermore, there was a striking reduction in staining for E-cadherin, involucrin and both connexins in the pathological lining epithelium of the periodontal pocket. These changes were associated with marked alterations of filamentous actin expression, collectively indicating profound perturbation of epithelial structure. The data support the concept that the ability of the pathological lining epithelium to function as an effective barrier against the ingress of microbial products into the tissues is severely compromised.

Keywords: E-cadherin; involucrin; connexins; F-actin; immunohistochemistry; advanced periodontitis.

2.2 INTRODUCTION

Periodontitis is a chronic inflammatory disease that is considered to arise in some gingivitis sites in association with failure of the epithelial attachment to the tooth, resulting in the development of a cleft or pocket lined by proliferating epithelial cells. The pocket epithelium functions as a critical interface with the microbial flora of the

pocket and as a rate limiting barrier to the ingress of microbial products into the tissues.

The source of the non-keratinizing squamous epithelial cells lining the pocket is not known. It is thought to arise from the junctional epithelium but there is also potential for contribution by developmental remnants (Spouge, 1980). Morphologically, the pocket epithelium appears abnormal, lacking an obvious layered structure and with tenuous intercellular contacts (Pinchback *et al.*, 1996). The biological basis of this altered differentiation is unknown although an understanding of the regulation of epithelial differentiation in the periodontal pocket is clearly of importance.

The structural and adhesion proteins, E-cadherin, involucrin, and the gap junction connexins are considered to be important markers of tissue integrity, differentiation and communication in squamous epithelium.

E-cadherin (epithelial cadherin), a subclass of cadherins found in stratified squamous epithelium, plays a crucial role in maintaining the structural integrity and function of both adherens and desmosomal epithelial intercellular junctions (Wheelock & Jenson, 1992). The expression of E-cadherin in normal oral epithelium, gingival carcinoma and other oral tumours has been widely investigated (Andrews *et al.*, 1997; Yamada *et al.*, 1997; Sakaki *et al.*, 1994; Sakaki *et al.*, 1993; Downer & Speight, 1993), but distribution in the reactive pocket epithelium of periodontitis has not been reported.

Involucrin, a specialized structure formed during terminal differentiation of stratified squamous epithelial cells, is considered to serve an essential barrier function at the surface of epithelia (Steinert & Marekov, 1997). The expression of involucrin in normal human skin and epidermal lesions has received much attention (Ekanayake-Mudiyanselage *et al.*, 1998; Crish *et al.*, 1998; Ishida-Yamamoto & Iizuka, 1995), but little is known concerning involucrin expression as a marker for keratinocyte terminal differentiation in gingival epithelia.

Gap junction mediated intercellular communication has been postulated to be an important mechanism by which cells exchange ions and other small molecules (< 900 Da) to maintain tissue homeostasis and to control growth and differentiation (Bennett *et al.*, 1991). Different types of connexins are expressed in many human and animal tissues, and have been widely studied in normal human tissue and cancer, with findings that the expression patterns of connexins 26 and 43 are distinctive (Wilgenbus *et al.*, 1992). However, connexin expression in gingival epithelium has not been reported.

Filamentous actin (F-actin) is important in cell structural integrity, cell attachment and migration and there is considerable evidence of association to link

F-actin structure to cytoplasmic membrane receptors, particularly E-cadherin through α -catenin (Yap *et al.*, 1997). There is a need to examine the expression of filamentous actin (F-actin) in the context of the reactive pocket epithelium as evidence from studies *in vitro* indicates that expression of F-actin in epithelial cells may be disrupted by specific bacteria that colonise the periodontal pocket (Lamont & Jenkinson, 1998; Fives-Taylor *et al.*, 1999).

This study was therefore undertaken to obtain further information concerning the regulation of the structural integrity and the behaviour of the pocket epithelium by examining the expression of E-cadherin, F-actin, involucrin, and connexins as key markers of cell maintenance, differentiation and communication.

2.3 MATERIALS AND METHODS

2.3.1 Tissue samples

Samples of gingival tissues were obtained with informed consent (and with approval of the Human Ethics Committees of Central and Western Sydney Area Health Services) from 48 adult patients with no record of previous periodontal therapy or relevant medical and drug histories, who were scheduled for extraction of premolar or molar teeth. All patients had a detailed record, including age, sex, dento-gingival site and probing depth. The tissue samples obtained from the buccal aspect were identified as either clinically healthy gingival tissue or advanced periodontitis on the basis of clinical and histological examination. Clinically healthy gingiva was defined by absence of bleeding on examination, a probing depth of 3 mm or less and histological evidence of only a small number of chronic inflammatory cells in the sub-epithelial connective tissues. Patients were matched for age, race and sex to the extent possible in groups listed below. Twenty specimens were obtained from 11 males, (37 to 72 years of age, average 46 years) and 9 females, (41 to 82 years of age, average 57 years), with probing depths of 2-3 mm. Advanced periodontitis lesions showed loss of attachment, formation of periodontal pockets, resorption of the alveolar bone, probing depth of more than 6 mm and numerous chronic inflammatory cells including infiltration of plasma cells throughout the connective tissue. Twenty eight specimens were obtained from 20 males, (30 to 90 years of age, average 57 years), and 8 females, (37 to 69 years of age, average 55 years), with probing depths of 6-10 mm. All biopsies yielded sections that were satisfactory in relation to tissue preservation and orientation.

Gingival tissues were snap-frozen in isopentane cooled in liquid nitrogen and 6 μ m sections cut. Slides with sequential sections were stored in sealed boxes at -70° C until required.

2.3.2 Immunohistochemistry

Sections were fixed in acetone at 4° C for 10 min. Sections were blocked with 3 per cent hydrogen peroxide for 5 min to suppress endogenous peroxidase activity and followed by 20 per cent horse serum in phosphate buffered saline (PBS) for 1 h at room temperature, and then incubated with monoclonal antibodies (1 μ g/ml) to E-cadherin (Transduction Laboratory, Lexington, KY, U.S.A.); involucrin (Sigma Chemical Co., MO, U.S.A.); or connexin 26, 32 and 43 (Zymed, CA, U.S.A.) for 1 h at room temperature in a humid chamber. All primary antibodies were diluted in 10 per cent foetal calf serum in PBS. Slides were washed in fresh PBS three times for 10 min each, with gentle agitation. Sections were incubated with goat anti-mouse antibody (DAKO, Denmark) conjugated with horseradish peroxidase for 1 h at room temperature, followed by three washes with PBS, then stained with enhanced diaminobenzidine (DAB) in stable peroxide buffer (Pierce Ltd). Sections were lightly counterstained with Mayer's haematoxylin and mounted. As negative controls, irrelevant isotype matched monoclonal antibodies (DAKO, Denmark) were used in place of the primary antibodies. Specificity of staining was confirmed by the expected distribution of marker proteins in the cornified regions of healthy gingival tissues.

2.3.3 Fluorescence staining of F-actin

Cryostat sections of tissue from 9 patients with advanced periodontitis and 6 with clinically healthy gingivae were stained for filamentous actin, identified by staining with Phalloidin from *Amanita phalloides*, labelled with Fluorescein Isothiocyanate (FITC) (Sigma Chemical Co., MO, U.S.A.). Prior to labelling, sections were fixed in ice-cold methanol for 4 min and blocked for 1 h with 20 per cent horse serum in PBS. Sections were incubated with FITC-labelled Phalloidin for 1 hr in a dark, humid chamber, then washed in PBS, dried and mounted in an antifade medium, Citifluor (Alltech, NSW, Australia).

2.3.4 Fluorescence microscopy

Fluorescence staining of tissues was visualised using a Leitz Orthoplan microscope equipped with a xenon arc lamp, appropriate dichroics and 25 $\times$ and 50 $\times$, NA 1.0 water-

immersion epifluorescence objectives. Confocal laser scanning was performed with the Optiscan F900e personal confocal system (Optiscan, Melbourne, Australia). Summed digital images were acquired for 2D analysis and 3D reconstruction using Voxblast software, version 1.3.1. (VayTek Inc., Iowa, U.S.A.).

2.3.5 Evaluation of immunolabelled tissues

The gingival epithelium was divided into four regions for clinically healthy gingiva (CHG) and advanced periodontitis (AP) respectively that is oral epithelium (OE), gingival crest (GC), gingival sulcus (GS) and junctional epithelium (JE, i.e. gingival attachment GA) or pocket epithelium (PE) (Fig. 2.1). To ensure reproducibility, sections from both healthy and diseased tissues were processed in batches under uniform, standardised conditions. The intensity of immunostaining of coded sections was graded on a scale of 0 to 4. All sections were scored by two independent observers who were calibrated to reference slides until consensus of > 95% was achieved.

Sections stained for fluorescence were examined using 2D images captured from multiple non-contiguous fields within each region. In addition, series of images were captured for 3D reconstruction and evaluation.

2.3.6 Statistical analysis

Non-parametric analysis of variance using the Kruskal-Wallis test was followed by Dunn's test to compare all regions in both tissue groups with the clinically healthy oral epithelial region, which served as a control. A level of $P < 0.05$ was accepted as statistically significant. The software package GraphPad Prism (GraphPad Software, San Diego, CA, U.S.A.) was used for all data analysis and preparation of graphs.

2.4 RESULTS

2.4.1 Immunostaining patterns for E-cadherin

In both clinically healthy gingiva ($n = 10$) and lesions of advanced periodontitis ($n = 13$), staining in stratified epithelial regions was present at the cell-cell junctions along the cell membrane (Fig. 2.3a). There was moderate staining in the basal layer and strong staining in the spinous layers. The upper spinous layers demonstrated weaker staining and there was no staining detected in the parakeratinized or keratinized layers. No reaction was observed in the control slides.

The intensity of staining for E-cadherin in the gingival sulcus (GS) and junctional epithelium (JE) appeared to be reduced. Pocket epithelium (PE), which displayed a loose structure with few apparent intercellular contacts, exhibited focal and heterogeneous immuno-reactivity (Fig. 2.3b).

Although the patterns of staining were similar in all sections, the quantification of the intensity of immunostaining revealed a decrease from the external oral epithelium (OE, GC) through the gingival sulcus (GS) to the junctional epithelium (JE) of clinically healthy gingiva (Fig. 2.2a, Fig. 2.4a $P < 0.01$). Similar but more pronounced reduction of staining was observed in the pocket epithelium (PE) (Fig. 2.2b, Fig. 2.4a $P < 0.001$).

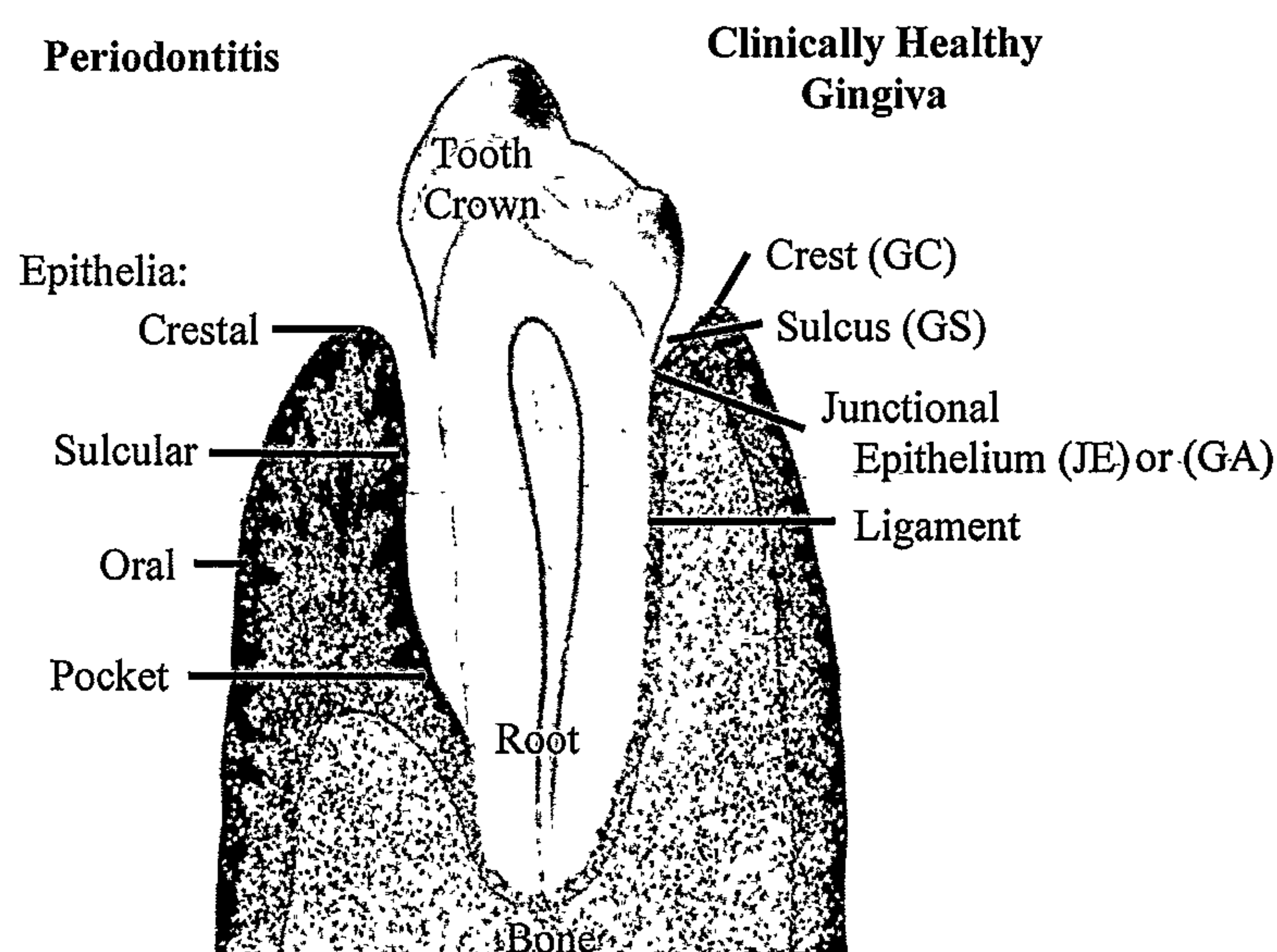


Fig. 2.1 Diagram comparing the anatomy of tissues that are clinically healthy with those affected by periodontitis.

In health, the gingival epithelium is attached to the tooth at the junction of the crown and the root (JE). With the accumulation of bacterial plaque in the gingival sulcus (GS), the gingival tissues become inflamed, establishing the lesion of gingivitis. In some patients, there is progression of gingivitis to periodontitis, with destruction of the supporting periodontal ligament, loss of alveolar bone, and pocket formation, occurring over a period of many years.

2.4.2 Immunostaining patterns for involucrin

Immunohistochemistry showed that the major localisation of involucrin was at the periphery of the cells in the lower spinous layers (Fig. 2.3c). Distinctive staining patterns for involucrin were seen in relation to different regions of gingival epithelium. Immuno-reactivity was consistently intense in OE, GC and GS of clinically healthy gingiva ($n=11$), as well as in OE, GC and GS of advanced periodontitis ($n=13$) (Fig. 2.2c,d), while in the JE staining was variable, with some biopsies demonstrating a weak and focal reaction (5/11), others displaying strong uniform reactivity (4/11). Involucrin expression in PE was absent in 7/13 biopsies (Fig. 2.2d, Fig. 2.3d), or weak and focal in 6/13 biopsies. The intensity of involucrin staining in PE was significantly different (Fig. 2.4b $P < 0.001$) when compared with all other regions in both tissue groups.

2.4.3 Immunostaining patterns for connexins 26, 32 and 43

Different staining patterns for connexin antibodies were evident in the sections of gingival epithelia. Reactivity for connexin 26 was expressed in a discontinuous pattern and in high density at the plasma membrane in the spinous layers only (Fig. 2.3e) in the different regions. Furthermore, there was a distinct appearance in each region of gingival epithelium from both clinically healthy ($n=8$) and advanced periodontitis ($n=13$) tissues. For instance, in oral epithelium (OE) connexin 26 antigen was not expressed in all the spinous layer cells. Variable reactivity was also noted in other regions. On the whole, connexin 26 expression in PE was limited to 5/13 biopsies with some focal staining (Fig. 2.3f), and was significantly reduced compared with OE (Fig. 2.4c $P < 0.001$). No staining for connexin 32 was observed in any biopsy of gingival tissue examined.

Clinically healthy tissue sections ($n=9$) stained for connexin 43 showed a moderate or strong reaction in the cytoplasm of the basal layer and spinous layers (Fig. 2.3g), weak reaction in the upper spinous layers, and with no stain detected in the parakeratinized or keratinized layers. The intensity of staining tended to decrease from OE, GC through GS to JE (Fig. 2.2e, Fig. 4d $P < 0.001$). Similar patterns of staining intensity for connexin 43 in advanced periodontitis ($n=13$) were observed as a progressive decrease from OE, GC, GS to PE (Fig. 2.2f, Fig. 2.4d $P < 0.001$). Specifically, in PE, there was no detectable expression of connexin 43 in the majority of biopsies studied (10/13 biopsies) and with only weak labelling observed in 3/13 samples (Fig. 2.3h).

Fig. 2.2 The low magnification ($\times 40$) photomicrographs show the changes of the staining intensity in clinically healthy gingiva (a,c,e) and advanced periodontitis (b,d,f) labelled sections.

(a,b) The intensity of staining for E-cadherin in clinically healthy gingiva tended to decrease from the external oral epithelium (OE, GC) through the gingival sulcus (GS) to the junctional epithelium (JE) (a). Similar results were observed in advanced periodontitis (b).

(c,d) The intense staining for involucrin in OE, GC and GS of clinically healthy gingiva (c) and advanced periodontitis (d), are in contrast to the weak reaction in JE (c) and the negative staining in PE (d). Involucrin expression in PE was absent in 7/13 biopsies.

(e,f) Similar patterns of staining intensity of connexin 43 were observed in clinically healthy gingiva (e) and advanced periodontitis (f); specifically a progressive decrease from OE GC through GS to PE. There was negative reaction in PE for the majority (10/13) biopsies studied.

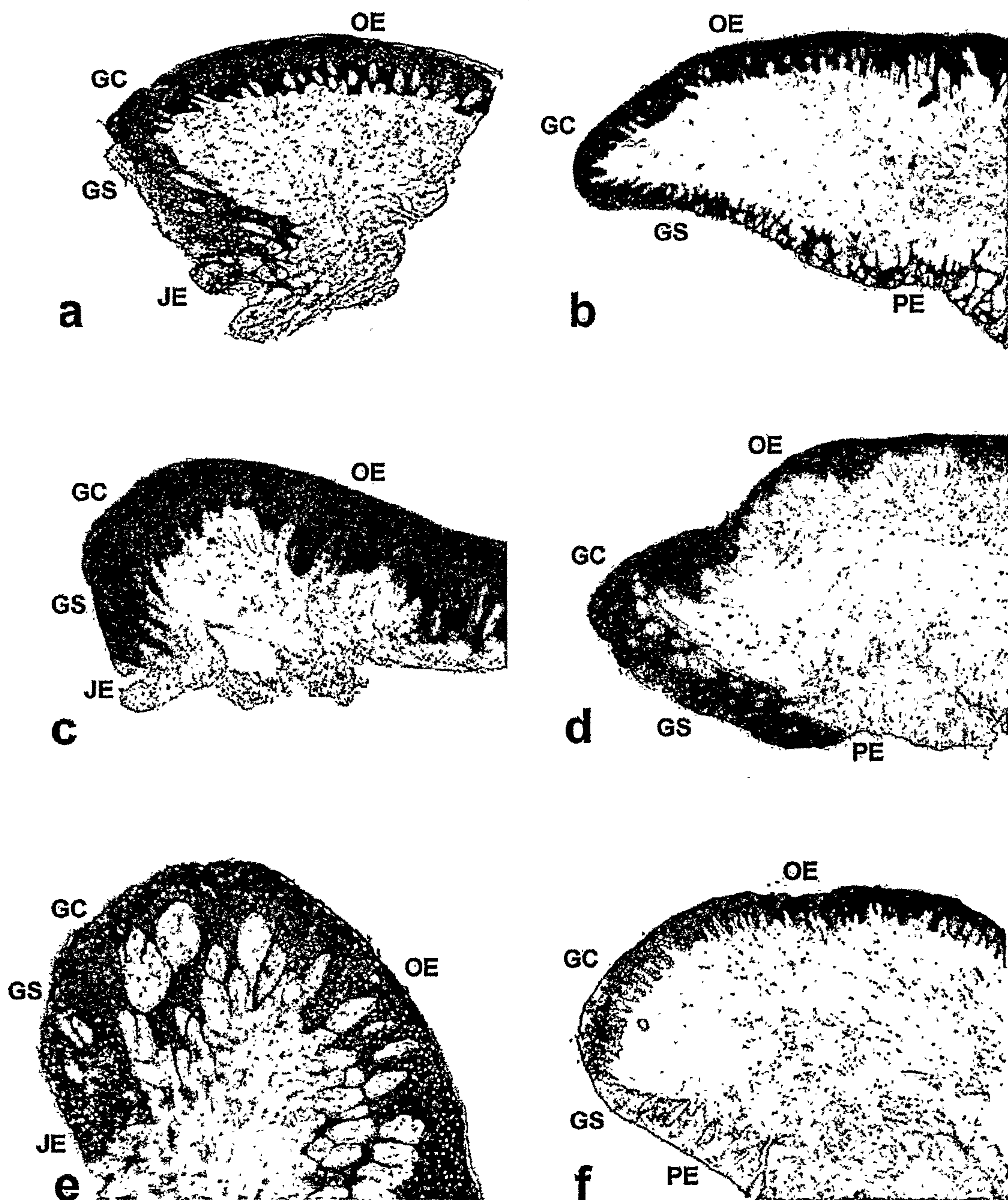


Fig. 2.3 Photomicrographs (×200) of external oral epithelia from clinically healthy (a,c,e,g) and pocket epithelia from advanced periodontitis (b,d,f,h) labelled sections.

(a) A typical staining pattern for E-cadherin in external oral epithelium showing uniform expression at cell-cell contact sites, with strongest staining in the spinous layers.

(b) E-cadherin expression in pocket epithelium displaying focal heterogeneity.

(c) Positive staining for involucrin in external oral epithelium was present at the margins of the cells, displaying strong staining in the lower stratum spinosum but absence of staining in the basal and parakeratinized layers.

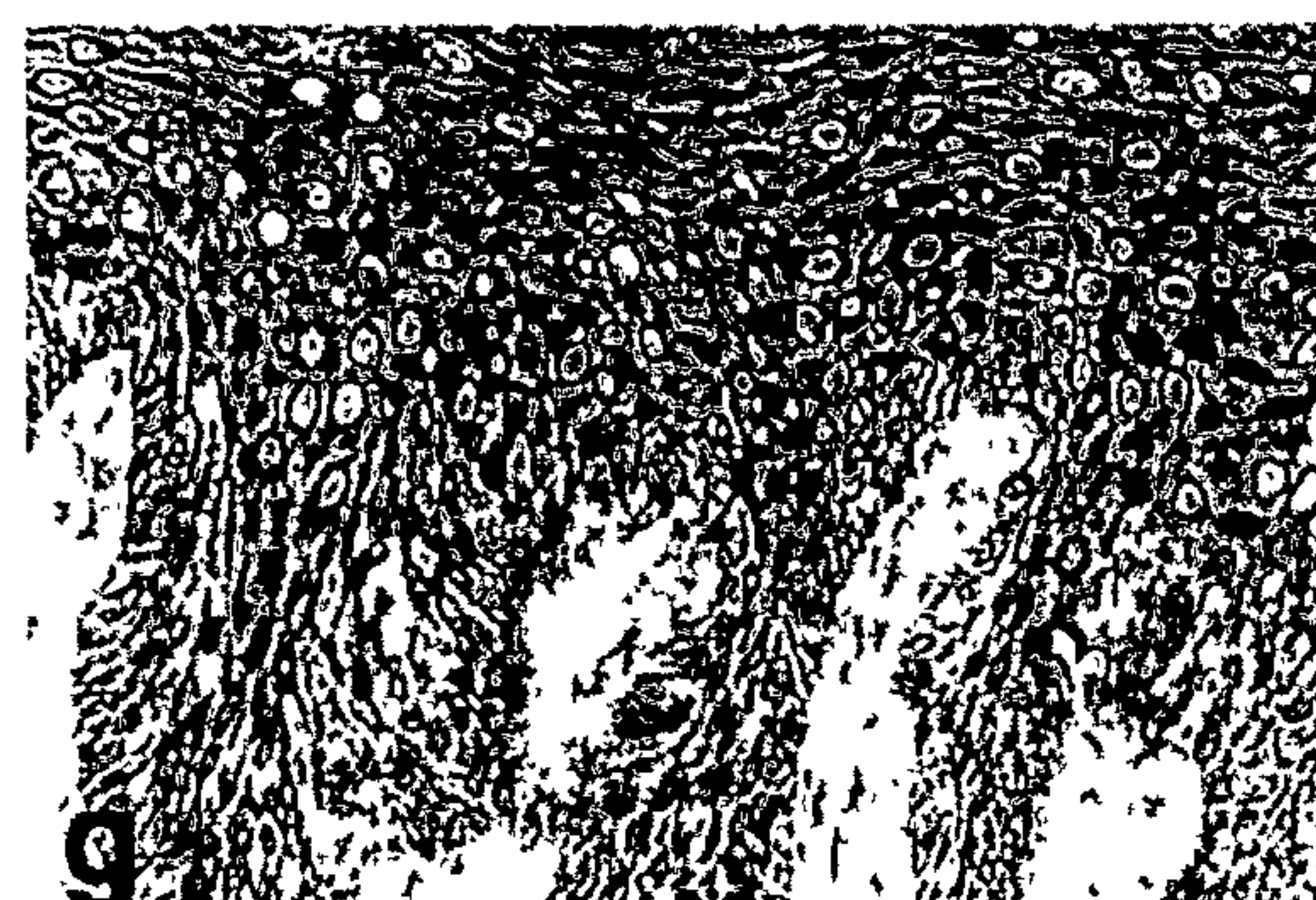
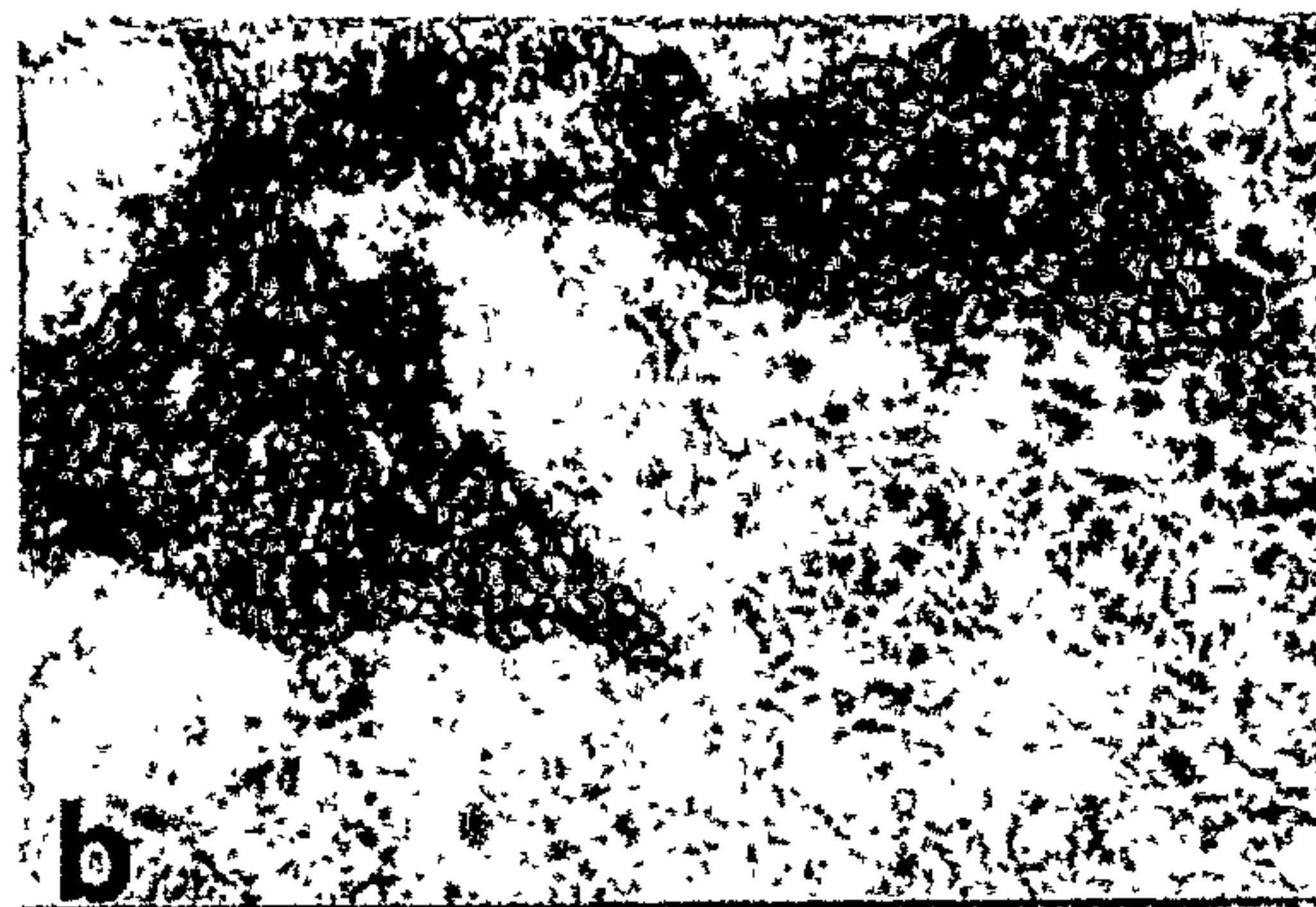
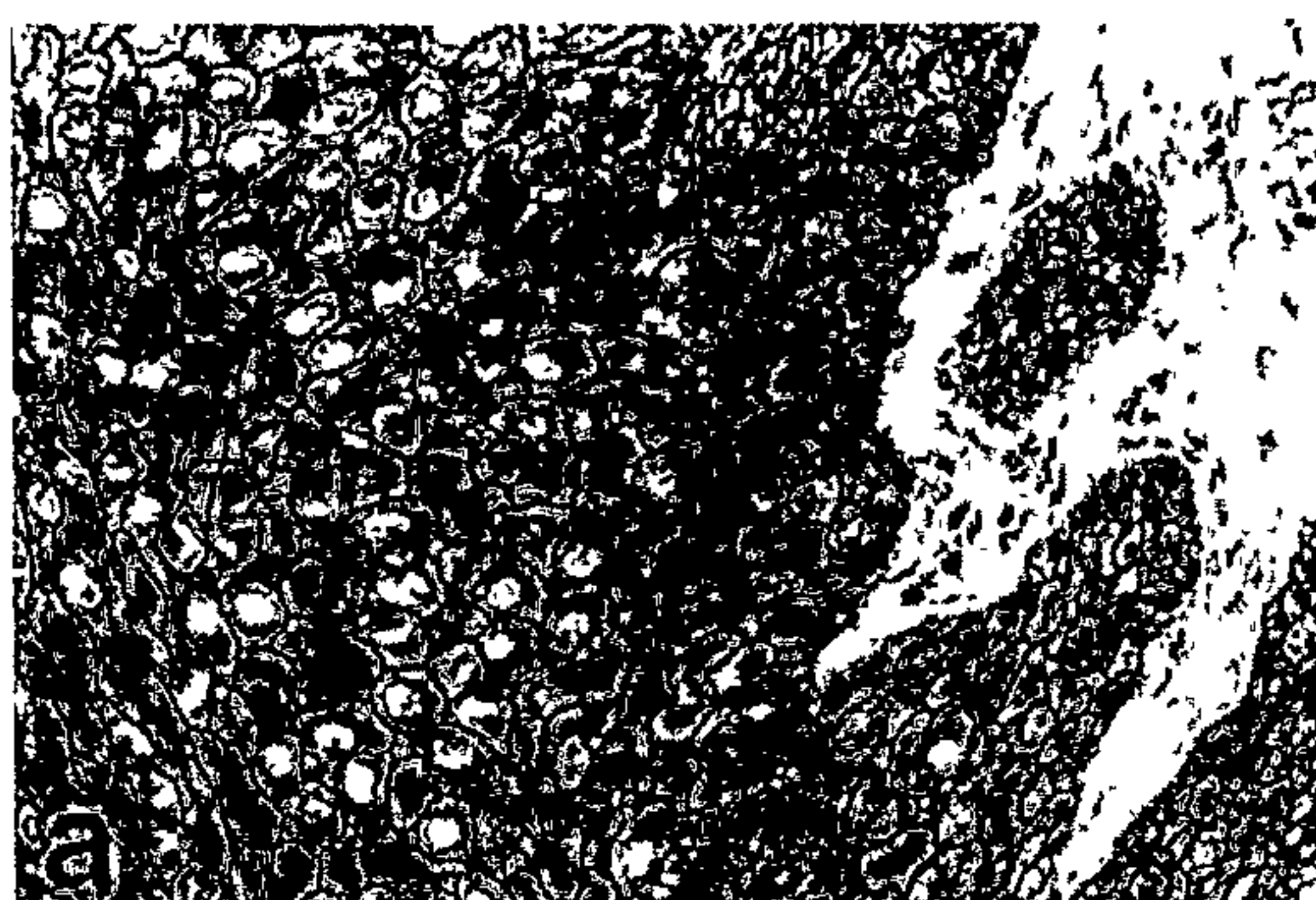
(d) In contrast, no staining for involucrin was detected in pocket epithelium in this biopsy.

(e) Staining for connexin 26 in external oral epithelium showing reactivity only in the spinous layers.

(f) A biopsy showing connexin 26 expression in pocket epithelium with heterogeneous staining and areas completely devoid of reactivity. Most biopsies were negative for this connexin.

(g) Staining for connexin 43 in external oral epithelium demonstrating marked expression in the cytoplasm of epithelial cells in the spinous layers and moderate staining in the basal layer.

(h) Weaker staining for connexin 43 in pocket epithelium.



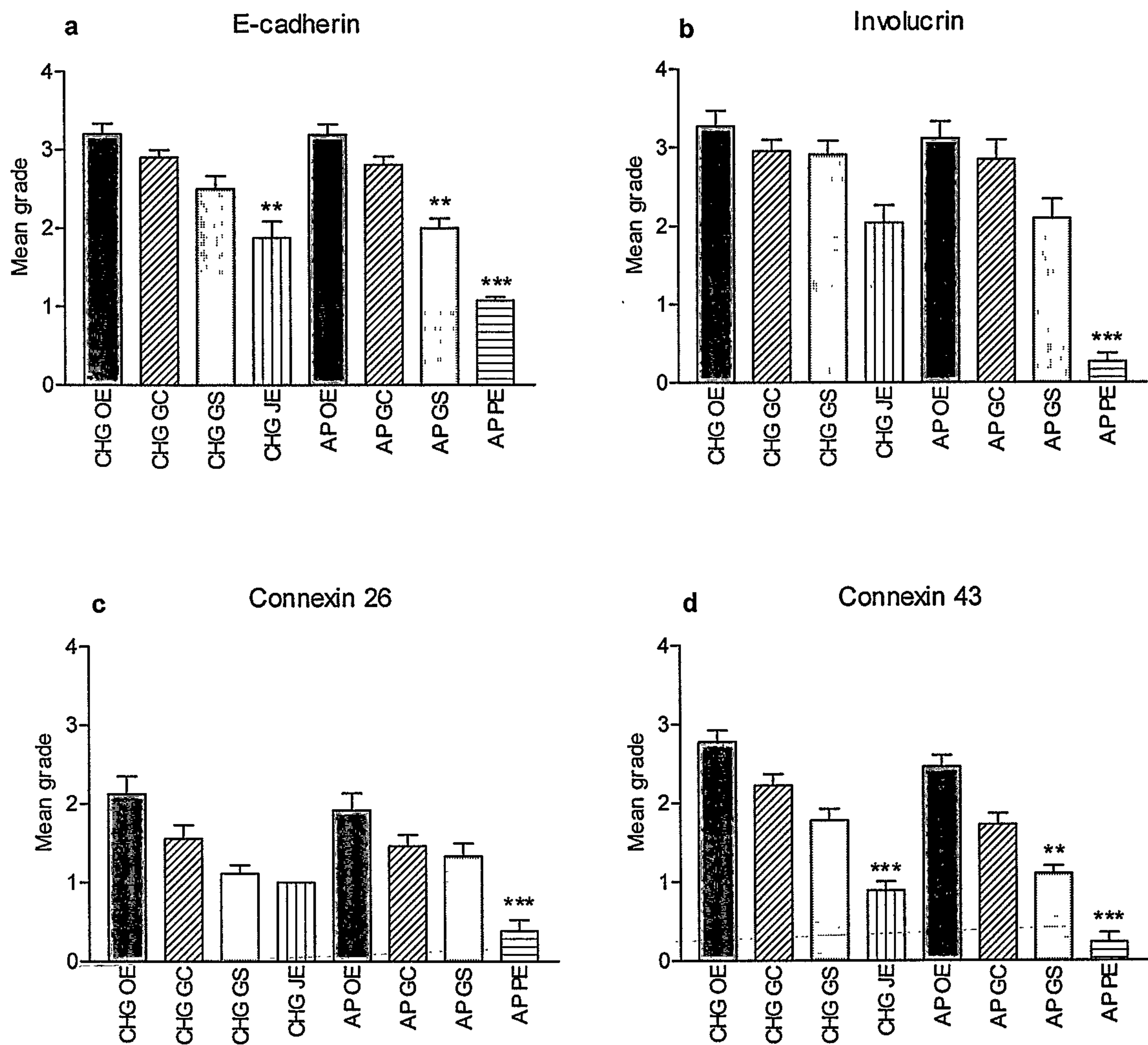


Fig. 2.4 Column graphs showing mean grade of intensity with standard deviations of staining in different regions of epithelium in both clinically healthy gingiva (CHG) and advanced periodontitis (AP) tissues.

Stained for (a) E-cadherin, (b) involucrin, (c) connexin 26, (d) connexin 43. Differences between groups were evaluated using Dunn's test, comparing each region in both sets of tissues to the oral epithelium of clinically healthy tissues, which served as a control. Significant differences are shown as ** = $P < 0.01$, *** = $P < 0.001$).

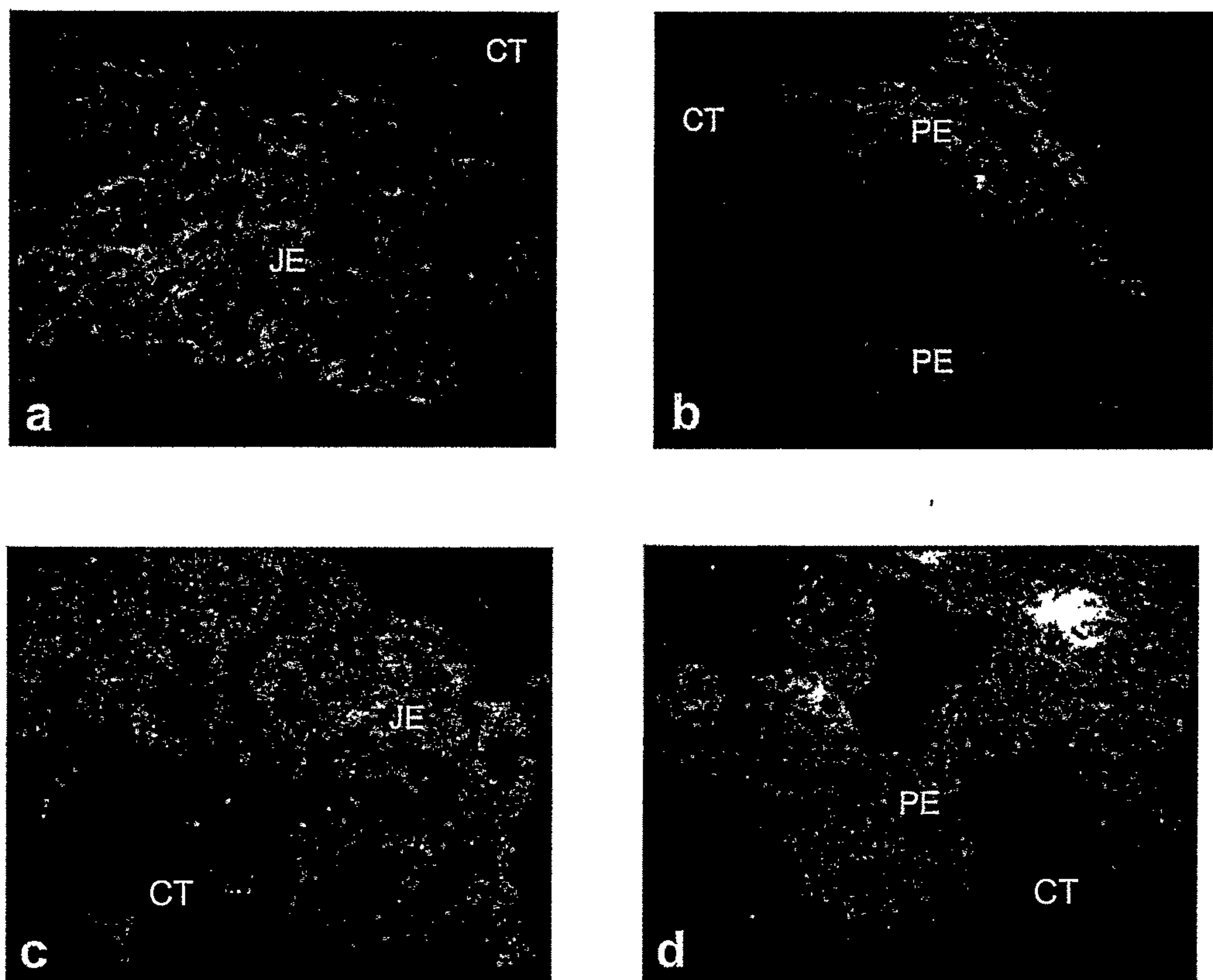


Fig. 2.5 Photomicrographs ($\times 600$) of gingival epithelium showing fluorescence localisation of F-actin, and adjacent connective tissue (CT) showing negative fluorescence staining.

(a) Clinically healthy gingiva showing a typical staining pattern in the region of junctional epithelium (JE).

(b) Periodontitis tissue showing a variable staining pattern with some regions of intense atypical reactivity and some areas devoid of staining, typical of pocket epithelium (PE).

(c, d) Higher magnification 3D reconstructions of F-actin labelled epithelium in the regions of (c) junctional epithelium (JE) and (d) pocket epithelium (PE).

2.4.4 Fluorescence staining for F-actin

Using FITC-labelled phalloidin for F-actin, clinically healthy tissue displayed uniform epithelial reactivity for F-actin throughout the OE, GC, GS and JE regions (Fig. 2.5a). The lining epithelia of periodontitis lesions also showed uniform staining for F-actin in OE, GC and GS, whereas the pocket epithelium was demonstrably different (Fig. 2.5b). Pocket epithelium (PE) contained areas of intense focal staining, often of groups of epithelial cells. These sites were interspersed with a more frequent weak reaction and

in some regions the absence of F-actin in epithelial cells. Within the cytoplasm of PE cells, F-actin distribution was typically asymmetrical and sometimes aggregated. 3D reconstruction of F-actin labelled gingival tissues showed a loose and poorly organised pocket epithelium (PE) in comparison with the junctional epithelium (JE) of healthy tissue (Fig. 2.5 c,d).

2.5 DISCUSSION

In this study, the expression of E-cadherin, involucrin, connexins 26 and 43, and F-actin, key markers of cell maintenance, differentiation and communication in clinically healthy gingival tissue and in periodontitis were examined. The expression of these markers was either weak or absent in the reactive pocket epithelium of periodontitis.

E-cadherin molecules are not only required for maintenance of tissue integrity, but also regulate many aspects of cell behaviour, including growth, differentiation, polarization and stratification of epithelial cells (Kandikonda *et al.*, 1996; Govze & Herzberg, 1993). The findings of the present study confirm the localization of E-cadherin in healthy gingiva as previously reported (Yamada *et al.*, 1997; Sakaki *et al.*, 1994; Sakaki *et al.*, 1993; Downer & Speight, 1993). Marked expression of E-cadherin in the basal cell layer (the main region of proliferating cells) of gingival epithelium was also found. However, the intensity of staining was reduced in the junctional epithelium (JE) of clinically healthy gingiva, and strikingly so in reactive pocket epithelia (PE) of periodontitis when compared with the external oral epithelia. In reactive pocket epithelia (PE), there was loss of the proteins responsible for the structural integrity and this may contribute to the lack of structural organisation of pocket epithelium.

Involucrin was expressed in the lower spinous layers of the external oral epithelia (OE, GC) and gingival sulcus (GS), as well as in the junctional epithelium (JE). Although the spinous layers undergo progressive intracellular remodelling in preparation for terminal differentiation, they contain viable and functioning cells (Eckert *et al.*, 1993). Therefore, the results confirm that involucrin expression can be identified as a marker of terminal differentiation in gingival epithelia and could function to protect the junctional epithelium from invasion by periodontal pathogens. The observed patterns of involucrin expression indicated a failure of the superficial cells of pocket epithelium to undergo terminal differentiation. In particular, there is the implication that the microbial flora of the pocket can interact directly with a responsive

epithelial surface. This adds credibility to the studies *in vitro* which have demonstrated the capacity of the periodontal pathogen *Porphyromonas gingivalis* to infect gingival epithelial cells (Lamont *et al.*, 1995).

Connexin 26 was expressed in the spinous layers with distinct appearance typical of gap junctional intercellular communication channels in the four regions of gingival epithelia and with individual variation. This result suggests that connexin 26 protein could be associated with different levels of differentiation of gingival epithelial cells. Nevertheless, reactivity for connexin 26 was only detected at trace levels in the reactive pocket epithelia (PE). Connexin 32 was not detected in any gingival tissues examined in accordance with the findings for most human epithelia in both normal and diseased tissues, such as skin, lung and oesophagus (Wilgenbus *et al.*, 1992). The reaction for connexin 43 was predominantly cytoplasmic rather than discontinuous cell surface staining. This pattern was not altered by fixation in methanol or paraformaldehyde. Studies in a mouse model have indicated that a predominantly cytoplasmic expression of connexin 43 in epithelial cells was related to the failure of this protein to contribute to gap junctional intercellular communication channels (Jongen *et al.*, 1991). Connexin 43 expression showed a progressive decrease from the external oral epithelia to the gingival sulcus and junctional epithelium of clinically healthy gingiva. Similar results were obtained in advanced periodontitis, with markedly reduced reactivity in pocket epithelia (PE).

While the presence of gap junction communication channels is defined by morphology, the presence of connexins in a surface location, demonstrated only for connexin 26 in this study, is generally accepted as indicative of these structural entities, which appear to be effectively absent in reactive pocket epithelia. These results suggest a communication deficiency between epithelial cells in pocket epithelia (PE) of advanced periodontitis, with scattered and low levels of connexins 26 and 43. The homeostatic function of gap junctions may influence important events during epithelial morphogenesis, including proliferation, differentiation and migration of keratinocytes. This change may provide an altered environment favourable for invasion by bacteria.

Previously reported investigations have demonstrated a correlation between the calcium-dependent cell adhesion molecule E-cadherin and gap junctional intercellular communication. In mouse epidermal cells, gap junction protein (connexin 43) function in intercellular channels is regulated by E-cadherin (Jongen *et al.*, 1991, Yamasaki & Naus, 1996) suggesting an important role for cell adhesion molecules in

the regulation of gap junctions. A possible correlation exists in the pathological lining epithelia of gingiva between E-cadherin and connexin 43. The expression pattern and functional regulation of connexins appears to be different in various pathological states and therefore the interpretation of the impact of the downregulation of these proteins in periodontitis remains speculative.

FITC-labelled phalloidin data indicated a correlation between the down-regulation of E-cadherin expression and atypical distribution of F-actin at the level of both tissue and cell for the pocket epithelium. There is considerable evidence of association to link filamentous-actin structure to cytoplasmic membrane receptors, particularly E-cadherin through α -catenin (Yap *et al.*, 1997). Accordingly, the manifest perturbation of structure of the pocket epithelium could be linked to either a disruption of F-actin leading to altered intercellular adhesion and communication, or to a disruption of cell adhesion. In this context, the periodontal pathogens *Porphyromonas gingivalis* and *Haemophilus actinomycetemcomitans* as well as *Treponema denticola* have been demonstrated to alter F-actin expression in gingival epithelial cells *in vitro* (Lamont & Jenkinson, 1998; Fives-Taylor *et al.*, 1999; DeFilippo *et al.*, 1995). In addition, the virulence of *P. gingivalis* is mediated by cysteine proteinases with trypsin-like specificity that have the capacity to degrade a wide variety of protein substrates, possibly including cell adhesion molecules. Other bacteria are known to alter F-actin (Theriot 1995). Successful escape from phago-lysosomes by two widely divergent bacteria *Shigella flexneri* and *Listeria monocytogenes* is associated with migration of these organisms in the cytoplasm mediated by “comet tails” of actin. Therefore, a linked perturbation of essential structural units could relate to either the endogenous action of invading bacteria or the exogenous effects of bacterial products.

In summary, the altered expression of the cell adhesion complexes and F-actin of the epithelial cells of the reactive pocket epithelium could reflect the action of periodontal pocket bacteria or their products. Alternatively, perturbation could be related to the poorly understood developmental origin of the pocket epithelium. Elucidation of the mechanisms involved will provide a basis for understanding this critical rate-limiting determinant of the pathogenesis of one of the most common destructive infections.



CHAPTER 3

DIFFERENTIAL EXPRESSION OF TRANSFORMING GROWTH FACTORS- β 1, - β 2, - β 3 AND THE TYPE I, II, III RECEPTORS IN THE LINING EPITHELIA OF INFLAMED GINGIVA

CHAPTER 3: DIFFERENTIAL EXPRESSION OF TRANSFORMING GROWTH FACTORS- β 1, - β 2, - β 3 AND THE TYPE I, II, III RECEPTORS IN THE LINING EPITHELIA OF INFLAMED GINGIVA

3.1 ABSTRACT

Background: Transforming growth factor- β isoforms have important functions including the control of cell migration, proliferation and differentiation and the regulation of inflammation and immunity. The present study determined, by immunohistochemistry, the expression patterns of TGF- β s and their receptors in the lining epithelia of inflamed gingiva. Frozen sections were obtained from 22 human gingival biopsies.

Results: TGF- β 1 was not detected in gingival epithelial cells in sections. TGF- β 2 was detected. There was a progressive reduction of staining from the external oral epithelium through to gingival sulcus and the gingival attachment or pocket epithelium. TGF- β 3 showed intense staining in all domains of both minimally inflamed gingiva and advanced periodontitis tissues. TGF- β RI was visualised as focal staining of the spinous layer in the external oral epithelium of both periodontitis lesions and minimally inflamed tissues. TGF- β RII was present throughout the strata but with progressive reduction in intensity from the oral epithelium to gingival attachment or pocket epithelium respectively while, conversely, TGF- β RIII showed an increase in diffuse staining intensity from external oral epithelium to pocket epithelium.

Conclusion: A distinct expression profile was observed within different individuals for TGF- β s and the corresponding receptors. These findings provide a basis for evaluation of the role of these growth factors in the pathogenesis of periodontitis.

Key words: TGF- β s; TGF- β receptors; immunohistochemistry; polyclonal antibodies; periodontitis

3.2 INTRODUCTION

Destructive periodontitis is characterised by a pathological migration of the epithelial attachment along the tooth root and bone surface concomitant with a dense infiltrate of inflammatory cells, loss of connective tissue matrix, formation of periodontal pockets

and resorption of alveolar bone. Gingival epithelial cells (keratinocytes) play an important role as the first barrier against the offending bacterial invasion in gingival tissues (Presland & Jurevic, 2002).

Epithelial morphogenesis is regulated by intra- and extra-cellular interactions such as cell adhesion molecules, cellular matrix and soluble factors, including transforming growth factor- α (TGF- α), - β (TGF- β), fibroblast growth factors (FGF) and platelet-derived growth factor (PDGF). Epithelial-mesenchymal interactions also influence cell differentiation and development of a variety of oral embryonic epithelia (Vaahtokari *et al.*, 1991). In preliminary studies (Ye *et al.*, 2000), reduced expression of proteins including E-cadherin, involucrin and the gap junction connexins, considered to be important markers respectively of the structural integrity associated with cell adhesion, differentiation and communication, in the lining epithelia of inflamed gingiva, was demonstrated. These findings are compatible with the loose, poorly structured morphology of the pocket epithelium (Ye *et al.*, 2000). Failure to regulate expression of these proteins in epithelial cells may contribute to a variety of perturbations including hyperplasia and immunopathology (Ye *et al.*, 2000; Downer & Speight, 1993).

TGF- β is a superfamily of regulatory proteins that includes five isoforms of TGF- β , only three of which, TGF- β 1, - β 2, and - β 3 have been identified in mammals. TGF- β s signals are expressed through the same serine-threonine kinase type I and type II cell surface receptors. The different isoforms of TGF- β s are encoded by different genes and have many biological activities. The action of TGF- β is multifunctional, not only contributing to epithelial cell migration, proliferation and differentiation (Ciacchi *et al.*, 1993; Massague, 1990; Moses *et al.*, 1994), but also facilitating formation of the connective tissue matrix proteins (Wahl *et al.*, 1993), and having an essential role in regulating immune aspects of disease pathogenesis (Letterio & Roberts, 1998). TGF- β regulates T cell function (Gorelik & Flavell, 2002) activation and apoptosis and also has a critical role in B cell function. It typically inhibits B cell proliferation and may induce apoptosis in both B cells and fully differentiated plasma cells (Letterio & Roberts, 1998; Lomo *et al.*, 1995). TGF- β induces a chemotactic response, in sites of injury or inflammation, in human peripheral blood monocytes and promotes leukocyte adhesion to endothelium and extracellular matrix. Thus, it has angiogenic and pro-inflammatory properties. In contrast, the actions of TGF- β on tissue macrophages are generally suppressive and balance an inflammatory response (Wrana *et al.*, 1992).

In addition, strong evidence indicates the role of TGF- β in the pathogenesis of

autoimmune disease. Studies on animal models such as collagen-induced arthritis and systemic lupus erythematosus demonstrated that TGF- β actively participates in binding IgG autoantibodies and promotes a suppressive effect on autoimmune diseases (Letterio & Roberts, 1998; Stach & Rowley, 1993).

The TGF- β genes have been identified at sites of inflammation in different disease lesions, including periodontal disease. The present study was undertaken by immunoperoxidase technique using polyclonal antibodies to probe gingival biopsy samples to explore the distribution of TGF- β s and their receptors I, II and III in gingival epithelia.

3.3 MATERIALS AND METHODS

3.3.1 Tissue samples

Samples of gingival tissues were obtained with informed consent from 22 adult patients with no record of treatment for periodontal disease, that is, the tissue biopsies reflected natural disease progression in this community; also no record of systemic diseases, including diabetes, known to influence progression of periodontal disease and no history of antibiotic use in the previous 6 months; who were scheduled for extraction of premolar or molar teeth. The tissue samples obtained from the buccal aspect were identified as minimally inflamed gingival tissue or advanced periodontitis on the basis of clinical record, radiographs and histological examination. Minimally inflamed gingiva (as clinically healthy gingiva) was defined by absence of bleeding on gentle probing, no clinical signs of inflammation, a probing depth of 3 mm or less and histological evidence of only a small number of chronic inflammatory cells in the sub-epithelial connective tissues. Nine specimens with probing depths of 2–3 mm were obtained from 5 males, (49–75 years of age, average 57 years) and 4 females, (50–82 years of age, average 64 years). The age, race and gender matched group of advanced periodontitis (severe generalized or localized) lesions showed loss of attachment (7–11 mm), formation of periodontal pockets with probing depth of more than 6 mm, resorption of the alveolar bone and numerous chronic inflammatory cells including infiltration of plasma cells throughout the connective tissue. Thirteen specimens with probing depths 6–8 mm were obtained from 9 males, (42–83 years of age, average 56 years) and 4 females, (45–69 years of age, average 58 years). All biopsies yielded serial sections that were satisfactory in relation to tissue preservation and orientation.

Gingival tissues were snap-frozen in isopentane cooled in liquid nitrogen and 6 μ m sections cut. Slides with sequential sections were stored in sealed boxes at -70°C until required.

3.3.2 Immunohistochemistry

Rabbit or goat polyclonal antibodies used were provided by Santa Cruz Biotechnology. The antibodies were raised against a peptide mapping at the carboxy terminus of the precursor form of TGF- β 1 (cat: sc-146), TGF- β 2 (cat: sc-90), TGF- β 3 (cat: sc-82), TGF- β RI (cat: sc-402), TGF- β RII (cat: sc-400) and TGF- β RIII (sc-6199) of human origin.

Serial sections taken from the same patient were used for each different antibody. Briefly, sections were fixed in acetone at 4°C for 10 min, and then blocked with 20 per cent horse serum in phosphate buffered saline (PBS) for 1 hr at room temperature. Sections were then incubated with affinity-purified rabbit or goat polyclonal primary antibodies for 1 hr at room temperature in a humid chamber. All primary antibodies were diluted in 10 per cent foetal calf serum in PBS. TGF- β 1, - β RI and - β RIII antibodies were used at 8 $\mu\text{g/mL}$. TGF- β RII antibody was used at 2 $\mu\text{g/mL}$. TGF- β 2 antibody was used at 1 $\mu\text{g/mL}$, and TGF- β 3 antibody was used at 0.5 $\mu\text{g/mL}$. Negative control slides were incubated with the IgG fraction of normal rabbit serum (DAKO, A/S, Denmark) at the same concentration as the primary antibody. Tissue slides were then rinsed in fresh PBS three times for 5 min each with gentle agitation. Sections were incubated with swine anti-rabbit or rabbit anti-goat secondary antibody conjugated with horseradish peroxidase diluted 1:50–100 (DAKO, A/S, Denmark), for 1 hr at room temperature, followed by three washes with PBS, then stained with enhanced diaminobenzidine (DAB) in stable peroxide buffer (Pierce Ltd). Sections were lightly counterstained with Mayer's haematoxylin and mounted. All positive reactions for TGF- β s and their receptors were confirmed by specific reduction in staining by pre-incubation of the antibody preparation with control peptide TGF- β 2 (cat: sc-90P), TGF- β 3 (cat: sc-82P), TGF- β RI (cat: sc-398P), TGF- β RII (cat: sc-400P) and TGF- β RIII (sc-6199P).

Rat small intestine and human skin from breast were used as positive controls. Simultaneously, relevant slides from patients were stained with H & E as a guide in this study. The immunohistochemical results obtained were consistent and reproducible.

3.3.3 Oral epithelial cell line (H413)

The epithelial cell line H413 (provided by Professor Prime and Dr Patterson, University of Bristol, U. K.) derived from a human oral squamous cell carcinoma (Prime *et al.*, 1990), displayed stratified epithelial cell morphology in culture. The cells were cultured in Dulbecco modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture (1:1) (Sigma, Sydney, Australia), 15mM HEPES (Sigma) and supplemented with 10% foetal bovine serum (CSL Limited, Victoria, Australia) and penicillin/streptomycin (100 IU/ml, ICN Biomedicals, Inc., Sydney, Australia) at 37°C with 5% CO₂.

3.3.4 H413 cell antigens

The H413 cells were subcultured every 2–3 days and harvested at a multilayer confluence (10^7 – 10^8 cells/3–4 mg total protein) in 175 cm² flasks (Sarstedt, Inc., Newton, NC). Briefly, cells were placed on ice, washed with ice cold Dulbecco's phosphate buffered saline (DPBS), containing a proteinase inhibitor cocktail (Sigma-P8340) and harvested by gentle scraping. After centrifugation, in preparation for Western blot analysis, cells were resuspended in 2ml PBS, then boiled in sodium dodecyl sulfate (SDS) sample buffer with 1% 2-mercaptoethanol for 10 minutes.

3.3.5 Western immunoblot

H413 antigens were separated by standard SDS-PAGE mini gels (5% stacking gel, 12% separating gel). Fractionated cell proteins were transferred onto nitrocellulose membranes (Bio-Rad, Australia), and blocked with 3% bovine serum albumin (BSA, Sigma) in 0.1M Tris-buffered saline (TBS, pH 7.5) overnight. Blotted H413 antigens were incubated with TGF- β s and receptor antibodies (Santa Cruz Biotechnology, Inc.) used at 1 μ g/mL. The secondary antibody employed was alkaline phosphatase (AP) conjugated goat anti-rabbit (DAKO) or mouse anti-goat IgG (Sigma) diluted 1:2,000 in 0.05% Tween 20/TBS. IgG antibody binding antigen was visualized with AP substrate (cat 170-6432, Bio-Rad).

3.3.6 Evaluation of results

Gingival epithelial sections (Fig. 3.1) were coded and examined as four regions for minimally inflamed gingiva (Min) and advanced periodontitis (Adv) respectively, that is, oral epithelium (OE), gingival crest (GC), gingival sulcus (GS) and gingival

attachment (GA, i.e. junctional epithelium JE) or pocket epithelium (PE). To ensure reproducibility, serial sections from both clinically healthy and diseased tissues were processed in batches under uniform, standardised conditions. The intensity of immunostaining was assessed on a visual analogue scale ranging from 0 to 3, that is, 0 = – (negative), no staining or very little was detectable; 1 = +, weak staining either in the cytoplasm or plasma membrane were observed with 30–60 per cent of epithelial cells; 2 = ++, moderate staining with 60–90 per cent of epithelial cells; 3 = +++, strong and intensive staining with more than 90 per cent of epithelial cells. All sections were scored by two independent observers who were calibrated to reference slides.

Non-parametric analysis of variance using the Kruskal-Wallis test was followed by Dunn's test (Prism Software, San Diego, CA, USA) to compare all regions in both tissue groups with each other. A level of $P < 0.05$ was accepted as statistically significant.

3.4 RESULTS

The positive control sections (rat small intestine tissue and human skin tissue) had clear immunoreactive labelling in epithelial cells. The negative control sections had no specific immunoreactivity in any region. All samples probed were categorized into the two groups for each antibody, that is advanced periodontitis ($n = 13$) and minimally inflamed gingiva ($n = 9$).

3.4.1 Immunostaining patterns for TGF- β 1, - β 2 and - β 3

TGF- β 1: No discernible epithelial staining was observed in any gingival epithelial biopsies (Fig. 3.2, A and B) or in human skin, but staining in blood vessel of the connective tissue was detected in some examined sections and in the positive control of rat small intestine. This result was confirmed by a second antibody, anti-human LAP (TGF- β 1) neutralizing antibody (R&D Systems Inc., NSW, Australia).

TGF- β 2: Reactivity was detected in the external oral epithelium (OE, GC) of minimally inflamed gingiva and in the four regions of advanced periodontitis tissues. Moderate staining was noted in the granular and spinous layers, and the basal layer in the cytoplasm and plasma membrane in stratified epithelium (Fig. 3.2, C and D). The intensity of immunoreactivity for TGF- β 2 showed a significant increase in the external oral epithelium (OE, GC) of advanced periodontitis compared with the GS (Fig. 3.5a, P

< 0.001) and GA (Fig. 3.5a, $P < 0.01$) of minimally inflamed gingiva. Further, there was a diverse profile observed in the gingival epithelium for different individuals. In minimally inflamed gingiva, TGF- β 2 expression in GS was absent in 7/9 biopsies, while GA demonstrated trace reaction in 4/9 biopsies. TGF- β 2 expression in PE was shown as weak or focal reaction in 7/13 biopsies (Fig. 3.2E, and Table 3.1).

TGF- β 3 expression differed from TGF- β 2. In both minimally inflamed gingiva and lesions of advanced periodontitis, staining in stratified epithelial regions was present at the plasma membrane in the basal layer and suprabasal layers with an intense reaction (Fig. 3.3, A and B). Although the patterns of staining for TGF- β 3 were similar in all sections, the distribution of TGF- β 3 indicated higher levels in the four regions of advanced periodontitis than that of minimally inflamed gingiva. There was a difference in the OE of advanced periodontitis compared with the GA of minimally inflamed gingiva (Fig. 3.5b, $P < 0.05$). Pocket epithelium (PE) displayed an increased focal and heterogeneous immunoreactivity (Fig. 3.3C, and Table 3.1).

3.4.2 Immunostaining patterns for TGF- β RI, - β RII and - β RIII

The staining for TGF- β RI was in a discontinuous pattern at the plasma membrane in the spinous layers in the gingival epithelium (Fig. 3.3, D and E). The reaction of TGF- β RI displayed weak or moderate reaction in external oral epithelium (OE, GC), and negative or very faint reaction in GS, GA and PE of both minimally inflamed gingiva and advanced periodontitis tissues. There was a distinct profile observed for different individuals. TGF- β RI expression in external oral epithelium (OE, GC) was detected in 6/13 biopsies in advanced periodontitis, while in 4/9 biopsies in minimally inflamed gingiva. There was no detectable expression of TGF- β RI in 10/13 biopsies in PE of advanced periodontitis (Fig. 3.3F), and none in GS and GA in biopsies ($n = 9$) of minimally inflamed gingiva (Table 3.1). However, no epithelial staining was observed in any gingival sections using an antibody to an internal domain of TGF- β RI (cat: sc-398) whereas staining was observed in the positive control rat intestine sections.

The staining pattern of TGF- β RII exhibited a moderate or weak reaction at the cell surface along the cell membrane in the keratinized or parakeratinized, granular and spinous layers (Fig. 3.4, A and B). In 8/9 and 7/9 biopsies of minimally inflamed gingiva, there was negative to faint staining in the GS and GA respectively, whereas in advanced periodontitis tissues, there was an increased level of positive cells in the OE compared with the GS and GA of minimally inflamed gingiva (Fig. 3.5c, $P < 0.05$).

Pocket epithelium displayed a loose structure with heterogeneous reactivity (Fig. 3.4C and Table 3.1). In addition, the mean intensities of staining of TGF- β RII for each region of the two sets of groups were at a relatively low level compared with that for TGF- β 2 and TGF- β 3 (Fig. 3.5).

In contrast, the staining of TGF- β RIII showed a reverse profile in the four regions of epithelium compared with other antibody reaction for TGF- β 2, - β RI and - β RII. The intensity of staining for TGF- β RIII in advanced periodontitis increased from negative (10/13) or trace (3/13) reaction in the external oral epithelium (OE, GC) (Fig. 3.4, D and E), through weak product in the gingival sulcus (GS, 6/13), and to moderate reactivity in the pocket epithelium (PE, 7/13) (Fig. 3.4F and Table 3.1). In minimally inflamed gingiva, only 3/9 biopsies had detectable staining in GS and GA (Table 3.1). The location of positive staining was at the cell membrane in basal and suprabasal layers in epithelia.

3.4.3 Western analysis for TGF- β s and their receptors in epithelial cells

H413 oral epithelial cells were extracted as described in Methods. There was no obvious reactivity for TGF- β 1, while TGF- β RI and RIII showed very weak reaction; however TGF- β 2, - β 3 and - β RII were strongly expressed (Fig. 3.6). Western blot analysis supported the immunohistochemical results.

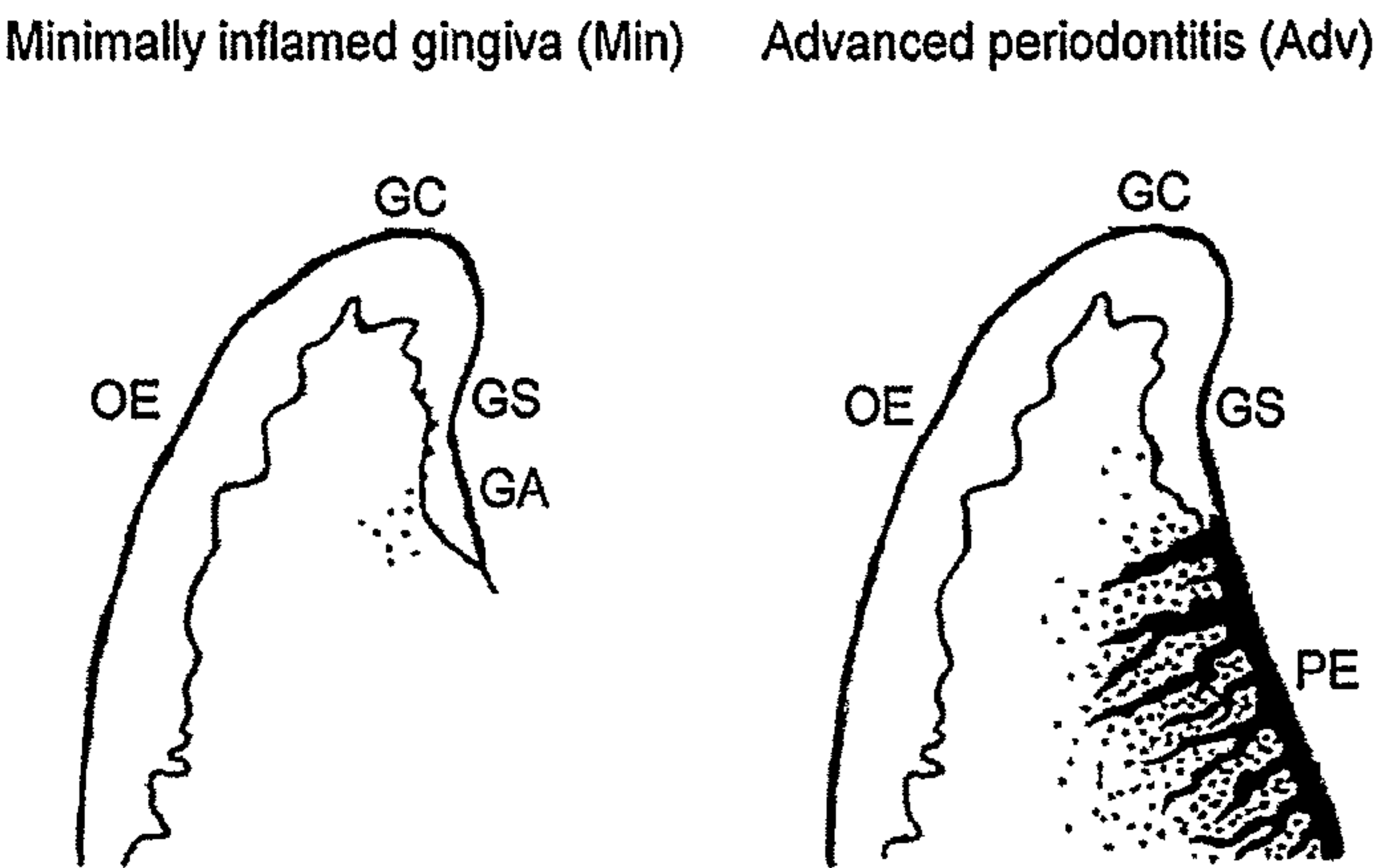


Fig. 3.1 Diagram showing regions of gingival epithelial sections.

Fig. 3.2 Photomicrographs of reactivity for TGF- β 1, (A and B), TGF- β 2, (C, D and E).

- (A) Negative staining for TGF- β 1 in gingival epithelium (OE, $\times 200$) in all examined sections.
- (B) No staining observed in pocket epithelium (PE, $\times 200$) for TGF- β 1.
- (C) Positive staining for TGF- β 2 in stratified oral epithelium (OE, $\times 200$) of advanced periodontitis. Similar patterns were observed in minimally inflamed gingiva.
- (D) Showing high magnification (box in C, $\times 400$) of TGF- β 2 expression pattern.
- (E) Staining for TGF- β 2 in pocket epithelium (PE, $\times 200$) showing weak and focal reactivity mainly in the basal layer in 7/13 biopsies.

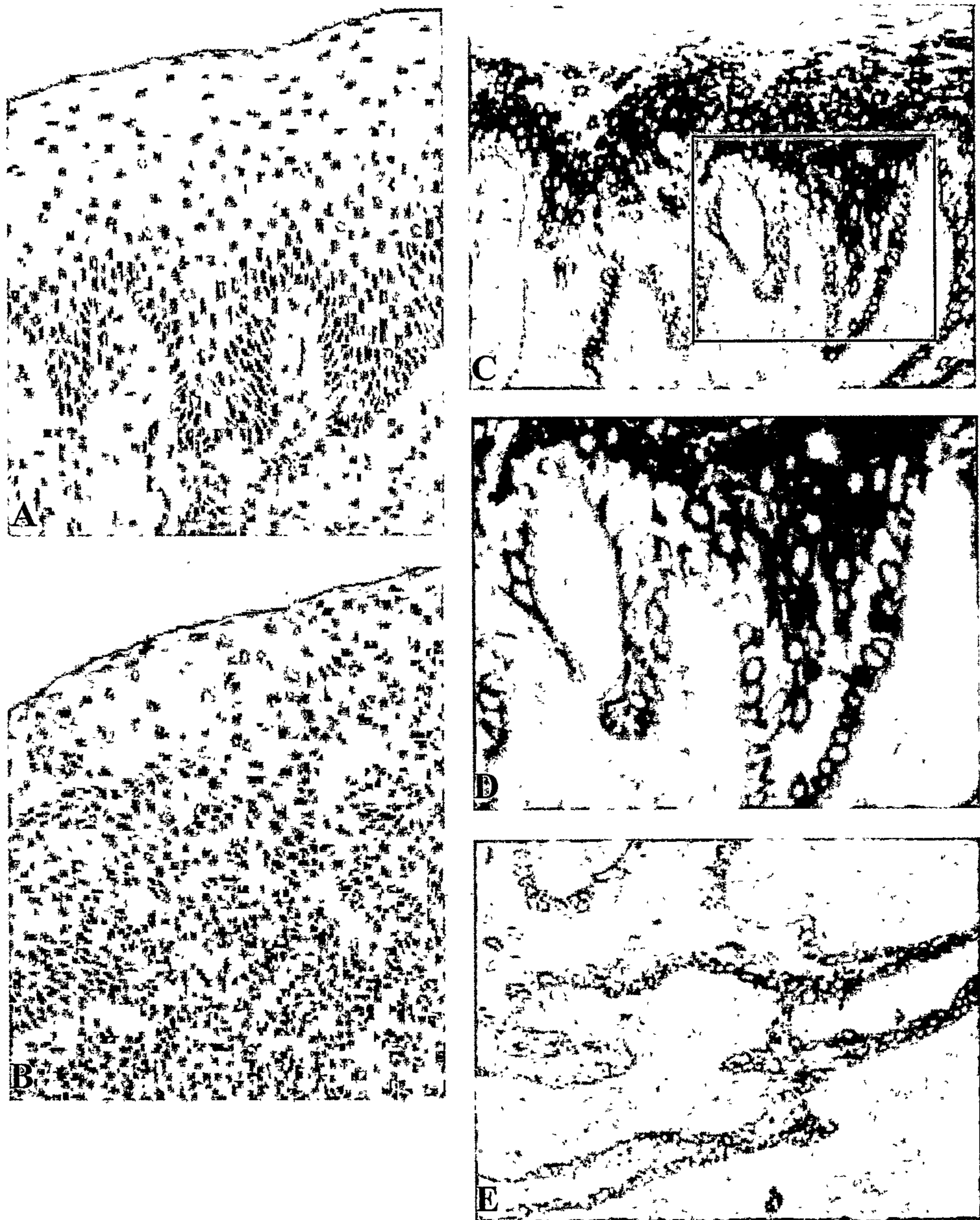


Fig. 3.3 Photomicrographs of reactivity for TGF- β 3, (A, B and C), TGF- β RI, (D, E and F).

- (A) A typical staining pattern for TGF- β 3 in stratified oral epithelium (OE, $\times 200$) of advanced periodontitis showing an intense reaction. A similar pattern was observed in minimally inflamed gingiva.
- (B) Showing high magnification (box in A, $\times 400$) of TGF- β 3 expression pattern.
- (C) TGF- β 3 expression in poorly structured pocket epithelium (PE, $\times 200$) showing intensively strong reactivity.
- (D) The staining pattern for TGF- β RI in the gingival epithelium (OE, $\times 200$).
- (E) Showing high magnification (box in D, $\times 400$) for TGF- β RI reaction pattern.
- (F) Negative to trace reaction in pocket epithelium (PE) for the majority (10/13) of biopsies studied.

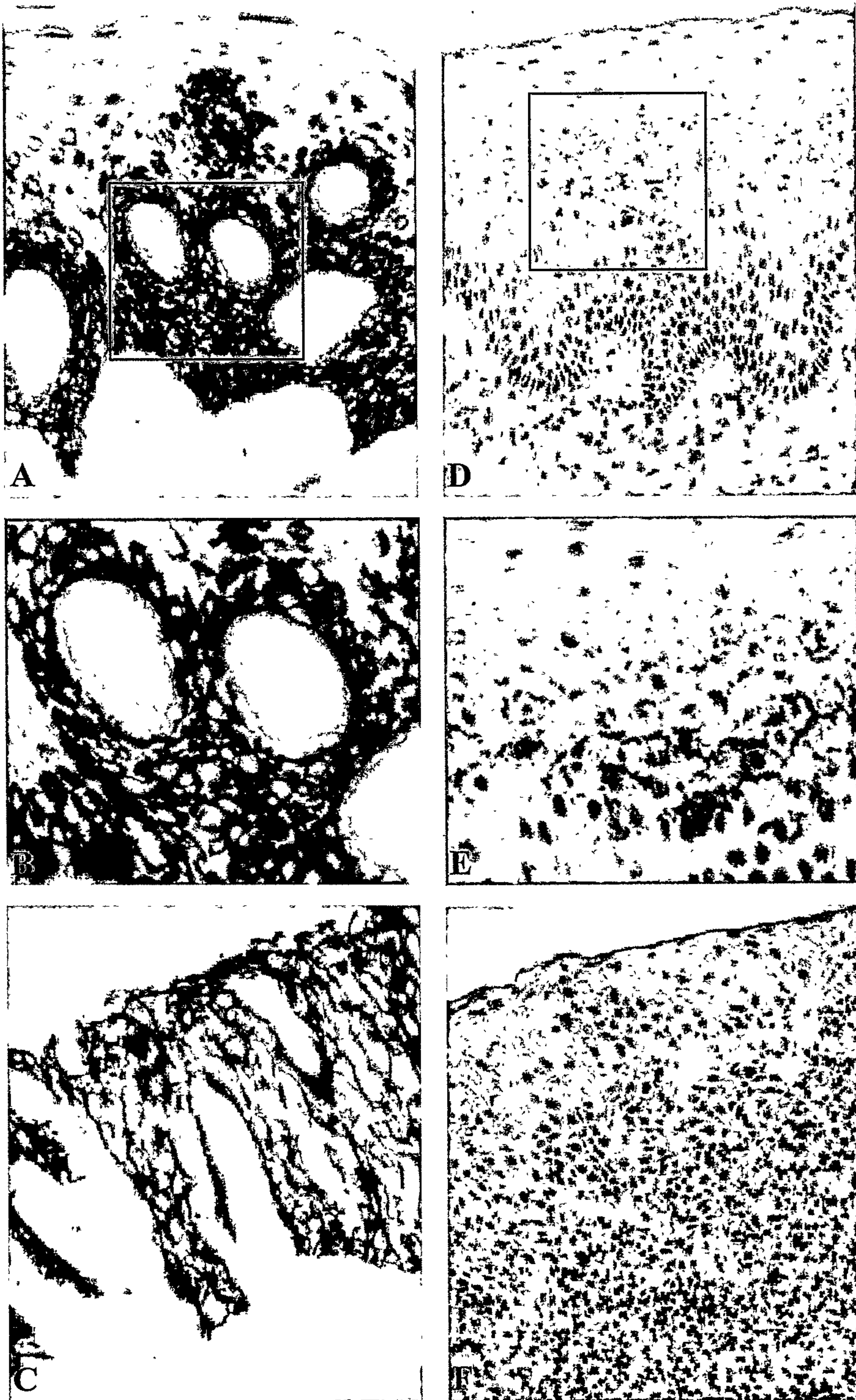
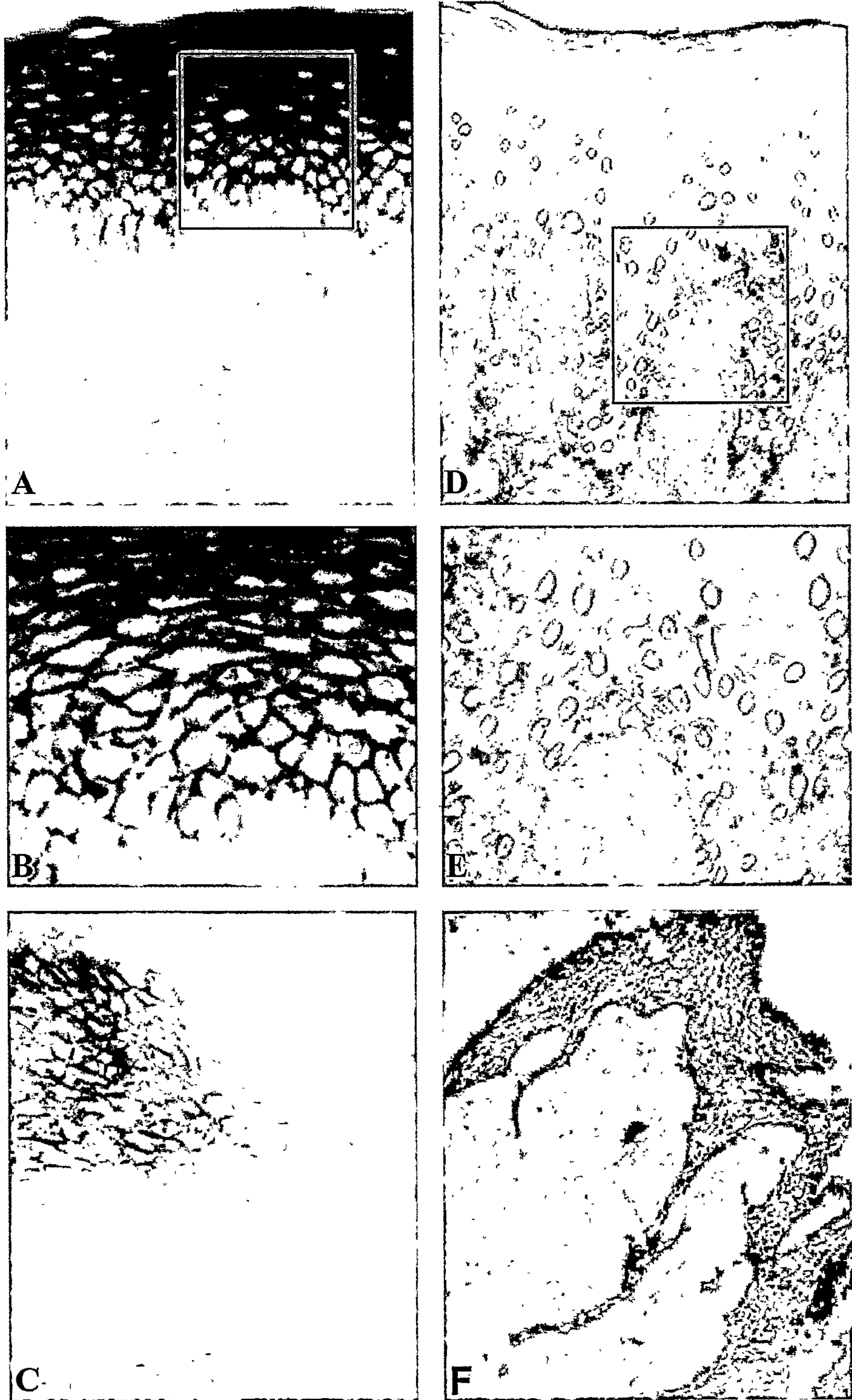


Fig. 3.4 Photomicrographs of staining patterns for TGF- β RII, (A, B and C), TGF- β RIII, (D, E and F).

- (A) A biopsy of advanced periodontitis stained for TGF- β RII; showing moderate reactivity in oral epithelium (OE, $\times 200$).
- (B) High magnification (box in A, $\times 400$) of TGF- β RII pattern.
- (C) TGF- β RII expression in pocket epithelium (PE, $\times 200$); the tissue displays a loose structure with heterogeneous reactivity.
- (D) TGF- β RIII staining pattern exhibiting weak reactivity in oral epithelium (OE, $\times 200$) of advanced periodontitis.
- (E) Showing high magnification (box in D, $\times 400$) for TGF- β RIII pattern.
- (F) Strong reaction in poorly structured pocket epithelium (PE, $\times 200$) for 7/13 of biopsies studied.



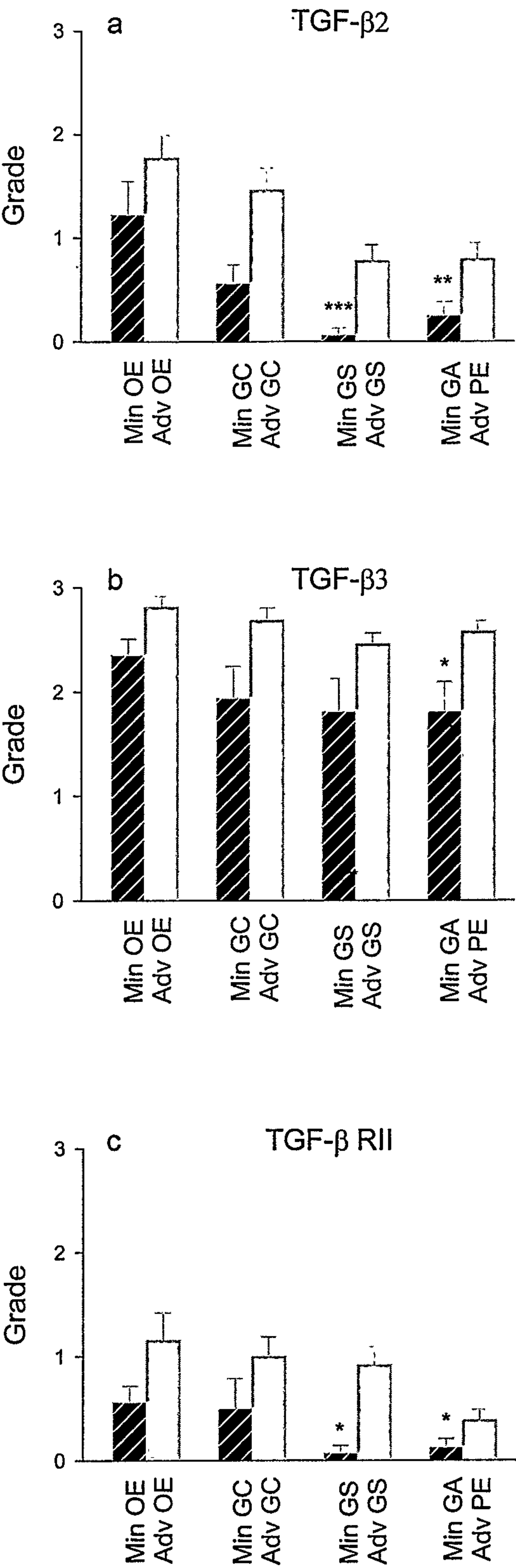


Fig. 3.5 Column graphs showing grade of average intensity with standard deviations of staining in different regions of epithelium in both minimally inflamed (Min, $n = 9$) and advanced periodontitis (Adv, $n = 13$) tissues.

Stained for (a) TGF- β 2, (b) TGF- β 3, (c) TGF- β RII. Differences between groups were evaluated using Dunn's test, comparing each region in both minimally inflamed tissues and advanced periodontitis lesions. Significant differences are shown as * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

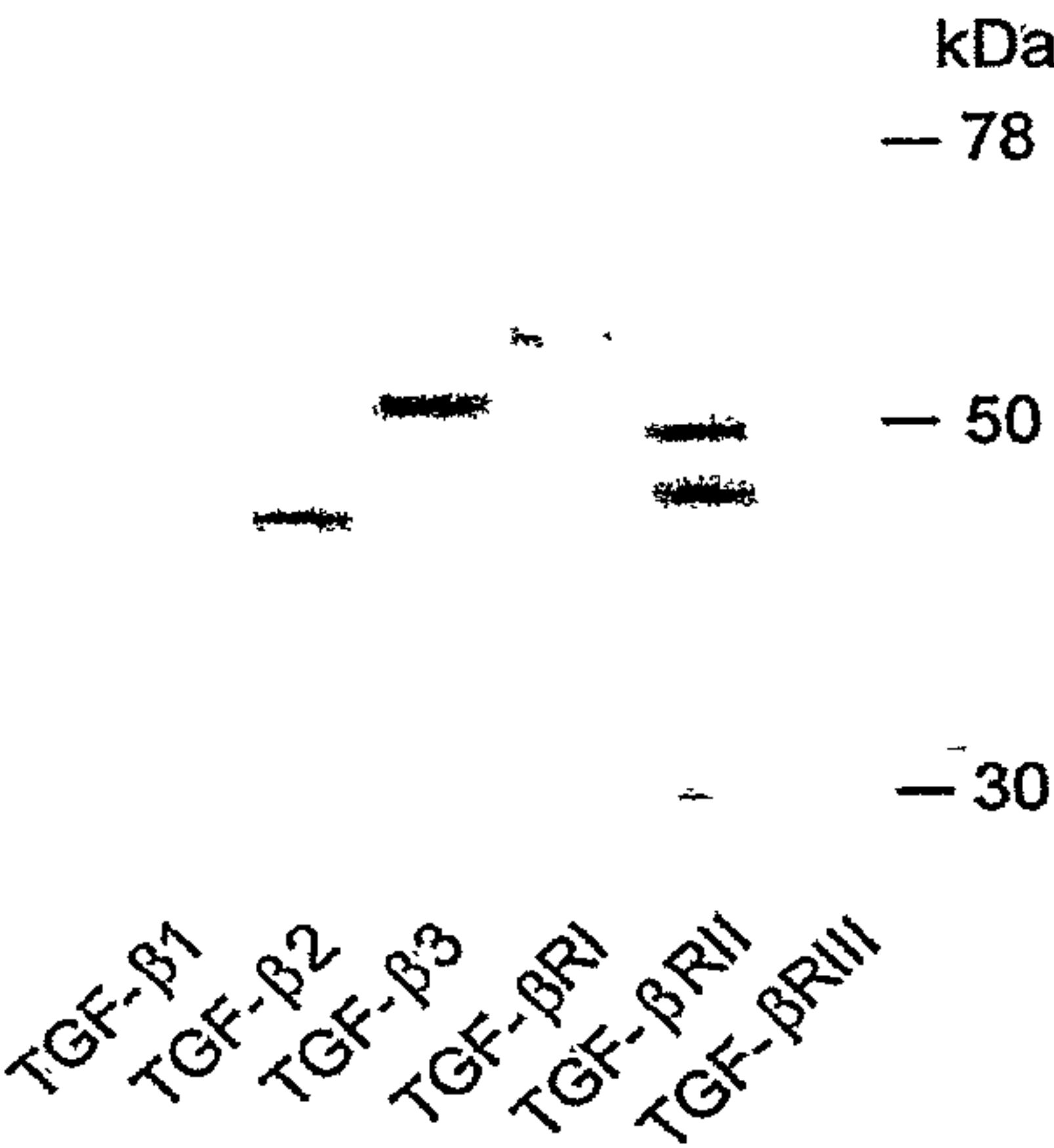


Fig. 3.6 The profile of antibodies to TGF- β s and receptors reacting with H413 antigens.

Western analysis for TGF- β s and their receptors in epithelial cells:

H413 oral epithelial cells were extracted as described in Methods. There was no obvious reactivity for TGF- β 1, while TGF- β RI and RIII showed very weak reaction; however TGF- β 2, - β 3 and - β RII were strongly expressed. Western blot analysis supported the immunohistochemical results.

Table 3.1 Expression of TGF-βs and receptors in the four regions of gingival epithelium.

Staining grade		Minimally inflamed gingiva (n = 9)				Advanced periodontitis (n =13)			
		OE	GC	GS	GA	OE	GC	GS	PE
TGF-β2	—	0	3	7	4	0	2	4	5
	+	6	6	2	5	4	4	9	7
	++	3	0	0	0	7	5	0	1
	+++	0	0	0	0	2	2	0	0
TGF-β3	—	0	0	0	0	0	0	0	0
	+	2	3	4	4	0	1	2	1
	++	2	3	2	2	2	1	2	2
	+++	5	3	3	3	11	11	9	10
TGF-βRI	—	5	5	9	9	7	7	10	10
	+	4	4	0	0	2	2	2	2
	++	0	0	0	0	3	3	1	0
	+++	0	0	0	0	1	1	0	1
TGF-βRII	—	4	5	8	7	2	3	4	8
	+	5	4	1	2	7	7	6	4
	++	0	0	0	0	3	3	3	1
	+++	0	0	0	0	1	0	0	0
TGF-βRIII	—	9	9	6	6	13	10	7	0
	+	0	0	3	3	0	3	6	6
	++	0	0	0	0	0	0	0	7
	+++	0	0	0	0	0	0	0	0

3.5 DISCUSSION

Patterns of expression of TGF-β isoforms and corresponding receptors indicate the potential for autocrine and paracrine regulatory loops within the gingival epithelia. Also suggested is the capacity of epithelial cells to both modify the adjacent inflammatory infiltrate and to respond to TGF-β isoforms released by the inflammatory matrix. The present study demonstrated increased expression of TGF-β2 and TGF-β3 in all regions of the gingival epithelium in tissues affected by chronic periodontitis compared with

clinically healthy gingiva. There was, however, no evidence for the expression at the protein level, of TGF β 1.

The isoforms of TGF- β are secreted as latent high molecular weight complexes (Pircher *et al.*, 1984), with the active species released by proteolytic cleavage. This could be enhanced by proteinases released directly from inflammatory cells or by proteolytic cascades resulting in the activation of tissue proteinases. Within the environment of the gingival epithelium in periodontal disease there is the additional potential for activation of the TGF's by the action of bacterial proteinases including the gingipains of *Porphyromonas gingivalis* (DeCarlo *et al.*, 1998). All three isoforms bind to the low affinity type III betaglycan receptor which may serve to create a depot of ligand that can be transferred to the high affinity type I/type II receptor complex (Massague, 1992). The type II receptor is an active serine/threonine kinase receptor that can bind TGF- β independently, but can not signal without the type I receptor (Wrana *et al.*, 1992). In turn the type I receptor can only bind TGF- β in the presence of the type II receptor (Ebner *et al.*, 1993). The type I receptor shows decreasing affinity for TGF ligands: TGF- β 1>TGF- β 2>TGF- β 3 (Cheifetz & Massague, 1989). This property linked with the regulation of receptor density could explain the observed differential responses to the three isoforms particularly in relation to potency. A further level of control is that of expression as indicated by the paucity of TGF- β 1 antigen expression in many epithelia (Wataya-Kaneda *et al.*, 1996; Nishida *et al.*, 1994). The findings of the present study do not support the synthesis of TGF- β 1 by gingival epithelial cells or the concentration of this isoform which may be released from the adjacent inflammatory response or by leukocytes transmigrating through the epithelium. This finding applied also to the foci of expression of TGF- β RIII in the pocket epithelium with potential to concentrate TGF- β 1. Co-location of TGF- β 2 and 3 with receptor III and I/II was restricted to areas within the pocket epithelium, suggesting that optimal reactivity was highly localised.

Another study reported that expression of TGF- β 1 mRNA was comparably weak in normal skin epithelia and basal cell carcinomas (Schmid *et al.*, 1996). However, Skaleric *et al.* reported that a significantly higher concentration of TGF- β 1 was observed in both the supernatants of the gingival homogenates and crevicular fluid samples obtained from gingival inflammatory sites compared to healthy sites (Skaleric *et al.*, 1997). The detection of TGF- β 1 may depend on rates of synthesis, processing, secretion and clearance.

TGF- β 1 is a potent regulator of immunity including autoimmune disease (Moses *et al.*, 1994; Shull *et al.*, 1992), generally acting as an immunosuppressant. The absence of TGF- β 1 may reduce suppression of T cells and lead to auto-reactivity in the affected animal. TGF- β 1 down-regulates interferon- γ -induced MHC class II antigen expression, which is thought to underpin the autoimmune state, in a variety of cells. Consequently, the absence of TGF- β 1 might result in presentation of self-antigens by inappropriate cells, thereby eliciting an autoimmune response (Shull *et al.*, 1992). This is compatible with the observed expression of MHC II antigens by keratinocytes in the pocket epithelium (Hedberg & Hunter, 1987) and of increased levels of antibodies reactive with epithelial antigens in patients with chronic periodontitis (Ye *et al.*, 2003).

TGF- β 2 is a potent regulator of osteoclastic bone resorption by modulating both osteoclastic migration and osteoclast differentiation (Sporn & Roberts, 1990). Increased expression of TGF- β 2 in epithelia related to periodontitis lesions compared to clinical healthy gingival epithelia is of note as the destruction of the supporting alveolar bone is considered to be an irreversible process in progressive periodontal disease. TGF- β RIII was not detected in the external oral epithelium, but this receptor was present in some pocket epithelia. It has been reported that expression of TGF- β RIII in vascular endothelial cells and endocardial cells increased responsiveness to TGF- β 2 (Martinez-Alvarez *et al.*, 2000; Bassing *et al.*, 1994); on this basis the lack of expression of TGF- β RIII by some gingival epithelial cells suggests lower responsiveness to TGF- β 2. An additional level of regulatory control could involve alternative splicing of the TGF- β receptor transcripts. For instance, the variant receptor T β RII-B mediated signalling of TGF- β 2 in a manner independent of TGF- β RIII (Wahl, 1992).

TGF- β 3 expression was evident at relatively high levels in the basal and suprabasal layers in both clinically healthy and lesion sites. TGF- β 3 exhibits a broad range of regulatory activities, including control of cell proliferation, migration and differentiation, regulation of extracellular matrix deposition and epithelial-mesenchymal transformation. TGF- β 3 plays a critical role in palatogenesis, the formation of Meckel's cartilage and wound healing (Shah *et al.*, 1995). This isoform has limited distribution in epithelia; bronchial epithelia and the medial edge epithelium of the secondary palate (FitzPatrick *et al.*, 1990). It is therefore of interest that it is strongly expressed in the pocket epithelium of chronic periodontitis, a tissue characterised by expression of

primitive keratins and which does not exhibit typical stratum differentiation (Hunter *et al.*, 2001). Potentially, this tissue is extensively contributed to by developmental remnants which persist in this region.

An important consideration in the epithelial cell response to changes in the environment is that bacterial products can stimulate the production of cytokines by epithelial cells (Wahl *et al.*, 1993). This could provide a basis to explain the finding that the expression of some TGF- β s in diseased gingival epithelia is greater than that of epithelia from non-diseased regions.

Expression of TGF- β is complicated not only through direct effects on target cells, but also by influencing the synthesis and action of other regulatory molecules. This report provides a basis for further analysis of the role of this important family of growth factors in regulating the disease process of chronic periodontitis.

CHAPTER 4

SERUM IgG ANTIBODIES
REACTIVE WITH
EPITHELIAL
COMPONENTS;
ASSOCIATION WITH
CLINICAL PARAMETERS
IN CHRONIC
PERIODONTITIS

CHAPTER 4: SERUM IgG ANTIBODIES REACTIVE WITH EPITHELIAL COMPONENTS; ASSOCIATION WITH CLINICAL PARAMETERS IN CHRONIC PERIODONTITIS

4.1 ABSTRACT

The pathological basis for disturbance of epithelial structure is not understood. In the present study, Western analysis of IgG from the sera of 22 patients with advanced periodontitis showed recognition of multiple epithelial components. In contrast, 12 periodontally healthy subjects displayed only trace recognition of epithelial antigens. Serum IgG antibodies were evaluated semi-quantitatively by immunodot blot analysis. Levels of anti-*Porphyromonas gingivalis* IgG of pre-treatment patients were significantly ($P < 0.05$) correlated with the disease index of attachment loss. Post-treatment patient sera IgG levels to epithelial antigens showed a significant reduction ($P < 0.05$) compared with pre-treatment patient sera IgG. It was found that during the course of periodontitis, subjects developed increased levels of antibodies reactive with epithelial antigens. Development of immuno-reactive autoantibodies may be related to perturbation of the epithelial attachment to the tooth in the pathogenesis of periodontitis. These autoantibodies could also present a risk for the subsequent development of some autoimmune diseases.

Keywords: serum IgG; anti-epithelial antibodies; *Porphyromonas gingivalis*;
autoimmunity; periodontitis

4.2 INTRODUCTION

Destructive periodontitis is considered to be the manifestation of a multi-factorial aetiology. The pathogenic processes of periodontal disease result from a host response to microbial products (Kinane, 2001). Although some microorganisms, including *Porphyromonas gingivalis*, *Prevotella intermedia*, *Bacteroides forsythus*, *Haemophilus actinomycetemcomitans* and *Treponema denticola*, have been considered as potential periodontal pathogens (Zambon, 1996), it has become apparent that pathogens are necessary but not sufficient for disease activity to occur (Socransky & Haffajee, 1992). Other risk factors include genetics, age, sex, smoking and some autoimmune diseases that affect the periodontal lesion by altering aspects of the

immuno-inflammatory response.

Periodontal disease prevalence increases with age (Albandar & Spencer, 1999) and severity of attachment loss is greater for males (Slade, 1995). Many studies suggest that tobacco smoking is an important risk factor for periodontitis. A consistent, positive correlation between smoking and attachment loss has been reported and tobacco smoking affects periodontal tissues more severely in men than in women (Calsina *et al.*, 2002; Haffajee & Socransky, 2001a; Kinane, 2001). A possible mechanism is the capacity of tobacco products to alter the immune response against periodontal pathogens (Barbour *et al.*, 1997). There are reports indicating that tobacco products do not directly affect the microflora of the periodontium (Darby *et al.*, 2000; Chen *et al.*, 2001).

About 30-33 percent of the Caucasian population is IL-1 genotype positive (Kornman *et al.*, 2000). Investigation suggests that genotype positive subjects more frequently have higher levels of subgingival plaque species that are known to be strongly associated with periodontal inflammation (Socransky *et al.*, 2000). There is evidence to support the importance of IL-1 genotype as a factor in severe adult periodontitis (McDevitt *et al.*, 2000; Kornman *et al.*, 1997; Kornman & di Giovine, 1998; see Chapter 1.3.1).

There is some evidence to support an association of periodontitis with autoimmune diseases. Rheumatoid factor is cross-reactive with epitopes on oral bacteria (The & Ebersole, 1996). Moreover, refractory periodontitis patients with insulin-dependent diabetes mellitus (IDDM) have elevated serum glutamic acid decarboxylase (GAD) autoantibody levels immuno-reactive with the oral bacterium *P. gingivalis* (Sims *et al.*, 2001). In addition, there was a significantly higher level of circulating immune complexes (CIC) in the sera of patients with localised juvenile periodontitis (Anil *et al.*, 1990).

Porphyromonas gingivalis, a Gram-negative anaerobic black-pigmented rod, is an important putative pathogen in different types of periodontitis (van Winkelhoff *et al.*, 1993). Many investigators have observed significantly elevated serum anti-*P. gingivalis* IgG levels in patients with rapidly progressive periodontitis and adult periodontitis (Kojima *et al.*, 1997). In the present study, *P. gingivalis* was used as a reference control in the investigation of the possible relationship between levels of antibodies reactive with epithelial components and indices of disease.

The aim of this study was to characterize the host systemic IgG antibody

response to epithelial antigens in pre-, post-treatment and periodontally healthy subjects as an indication of risk factors for periodontal disease. Findings are reported that indicate the recognition of multiple epithelial antigens as a consistent finding in periodontitis patients. Levels of these antibodies were associated with disease severity, indicating a possible mechanism for progressive periodontal disease.

4.3 MATERIALS AND METHODS

4.3.1 Clinical data, serum and tissue samples

Pre-treatment patients: Blood samples were obtained with informed consent and prior to commencement of therapy from 22 adult chronic periodontitis patients (14 males, 8 females, 35–68 years of age, average 48 years) with no history of systemic disease or medications within the past 6 months and no record of periodontal therapy including subgingival scaling, root planing or relevant surgery, in the previous three years. Participants were examined clinically and radiographically then given a diagnosis score according to the template provided by the American Society of Periodontology which is a measure of overall status for periodontal disease, as follows: Score of 0 = none, 1 = mild local, 2 = mild general, 3 = moderate local, 4 = moderate general, 5 = severe local, 6 = severe general. All patients showed signs of gingival inflammation (diagnosis score: 1–6), loss of attachment (5 –11mm) which measures long-standing disease activity, formation of periodontal pockets which is a measure of current disease status with probing depths of 4 –11mm and resorption of alveolar bone (determined by examination of radiographs).

Post-treatment patients: Blood samples were collected from 15 patients (11 males, 4 females, 35–63 years of age, average 47 years) of above the same group within a time frame of three weeks to eight months after successful treatment by scaling and root planning (Table 4.1).

Periodontally healthy subjects: Blood was also collected from 12 race, age and gender matched periodontally healthy subjects (7 males, 5 females, 27–64 years of age, average 46 years) characterised by no clinical signs of gingival inflammation, probing depths of 3 mm or less, no sites with significant attachment loss, no evidence of radiographic bone loss and no history of systemic disease.

Sera were separated from clotted blood by centrifugation at 1000g for 20 minutes and stored at – 70°C until used.

Tissue samples: Samples of gingival tissues were also obtained pre-treatment from the same group of 22 periodontitis patients. Gingival tissues were snap-frozen in isopentane cooled in liquid nitrogen and 6 µm sections cut. Slides with sequential sections were stored in sealed boxes at -70° C until required.

Patients clinical data are summarised in Tables 4.1 and 4.2.

4.3.2 Oral epithelial cell lines

For use in this study, the mucosal epithelial cell line (CCL-4) derived from normal oral mucosa in a laboratory rat (Birkedal-Hansen *et al.*, 1981), displayed typical epithelial morphology in culture, such as coherent monolayer sheets and stratification of epithelial cells. It shares with human keratinocytes: properties of proliferation and terminal differentiation including keratinization markers of keratin filaments, cornified envelopes, increased plasma membrane permeability and cytoplasmic and nuclear digestion. The cells were cultured in Dulbecco modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture (1:1) (Sigma, Sydney, Australia) 15mM HEPES (Sigma) and supplemented with 10% foetal bovine serum (CSL Limited, Victoria, Australia) and penicillin/ streptomycin (100 IU/ml, ICN Biomedicals, Inc., Sydney, Australia) at 37°C with 5% CO₂. The CCL-4 cells were subcultured every 2–3 days and harvested at a multilayer confluence in 175 cm² flasks (Sarstedt, Inc., Newton, NC). Another epithelial cell line (H413) derived from a human oral squamous cell carcinoma (Prime *et al.*, 1990), displayed a stratified epithelial cell morphology in culture, and was cultured as for CCL-4.

4.3.3 Epithelial cell antigens

CCL-4 and H413 cells were collected in the presence of a proteinase inhibitor cocktail (Sigma-P8340) from confluent cultures (10⁷ – 10⁸ cells/3-4 mg total protein). Briefly, the cells were washed 3 times in pre-cooled Dulbecco phosphate buffered saline (PBS) and harvested by gentle scraping on ice. After centrifugation, in preparation for Western blot analysis, cells were resuspended in 2ml PBS, then boiled in sodium dodecyl sulfate (SDS) sample buffer with 1% 2-mercapthoethanol for 10 minutes. For dot blot analysis, resuspended cells in 0.1M Tris-buffered saline (TBS, pH 7.5) containing 3% Triton X-100 (AJAX chemicals, Australia), were gently vortexed and placed on ice for one hour. The samples were stored at -20°C for up to 2 months.

Table 4.1 Patients' Clinical Data

Patient no (Pre-treatment)	File no	Sex	Age	Patient no (Post-treatment)	Months (After treatment)	Diagnosis score	Pocket depth (mm)	Attach- ment loss (mm)	Inflammat- ion score	Health a tissue site	Disease tissue site	b Disease types	smoke
1	274828	m	38			5	7	10	1	34 db	27 b	general	y
2	279251	m	43	1	8	4	10	10	2	45 db	48 b	general	y
3	254136	m	57			6	8	9	3	45 mb	36 db	general	
4	277715	m	68			4	4	6	2	44 mb	17 b	general	y
5	181139	m	63	2	2-	6	5	10	3	34 db	32 mb	general	
6	182908	m	43	3	7+	6	4	7	3	14 mb	15 db	general	y
7	232804	m	41	4	3-	3	6	6	2	36 db	37 mb	general	
8	279856	f	55	5	6+	5	11	11	3	45 db	16 mb	local	
9	279894	f	41	6	6+	6	10	10	3	44 db	15 db	general	y
10	278169	m	41			5	9	11	3	45 db	46 b	local	
11	148462	f	58	7	8	4	6	8	3	34 db	26 db	general	
12	279821	f	47	8	5	6	7	8	3	46 mb	27 mb	general	
13	142715	m	43	9	3	5	5	10	3	45 db	44 mb	local	y
14	281351	f	50	10	1-	5	8	5	2	45 db	25 db	local	
15	281149	f	55	11	2-	4	4	7	3	35 mb	16 db	local	
16	282194	m	58	12	2-	3	6	6	3	45 mb	36 db	general	y
17	218875	m	45	13	1+	1	6	6	2	44 db	13 db	local	
18	251936	m	35	14	1-	4	6	6	3	46 mb	47 db	general	y
19	283430	m	52	15	3	6	7	9	3	47 mb	45 db	general	
20	282866	m	42			5	7	8	2	25 db	26 b	local	
21	172058	f	39			6	5	7	3	36 db	35 db	general	y
22	267062	f	47			6	5	7					

^a tissue site: db = distal-buccal; mb = mesio-buccal; b = buccal.

^b Disease types: generalised or localised periodontitis.

Table 4.2 The clinical data of periodontitis patients and controls

Classification	n	Years Average(range)	Gender M : F	Race Caucasian : Asian	Attachment loss (mm)	Pocket depth (mm)	Diagnosis (score)	Smoker	Classification generalised localised
Pre-treatment patients	22	48 (35-68)	14 8	17 5	5-7 8-11 n = 8 n = 14	4-6 7-11 n = 9 n = 13	1-4 5-6 n = 12 n = 10	n = 9	n = 14 n = 8
Post-treatment	15	47 (35-63)	11 4	11 4	5-7 8-11 n = 7 n = 8	4-6 7-11 n = 9 n = 6	1-4 5-6 n = 6 n = 9	n = 5	n = 9 n = 6
Controls	12	46 (27-64)	7 5	8 4	<3	<3	≈ 0		

4.3.4 *P. gingivalis* antigens

P. gingivalis strain (ATCC 33277) from stock, was inoculated into enriched 1 L CDC anaerobic broth (Trypticase peptone 10 g, Trypticase soy broth 10 g, Yeast extract 10 g, NaCl 5 g, L-cysteine 0.4 g), supplemented with haemin (5 µg/ml, Sigma), menadione (5 µg/ml, Sigma) and grown in an anaerobic chamber (85% N₂, 5% CO₂, and 10% H₂) for primary culture. Bacterial numbers were estimated by reference to the standard curve determined by absorbance at 600 nm and collected in late exponential phase and washed with PBS in the presence of a proteinase inhibitor cocktail (Sigma) by centrifugation prior to protein extraction. Components were extracted by sonication at full power for 5 min on ice using a Branson Sonifier 450 (Branson Ultrasonics, USA). The extract was clarified by centrifugation at 15,000g for 10 min at 4°C and the supernatant collected.

4.3.5 SDS-PAGE and immunoblots

Proteins from the CCL-4 and H413 cell lines were separated by a standard SDS-PAGE mini gel (10% polyacrylamide, 30 µg protein per mini gel). A pre-stained broad range molecular weight marker set (Bio-Rad) was run in an outer lane of each gel. Fractionated proteins were transferred onto nitrocellulose membranes (Bio-Rad) for Western immunoblots. After blocking with 3% bovine serum albumin (BSA, Sigma) in TBS overnight, the nitrocellulose sheet was assembled in a 24-channel MilliBlot-MP membrane probe device (Millipore Corp., Bedford, MA). The membrane was divided into 24 lanes, each lane allowed the introduction of one patient's serum. Blotted proteins were incubated with titrations of pre-treatment patient sera ($n = 22$), post-treatment patient sera ($n = 15$), or clinically healthy subject sera ($n = 12$). The secondary antibody employed was alkaline phosphatase (AP) conjugated γ -chain-specific goat anti-human IgG (Sigma) diluted 1:30,000 in 0.05% Tween 20/TBS. IgG antibody binding antigen was visualized with AP substrate (cat 170-6432, Bio-Rad).

For immunodot blots, CCL-4 antigens and *P. gingivalis* antigens diluted in TBS to a concentration of 1µg/spot, were applied to pre-wetted nitrocellulose membranes (0.45µm, 9x12 cm size, Bio-Rad) and filtered by a vacuum microfiltration apparatus (96-well format, Bio-Rad). Blotted antigens were blocked with 1% BSA/TBS. All membranes were immunodetected by pre-treatment patient sera ($n = 22$), post-treatment patient sera ($n = 15$), and clinically healthy subject sera ($n = 12$) respectively. Bound antibodies were detected as described above. Triplicate blots were performed for each experiment.

4.3.6 Immuno-fluorescence labelling of epithelium

CCL-4 cells were passaged and seeded into chamber slides (4 well glass slide, Nalgene Nunc, International Corp., 2000 N Aurora Rd, Naperville, IL 60563-1796 USA) with 1.0 ml of cell suspension per well, then incubated at 37°C, 5% CO₂ for 2 days until the cells were 70–80% confluent. CCL-4 cells in duplicate chamber slide cultures and tissue sections, including diseased sites and periodontally healthy sites from the pool of volunteers were fixed in ice-cold methanol for 4 min and blocked for 1 h with 1% BSA in PBS at room temperature. Duplicate chamber slide cultures and tissue sections were incubated with titrations (1:10–1000) of pre-treatment patient sera pool or periodontally healthy sera pool, or random individual subject serum for 1 h at room temperature in a humid chamber. Slides were washed in fresh PBS three times for 5 min each, with gentle agitation. Cells and sections were incubated with TRITC-labeled rabbit anti-human IgG (λ -chains) or FITC-labeled rabbit anti-human IgG (γ -chains) for 1 h in a dark, humid chamber at room temperature, then washed in PBS three times, coverslipped and detected under incident fluorescence microscopy.

4.3.7 Scanning laser confocal microscopy (SLCM)

Cells and tissues were evaluated with an Optiscan F900e personal confocal system (Optiscan, Melbourne, Australia) equipped with an air-cooled argon laser and fitted to an Olympus BX-60 microscope. The lens used was an Olympus Uplan Apo 60x/1.20 water-immersion epi-fluorescence objective. Single channel fluorescence imaging was performed using an excitation wavelength of 488 nm (blue), with detection using a 515–550 nm band-pass filter (green) for FITC or an excitation wavelength of 512 nm (green), with detection using a 515–550 nm band-pass filter (red) for TRITC. Accumulation of digital images was acquired for three-dimensional (3-D) analysis and reconstruction using Voxblast software, version 1.3.1. (VayTek Inc., Iowa, U.S.A.).

4.3.8 Data analysis

Semi-quantification of dot blots was performed using densitometric scanning (Scion Image software, Scion Corporation, Frederick, MD). The graphs and data analysis of linear regression and t-tests were performed by SigmaPlot software 3.0 (Jandel). The comparison of three or more groups was assessed by Kruskal-Wallis test analysis of variance (GraphPad software, San Diego, CA, USA). A value of $P < 0.05$ was considered significant.

4.4 RESULTS

4.4.1 Serum antibody responses to epithelial components

Western blots displayed distinct immuno-reactive characteristics for individual pre-treatment patient sera IgG and post-treatment patient sera IgG (Fig. 4.1a and b) and periodontally healthy sera IgG (see Chapter 5.4.1, Fig. 5.1b and c) with CCL-4 antigens; however, there was also observed an additional immuno-recognition of pre-treatment and periodontally healthy sera IgG with the human cell line H413 (Fig. 4.2a and b). These data suggested that serum antibodies immuno-reactive with epithelial components are present in proportion to existing periodontal disease pathology.

4.4.2 The relationship of periodontal disease indices to serum antibody immuno-reactive with epithelial (CCL-4) antigens and *P. gingivalis* antigens

Using a semi-quantitative dot blot assay pre-treatment and post-treatment periodontitis patient sera and periodontally healthy subject sera were evaluated for IgG antibody levels against epithelial cell (CCL-4) antigens and for responses against an extract from the periodontal pathogen *Porphyromonas gingivalis* as a reference control.

The linear regressions (Fig. 4.3a) indicated a positive trend ($r = 0.42$, $P > 0.05$) between the 95% confidence interval for pre-treatment patient sera ($n = 20$) IgG levels against epithelial antigens and related to attachment loss. In contrast, there was a negative correlation ($r = 0.52$, $P < 0.05$) between the 95% confidence interval for pre-treatment patient sera ($n = 20$) IgG levels against *P. gingivalis* antigens and attachment loss (Fig. 4.3b).

Although there was no significant difference in the 95% confidence interval of pre-treatment patient sera IgG to epithelial (CCL-4) antigens and *P. gingivalis* antigens related to pocket depth and diagnosis score, the trends were towards elevated IgG levels and decreased IgG levels respectively (Fig. 4.3c, d, e, f) as these indices of disease increased.

4.4.3 The relationship of periodontal disease risk factors and disease classification to serum antibody immuno-reactive with epithelial (CCL-4) antigens and *P. gingivalis* antigens

Data indicated a negative trend between increase in age and decrease in the 95% confidence interval for pre-treatment patient sera ($n = 20$, $r = 0.41$, $P > 0.05$, Fig. 4.4a) anti-CCL-4 IgG, and the 95% confidence interval for pre-treatment patient sera ($n = 21$,

$r = 0.42$, $P > 0.05$, Fig. 4.4b) anti-*P. gingivalis* IgG. For periodontally healthy subject sera ($n = 12$, Fig. 4.4c, and d), there was a negative trend between IgG antibody levels to CCL-4 antigens and increased age, but conversely, a positive trend between control subject sera anti-*P. gingivalis* IgG and increased age was observed. These results indicated that autoimmune reactivity against epithelial components was associated with periodontitis patient characteristics, raising questions of the origin and pathogenic significance of these antibodies.

There were no significant differences for anti-CCL-4 IgG or anti-*P. gingivalis* IgG between smokers ($n = 9$) and non-smokers ($n = 13$), or generalised ($n = 14$) and localised periodontitis ($n = 8$) related to clinical parameters. These were, however, based on small sample size. There was a trend towards a positive correlation for anti-CCL-4 IgG or a negative correlation for anti-*P. gingivalis* IgG within smokers and generalised periodontitis patients related to attachment loss compared to non-smokers or localised periodontitis patients (data not shown).

4.4.4 Comparison of the levels of pre-, post-treatment and control group sera IgG against CCL-4 antigens and *P. gingivalis* antigens

After treatment, only 15 patients reported for follow up and blood samples were obtained. Box plots summarising the range and median IgG levels for different groups are shown in Fig. 4.5. For post-treatment patient sera IgG levels to CCL-4-antigens showed a significant reduction ($P < 0.05$, t test) compared with pre-treatment patient sera IgG. There was no difference between pre- and post- treatment patient sera IgG levels to *P. gingivalis* antigens. Periodontally healthy subjects showed only low anti-CCL-4 and anti-*P. gingivalis* IgG levels ($P < 0.001$, Kruskal-Wallis test) compared to pre- and post-treatment patient sera anti-CCL-4 and anti-*P. gingivalis* IgG levels respectively. These data suggested that high levels of serum antibodies immuno-reactive with epithelial components are present in proportion to existing periodontal disease pathology and might relate to host autoimmunity.

4.4.5 Serum IgG responses to epithelial cells and tissues analysed by scanning laser confocal microscopy (SLCM)

Sections of periodontitis lesions and minimally inflamed gingival biopsies uniformly showed only trace and focal epithelial reaction with fluorochrome-labeled anti-human immunoglobulin. Positive staining was observed in pocket epithelia where strong

reaction in the cytoplasm of keratinocytes was noted (Fig. 4.6A and B). IgG deposition on keratinocyte cell surfaces was also observed at the epithelial surface in periodontitis patients (Fig. 4.6C and D). This pattern was markedly reduced in intensity when a pool of sera from periodontally healthy individuals was employed (data not shown).

A comparison of reactivity to pooled periodontitis patient sera in clinically healthy sites (Fig. 4.7A, C and E) and diseased site (Fig. 4.7B, D and F) is shown in Fig. 4.7. There was a more intense reaction in pocket epithelium (Fig. 4.7B) compared to junctional epithelium (Fig. 4.7A). In Fig. 4.8 the reactivity of CCL-4 epithelial cells stained by random patients' sera IgG (A: patient number 7; B: patient number 16) and control subject number 1 (C) is displayed. The staining characteristics indicated the dominant reaction of cytoplasmic components and the brighter reaction with patient sera versus periodontally healthy serum.

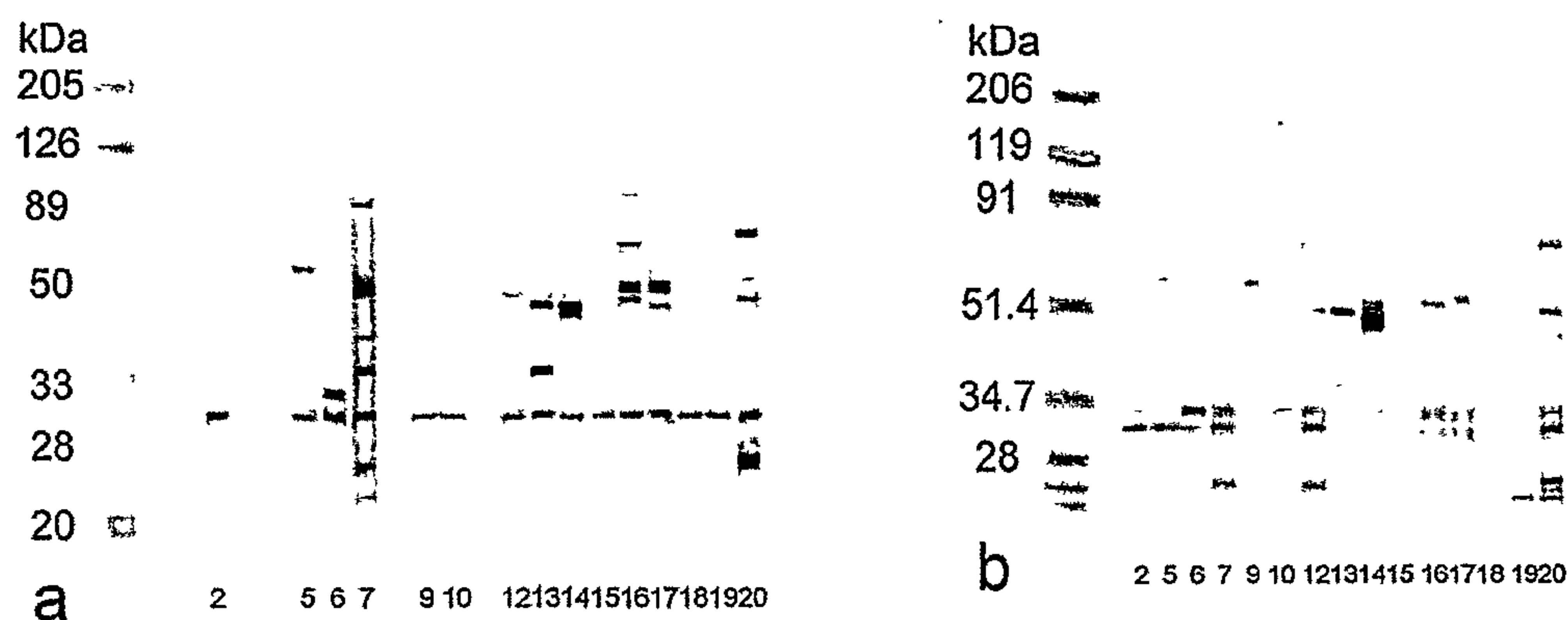


Fig. 4.1 Recognition patterns for CCL-4 epithelial antigens.

Western immunoblots of CCL-4 epithelial antigens separated on an SDS-PAGE gel (see Chapter 5.4.1, Fig. 5.1a). Blotted CCL-4 proteins were incubated with optional titrations of pre-treatment patient sera ($n = 22$, see Chapter 5.4.1, Fig. 5.1b), post-treatment patient sera ($n = 15$), or clinically healthy subject sera ($n = 12$, see Chapter 5.4.1, Fig. 5.1c). The secondary antibody employed was alkaline phosphatase (AP) conjugated γ -chain-specific goat anti-human IgG (Sigma) diluted 1:30,000 in 0.05% Tween 20/TBS. IgG antibody binding antigen was visualized with AP substrate (cat 170-6432, Bio-Rad).

The profile for comparison of pre-treatment patient sera IgG ($n = 15$, a) and for the same group after treatment (post-treatment) sera IgG (b) against CCL-4 antigens are shown. There was a weaker reaction for post-treatment sera IgG compared to those of pre-treatment patients.

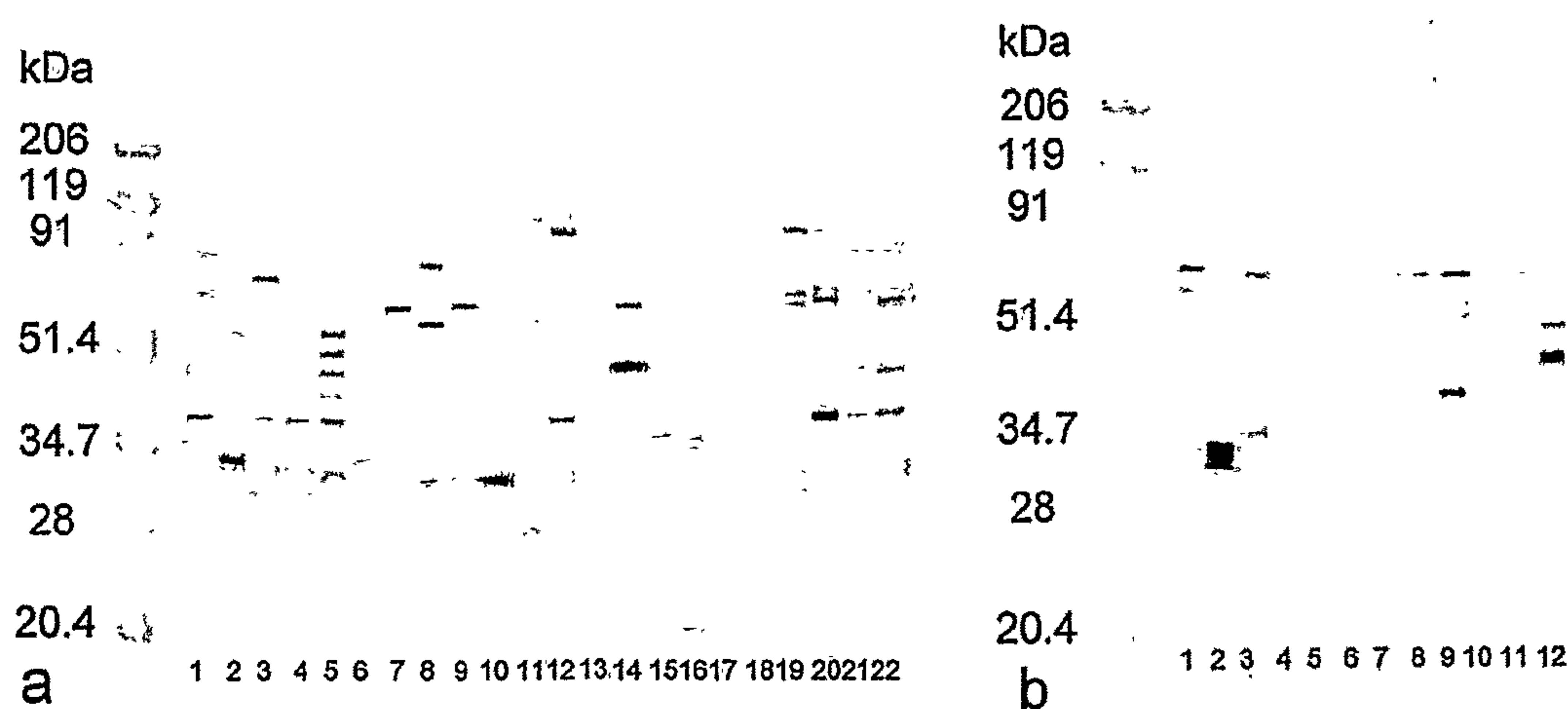


Fig. 4.2 Recognition patterns for H413 epithelial antigens.

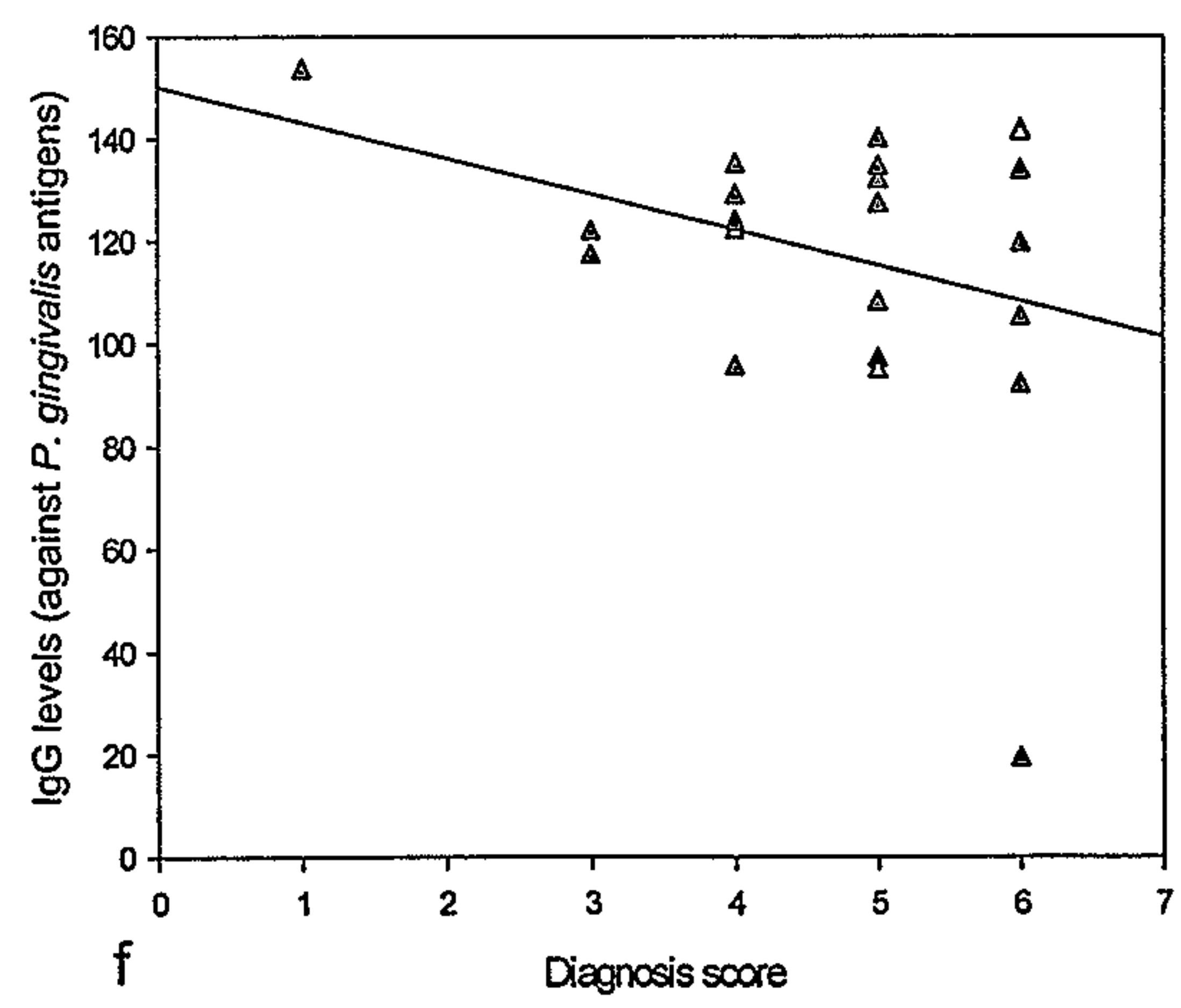
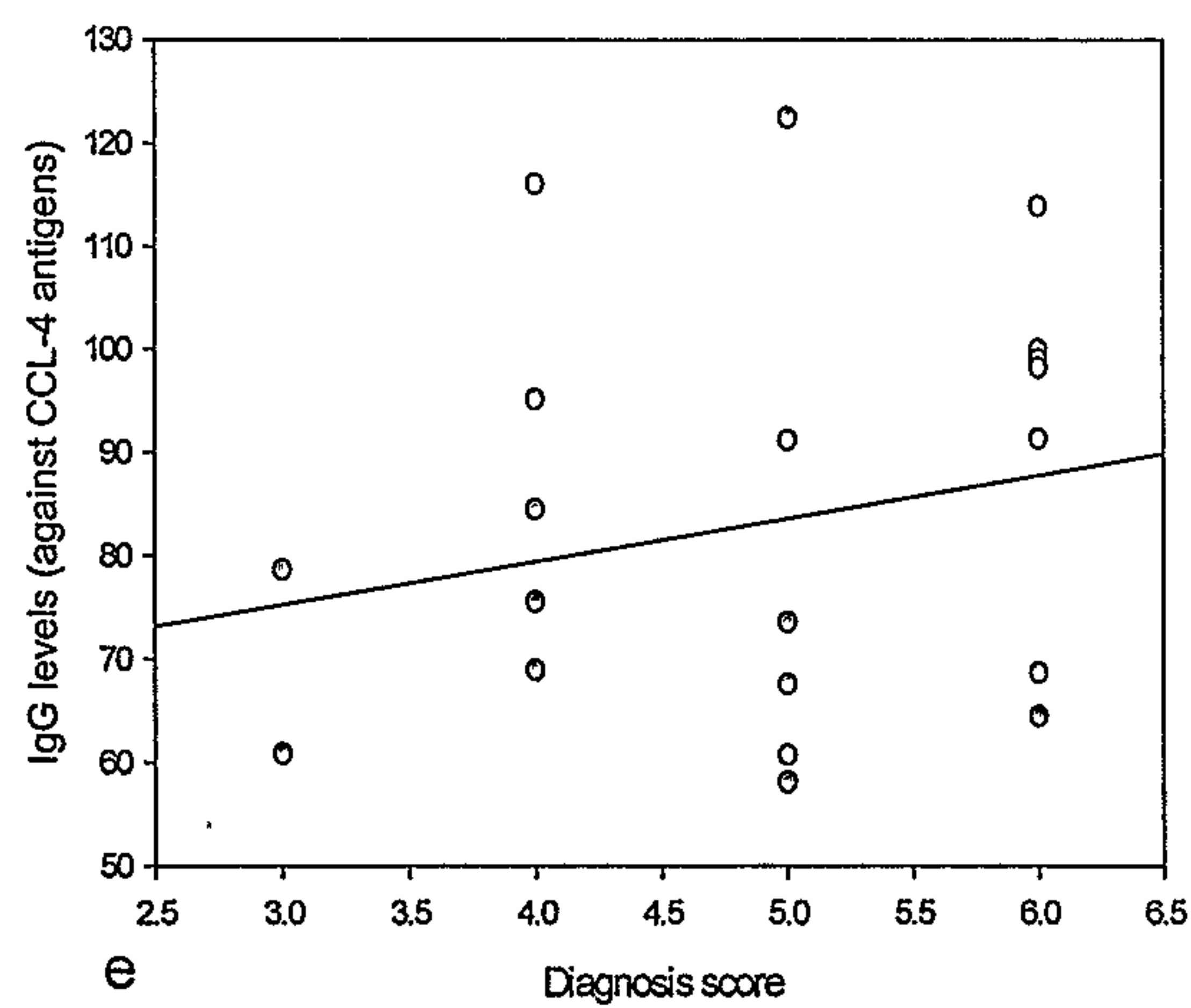
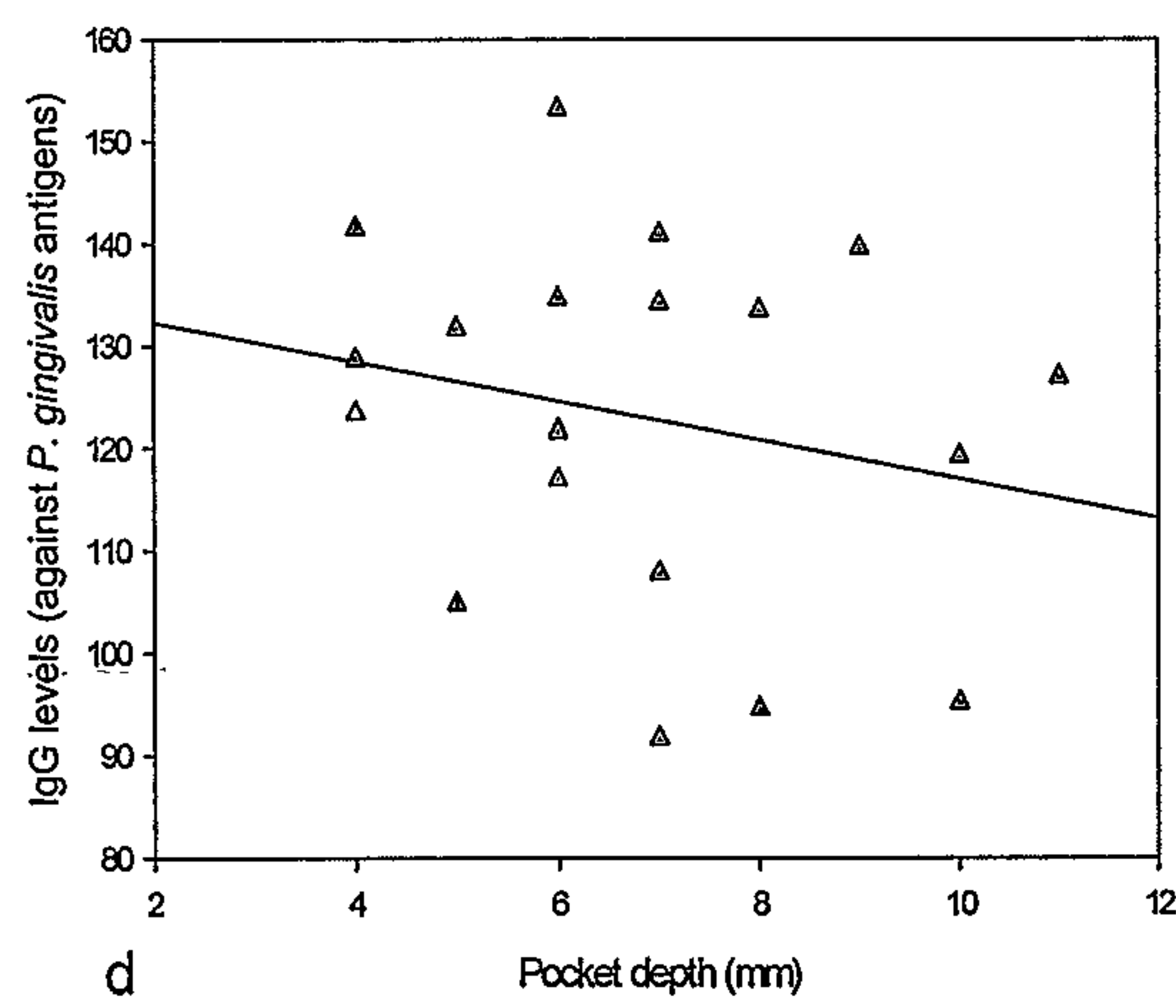
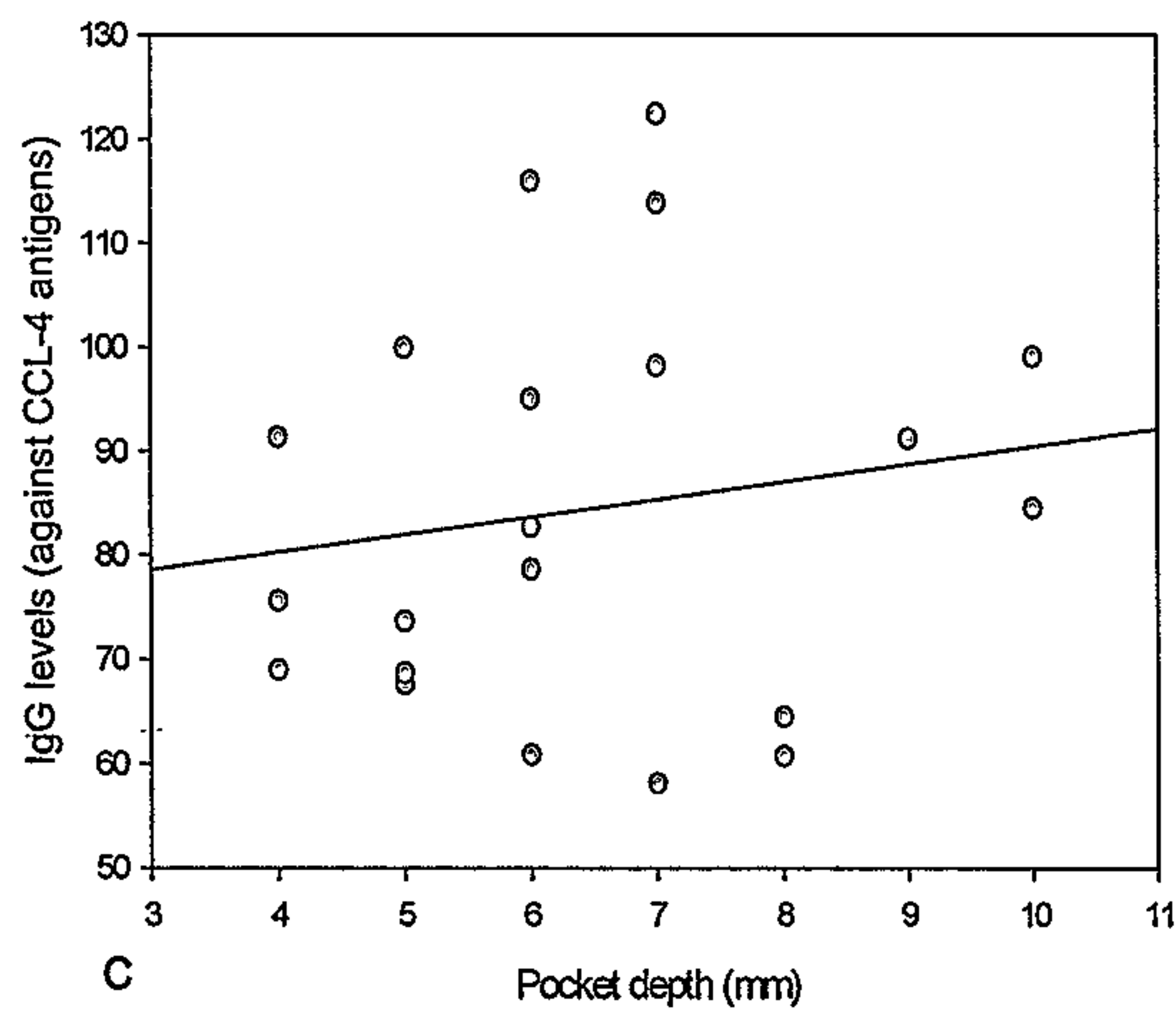
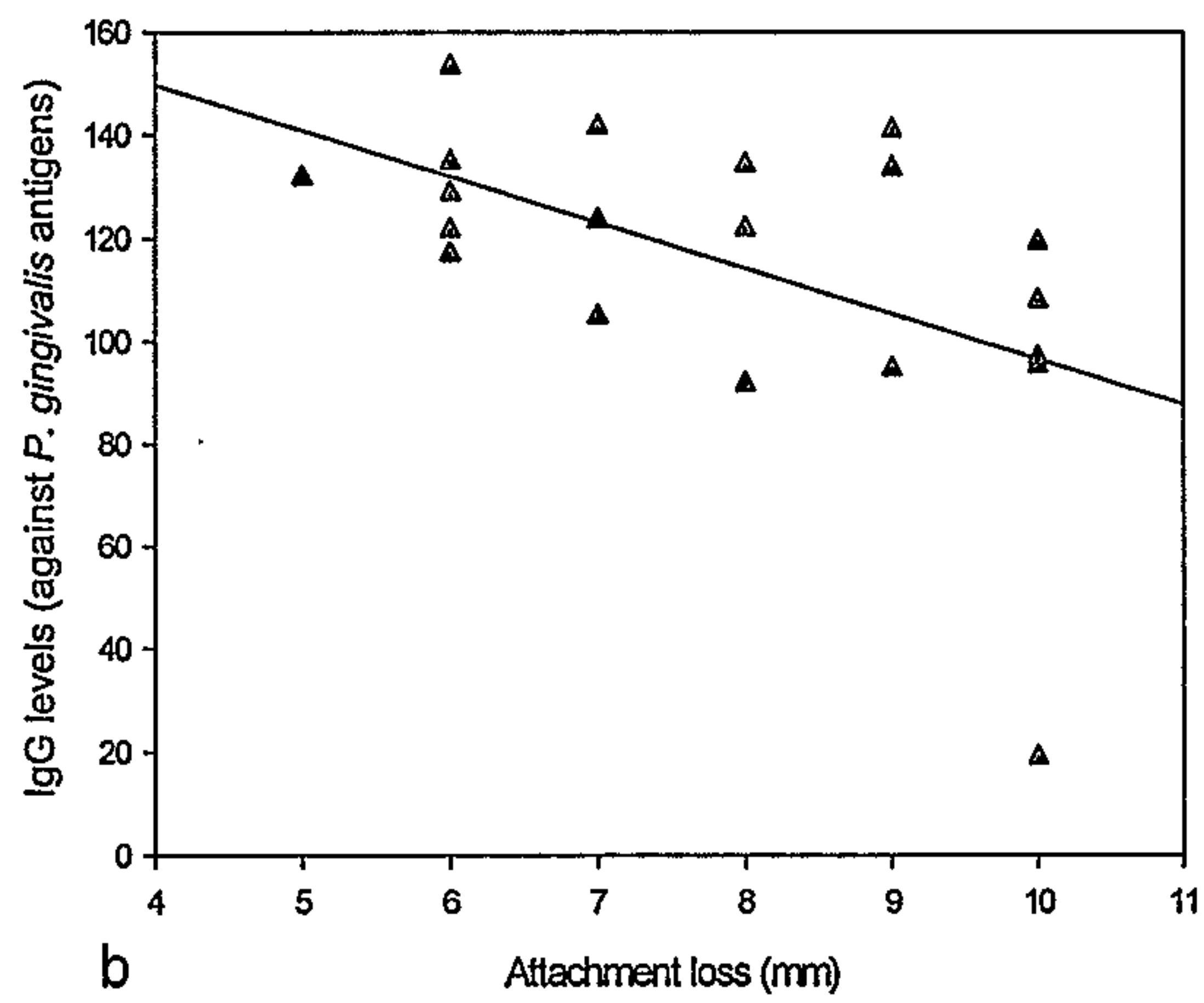
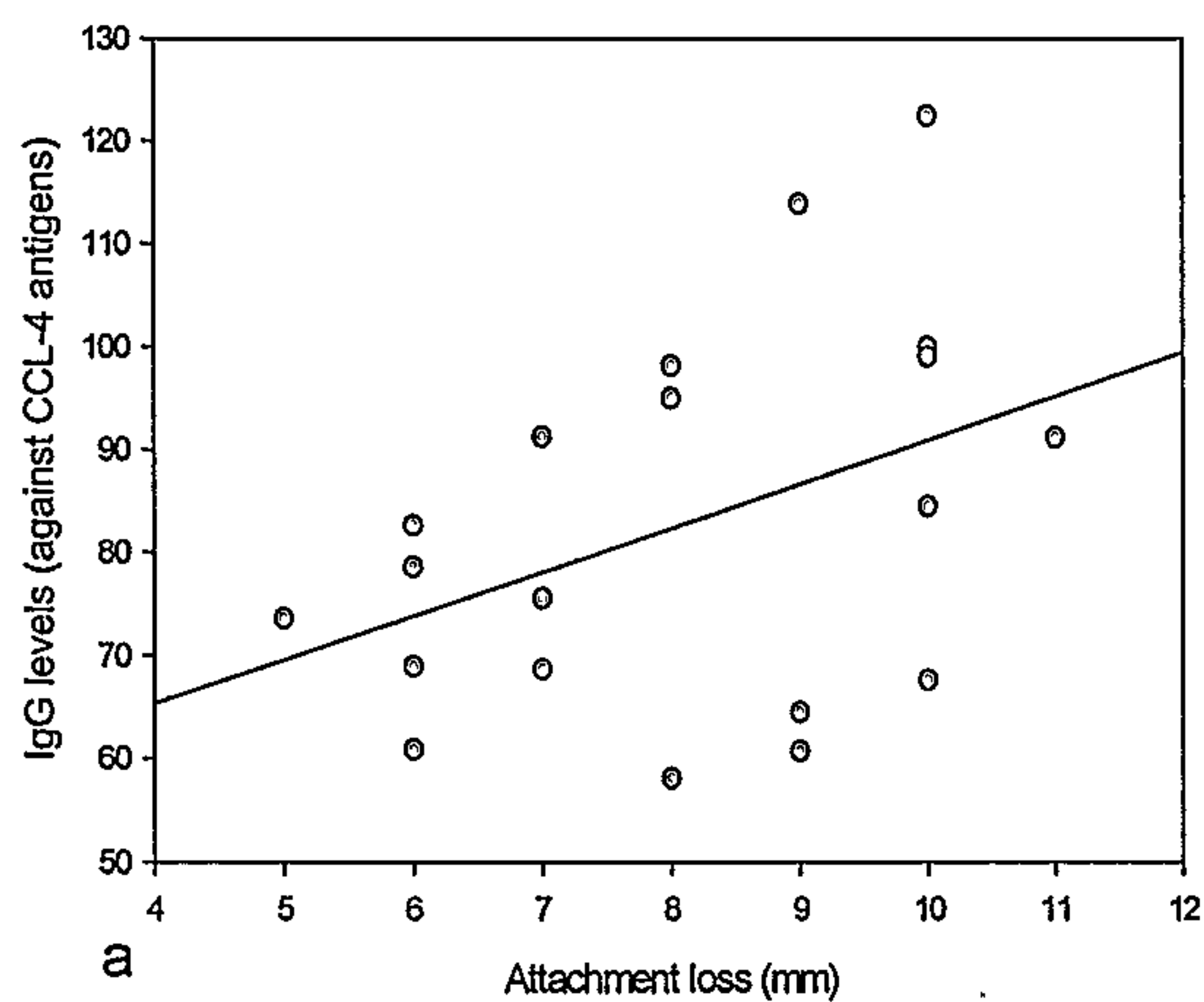
Western immunoblots of H413 epithelial antigens separated on an SDS-PAGE gel. Blotted H413 proteins were incubated with optional titrations of pre-treatment patient sera ($n = 22$) and clinically healthy subject sera ($n = 12$). The secondary antibody employed was alkaline phosphatase (AP) conjugated γ -chain-specific goat anti-human IgG (Sigma) diluted 1:30,000 in 0.05% Tween 20/TBS. IgG antibody binding antigen was visualized with AP substrate (cat 170-6432, Bio-Rad).

(a) 86% ($n = 19$, except patients 13, 17 and 18) of pre-treatment patients demonstrated specific reaction bands.

(b) The weak reaction of periodontally healthy sera IgG was observed except lane 1, 2, 9 and 12.

Fig. 4.3 Linear-regression indicating correlation between sera IgG levels of pre-treatment patients immuno-reactive with CCL-4 antigens (a, c and e) or *P. gingivalis* antigens (b, d and f) and disease indices.

- (a) A positive trend with attachment loss, $n = 20$, $r = 0.42$, $P > 0.05$.
- (b) Significant negative correlation with attachment loss, $n = 20$, $r = 0.52$, $P < 0.05$.
- (c) Pocket depth, $n = 21$, $r = 0.16$, $P > 0.05$.
- (d) Pocket depth, $n = 20$, $r = 0.23$, $P > 0.05$.
- (e) Diagnosis score, $n = 21$, $r = 0.21$, $P > 0.05$.
- (f) Diagnosis score, $n = 22$, $r = 0.32$, $P > 0.05$.



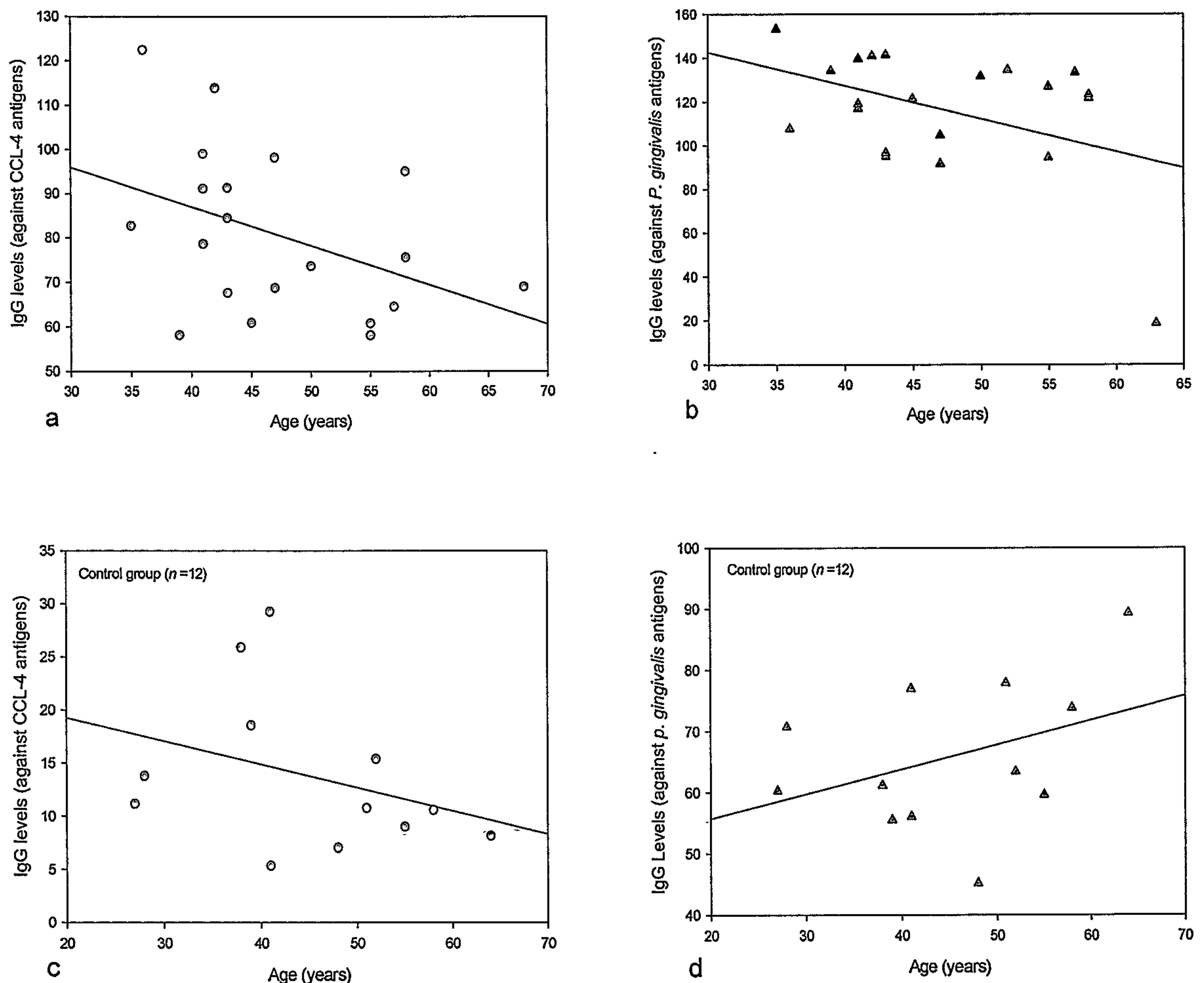


Fig. 4.4 Linear-regression indicating a trend between sera IgG levels and increased age of pre-treatment patients (a) and controls (c) immuno-reactive with CCL-4 antigens or *P. gingivalis* antigens (b and d).

- (a) A negative trend between sera IgG levels and increased age of pre-treatment patients immuno-reactive with CCL-4 antigens ($n = 20$, $r = 0.41$, $P > 0.05$).
- (b) A negative trend between sera IgG levels and increased age of pre-treatment patients immuno-reactive with *P. gingivalis* antigens ($n = 21$, $r = 0.42$, $P > 0.05$).
- (c) For periodontally healthy subject sera, there was a negative trend between IgG antibody levels to CCL-4 antigens and increased age ($n = 12$, $r = 0.34$, $P > 0.05$).
- (d) Conversely, a positive trend between control subjects sera anti-*P. gingivalis* IgG and increased age was displayed ($n = 12$, $r = 0.38$, $P > 0.05$).

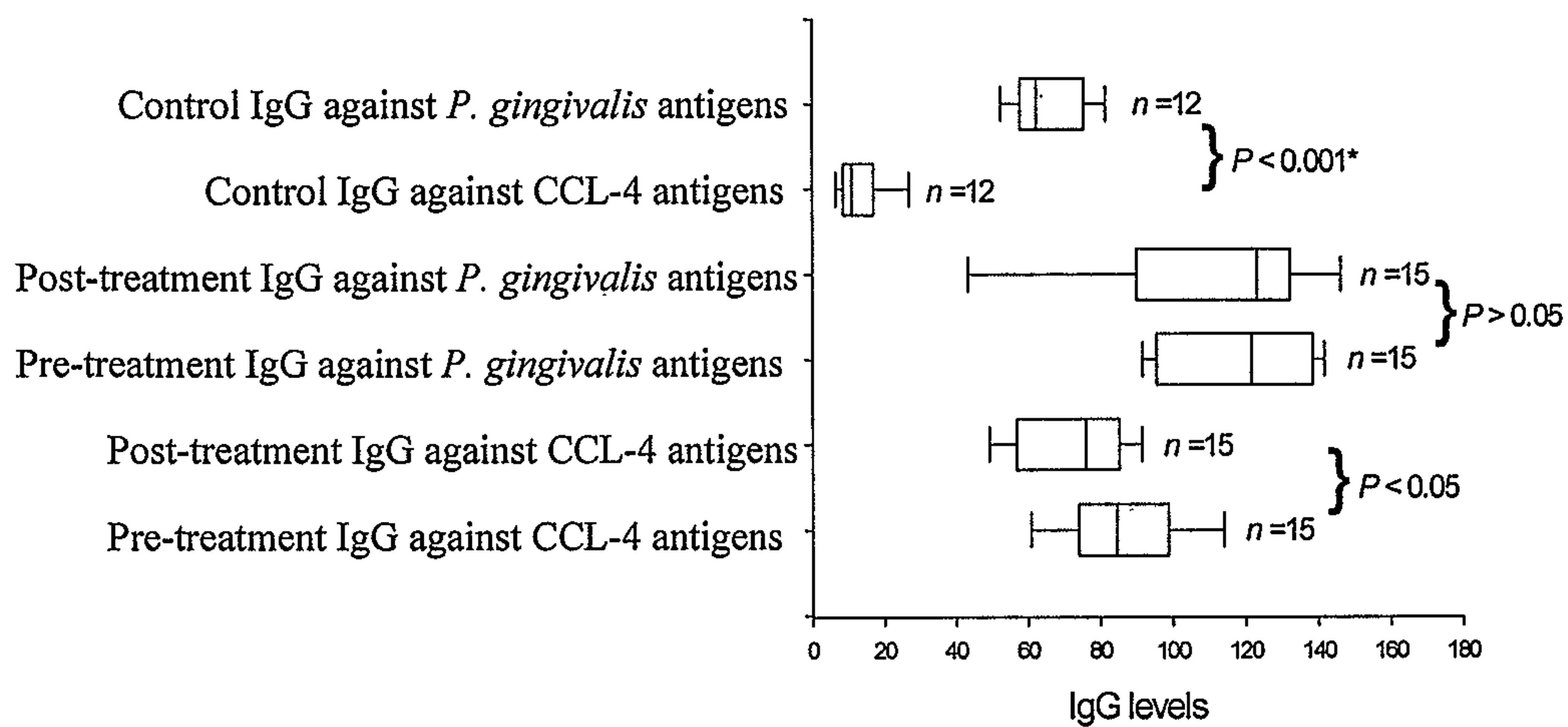


Fig. 4.5 Summary of the range and median of IgG levels.

* Control subjects showed very low anti-CCL-4 and anti-*P. gingivalis* IgG levels ($P < 0.001$, Kruskal-Wallis test) compared to pre- and post- treatment patient sera anti-CCL-4 and anti-*P. gingivalis* IgG levels respectively.

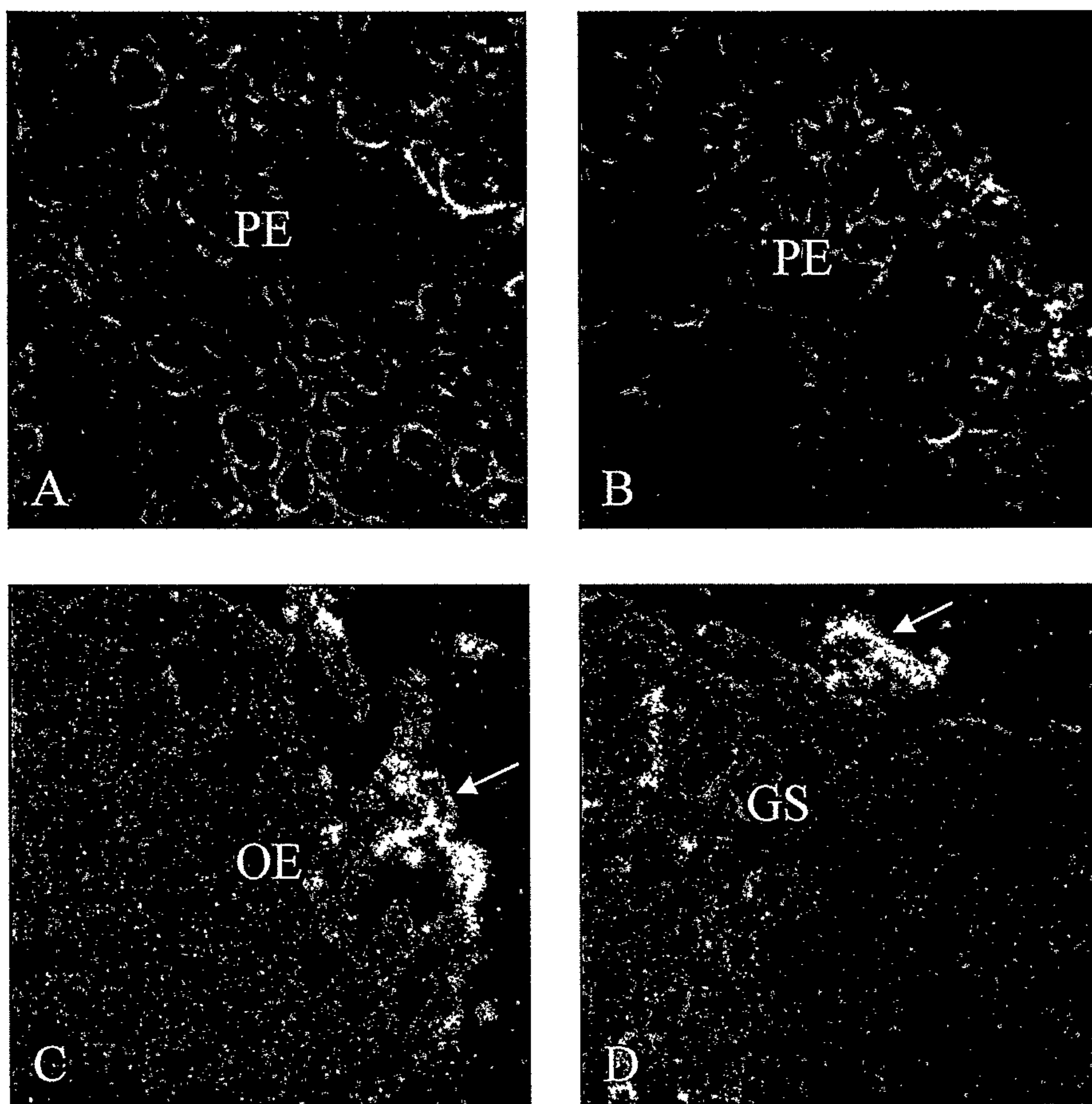


Fig. 4.6 Confocal images of morphology of active pocket epithelia.

A series of 8 optical slices (0.5 μm apart) of sections from two random patients number 3 (A and C) and number 7 (B and D) with re-construction of 3-dimensional (3-D) image, and detected in the blue channel (green colour). The bright areas were present in the cytoplasm of epithelial cells (A and B). Tissue sections in duplicate were incubated with titrations (1:10–1000) of subject serum for 1 h at room temperature in a humid chamber. After washing with PBS the sections were incubated with FITC-labeled rabbit anti-human IgG (γ -chains) for 1 h in a dark, humid chamber at room temperature, then washed in PBS three times, coverslipped and detected under incident fluorescence microscopy ($\times 600$).

(A) Showing a discontinuous pattern in the pocket epithelium (PE). (B) Showing a clustered and disordered profile in PE. IgG deposition on keratinocyte cell surfaces (arrows) was observed in the external gingival mucous membrane (OE, C) and gingival sulcus (GS, D) in periodontitis patients.

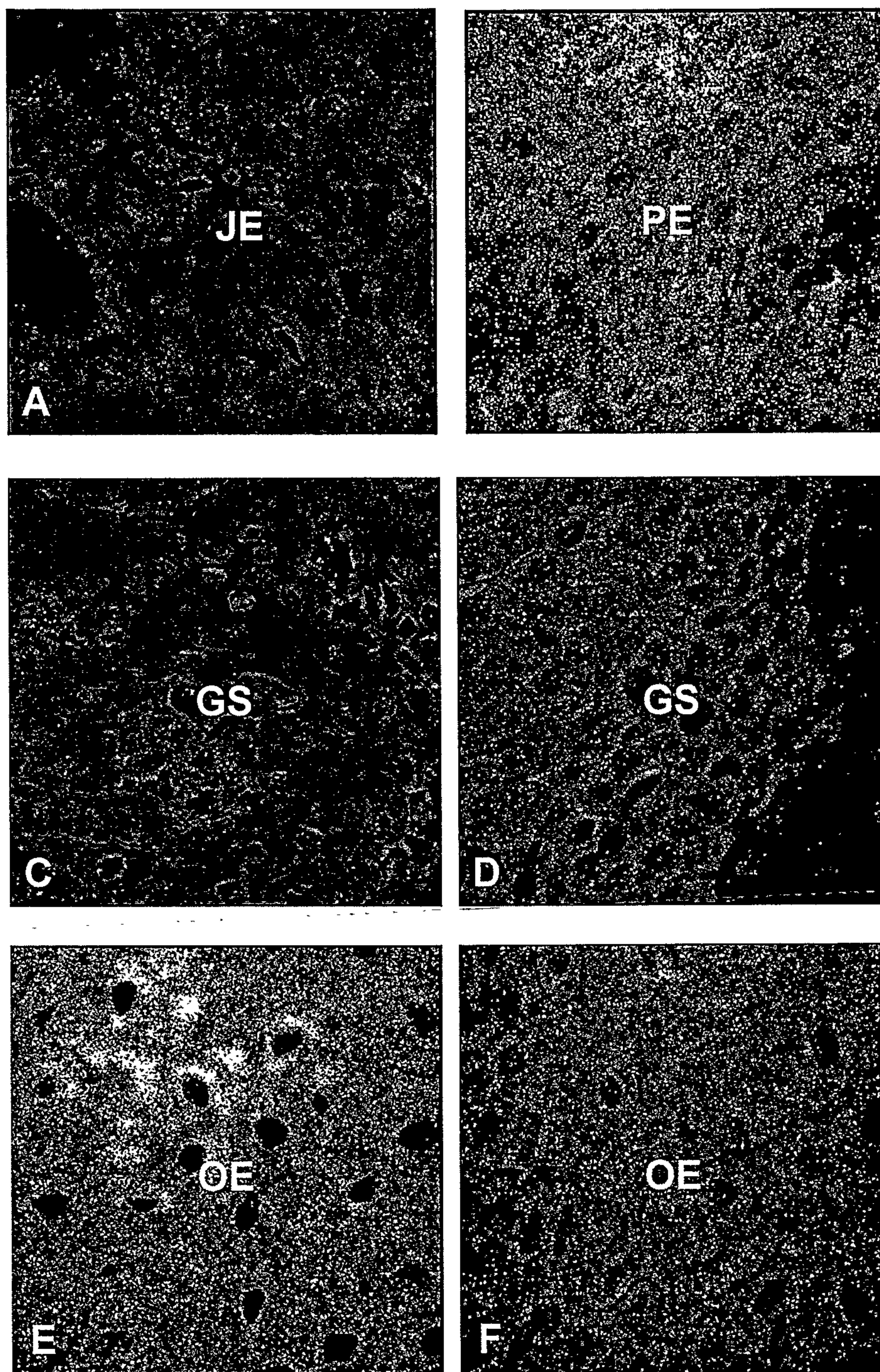


Fig. 4.7 Patterns ($\times 600$) of comparison of reactivity to pooled periodontitis patient sera in a clinical healthy site (A, C and E) and diseased site (B, D and F).

(A) Junctional epithelium (JE) displays the tight cell structure compared to pocket epithelium (PE).

(B) Pocket epithelium (PE) shows a greater brightness compared to JE (A).

(C) and (D) Gingival sulcus (GS) exhibits a similar stain density, but GS in (D) shows

a loose structure compared to GS in (C).

(E) and (F) Oral epithelium (OE) shows a similar pattern.

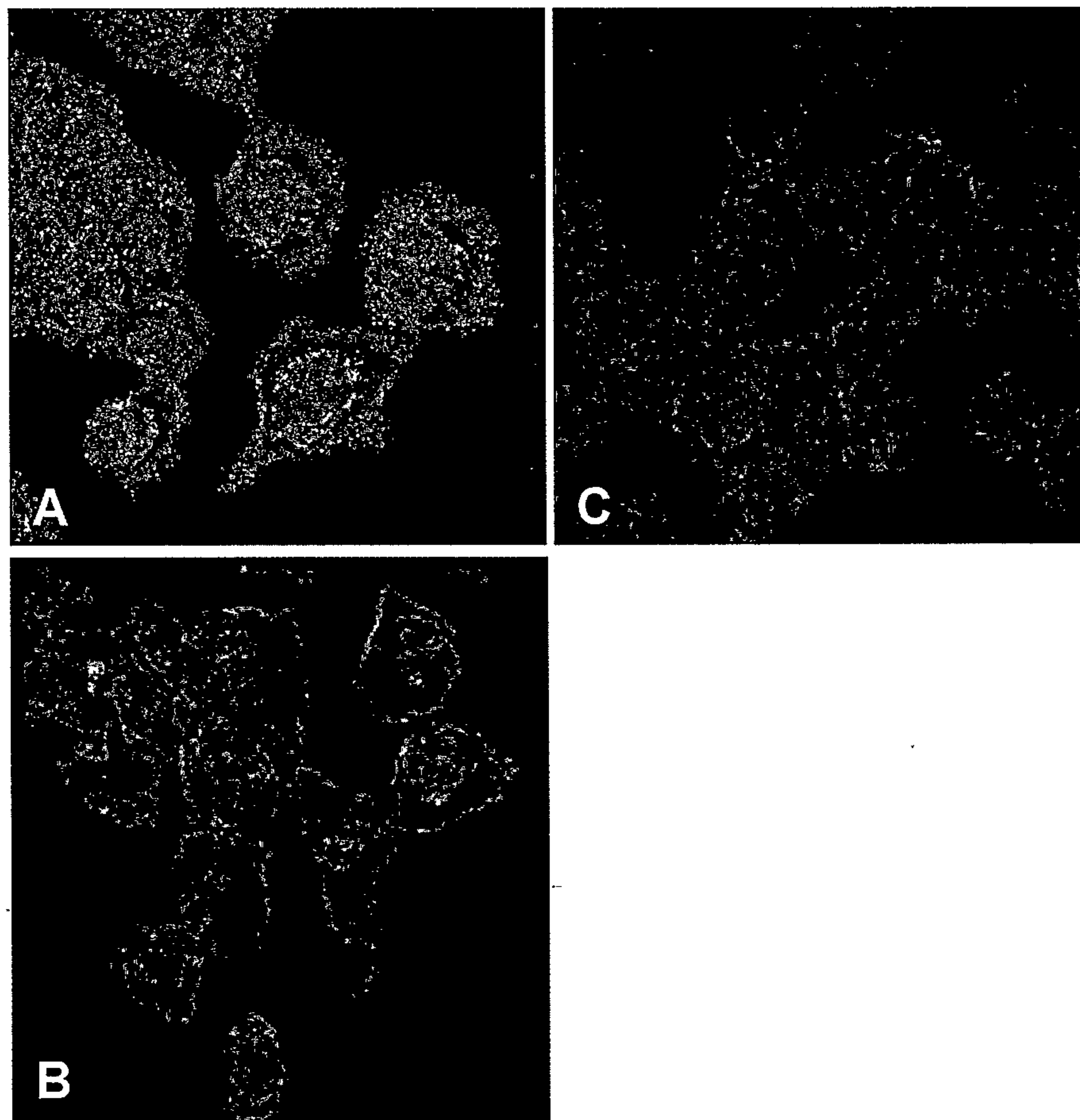


Fig. 4.8 CCL-4 epithelial cell pattern (×600) stained by random patient sera IgG (A: no 7; B: no 16) and control subject no 1 (C). A brighter reaction of periodontitis patients (A and B) was observed than that of control subject (C) in the same dilution of serum.

4.5 DISCUSSION

The findings demonstrated that periodontitis is not only associated with antibody responses to periodontal bacterial pathogens (e.g. *P. gingivalis*), but also to epithelial components. In contrast, there was a decrease in anti-epithelial IgG levels in control subjects and post-treatment patients respectively.

Serum IgG antibodies were evaluated semi-quantitatively by immunodot blots to

indicate that the sera anti-epithelial IgG levels tended to be greater in accordance with the increase of disease indices. Conversely, serum anti-*P. gingivalis* IgG levels decreased with increased disease severity.

The data indicated a negative trend between sera anti-epithelial IgG levels and increased age of control subjects and periodontitis patients, but interestingly, a positive trend between sera anti-*P. gingivalis* IgG levels and increased age of control subjects.

It is recognized that smoking is a risk factor for periodontal disease, but there are conflicting reports on whether or not smoking has an effect on the periodontal microflora (Chen *et al.*, 2001; Darby *et al.*, 2000; Haffajee & Socransky, 2001b). Direct correlations between tobacco use and increased attachment loss and pocket depth have been reported (Chen *et al.*, 2001; Haffajee & Socransky, 2001a). The data (not shown) in this study indicated that periodontitis patients who are smokers have increased IgG levels related to attachment loss compared to periodontitis patients who are non-smokers. The possible explanation is that tobacco-exposed B- and T-lymphocytes exhibit reduced proliferative capacities which could limit the production of protective immunoglobulins against oral pathogens (Barbour *et al.*, 1997).

The periodontitis classification also relates to clinical parameters, such as attachment loss. In this study, there was a trend towards a positive correlation for anti-epithelial IgG and a negative correlation for anti- *P. gingivalis* IgG in generalised periodontitis patients related to severity of attachment loss compared to localised periodontitis patients (data not shown). This suggests systemic immuno-responses play an important role in the progression of periodontitis.



CHAPTER 5

**STREPTOCOCCI AND
ACTINOMYCES INDUCE
ANTIBODIES CROSS-
REACTIVE WITH
EPITHELIAL
ANTIGENS IN
PERIODONTITIS**

CHAPTER 5: STREPTOCOCCI AND *ACTINOMYCES* INDUCE ANTIBODIES CROSS-REACTIVE WITH EPITHELIAL ANTIGENS IN PERIODONTITIS

5.1 ABSTRACT

Perturbation of epithelial structure is a prominent but poorly understood feature of the immunopathological response to bacterial antigens which characterizes the destructive lesion of periodontitis. Western analysis of sera from 22 patients with periodontitis detected multiple antigens in extracts of epithelial cells whereas sera from 12 periodontally healthy subjects displayed only trace reaction with epithelial antigens. To investigate a possible relationship between the bacterial flora adjacent to diseased sites and the presence of antibodies reactive with epithelium, subgingival plaque samples were taken from deep periodontal pockets and cultured anaerobically. Gram positive bacteria containing antigens cross-reactive with epithelial cells were reproducibly isolated by probing membrane colony-lifts with affinity-isolated (epithelium-specific) antibodies and identified by 16S rDNA sequence homology as streptococci (*S. mitis*, *S. constellatus* and two *S. intermedius* strains) and *Actinomyces* (*A. georgiae*, and *A. sp. oral clone*). Conversely, when serum from patients with periodontitis was absorbed with the captured bacterial species the number of epithelial antigens recognised was specifically reduced. It was concluded that development of cross-reactive antibodies related to these organisms may contribute to perturbation of the epithelial attachment to the tooth and the progression of periodontitis. These autoreactive antibodies could also be a contributing factor in other diseases affecting epithelia.

Keywords: epithelium; cross-reactive antibodies; plaque bacteria; periodontitis

5.2 INTRODUCTION

Destructive periodontitis is considered to represent an immunopathological response to a complex microflora that develops adjacent to the affected tissues. A hallmark of the progressing lesion is the failure of the epithelial attachment to the tooth and the migration of epithelial components down the tooth root to form a cleft or pocket creating a favourable environment for an abundant anaerobic microbial flora. Perturbation of the structural integrity and functional differentiation of the pocket epithelium in relation to inflammatory changes associated with destructive periodontitis

was reported previously (see Chapter 2 and Ye *et al.*, 2000) .

Serum antibodies reacting with tissue components, including type I collagen and epithelial desmosomal proteins, have been reported to occur with greater frequency and in higher levels in periodontitis patients than in periodontally healthy subjects (Anusaksathien *et al.*, 1992b; Govze & Herzberg, 1993). In this context, a contribution of autoimmunity to the immunopathology of destructive periodontitis has been proposed (Anusaksathien & Dolby, 1991; Ranney, 1991).

The bacterial aetiology of periodontal disease is complex, with a variety of organisms responsible for the initiation and progression of disease. Although some 500 bacterial species have been detected in the human oral cavity, and over 400 species in the subgingival plaque (Paster *et al.*, 2001), only a limited number have been implicated as periodontal pathogens. Many of these organisms may also be present in periodontally healthy individuals and can exist in commensal harmony with the host.

For this study, a cell line (CCL-4) derived from the normal oral mucosa of a laboratory rat was selected as a source of epithelial antigens (Birkedal-Hansen *et al.*, 1981). This cell line is well characterised and has been utilised as a model in investigation of microbial pathogenicity related to gingival inflammation (DeCarlo *et al.*, 1998). In the context of the reported findings, use of the CCL-4 cell line overcame the potential for contamination by adherent bacterial antigens in primary oral epithelial cultures. The majority of established human epithelial lines were contaminated by HeLa cells while many human oral carcinoma-derived lines express viral antigens or are insufficiently characterised (Yeudall *et al.*, 1995), rendering them unsuitable for this purpose.

Of clinical importance is the failure of the epithelial attachment to the tooth which signals progression of the lesion from stable non-destructive marginal gingivitis. The determinants of the host-parasite interaction leading to altered epithelial behaviour remain unknown. On this basis, the objective of the study was to investigate the relationship between potential autoreactive antibodies, oral epithelial antigens and subgingival plaque bacteria. Findings are reported which indicate that bacterial plaque from diseased sites contains Gram positive organisms with antigens that display cross-reactivity with epithelial components.

5.3 MATERIALS AND METHODS

5.3.1 Clinical data and serum samples

Blood samples were obtained with informed consent from 22 adult chronic periodontitis patients (14 males, 8 females, 35–68 years of age, average 48 years), including 14 with generalised periodontitis (7 patients were tobacco smokers) and 8 with localised chronic periodontitis, with no history of systemic disease or of medications including antimicrobials within the past 6 months and no record of periodontal therapy including subgingival scaling, root planing or relevant surgery, in the previous year. All patients as pre-treatment had a diagnosis of moderate to severe generalised or localised periodontitis with loss of attachment (5–11 mm), formation of periodontal pockets with probing depths of 4–11 mm and resorption of alveolar bone. As a control group, blood was also collected from 12 race, age and gender matched periodontally healthy subjects (7 males, 5 females, 27–64 years of age, average 46 years) all non-smoking and with no use of anti-microbial mouthwashes within the past 6 months; characterised by no clinical signs of gingival inflammation, probing depths of 3 mm or less, no sites with significant attachment loss, no evidence of radiographic bone loss and no history of systemic disease. Sera were separated from clotted blood by centrifugation at 1000g for 20 minutes and stored at -70°C until used.

5.3.2 Epithelial cells

For use in this study, the epithelial cell line (CCL-4) derived from normal oral mucosa in a laboratory rat (Birkedal-Hansen *et al.*, 1981), displayed typical epithelial morphology in culture, such as coherent monolayer sheets and stratification of epithelial cells. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture (1:1) (Sigma, Sydney, Australia), 15 mM HEPES (Sigma) and supplemented with 10% foetal bovine serum (CSL Limited, Victoria, Australia) and penicillin/ streptomycin (100 IU/ml, ICN Biomedicals, Inc., Sydney, Australia) at 37°C with 5% CO_2 .

5.3.3 CCL-4 cell antigens

The CCL-4 cells were subcultured every 2–3 days and harvested at a multilayer confluence (10^7 – 10^8 cells/3–4 mg total protein) in 175 cm^2 flasks (Sarstedt, Inc., Newton, NC). Briefly, cells were placed on ice, washed with ice cold Dulbecco's

phosphate buffered saline (DPBS), containing a proteinase inhibitor cocktail (Sigma-P8340) and harvested by gentle scraping. After centrifugation, in preparation for Western blot analysis, cells were resuspended in 2ml PBS, then boiled in sodium dodecyl sulfate (SDS) sample buffer with 1% 2-mercapthoethanol for 10 minutes. For dot blot analysis, resuspended cells in 0.1M Tris-buffered saline (TBS, pH 7.5) containing 3% Triton X-100 (AJAX chemicals, Australia), were gently vortexed and placed on ice for one hour. The samples were stored at -20°C for up to 2 months.

5.3.4 Western immunoblot for patient and control subject IgGs against CCL-4 antigens

CCL-4 antigens were separated by standard SDS-PAGE mini gels (5% stacking gel, 10% separating gel). Fractionated CCL-4 cell proteins were transferred onto nitrocellulose membranes (Bio-Rad, Australia), and blocked with 3% bovine serum albumin (BSA, Sigma) in TBS overnight. Blotted CCL-4 antigens were incubated with optional titrations for patient sera ($n = 22$) and clinically healthy subject sera ($n = 12$). The secondary antibody employed was alkaline phosphatase (AP) conjugated γ -chain specific goat anti-human IgG (Sigma) diluted 1:30,000 in 0.05% Tween 20/TBS. IgG antibody binding antigen was visualized with AP substrate (cat 170-6432, Bio-Rad). Triplicate blots were performed for each experiment.

5.3.5 Affinity-isolation of antibodies from CCL-4 (CCL-4-specific antibodies)

The validity of the affinity-isolation procedure was established in this study. CCL-4 cells were passaged into tissue culture plates (24-well-Sarstedt) and incubated at 37°C , 5% CO_2 for 2 days until the cells were 70–80% confluent. CCL-4 cell layers were fixed with pre-cooled methanol for 3 minutes and blocked with 1% bovine serum albumin (BSA, Sigma) in PBS overnight at 4°C . Patient sera, plus one blank control and one reference serum were diluted empirically with 0.05% Tween 20/TBS, added into 24-well plates and then incubated for one hour at room temperature. Unbound antibodies were removed by washing 3 times with 0.05% Tween 20/TBS. Bound antibodies were eluted in 0.05 M glycine-HCl buffer (pH 2.3) and rapidly neutralized with 1M Tris (pH 8.0) and 10% foetal calf serum (FCS). Affinity-isolated (CCL-4-specific) antibodies were prepared from all patient sera and used immediately in further analysis.

5.3.6 Subgingival plaque samples and growth conditions

Sixteen subgingival plaque samples were taken with paper points from advanced periodontal pockets (> 6mm probing depth) of 8 adult periodontitis patients at diagnosis. Sterile paper points were inserted into the base of pockets for 20 seconds for each of two sites per patient and then placed into microcentrifuge vials containing 100 µl reduced transport fluid (RTF) medium and kept on ice. The plaque samples were transferred to an anaerobic chamber (85% N₂, 5% CO₂, and 10%H₂) for primary culture. Each sample was dispersed by vortexing for 10 seconds to break up bacterial aggregates. Tenfold serial dilutions (10⁻¹ to 10⁻³) were prepared in RTF; 100µl of the dilutions were plated onto CDC anaerobic blood agar containing 5% defibrinated sheep blood (BBL, Baltimore Biological Laboratories, Cockeysville, MD) and cultured at 37°C. Processing was completed within 24 hours.

5.3.7 Isolation of bacterial colonies from plaque

Nylon membranes (Boehringer Mannheim, Germany) were used to lift colonies from plated plaque samples cultured anaerobically. Briefly, 2 nylon membranes with lifted bacterial colonies from one plate were prepared at the same time. Membranes were dried and fixed in pre-cooled methanol for 3 minutes and then blocked in 5% skim milk powder in TBS overnight. Two nylon membranes were probed separately with a selected patient serum and matched affinity-isolated (CCL-4-specific) antibodies for immunodetection. Each plaque sample was tested with at least 5 different patient sera. Colonies reacting with affinity-isolated (CCL-4-specific) antibodies were picked and inoculated into CDC anaerobic broth, supplemented with haemin (5µg/ml, Sigma), menadione (5µg/ml, Sigma) and CDC blood agar (Trypticase peptone 10 g, Trypticase soy broth 10 g, Yeast extract 10 g, NaCl 5 g, L-cysteine 0.4 g, bacterial agar 20 g), and 3% horse serum (Amyl Media Pty Ltd, Victoria, Australia), for sub-culture under both anaerobic and microaerophilic conditions. Colony purity was checked routinely by Gram-stain with slides fixed in the anaerobic chamber (Johnson *et al.*, 1995). Growth curves for target organisms were established under anaerobic conditions. Bacterial numbers were estimated by reference to the standard curve determined by absorbance at OD 600 nm.

5.3.8 Identification of bacteria

Only the positive colonies reacting both with serum IgG and affinity-isolated (CCL-4-

specific) antibodies were identified by colonial morphology, Gram staining and conventional biochemical tests (RapID ANA II System, REMEL Inc., Lenexa, KS), and confirmed by 16S rDNA PCR. Biochemical test system (RapID ANA II System, REMEL Inc., Lenexa, KS) is a qualitative micromethod employing conventional chromogenic substrates for the identification of anaerobic bacteria of human origin. Test organisms on plates were grown anaerobically in pure culture, for no more than 72 hours. The procedure was performed according to the instructions provided by the manufacturer.

16S rDNA PCR provided a reliable and rapid method to distinguish these species. Bacteria were grown in CDC broth under anaerobic conditions, and the cells (10^8 – 10^9) harvested by centrifugation at $14,000 \times g$, 18 – 20°C for 2 minutes. Pellets were resuspended in $200\mu\text{l}$ 10 mM phosphate buffer ($\text{pH } 6.7$) containing $400\mu\text{g}$ lysozyme (Boehringer Mannheim), 200 U mutanolysin (Sigma) and $400\mu\text{g}$ proteinase K (Qiagen Pty Ltd., Clifton hill, Victoria, Australia) and incubated at 56°C for 40 minutes. Cells were lysed by adding 1% SDS. Following the addition of $200\mu\text{g}$ RNase (Sigma), the samples were incubated for a further 10 minutes at 37°C and purified using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA concentrations were measured at A_{260} in a spectrophotometer (Beckman, Instruments). Isolated DNA was used as a template to generate PCR fragments based on universal primers recognizing 16S rDNA sequences as described previously (Nadkarni *et al.*, 2002). PCR products were purified by Wizard PCR preps DNA purification system (Promega Corporation, Annandale, Australia) and detected by electrophoresis of a $5\mu\text{l}$ sample for 30 minutes at $5\text{V}/\text{cm}$ in 2% agarose in Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide ($0.5\mu\text{g}/\text{ml}$) and visualized under UV illumination. Purified PCR products (50 – 100 ng) were sequenced using standard dye-terminator chemistry (Prince Alfred Macromolecular Analysis Centre, University of Sydney, NSW, Australia).

The 16S rDNA gene sequences from each bacterium were searched against the NR nucleic database on WebANGIS (Australia National Genomic Information Service) by FastA program and further compared with Gap program. The accession numbers of the 16S rDNA gene sequences were obtained from the EMBL Data Library as shown in Table 5.1.

5.3.9 Bacterial cell antigens

Each bacterial culture was harvested in late exponential phase and washed with ice-cold PBS in the presence of a proteinase inhibitor cocktail (Sigma), and collected by centrifugation prior to protein extraction. Components were extracted by sonication on ice, and then boiled in reduced SDS sample buffer.

5.3.10 SDS-PAGE for bacterial antigens

Proteins of each identified bacterium were separated by standard SDS-PAGE mini gels (5% stacking gel, 12% separating gel) respectively. A pre-stained broad range molecular weight marker (code: 161-0318, BIO-RAD) was run in an outer lane. Electrophoresis was carried out at constant voltage 80V for stacking gel, followed by 100V for separating gel. Only the separating gel was used for electroblotting.

5.3.11 2-D electrophoresis for bacterial antigens

1) Sample preparation

(1) Bacterial cells were cultured anaerobically in 200 – 500 ml CDC medium, and harvested when they were at late log phase by centrifuging for 15 min at $20,000 \times g$ (8000 rpm), 4°C. Supernatant was decanted and the cells were washed three times in pre-cooled PBS containing a protease-inhibitor cocktail (Sigma-P8340). After centrifuging, cells were frozen at – 80°C for 1 hour and freeze-dried overnight.

(2) Cell disruption:

Twenty five mg of dry pellet was resuspended in 2 ml mutanolysin buffer (final concentration: 10 mM K_2HPO_4 , 10 mM $MgCl_2$ and 1 mM $CaCl_2$) containing 500 U mutanolysin, then incubated for 40 min in a 60°C water bath with occasional gentle shaking. Salt was removed by dialysis against 4 L Milli-Q H_2O at 4°C for 2 hours, and the water changed for a further 2 hours of dialysis. The sample was transferred from dialysis tubing to microcentrifuge tubes, then centrifuged for 30 min at $15,000 \times g$ (13,000 rpm) at 4°C. Cell pellets were frozen at – 80°C for 1 hour, and freeze-dried for overnight.

(3) Precipitation with trichloroacetic acid (TCA) in acetone:

To weighted pellets, 1 ml of 10% TCA in cold acetone with 0.07% 2-mercaptoethanol was added and the pellets were resuspended by pipetting. Samples were held at –20°C for 45 min, with gentle shaking three times every 15 min. Precipitated proteins were collected by centrifugation for 30 min at $15,000 \times g$ (13,000

rpm) at 4°C. One ml cold acetone containing 0.07% 2-mercaptoethanol was added to wash each pellet at -20°C for 45 min, with gentle shaking three times every 15 min. Samples were re-precipitated and the wash step repeated. Residual acetone was removed by air drying pelleted proteins for at least 15 min.

2) IEF

Isoelectric focusing (IEF) was used in the first dimension and performed with the IPGphor system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Immobiline pH 4–7 linear DryStrips (18cm in length, Pharmacia Biotech) were used in this study. Briefly, re-hydrated solution was prepared with final concentrations of 9 M urea, 2 M thiourea, 4% CHAPS w/v, 60 mM DTT, a trace of bromophenol blue in 0.5% IPG buffer w/v pH 4–7 (Bio-Rad). The re-hydrated solution was added to samples (15 mg respectively) to make the total volume up to 350 µl. An aliquot (340 µl) of the mixture was pipetted into an IPG strip-holder channel, IPG strips were placed with gel-side down and covered with re-hydrated solution with gentle mixing. After re-hydration for 12 hours, IEF was performed under paraffin oil at 20°C. Focusing by voltage gradients was as follows: 3h at 300V, 2.5h at 1000V, and 2.5h at 2500V and 18h at 3500V. Current was limited to 0.05 mA per IPG strip. The electro-focused strip was stored at -80°C until the second-dimension of electrophoresis.

3) 2-D SDS-PAGE

Before SDS-PAGE, strips were equilibrated for 10 min in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% w/v glycerol, 2% SDS and 100 mM DTT and further incubated for 10 min in the above equilibration solution that contained 400 mM iodoacetamide instead of 100 mM DTT. Second-dimension electrophoresis was carried out with gradient gels (8–18% T) run in the Multiphor system (Bio-Rad) at room temperature, 10 h at 3 mA, 12 h at 18 mA and 1 h at 18 mA.

4) Silver staining

Protein spots were visualized by silver staining. Briefly, the gels were fixed with 50% methanol/ 5% acetic acid for 20 min, followed by a wash with 50% methanol for 10 min and with water for 10 min. The gel was sensitised with 0.02% sodium thiosulfate for 1 min, followed by 2 water rinses (1 min each). The gels were submerged in 0.1% silver nitrate for 20 min, and washed with water (2×, 1 min each). The gels were developed with 2% sodium carbonate/0.4% formalin until the desired intensity of staining occurred. Staining was stopped with 5% acetic acid and rinsed with a 5-min water. The gels were ready to be scanned.

5.3.12 Western immunoblot for patient IgGs and affinity-isolated (CCL-4-specific) IgGs against oral bacterial antigens

Antigens from the identified bacterial strains recognised by affinity-isolated (CCL-4-specific) antibodies were separated by 12% polyacrylamide mini gels respectively, then transferred onto nitrocellulose membranes (Bio-Rad) and blocked with 3% bovine serum albumin (BSA, Sigma) in TBS overnight. The membranes were immuno-detected by titrated patient sera ($n = 22$) and matched affinity-isolated (CCL-4-specific) antibodies, and then bound antibodies were detected as described above. Triplicate blots were performed for each experiment.

5.3.13 Sera absorption

Sera from 22 periodontitis patients were absorbed individually to each of the identified bacteria, or a combination of all of the identified bacteria; and other bacteria: *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus casei* subsp. *rhamnosus* ATCC 7469 as controls. Briefly, bacterial cells were harvested at late exponential phase, washed in PBS by centrifugation, resuspended in 0.05% Tween 20/TBS to approximately 10^8 bacteria per ml (OD at 600nm =1.0) and then aliquoted, each bacterial suspension or pooled bacterial suspension to 22 vials respectively, for individual patient sera diluted empirically. The suspensions were mixed and shaken overnight at 4°C. The cells were removed by centrifugation at 13,000 rpm (15,000 x g) for 5 minutes, and the supernatants used for immunoblots.

5.3.14 Immunoblots against CCL-4 antigens following absorption with identified bacteria

For Western immunoblot, CCL-4 antigens were separated by 12% polyacrylamide mini gels, then transferred onto nitrocellulose membranes (Bio-Rad) and blocked with 3% bovine serum albumin (BSA, Sigma) in TBS overnight. For dot blot, CCL-4 antigens diluted in TBS to a concentration of 1µg/spot, were applied to pre-wetted nitrocellulose membrane (0.45µm, 9x12 cm size, Bio-Rad) and filtered by a vacuum microfiltration apparatus (96-well format, Bio-Rad). Blotted antigens were blocked with 1% BSA/TBS. All membranes were immunodetected by patient sera depleted by absorption with the identified bacteria or by control bacteria, and compared with non-absorbed sera respectively. Bound antibodies were detected as described above. Triplicate blots were performed for each experiment.

5.3.15 Data analysis

The semi-quantification of dot blots was measured using densitometric scanning (Scion Image software, Scion Corporation, Frederick, MD). A value of $P < 0.05$ considered significant was assessed by Kruskal-Wallis test analysis of variance (GraphPad software, San Diego, CA, USA).

5.4 RESULTS

5.4.1 Patient and control subject IgG responses to CCL-4 epithelial components

The protein profile of extracted CCL-4 antigens is shown in Fig. 5.1a. A wide distribution of bands was observed between 21–200 kDa. Western blots displayed distinct immunoreactive characteristics for individual patient sera with CCL-4 antigens (Fig. 5.1b); however, there existed a common recognition of a 30 kDa band. Only trace recognition of epithelial antigens in periodontally healthy sera was observed including common recognition of the 30 kDa band (Fig. 5.1c). Profiles for reactivity of individual sera were entirely reproducible over three consecutive experiments using separately prepared antigenic extracts in each case. These data suggested that detectable levels of serum antibodies reactive with epithelial components were present in relation to existing inflammatory periodontal disease.

5.4.2 Isolation of bacteria with cross-reactivity to epithelial antigens from subgingival plaque samples

Nylon membranes used to lift colonies from plated plaque samples grown anaerobically were probed with random serum from reactive patients (Fig. 5.2, lane 7 and 8; Fig. 5.2A and C) and with matched affinity-isolated (CCL-4-specific) antibodies (Fig. 5.2B and D). Only a small number of colonies (1–5% of the total) were recognized by affinity-isolated (CCL-4-specific) IgG antibodies. This test was repeated using different patient sera and similar results were noted. Affinity-isolated antibodies could not be prepared from periodontally healthy subject sera, in accordance with the lack of reactivity observed for CCL-4 epithelial antigens.

5.4.3 Identification of colonies with cross-reactivity for epithelial antigens

All positive colonies which reacted both with patient serum IgG and affinity-isolated (CCL-4-specific) IgG were isolated from the plaque samples, and identified by 1)

colony morphology (Table 5.1), 2) Gram-stain (under anaerobic conditions, Fig. 5.3), 3) conventional Biochemical test and 4) 16S rDNA PCR (Table 5.1 and Fig. 5.4). Six bacterial strains were isolated from the plaque samples and identified by closest homology to the reference organisms (Appendix B)

The frequency of detection in sixteen plaque samples of these six bacteria is summarized in Table 5.2 and ranged from 6–25%.

5.4.4 Cross-reactivity between affinity-isolated (CCL-4-specific) antibodies and streptococci / *Actinomyces* antigens

The protein patterns from the 6 isolated bacterial strains are displayed in SDS-PAGE (Fig. 5.5) and 2-D electrophoresis (Fig. 5.6).

Nylon membranes used to lift colonies from plated purified bacterial strains respectively grown anaerobically were probed with random serum from reactive patients (Fig. 5.7A-F) and with matched affinity-isolated (CCL-4-specific) antibodies (Fig. 5.7D and G).

Heterogeneous profiles for the 6 bacteria were identified by serum IgG from each of the 22 patients (Fig. 5.8a, c, e, g, i and k), and matched affinity-isolated (CCL-4-specific) IgGs (Fig. 5.8b, d, f, h, j, and l). Figure 5.8k shows a non-specific ladder-like (polysaccharide) pattern of reactivity to *Actinomyces* spp. with close homology to oral clone (BL008); however, after affinity-isolation (CCL-4-specific) of antibody, the ladder-like pattern of reactivity was not shown while a specific reactive band at 72 kDa was observed (Fig. 5.8l). This could be attributed to the concentrating effect of affinity isolation. Patterns of reactivity for affinity-isolated (CCL-4-specific) IgGs varied for the different bacteria; however, cross-reacting antigens at 27, 30, 35, 38 and 50 kDa among two or more bacterial strains were noted indicating the potential for common antigens (Table 5.3). In contrast, sera IgGs from periodontally healthy subjects showed low levels of recognition of antigens from this group of organisms (data not shown). Fig. 5.9 showed molecular weight determination of cross-reactive antigens for the six bacteria (Bollag *et al.*, 1996).

5.4.5 Removal of cross-reactive antibodies by serum absorption to *Streptococcus*/ *Actinomyces* spp.

To confirm the existence of cross-reactivity of serum antibodies between epithelial components and bacterial antigens, sera were absorbed with the four streptococcal and

two *Actinomyces* strains. This partially removed cross-reactivity with epithelial antigens as demonstrated by analysis of semi-quantitative dot blots ($P < 0.05$, Kruskal-Wallis test, Fig. 5.10a). Also this reactivity was confirmed by Western blot analysis (Fig. 5.10b). When the effect of absorption by all six bacteria was assessed by Western blots, anti-epithelial reactivity was either absorbed or substantially reduced for all patients (Fig. 5.10c). The reduction of cross-reactivity for serum IgG was specific for the captured organisms with only minor effect detected following pre-incubation with other Gram positive bacteria: either *Lactobacillus acidophilus* ATCC 4356 or *Lactobacillus casei* subsp. *rhamnosus* ATCC 7469 (data not shown).

Table 5.1 Identification of cross-reactive bacteria isolated from plaque samples

Colony morphology	Analyzed 16S rDNA sequences			Biochemical test	
	Species	Strain ^a /clone	Accession No.	Identity (%)	Species identity (%)
Gram-positive cocci, growth in both anaerobic and microaerophilic conditions. (Two-day cultures) Large size 1.0-3.0 mm diameter; round, white and bright colonies; Gram staining in long chains (average 10 cells per chain). Medium size 1.0-1.5 mm diameter; round and white colonies; Gram staining in pairs, short chains (3-6 cells per chain) or cuboidal packets. Small size 0.5-1.0 mm diameter; white, rough or heaped colonies; Gram staining in pairs, short chains, clusters or tetrads. Small size 0.2-0.8 mm diameter; white, smooth colonies; Gram staining in pairs, tetrads, or clusters. (Four-day cultures) Medium size 1.0-2.0 mm diameter; round, heaped but smooth colonies; one week old culture changes to red colonies; Gram-variable rods, vibrioid or straight, but variation of cells' size; after grow under anaerobic conditions can be subcultured in microaerophilic conditions. Small size 0.2 mm diameter; white and round colonies; Gram-positive rods, long, bow-shaped or C-shaped cells with pigment; grows only under anaerobic conditions.	<i>Streptococcus mitis</i>	NCTC12261	D38482	100.000	^b
	<i>Streptococcus constellatus</i>	206	AF104677	99.774	99.9
	<i>Streptococcus intermedius</i>	ATCC27335	AF104671	99.111	99.9
	<i>Streptococcus intermedius</i>	488	AF104673	98.670	99.9
	<i>Actinomyces georgiae</i>	DSM 6843	X80413	97.539	57.3
	<i>Actinomyces</i> sp. oral clone	BL008	AF385553	98.658	57.3

^a NCTC, National Collection of Type Culture, UK; ATCC, American Type Culture Collection, USA; DSM, Deutsch Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

^b No reference to this organism in the RapID ANA II Code Compendium for anaerobic bacteria.

Table 5.2 The frequencies of detection of cross-reactive bacteria from 16 plaque samples

Bacterial strains/clone ^a	Anaerobes n (%)
<i>Streptococcus mitis</i> (NCTC 12261)	2 (12.5)
<i>Streptococcus constellatus</i> (206)	3 (18.6)
<i>Streptococcus intermedius</i> (ATCC 27335)	4 (25.0)
<i>Streptococcus intermedius</i> (488)	3 (18.6)
<i>Actinomyces georgiae</i> (DSM 6843)	1 (6.0)
<i>Actinomyces</i> sp. oral clone (BL005)	1 (6.0)

^a Reference strains and clone with closest homology to the isolates are indicated in parentheses.

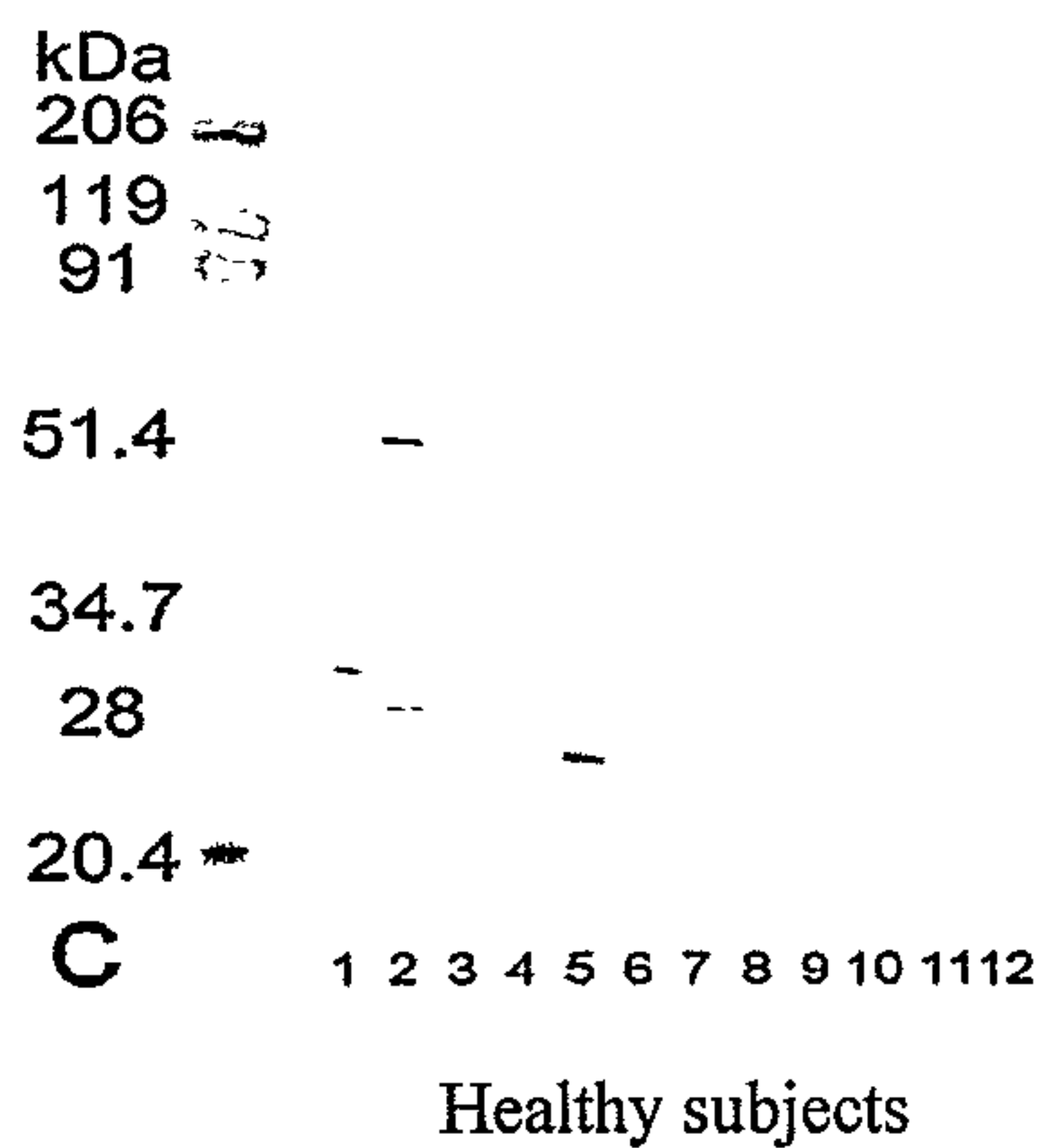
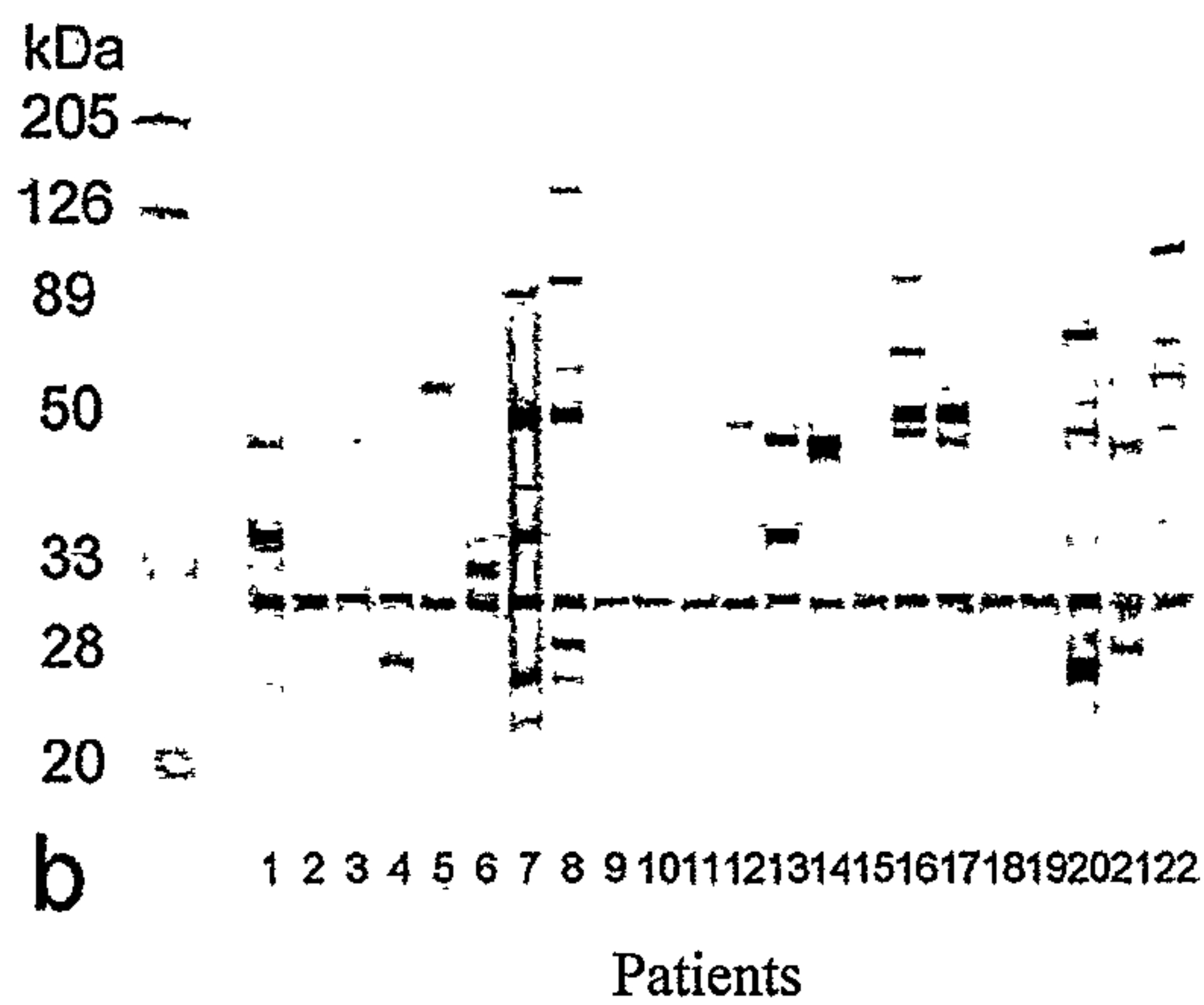
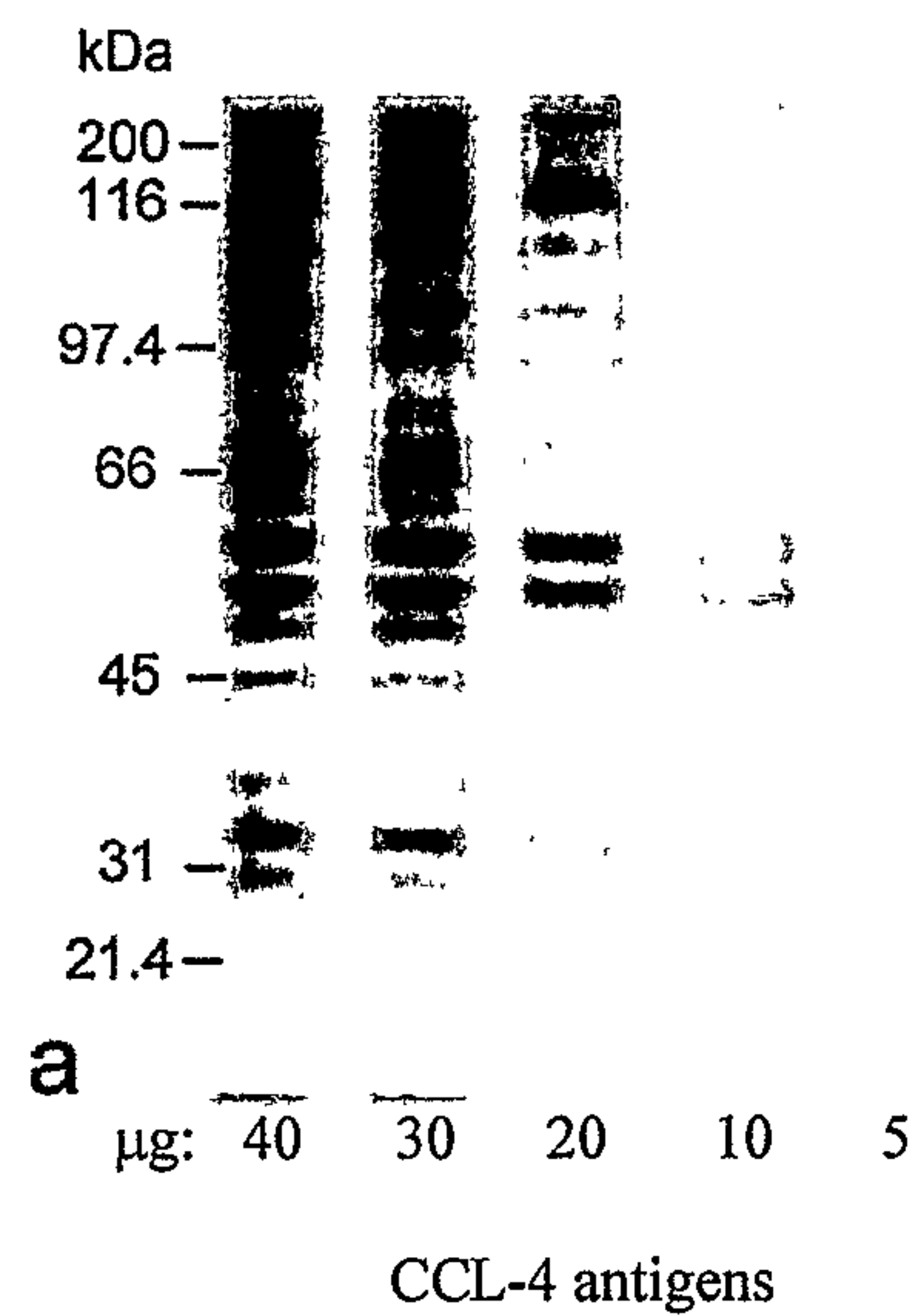
Table 5.3 Recognition of cross-reactive antigens in six bacterial strains ^a

Kilodalton	Streptococci				Actinomycetes	
(kDa) clone	<i>S. mitis</i> (NCTC 12261)	<i>S. constellatus</i> (206)	<i>S. intermedius</i> (ATCC 27335)	<i>S. intermedius</i> (488)	<i>A. georgiae</i> (DSM 6843)	<i>A. sp.oral</i> (BL008)
86		+				
78		+				
72						+
50		+	+	+		
42		+				
38	+	+		+		
35	+				+	
30	+	+		+		
27	+	+				

^a Reference strains with closest homology to the isolates are indicated in parentheses.

Fig. 5.1 Recognition patterns for epithelial antigens.

- (a) The profiles in dilution steps (5–40 µg/lane) of extracted epithelial cell (CCL-4) antigens separated by SDS-PAGE and stained with Coomassie Blue. A wide distribution of bands (21–200 kDa) is present.
- (b) Western immunoblots exhibit distinct immunoreactive characteristics for individual patient sera IgG ($n = 22$) against CCL-4 antigens. Lane (i.e. patient No.) 1, 7, 8, 13, 14, 16, 17, 20 and 22 showed strong reactive bands ranging from 25 to 90 kDa; remaining patients showed only trace to moderate recognition of antigens other than the 30 kDa band recognized by all patients. There were no clear differences between 14 generalised (lane 1–7, 9, 11, 12, 16, 19, 20 and 22) and 8 localised periodontitis subjects (lane 8, 10, 13–15, 17, 18 and 21), or between smokers (lane 1, 2, 4, 6, 9, 16 and 22) and non-smokers.
- (c) Only weak recognition of the 30 kDa band was observed in healthy subjects.



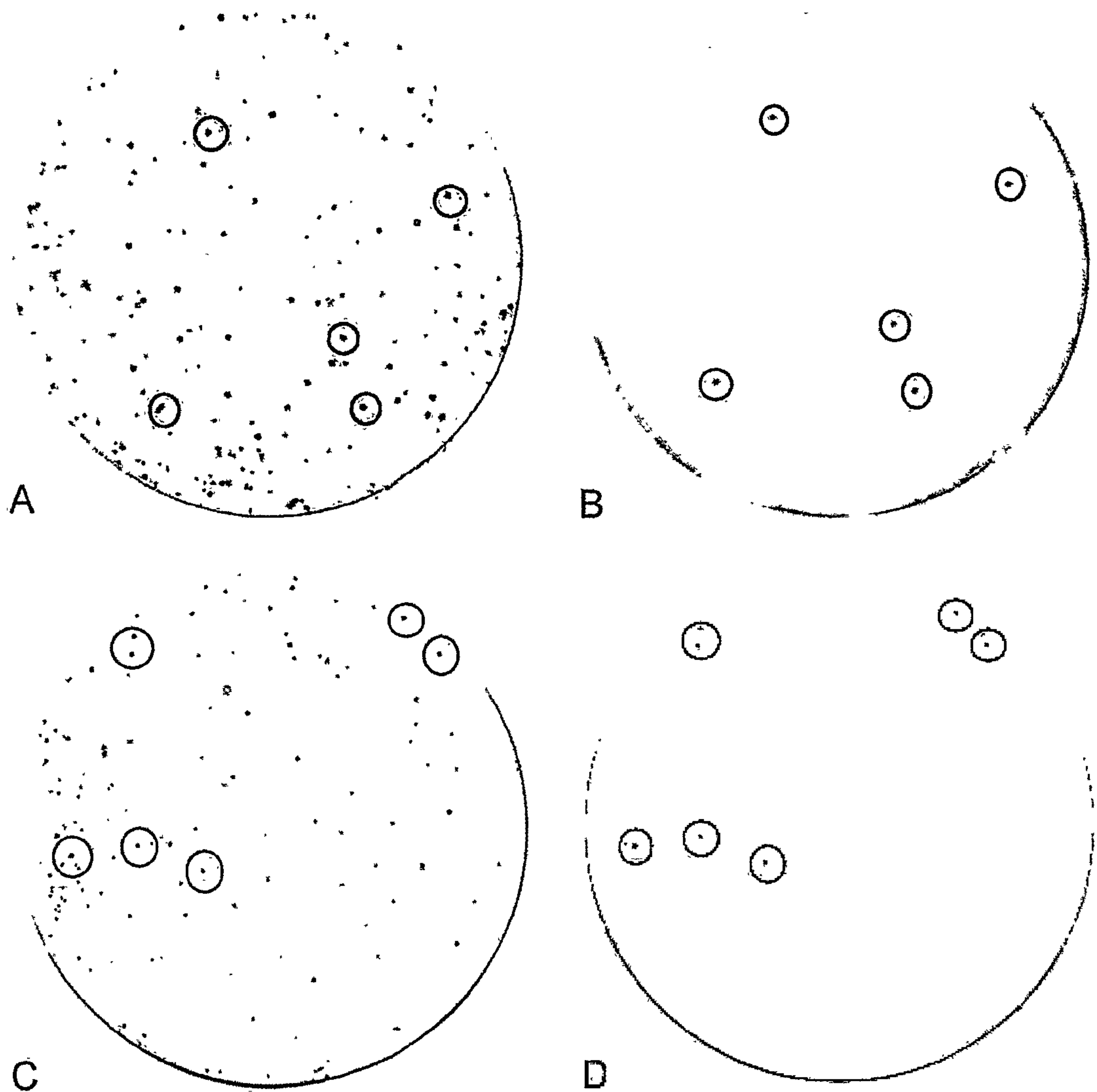


Fig. 5.2 Detection of dental plaque bacteria with immunocross-reactivity for epithelial antigens. Anti-IgG immunodetection following colony-lift techniques. Colonies were lifted onto 2 sheets of nylon membrane from each plated plaque sample.

(A and C) Probed with a serum from patient No.7 and 8.

(B and D) Probed with a matched affinity-isolated (CCL-4-specific) antibody from patient No.7 and 8. A small number of colonies was recognized.

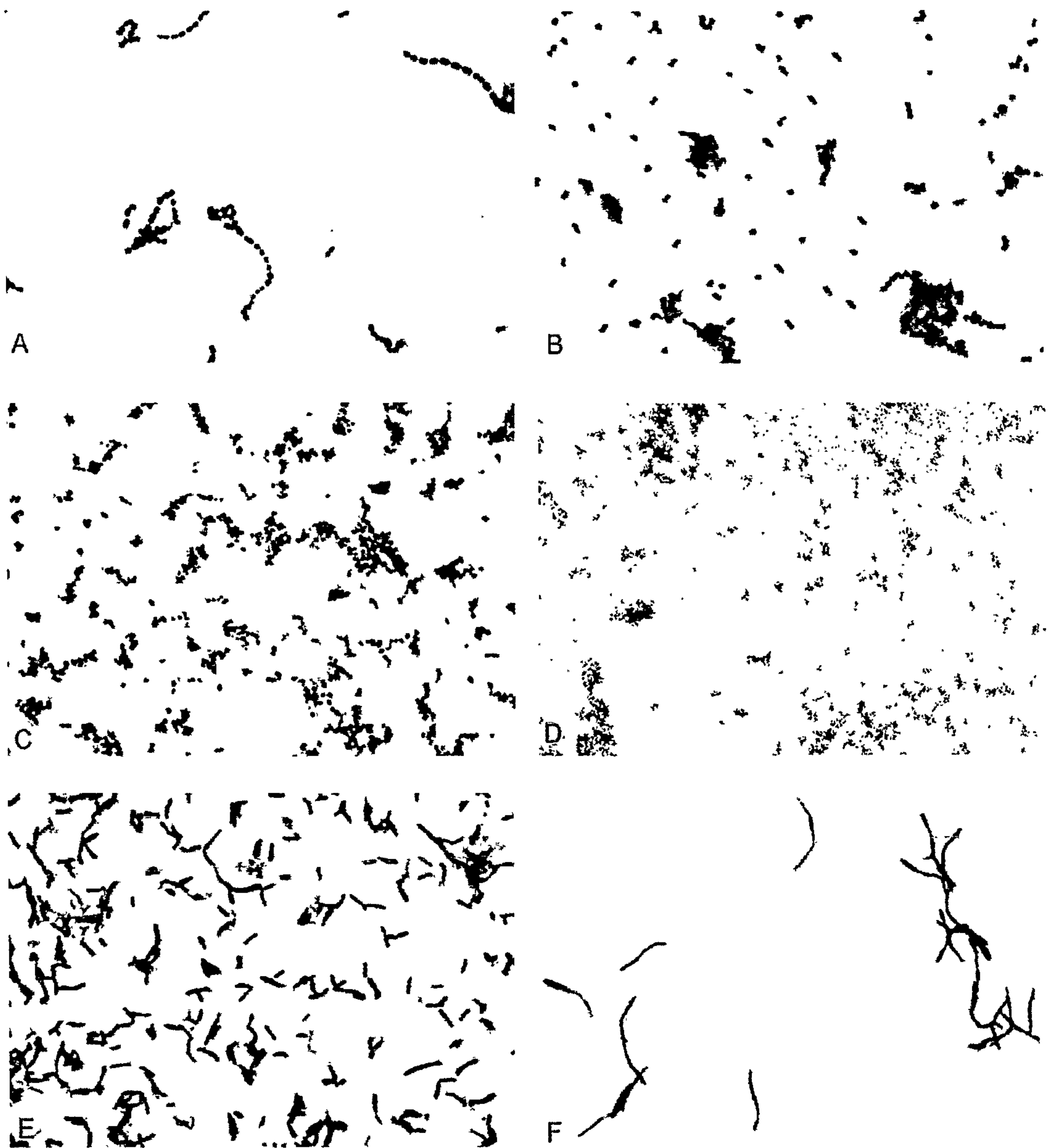


Fig. 5.3 Gram-stain exhibited morphology of *Streptococcus* and *Actinomyces* spp. by electronic microscope (40X). Reference strains with closest homology to the test organisms are shown in parentheses.

- (A) Gram-positive *Streptococcus mitis* (NCTC 12261).
- (B) Gram-positive *Streptococcus constellatus* (206).
- (C) Gram-positive *Streptococcus intermedius* (ATCC 27335).
- (D) Gram-positive *Streptococcus intermedius* (488).
- (E) Gram variable *Actinomyces georgiae* (DSM 6843).
- (F) Gram-positive *Actinomyces* sp. oral clone (BL005).

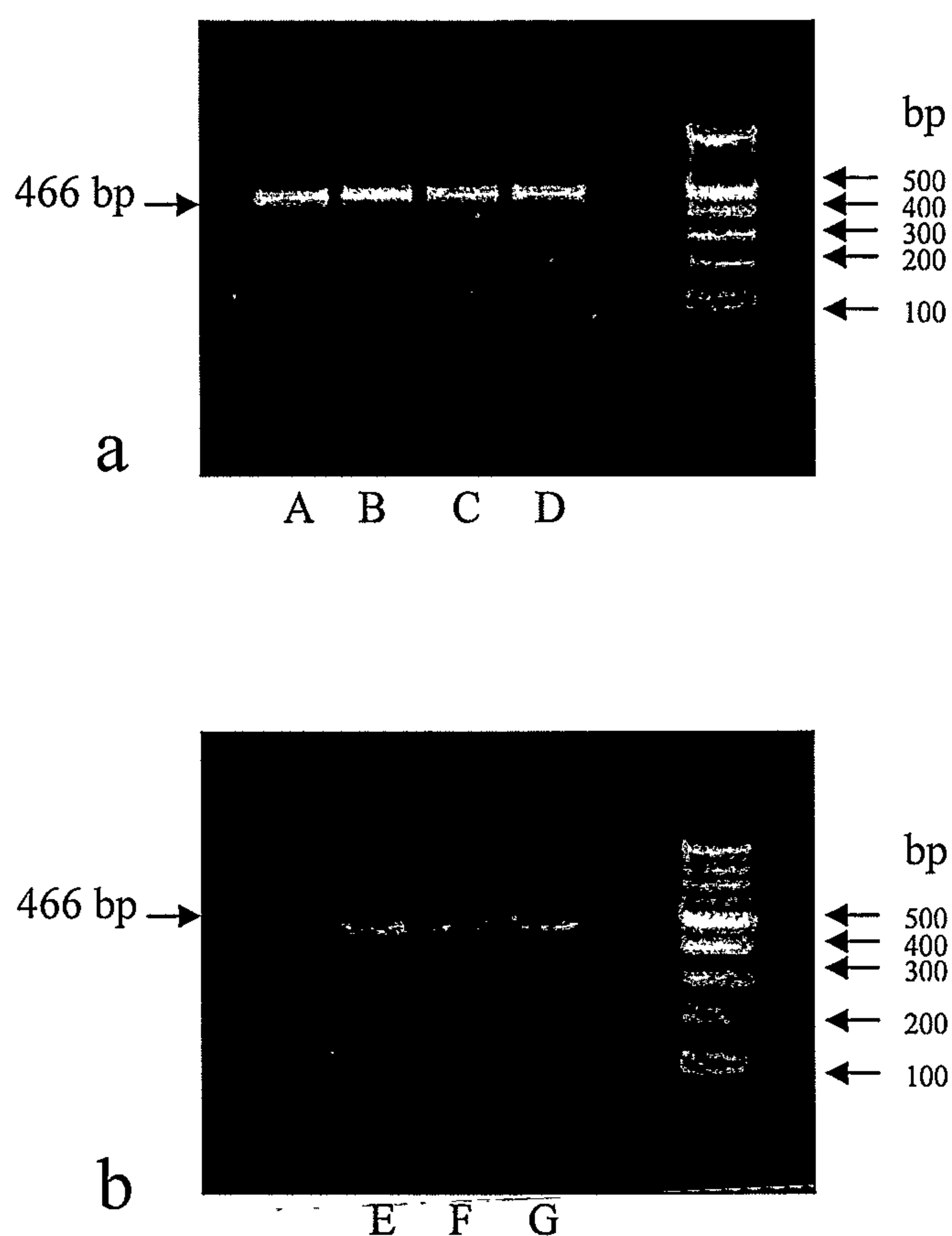


Fig. 5.4 PCR products (466 bp) from isolated purified colonies of dental plaques with cross-reactivity to epithelial antigens.

(a) Four *Streptococcus* species A-D.

(b) Three *Actinomyces* species E-F.



Fig. 5.5 Protein profiles of the isolated bacterial species with immunocross-reactivity to epithelial antigens.

The profiles of the 6 extracted bacteria were separated by SDS-PAGE (12%) and stained with Coomassie Blue. Profiles are representative of extracts from standardized (OD) bacterial suspensions grown to late exponential phase. Reference strains with closest homology are shown in parentheses.

- A: *Streptococcus mitis* (NCTC 12261),
- B: *Streptococcus constellatus* (206),
- C: *Streptococcus intermedius* (ATCC 27335),
- D: *Streptococcus intermedius* (488),
- E: *Actinomyces georgiae* (DSM 6843),
- F: *Actinomyces* sp. oral clone (BL005).

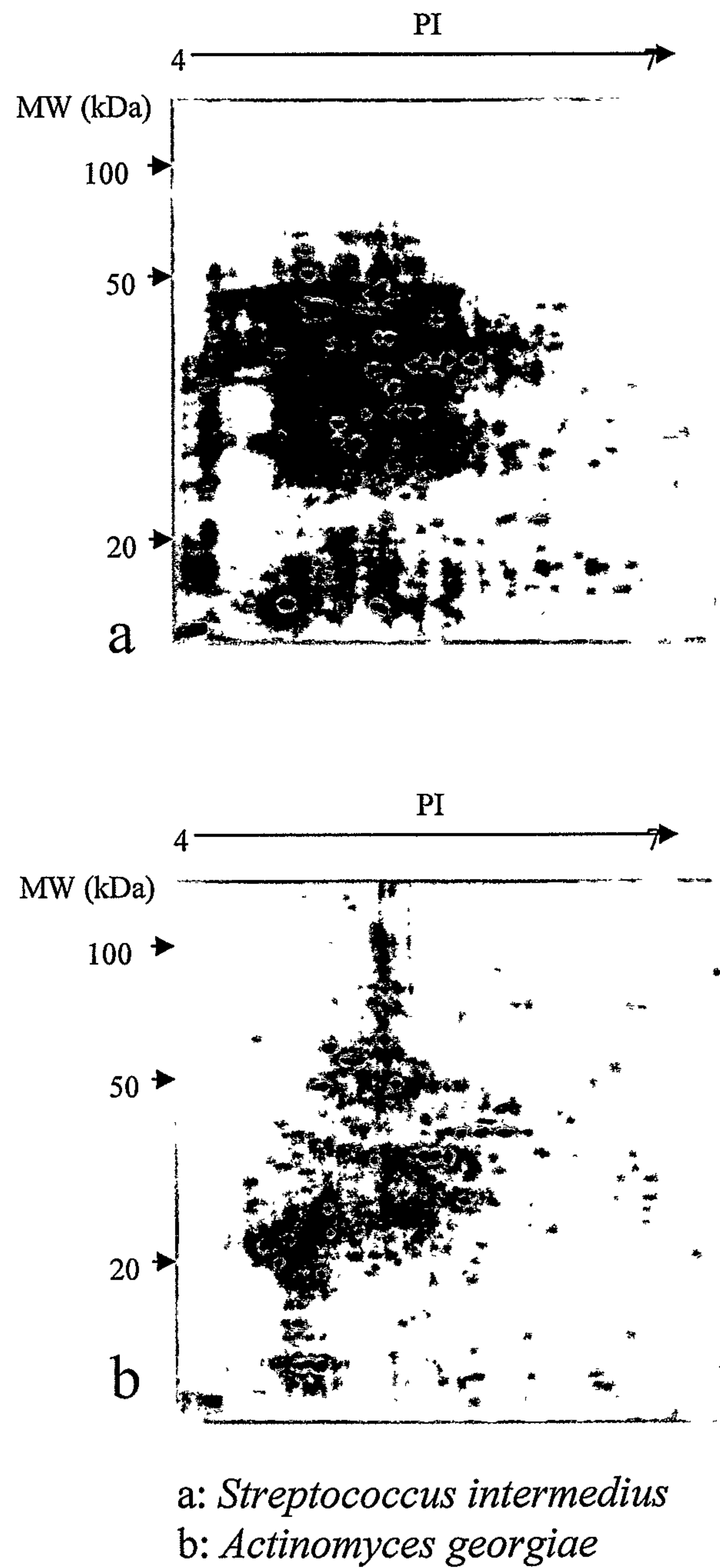


Fig. 5.6 The profile of 2-D gels showed identified bacterial species from plaques cross- reactivity to epithelial antigens.

2-D gels of protein separated on pH 4–7 linear IPG strips in the first dimension with two different samples. (a) *Streptococcus* sp. (b) *Actinomyces* sp.

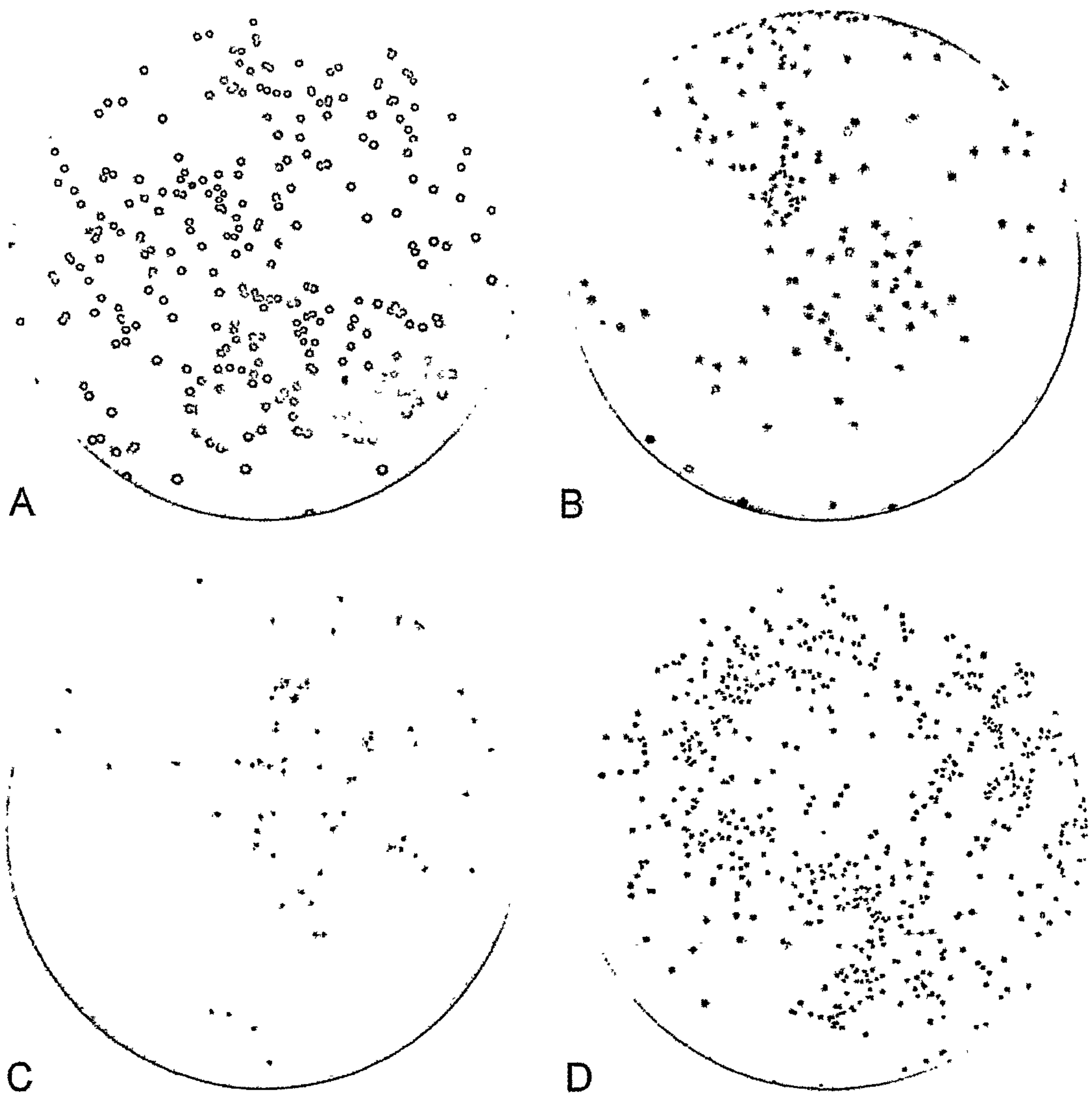
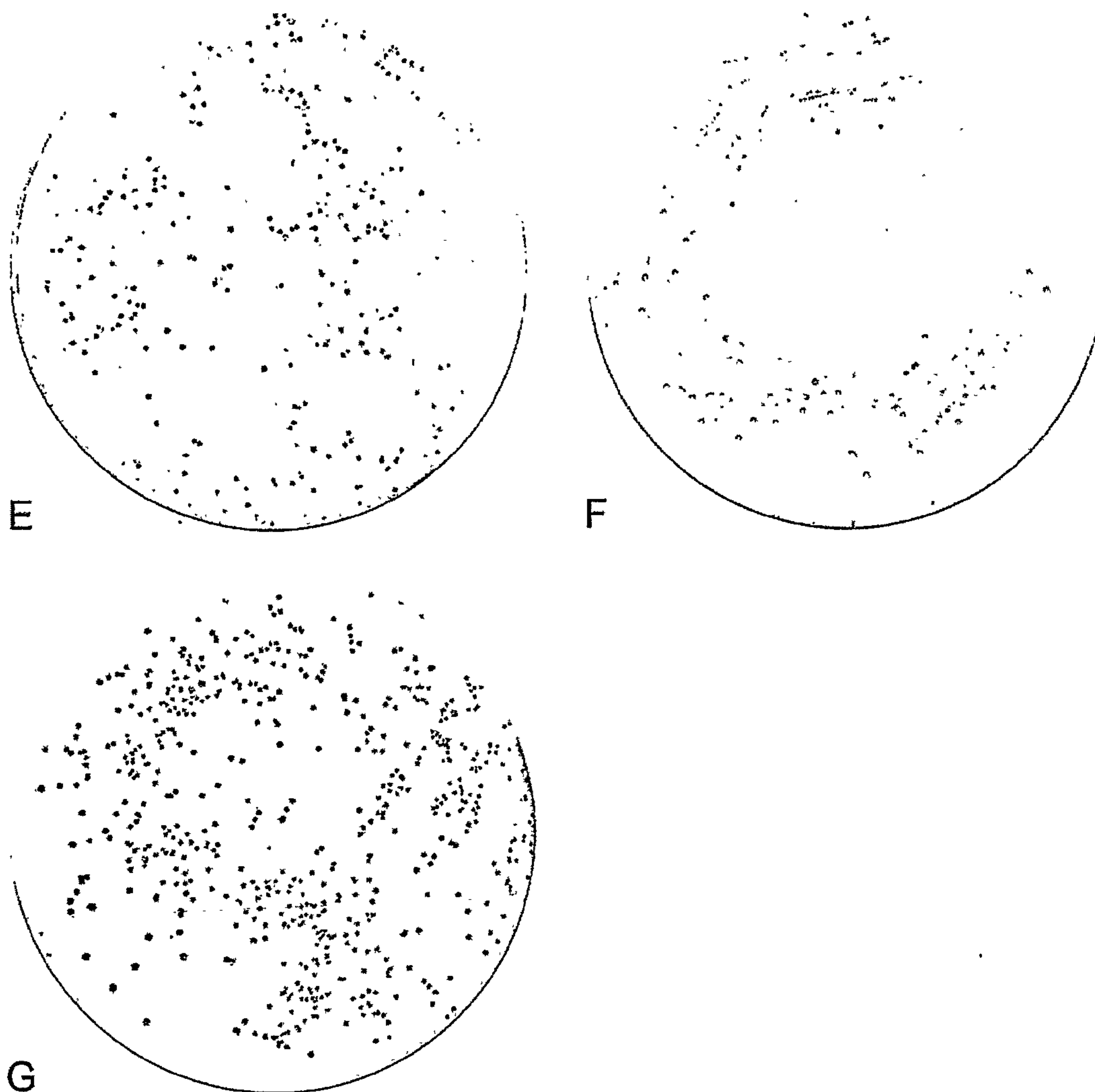


Fig. 5.7 Colony-lift techniques confirmed purity and reliable of identified bacteria with cross-immunoreactivity to epithelial antigens from dental plaque.

Purified colonies were lifted onto 2 sheets of nylon membrane from one bacterial sample respectively. These were probed with a randomly chosen serum from patient and with a matched affinity-isolated (CCL-4-specific) antibody from the same patient. Reference strains with closest homology to the test organisms are shown in parentheses.

- (A) *Streptococcus mitis* (NCTC 12261).
- (B) *Streptococcus constellatus* (206).
- (C) *Streptococcus intermedius* (ATCC 27335).
- (D) *Streptococcus intermedius* (488).



(E) *Actinomyces georgiae* (DSM 6843).

(F) *Actinomyces* sp. oral clone (BL005).

(G) *Streptococcus intermedius* (488) probed with a matched affinity-isolated (CCL-4-specific) antibody from the same patient displayed exactly the same pattern with Fig. 5. 7D. Similar results observed for other bacteria (data not shown).

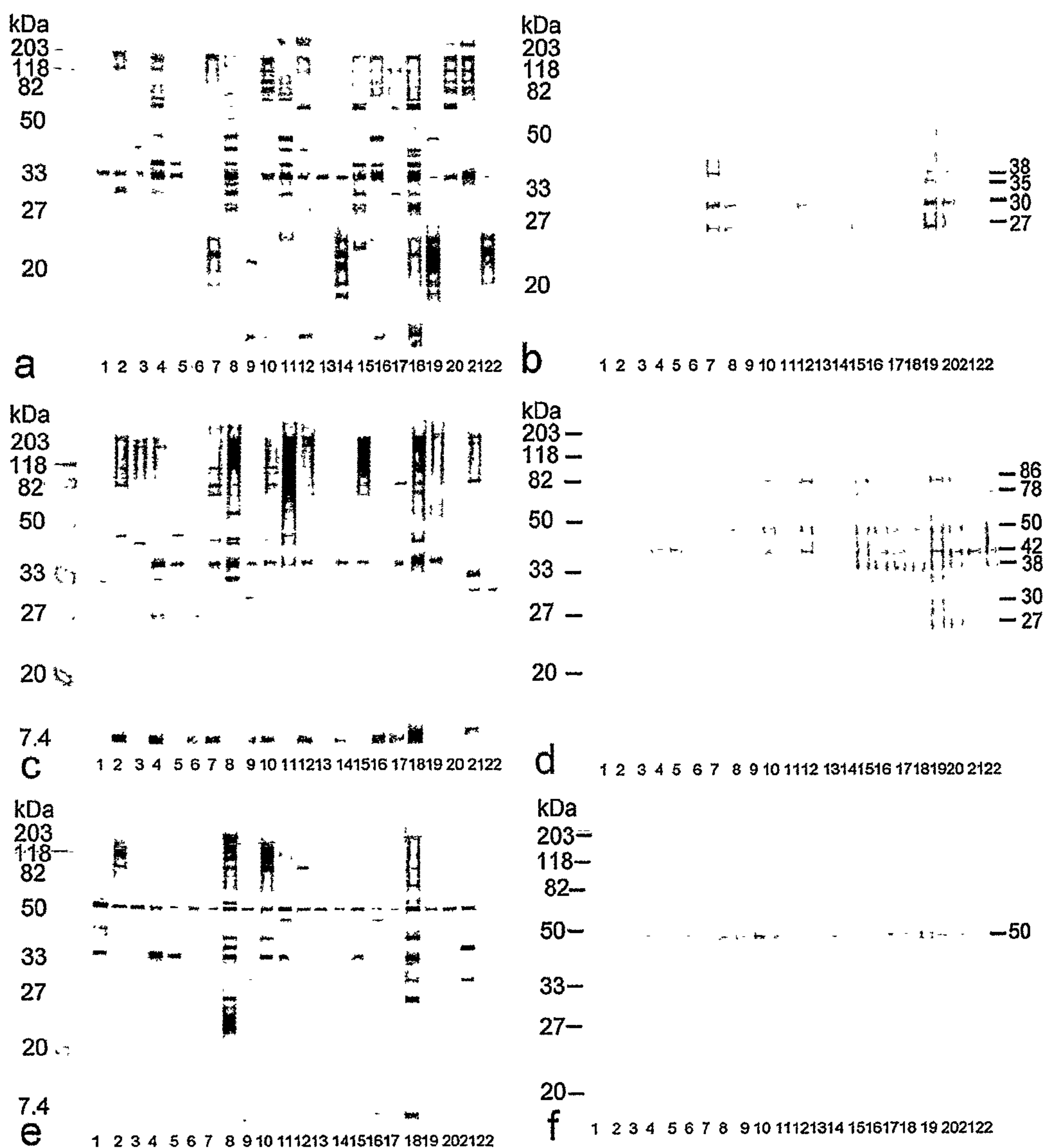


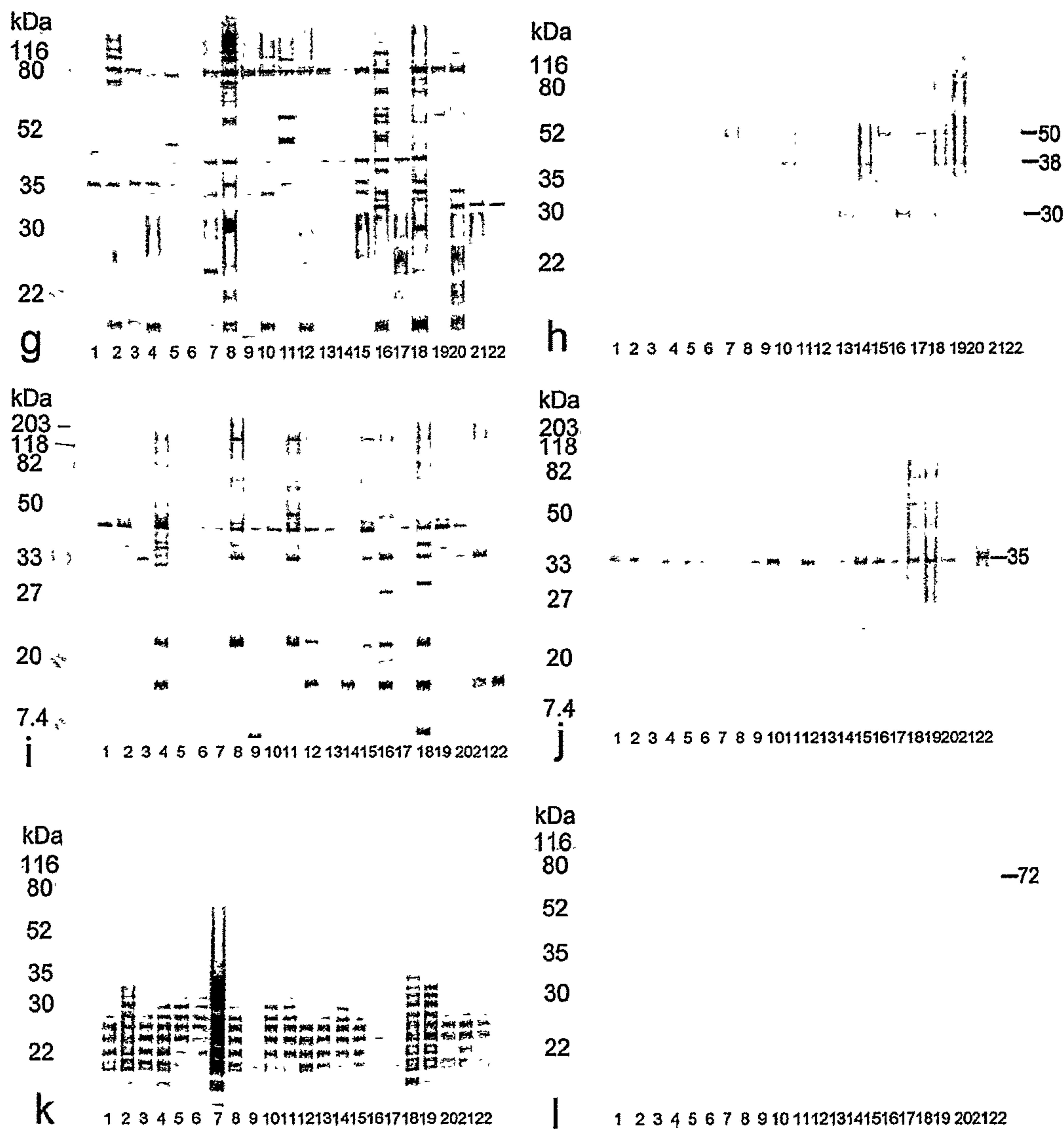
Fig. 5.8 Western immunoblots with patient sera against antigens of 6 isolated plaque bacteria.

Immunoreactive patterns of total patient serum IgG are represented in panels a, c, e, g, i, and k ($n = 22$). Immuno-cross reactive patterns of matched affinity-isolated (CCL-4-specific) IgG are represented in panels b, d, f, h, j, and l ($n = 22$). Reference strains with closest homology to the test organisms are shown in parentheses.

(a & b) *Streptococcus mitis* (NCTC 12261) cell antigens.

(c & d) *Streptococcus constellatus* (206) cell antigens.

(e & f) *Streptococcus intermedius* (ATCC 27335) cell antigens.



(g & h) *Streptococcus intermedius* (488) cell antigens.

(i & j) *Actinomyces georgiae* (DSM 6843) cell antigens.

(k & l) *Actinomyces* sp. oral clone (BL005) cell antigens.

Fig. 5.9 Molecular weight determination of six bacterial antigens.

Following gel electrophoresis and staining, the distance of migration of the pre-stained proteins as well as that of the tracking dye (bromophenol blue) was used to prepare standard curves. Distance of migration was measured from the beginning of the separating gel to the leading edge of a protein band.

$$R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

The molecular weight of the unknown protein was estimated from the graph based on its R_f value.

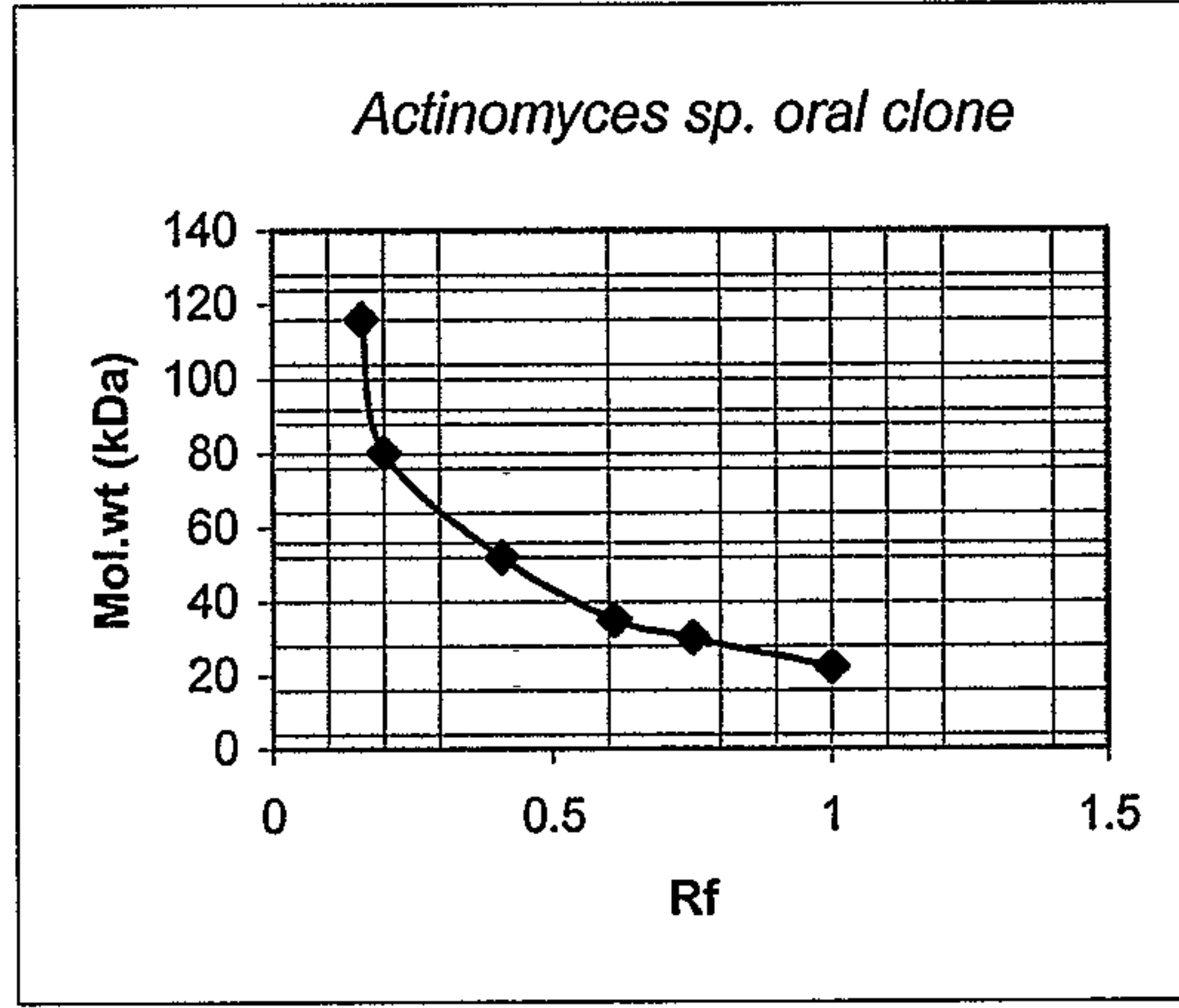
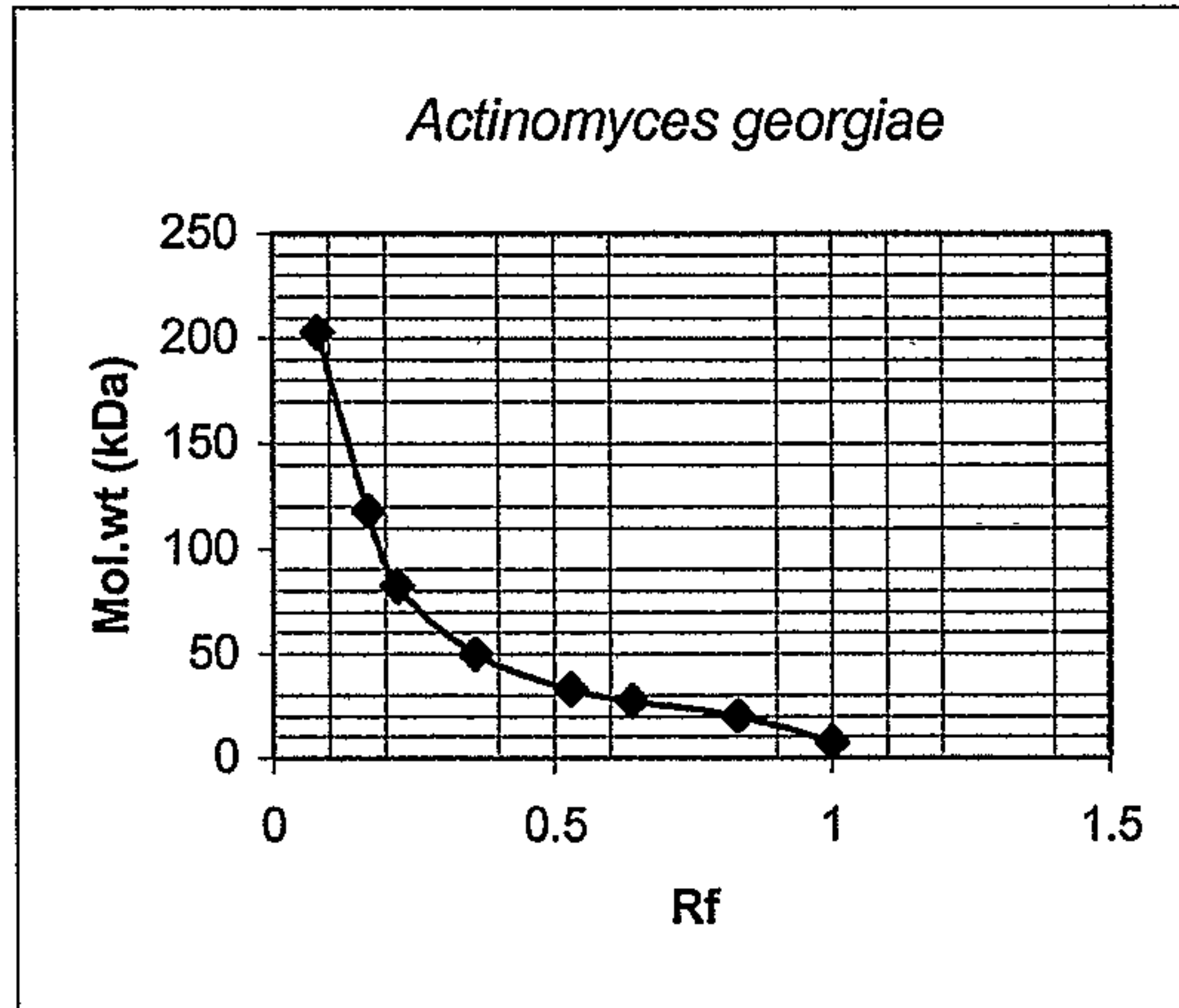
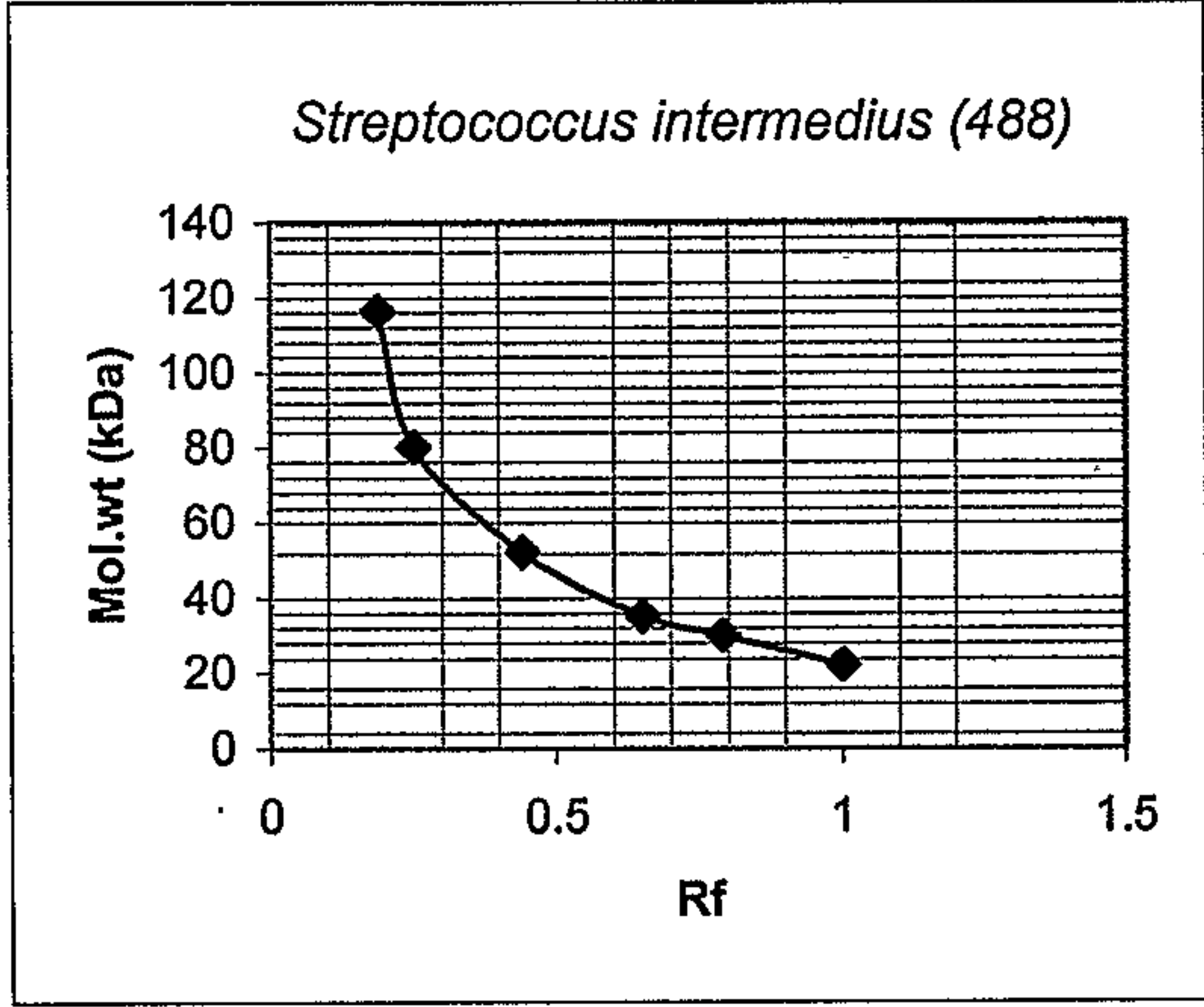
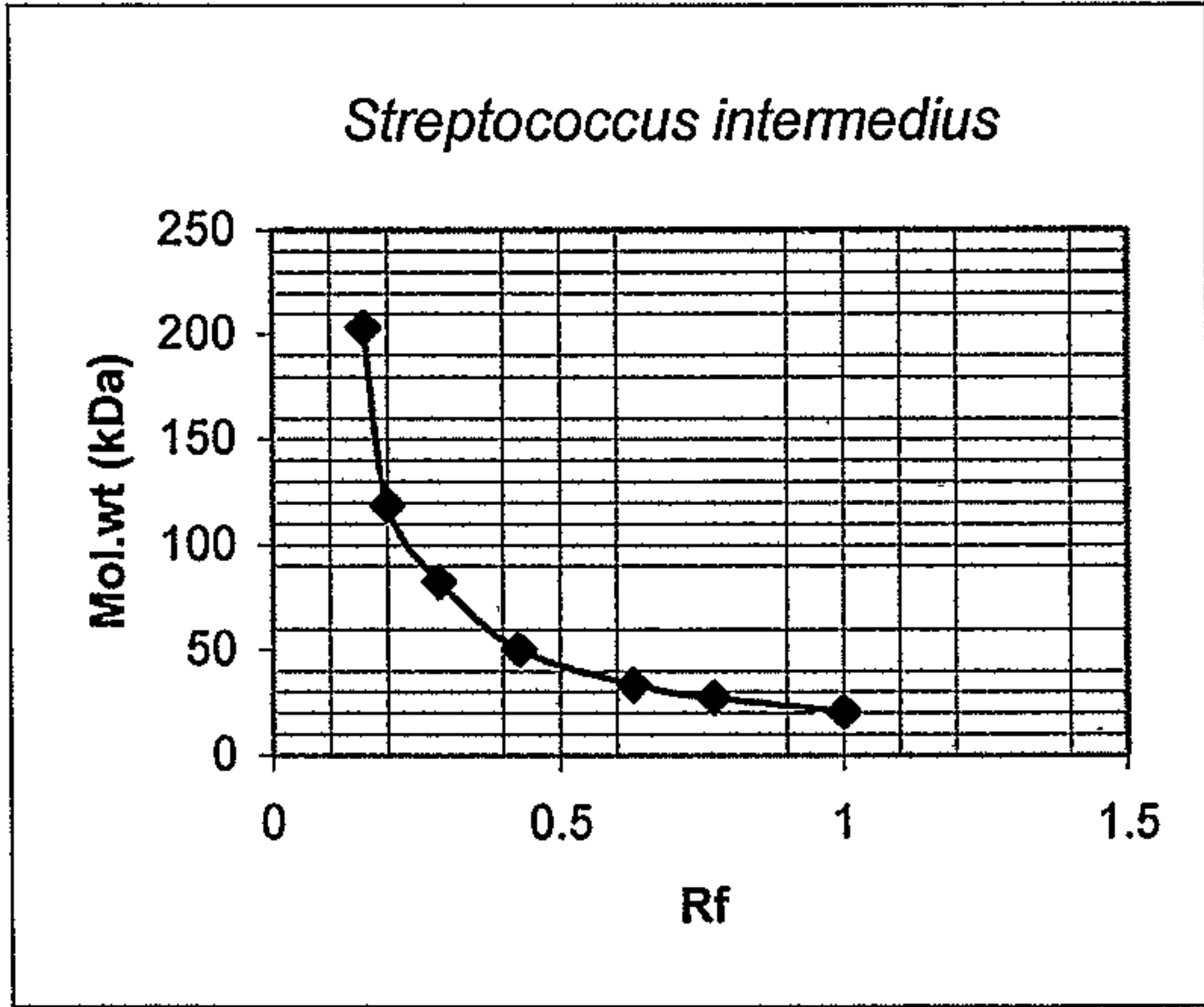
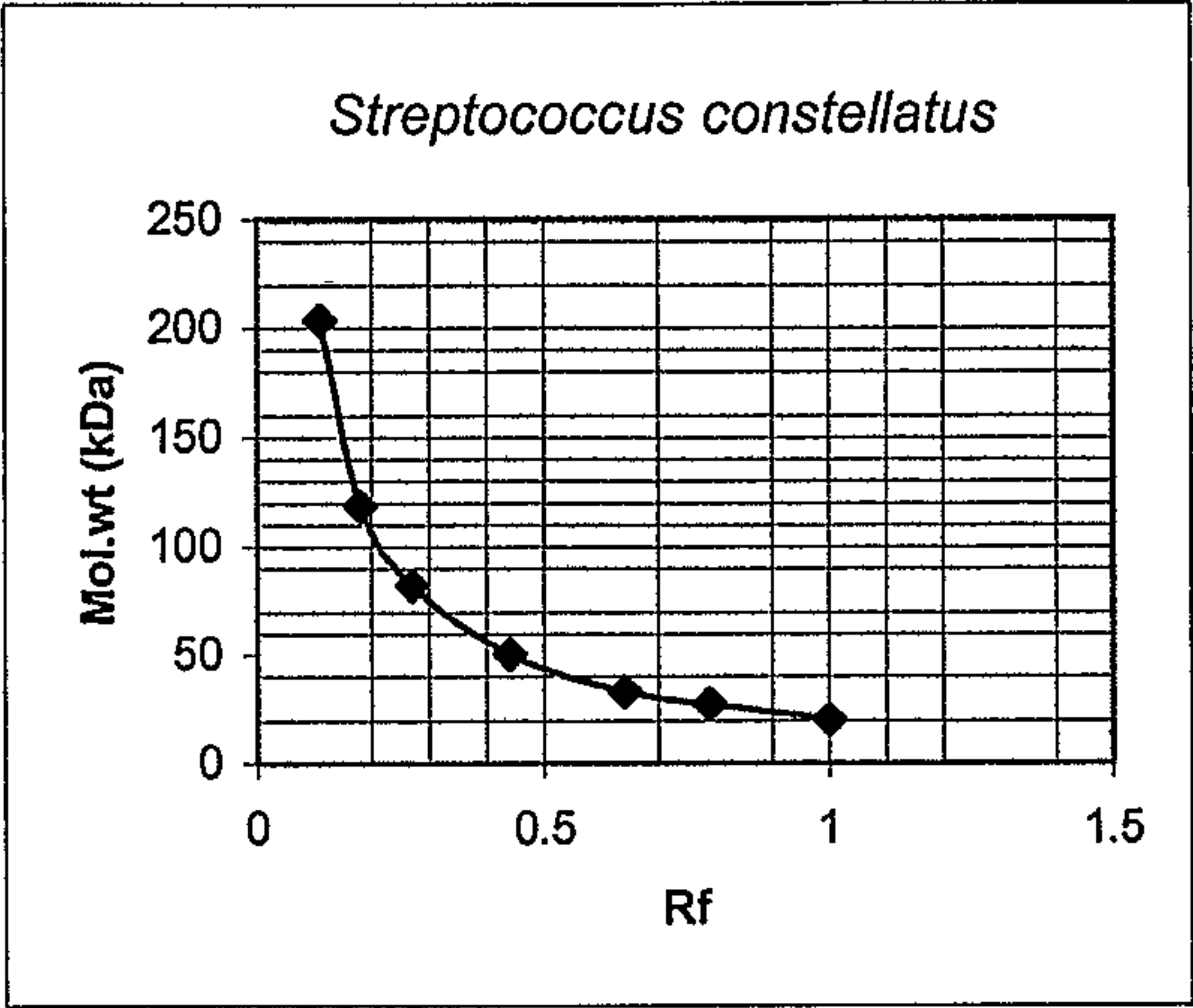
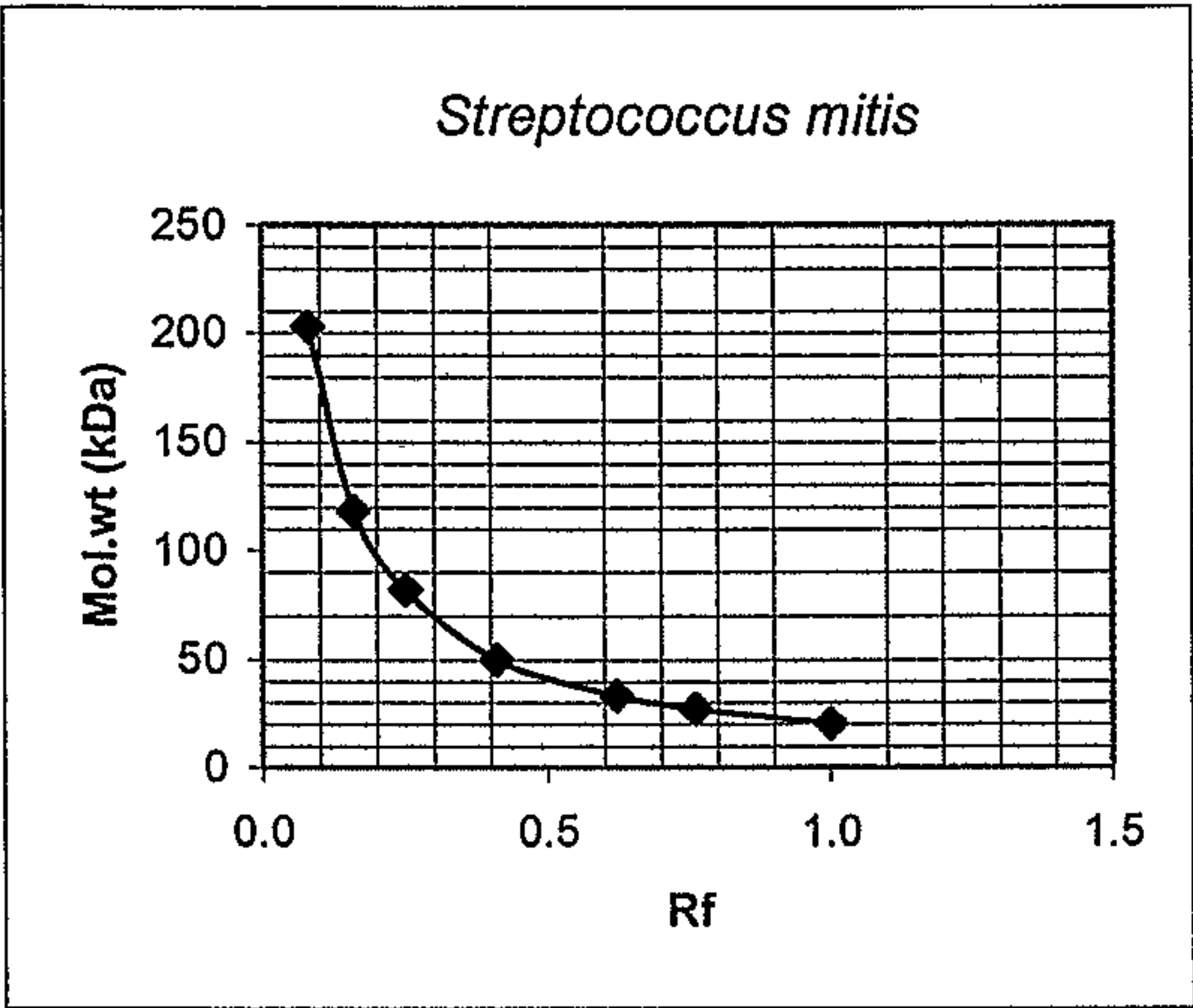
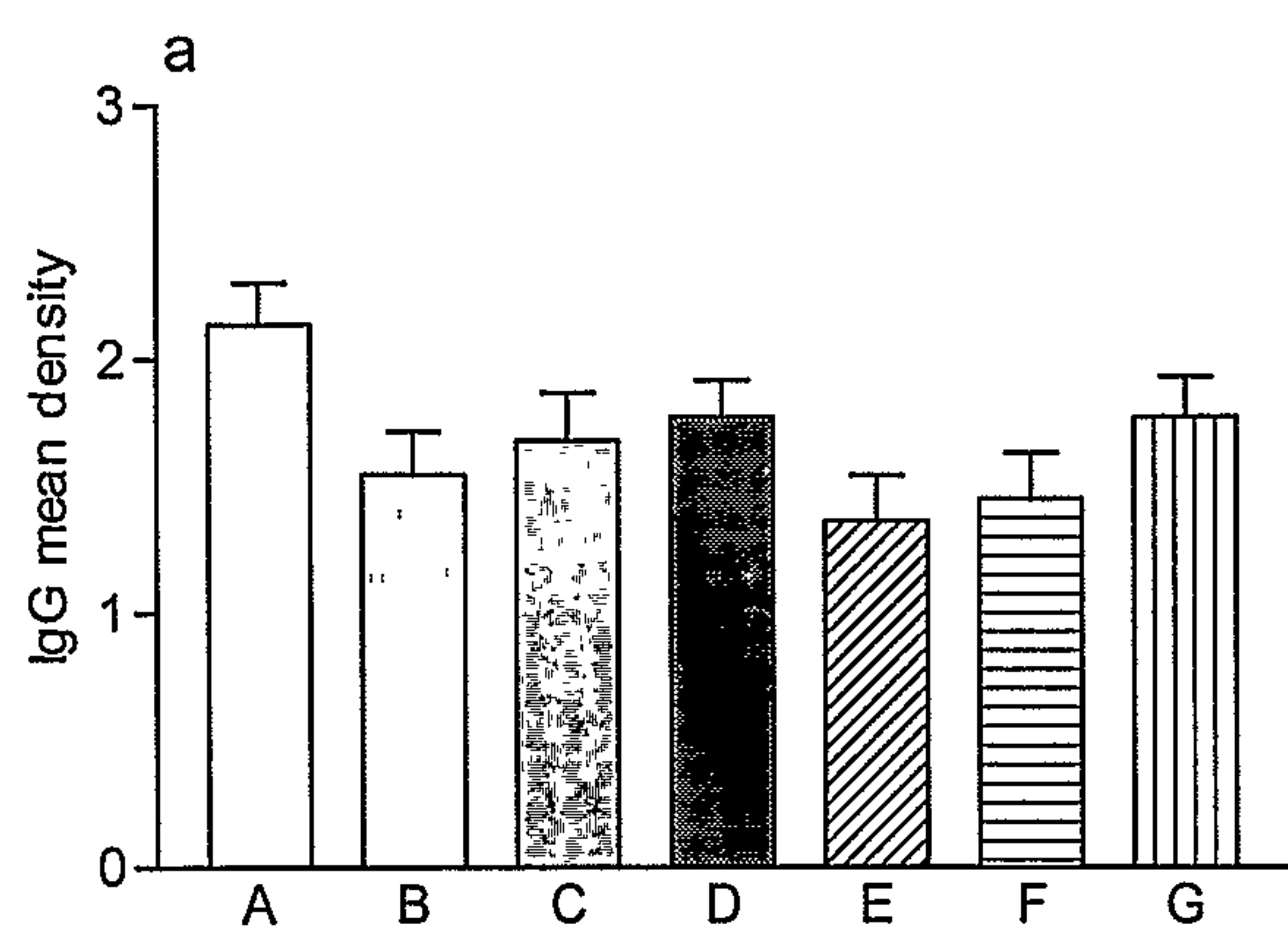
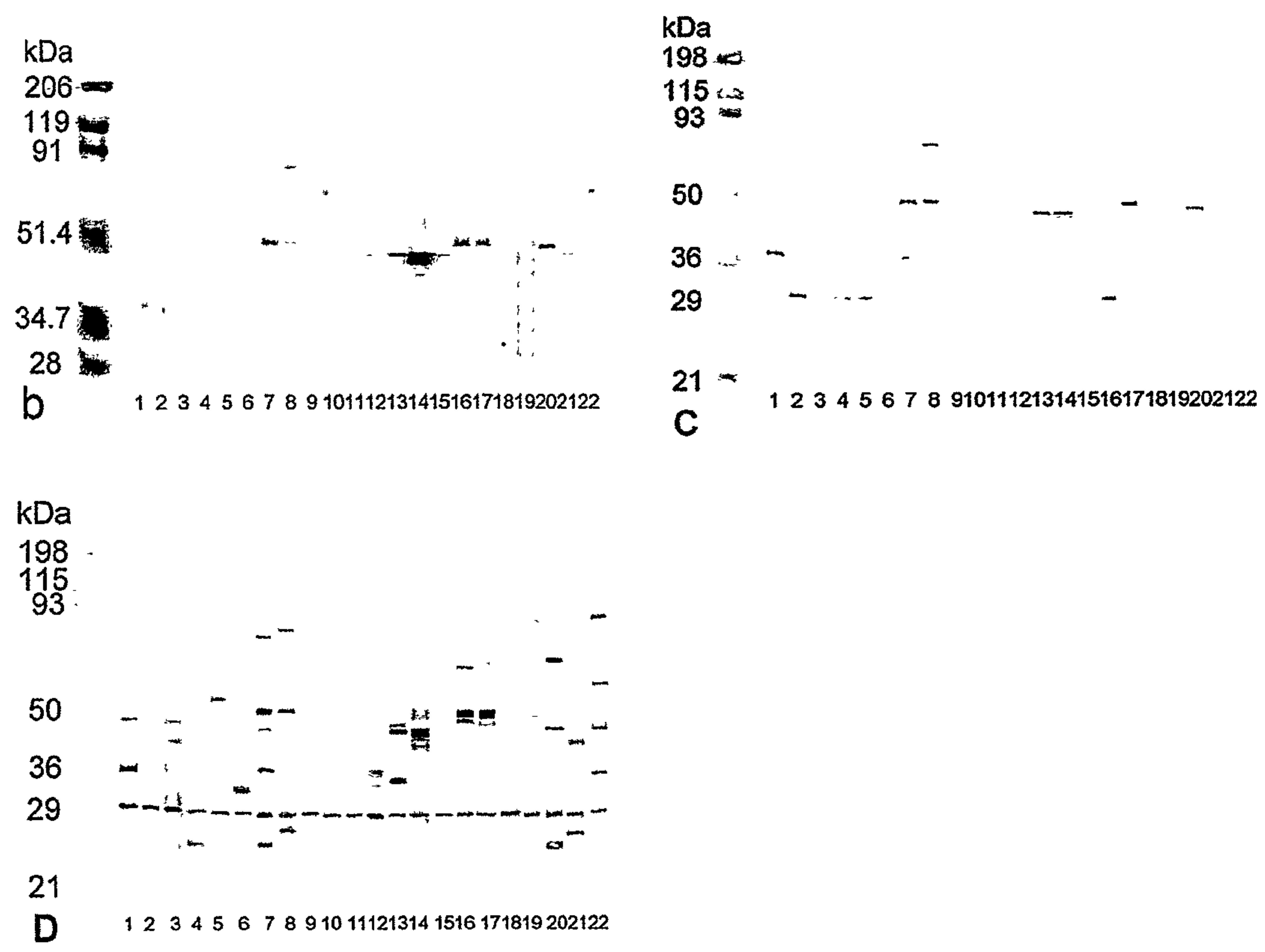


Fig. 5.10 Demonstration by immunoblot of a removal of epithelial cross-reactive antibodies. Reference strains with closest homology are shown in parentheses.

- (a) Mean density in dot blot analysis of IgG reactivity with CCL-4 antigens by patient sera ($n = 22$) non-absorbed (A) or absorbed by each identified bacterium (B–G) respectively. An overall P value < 0.05 (Kruskal-Wallis test), was considered significant among these groups.
- (b) Sera absorption ($n = 22$) with *S. intermedius* (488): showing reduced detection of CCL-4 antigens by Western analysis. For other isolated strains of *Streptococcus* and *Actinomyces* spp., similar results were observed (data not shown).
- (c) Absorption of titrations of patient sera ($n = 22$) by pooled cross-reactive bacteria was performed by Western analysis. The figure shows the result for maximum serum concentration (1: 20) with the indication of removal or reduction of cross-reactive bands compared to non-absorbed controls (data not shown). Further dilution of sera resulted in abolition of all cross-reactive bands.
- (d) Patient sera absorption ($n = 22$) with *Lactobacillus acidophilus* ATCC 4356 did not result in detectable removal of reactivity for any patient when compared with non-absorbed profiles run in parallel (data not shown). This finding was reproduced over three consecutive experiments. The apparent overall reduction of staining intensity compared with the experiments described in Fig.1 can be explained by the reduction in titre of low levels of polyclonal auto-reactive antibodies following prolonged storage (18 months) of the serum samples over the time course of this study. Identical findings were observed for *Lactobacillus casei* subsp. *ramnosus* ATCC 4356 (data not shown).



A: Non-absorbed sera
B: *Streptococcus mitis* (NCTC 12261)
C: *Streptococcus constellatus* (206)
D: *Streptococcus intermedius* (ATCC 27335)
E: *Streptococcus intermedius* (488)
F: *Actinomyces georgiae* (DSM 6843)
G: *Actinomyces* sp.oral clone (BL005)



5.5 DISCUSSION

It is possible that auto-reactive antibody production at a low level is a normal mechanism in health in response to products of damaged cells. In the environment of inflammatory disease, recognition of streptococcal and *Actinomyces* antigens by affinity-isolated (epithelial-specific) antibodies from periodontitis subjects and the capacity of these antigens to conversely absorb serum antibodies reactive with epithelial components were indicative of true immunological cross-reactivity. The almost complete removal of antibodies reactive with epithelium by the summed effect of the consortium of bacteria identified implied that it is unlikely that unrelated or uncultured organisms also contributed to this effect. Specific association with the disease state was demonstrated by the lack of reactivity in sera from periodontally healthy subjects.

The findings suggest that antibodies cross-reacting with epithelial antigens may arise by structural mimicry as a consequence of the host's immune response against subgingival plaque bacteria. In addition, cross-reactive antigens were detected at common apparent molecular weights of 27, 30, 35, 38 and 50 kDa, that were shared between two or more bacterial strains within the consortium and possibly in related organisms not identified in this study.

All patients had antibodies that recognised a 30 kDa epithelial antigen. Control subjects also showed trace recognition of this antigen. While the identity of this component is unknown at present it is of interest as a common factor. Based on the evidence that recognition of the 30 kDa epithelial antigen is probably related to cross-reactivity with the consortium of bacteria, it is possible that in turn, a common bacterial antigen is responsible.

The substantial enhancement of resolution obtained by 2-D electrophoresis of proteins extracted from representative Gram positive organisms is indicated in Fig. 5.6a and b. There is potential for greater resolving power and enhanced display by employing narrow range pH gradients in the first dimension. It was apparent that this approach could be used in Western analysis to provide near unequivocal identification of cross-reactive bacterial proteins. Maximum resolution requires large gels and unfortunately this placed too much demand on the available reserves of patient sera.

An alternative hypothesis is that recognition of epithelial antigens is a primary event in patients with periodontitis, with induced auto-antibodies demonstrating cross-reactivity for antigenic components from the consortium of bacteria. In this context,

tobacco smoking is recognised as an important risk factor for the development of periodontitis (Barbour *et al.*, 1997). One aspect could be that accumulating mutations within the oral epithelia facilitate auto-immunisation of the subject. This could be best analysed by studies of tobacco smoke effects in germ free animals, but there appears to be a lack of supportive literature. Further, analysis of the patterns of epithelial recognition (Fig. 5.1b) failed to support clear differences between smokers and non-smokers within the generalised periodontitis group.

Amongst the isolated strains, *S. intermedius* and *S. constellatus* belong to the *Streptococcus milleri* group (*S. anginosus*, *S. intermedius*, *S. constellatus*). Members of this diverse group include Lancefield groups C and G, known to express protein G (Willcox *et al.*, 1993), a 30 kDa receptor which binds to a common determinant on IgG and which could produce spurious apparent cross-reactivity (Bjorck & Kronvall, 1984). This is unlikely to contribute to the data obtained for the *S. milleri* group in this study. Boiling of extracted bacterial products in SDS sample buffer for Western analysis would be expected to inactivate protein G. Further, periodontally healthy subjects showed weak recognition of bacterial extracts and the pattern of recognition by affinity-isolated antibodies was complex with reactive bands present at a range of molecular weights.

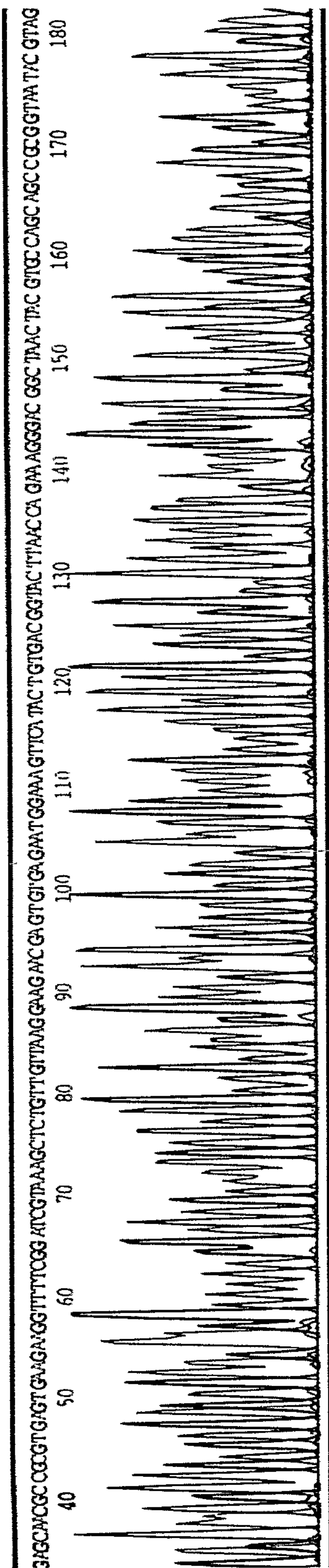
In the periodontitis lesion site, pathogenic attributes have been assigned principally to a consortium of anaerobic Gram negative bacteria, particularly *Porphyromonas gingivalis* (Socransky *et al.*, 1998). In this regard, it was of interest that subjects with periodontitis had much greater recognition of the streptococci and *Actinomyces* identified in this study than did periodontally healthy subjects. *Actinomyces* and *Streptococcus* spp., occur in human dental plaque and may contribute to various disease states including gingivitis (Ochiai *et al.*, 1993). *Streptococcus mitis* is considered among the pathogenic species of viridans streptococci, associated with an extensive range of infections including meningitis, endocarditis, urinary tract infection as well as liver, lung and epidural abscesses (Cunney *et al.*, 1993; Hirota *et al.*, 1995; Ong *et al.*, 1998; Shea *et al.*, 1995; Carrascosa *et al.*, 1994; Martin M & Lee, 1995).

Streptococcus constellatus and *S. intermedius* were strongly associated with indices of periodontal inflammation in interleukin-1 genotype(s) (IL-1A and IL-1B) positive subjects (Socransky *et al.*, 2000), suggesting the immuno-modulatory potential of these organisms. Further, higher proportions of *Streptococcus* spp., particularly *S. constellatus* and *S. intermedius* have been detected in subgingival plaques of refractory

periodontitis (Colombo *et al.*, 1998a; Magnusson & Walker, 1996), and titres of serum antibodies to these organisms were positively correlated with disease while periodontally healthy subjects had low levels of serum antibodies reactive with these organisms (Colombo *et al.*, 1998b) similar to the findings reported here. In addition, some members of the *S. milleri* group have been reported to be significant pathogens in a range of infections (Han & Kerschner, 2001; Goldstein *et al.*, 2001; Dorvilus & Edoo-Sowah, 2001; Yamamoto *et al.*, 1999).

Antibodies reactive with epithelial components and related to the consortium of captured bacteria, were a consistent feature in the periodontitis subjects studied. It remains to be determined if the cross-reacting antibodies are pathogenic in the context of human gingival epithelium.

Streptococci and *Actinomyces* are common components of dental plaque and some species appear to contain antigens cross-reactive with epithelia. Molecular mimicry by these organisms could be involved in the generation of autoreactive immune responses during the development of periodontitis lesions with implications for both the pathogenesis of this disease and also for systemic manifestations of autoimmunity.



CHAPTER 6

IDENTIFICATION OF AUTO-ANTIGENS RECOGNISED BY SERUM ANTIBODIES FROM PATIENTS WITH CHRONIC PERIODONTITIS

CHAPTER 6: IDENTIFICATION OF AUTO-ANTIGENS RECOGNISED BY SERUM ANTIBODIES FROM PATIENTS WITH CHRONIC PERIODONTITIS

6.1 ABSTRACT

Background: In studies reported in Chapter 5, a possible relationship between the bacterial flora adjacent to diseased sites and the presence of epithelial-reactive antibodies was investigated. Gram positive bacteria containing antigens potentially cross-reactive with epithelial cells were isolated. The bacteria were identified as streptococci (*S. mitis*, *S. constellatus* and two *S. intermedius* strains) and *Actinomyces* (*A. georgiae*, and *A. sp. oral clone*) by 16S rDNA sequence homology. The aim of the investigation reported in this Chapter was to identify auto-antigens from epithelial cells and to determine the relationship of these to the cross-reactive bacteria reported in Chapter 5.

Methods: Lambda cDNA expression libraries displayed on lambda phage have been successfully employed to identify partners involved in antibody-antigen, protein-protein and DNA-protein interactions and represent a novel approach to functional genomics. Pooled IgG immunoglobulins derived from 22 patients with chronic periodontitis were used to screen a Lambda cDNA expression library derived from human keratinocytes in order to identify the disease relevant auto-antigens.

Findings: Four antigens corresponding to known polypeptides (CD24, NFAT5, LDH-A and AOP2) and a product of a gene located in chromosome 18 and products of two novel genes located in chromosome 1 were identified. Among the identified auto-antigens, CD24 antigen was strongly recognized by sera from 3 patients, AOP2, NFAT5 and LDH-A antigens were recognized by 4 sera respectively. Chromosome 18 and chromosome 1 antigens were recognized by 5 and 8 sera respectively. The six different antigens were recognised by one control subject (healthy periodontium). The bacteria previously identified (Chapter 5) were demonstrated to absorb, in patterns varying between individuals, antibodies reactive with identified auto-antigens.

Conclusion: This approach enabled the identification of some of the auto-antigens recognized by serum antibodies from patients with chronic periodontitis. Further, the relationship of these antigens to apparent cross-reactive peptides in the bacteria previously studied, was indicated.

Keywords: antigen; antibody; CD24; AOP2; LDH-A; NFAT5; autoimmunity; periodontitis

6.2 INTRODUCTION

Profound perturbation of epithelial structure is a characteristic feature of the immunopathological response to bacterial antigens considered to be central in the pathogenesis of the destructive lesion of periodontitis.

cDNA expression libraries displayed on lambda phage have been successfully employed to identify genes involved in antibody-antigen, protein-protein and DNA-protein interactions and represent a novel approach to functional genomics. Accordingly, in the present study, a keratinocyte cDNA expression library was used to investigate the identity of auto-antigens.

The hypothesis to be further tested was that perturbation of epithelial structure in chronic periodontitis occurs when the subject recognises antigenic determinants that are shared between exogenous agents (e.g. bacteria) and host-cell components.

6.3 MATERIALS AND METHODS

6.3.1 Serum samples

Twenty-two adult patients (14 males, 8 females, 35–68 years, and average 48 years) with chronic periodontitis were recruited for the study. Diagnosis of the disease was based on clinical indicators which indicated from moderate to severe generalised or localised periodontitis as detailed in Chapter 4. 3.1 and Table 4.1. As controls, sera from a group of age, race, and gender-matched periodontally healthy individuals ($n = 12$, 7 males, 5 females, 27–64 years, and average 46 years) were used (Chapter 4. 3.1 and Table 4.2).

6.3.2 Library immunoscreening using sera as probes

To define antigens recognized by sera from patients with periodontal disease, a human keratinocyte cDNA library, which displays keratinocyte peptides/proteins, size range 0.6–3.0 kb, was purchased. This library (Contech Laboratories, Inc., USA) used the cloning vector by λ gt11 and a cloning site in *EcoRI* (Fig. 6.1). The library was screened with pooled IgG from the sera of 22 patients with periodontitis, according to the manufacturer's instructions. Briefly, 0.1 ml diluted library in $1 \times$ lambda dilution

buffer (1: 250,000) containing 10^4 pfu of λ gt11 phage, was combined with 0.2 ml *E. coli* (Y1090r⁻) host strain ($OD_{600} \approx 2.0$) in LB broth (containing 10 mM $MgSO_4$ and 0.2% maltose) at 37°C for 15 min; mixed with 3 ml LB soft top agarose (containing 10 mM $MgSO_4$) and poured onto 90 mm LB agar (containing 10 mM $MgSO_4$) plates; incubated at 42°C for 3.5 h to allow growth of lytic phage. IPTG-saturated nitrocellulose filters were placed over the top of the agarose and the plates were incubated at 37°C for 3.5 h until growth was near confluence. The filters were removed and rinsed in 0.05% Tween 20/TBS and blocked in 20% foetal calf serum (FCS) with 0.05% Tween 20/TBS for overnight. Plaques were incubated at room temperature for 1 hr with pooled sera previously absorbed with *E. coli* strain Y1090r⁻ lysates (see below), using a 1:100 dilution in 0.05% Tween 20/TBS, in 5 ml for each 82-mm filter. Filters were washed 3 times in 0.05% Tween 20/TBS, and incubated with secondary antibody alkaline phosphatase (AP) conjugated γ -chain specific goat anti-human IgG (Sigma) diluted 1:30,000 in 0.05% Tween 20/TBS. Reactivity was developed with AP substrate to locate positive plaques. After eight sequential rounds of experiments, 20 filters were screened by antibody probes.

6.3.3 Positive phage stock preparation

The filters were aligned with the plates to select each positive plaque. These were transferred to microcentrifuge tubes containing 200 μ l of 1 $\times$ Lambda dilution buffer. A drop of chloroform was added and the tube vortexed briefly. Phage was eluted at 4°C overnight and centrifuged at 10,000 rpm ($8,000 \times g$) for 2 min to remove debris. Phage stock was prepared according to the manufacturer's instructions; briefly, titred positive plaques were plated at 10^4 pfu on LB agarose containing 10 mM $MgSO_4$. Following incubation at 37°C for 5-7 hr, 5 ml of 1 $\times$ lambda dilution buffer was added, and the plates incubated at 4°C overnight. A few drops of chloroform were added to the plate and swirled briefly. The liquid containing phage lysate from the plate was poured into a sterile 50-ml polypropylene tube. Two ml of chloroform was added to the tube, which was then vortexed for 2 min, and centrifuged at 8,500 rpm ($10,000 \times g$) for 10 min. Supernatants were stored at 4°C for up to three months.

6.3.4 Isolation of DNA from positive plaques

To the collection of positive plaque supernatants, DNase I to 1 μ g/ml and RNase A to 5 μ g/ml were added and incubated at room temperature for 30 min. Chloroform was

added to a final concentration of 5%, and vortexed for 30 sec, followed by centrifugation at 8,500 rpm ($10,000 \times g$) for 10 min at 4°C to pellet the debris. The aqueous phage was transferred to new centrifuge tubes and mixed with an equal volume of 20% PEG/2.0 M NaCl, incubated on ice for at least 1 hr or overnight and centrifuged at 8,500 rpm ($10,000 \times g$) for 15 min at 4°C. The supernatant was discarded and the isolated phage DNA identified as a grayish smear on the side of each tube.

6.3.5 DNA extraction

The phage pellet was resuspended in 500 µl of 1× lambda dilution buffer. To the phage DNA, EDTA to 20mM, SDS to 0.5%, and proteinase K to 50 µg/ml final concentration were added. Following incubation at 65°C for 1 hr, an equal volume of phenol:chloroform was added, and mixed by gentle inversion for 10 min. The mixture was then centrifuged at 7,000 rpm ($7,200 \times g$) for 10 min at room temperature. The supernatant was collected and the procedure repeated until the interface was clean (usually one extraction was sufficient). The above steps were repeated with chloroform only to remove any residual phenol (the small amount of aqueous material left at the interface was collected separately in 1.5-ml microcentrifuge tubes, centrifuged, and combined with the larger fraction). 1/10 volume of 3 M NaOAc and 2.5 volumes of 95% ethanol were added and the preparation held at -20°C for at least 1 hr or overnight to allow DNA precipitation. The DNA pellet was collected by centrifugation at 15,000 rpm ($20,000 \times g$) for 15 min and washed with 70% ethanol for 5 min at room temperature. The pellet was allowed to dry until the edges turned clear; it was then resuspended in 50 µl of TE buffer and stored at 4°C or visualised on a 0.7% agarose gel in TAE buffer with ethidium bromide after electrophoresis at 100 V for 40 min.

6.3.6 Insert size determination by PCR

5µl of λ phage samples or 1µl of DNA extraction from λ phage was utilised for PCR reactions with primers (Fig. 6.2) 5'-AGGCACATGGCTGAATATCGA-3' and primer 5'-CCAGACCAACTGGTAATGGTAGC-3'. The PCR conditions were: 95°C for 10 min for one cycle, followed by 40 cycles of 95°C for 15 sec and 60°C for 5 min using a Corbett Research FTS-320 Thermal Cycler (Australia). PCR products (0.6–1.3 kb) were purified by Wizard PCR preps DNA Purification System (Promega Corporation, Annandale, Australia), and visualised on a 0.7% agarose gel in TAE buffer with ethidium bromide after electrophoresis at 100 V for 40 min.

6.3.7 DNA sequencing

DNA sequencing reactions were performed using the Applied Biosystems 'Big-Dye' Terminator (vers. 3) chemistry (Westmead Hospital). The results were analysed with BLAST (Standard nucleotide-nucleotide BLAST search tool program) via the National Centre for Biotechnology Information (NCBI) BLAST network service.

6.3.8 Recognition of identified antigens for individuals by dot blot

Preparation of *E. coli* lysates for absorption of anti-*E. coli* sera. *E. coli* strain Y1090r⁻ was grown to stationary phase ($OD_{600} = 1.4$) in 100 ml aliquots in LB medium. Cells were harvested by centrifugation at 5000 rpm ($4000 \times g$) for 10 min at 4°C and washed 3 times with PBS. Cells were resuspended in 3 ml of 50 mM borate buffer (pH 8.0); the suspension was frozen and thawed 5 times, and sonicated at full power for 6 periods of 20 seconds each on ice by Branson Sonifier 450 (Branson Ultrasonics, USA). The extract was clarified by centrifugation at 13,000 rpm ($15,000 \times g$) for 10 min at 4°C. The supernatant (containing protein 8 mg/ml) was transferred to a clean tube and stored at -20°C up to two months. *E. coli* proteins were bound to NHS-activated Sepharose 4 Fast Flow (Pharmacia) according to the manufacturer's instructions. Briefly, 4 ml of a 50% (v/v) slurry was washed on a sintered glass filter with 100 ml of 1 mM HCl. The gel was suspended with an equal volume (2 ml) of 50 mM borate buffer (pH 8.0), and 1 ml *E. coli* extract (8 mg protein) added. The mixture was incubated by end-over-end rotation overnight at 4°C. Non-reacted groups on the matrix were blocked by Tris buffer (pH 8-9) for 4 hours at 4°C using end-over-end rotation. This was followed by washing with Tris buffer (pH 8-9) and acetate buffer (pH 3-4) alternatively for 3 times. The bound matrix was resuspended in an equal volume of 0.05% Tween 20 in TBS and aliquoted into 36 vials. To deplete *E. coli* reactive antibodies, patient ($n = 22$), control subject serum ($n = 12$) and one reference control and one blank were added to final concentrations of 1:50 dilution. The sera were incubated with rotation overnight at 4 °C. The absorbed antibodies were ready for use and were stored at 4 °C in the presence of 0.05% sodium azide until used for immunodetection.

For immunodot blots, positive plaque stock antigens diluted in TBS to a concentration of 1 µg/spot, were applied to pre-wetted nitrocellulose membranes (0.45µm, 9 × 12 cm size, Bio-Rad) and filtered in a vacuum microfiltration apparatus (96-well format, Bio-Rad). Blotted antigens were blocked with 1% BSA/TBS. All membranes were immunodetected by pre-absorbed patient sera depleted of anti-*E. coli* (Y1090r⁻) reactivity ($n = 22$), and clinically healthy subject similarly depleted ($n = 12$)

respectively. The secondary antibody employed was alkaline phosphatase (AP) conjugated γ -chain specific goat anti-human IgG (Sigma) diluted 1:30,000 in 0.05% Tween 20/TBS. IgG antibody binding antigen was visualized with AP substrate (cat 170-6432, Bio-Rad). Triplicate blots were performed for each experiment.

6.3.9 Capacity of cross-reacting bacteria to absorb reactivity for auto-antigens

Sera identified as recognizing recombinant antigens were absorbed individually to each of streptococci (*S. mitis*, *S. constellatus* and two *S. intermedius* strains) and *Actinomyces* (*A. georgiae*, and *A. sp. oral clone*) bacteria (Ye *et al.*, 2003). Briefly, bacterial cells were harvested at late exponential phase, washed in PBS by centrifugation, resuspended in 0.05% Tween 20/TBS to approximately 10^8 bacteria per ml (OD at 600 nm =1.0) and then aliquoted. The suspensions of bacteria with dilutions of serum were mixed by rotation overnight at 4°C. The cells were removed by centrifugation at 13,000 rpm ($15,000 \times g$) for 5 minutes, and the supernatants used for immunoblots as described above.

6.4 RESULTS

6.4.1 Immunoscreening for positive plaques

Fig. 6.3a shows immunoscreening using pooled patient sera as probes for selecting positive plaques. Fourteen positive isolates were picked from 20 filters, and were checked by dot blots using pooled sera again as probes (Fig. 6.3b).

6.4.2 DNA extraction

The phage DNA from positive plaques were extracted and electrophoresed on agarose gel; the DNA should form a sharp band with some contaminating RNA (Fig. 6.4). Spectrophotometer readings indicated the $A_{260/280}$ ratio to be > 1.5 .

6.4.3 Insert size determination by PCR and DNA sequencing analysis

Fig. 6.5a shows insert size (from 0.3 to 1.0 kb) determination of PCR products; Fig. 6.5b depicts purification of the insert. Eleven selections were sequenced and amongst these (Fig. 6.5c), 6 positive clones were found to encode protein fragments corresponding to polypeptides expressed by the library. After searching the GenBank database four antigens (CD24, NFAT5, LDH-A and AOP2) and one gene in

chromosome 18 and two novel genes in chromosome 1 were identified (Table 6.1 and Appendix C).

6.4.4 Recognition of identified antigens by individual sera

After immunodot blot analysis, among the identified auto-antigens, CD24 antigen was strongly recognized by sera from 3 patients, AOP2, NFAT5 and LDH-A antigens were recognized by 4 sera respectively. Chromosome 18 and chromosome 1 antigens were recognized by 5 and 8 sera respectively. The six different antigens were recognised by one control subject. The results were summarised in Table 6.1.

6.4.5 Identified antigens contain shared structure/ epitopes cross-reactive with oral bacteria

Data summarised in Table 6.2 indicate that the capacity of particular bacterial strains to absorb serum reactivity to defined epithelial antigens provided the opportunity to screen Western blot reactivity patterns for common antigens (Chapter 5). Examination did not, however, clearly indicate identity for different patients. This could be explained either by the lack of resolution of single dimensional electrophoresis or by the expression of epitopes cross-reactive with an epithelial protein in more than one bacterial protein.

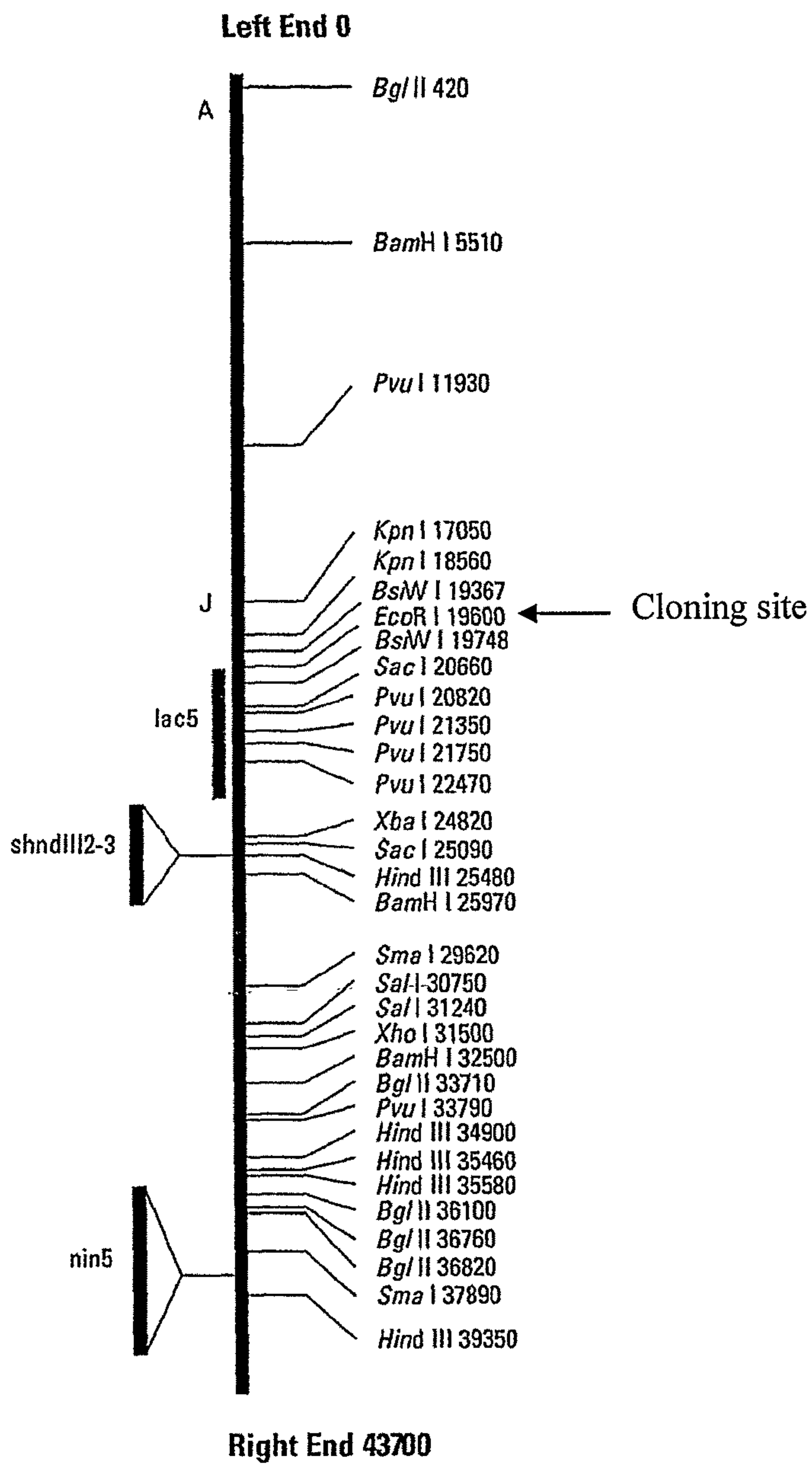


Fig. 6.1 Vector map of λ gt11.

841 GTCTTCCCGA GCGAAAACGG TCTGCGCTGC GGGACGCGCG AATTGAATTA TGGCCACAC
CAGAAGGGCT CGCTTTTGCC AGACGCGACG CCCTGCGCGC TTAACCTAAT ACCGGGTGTG

901 *BstX I* *RleA* *AluNI*
CAGTGGCGCG GCGACTTCCA GTTCAACATC AGCCGCTACA GTCAACAGCA ACTGATGGAA
GTCACCGCGC CGCTGAAGGT CAAGTTGTAG TCGGCGATGT CAGTTGTCGT TGACTACCTT

961 *Bsg I* *5' insert*
ACCAGCCATC GCCATCTGCT GCACGCGGAA GAAGGCACAT GGCTGAATAT CGACGGTTTC
TGGTCGGTAG CGGTAGACGA CGTGCGCCTT CTTCCGTGTA CCGACTTATA GCTGCCAAAG

1021 *Nde I* *Xcm I* *Gsu I* *EcoR I* *Esp I*
CATATGGGGA TTGGTGGCGA CGACTCCTGG AGCCCGTCAG TATCGGCGGA ATTCAGCTG
GTATACCCCT AACCACCGCT GCTGAGGACC TCGGGCAGTC ATAGCCGCCT TAAGGTCGAC

1081
AGCGCCGGTC GCTACCATTA CCAGTTGGTC TGGTGTCAAA AATAATAATA ACCGGGCAGG
TCGCGGCCAG CGATGGTAAT GGTCAACCAG ACCACAGTTT TTATTATTAT TGGCCCGTCC

1141 *3' insert*
CCATGTCTGC CCGTATTTTCG CGTAAGGAAA TCCATTGTAC TGCCGGACCA CCGACTGTGA
GGTACAGACG GGCATAAAGC GCATTCCTTT AGGTAACATG ACGGCCTGGT GGCTGACACT

1201 *Xcm I*
Bgl I *Nco I* *BsNI* *BspMI* *Age I* *Rsr II*
GCCACTCCGG CCATGGCGTA CGCACTGACC TGCTTACTGA TTTGTAAAAC CGGTCCGGCC
CGGTGAGGCC GGTACCGCAT GCGTGACTGG ACGAATGACT AACATTTTG GCCAGGCCGG

Fig. 6.2 λ gt11: Nucleic acid sequence around *BstX I*–*RsrII* region.

Green square: *EcoR I* site is at position 1070.
Red square: insert position.

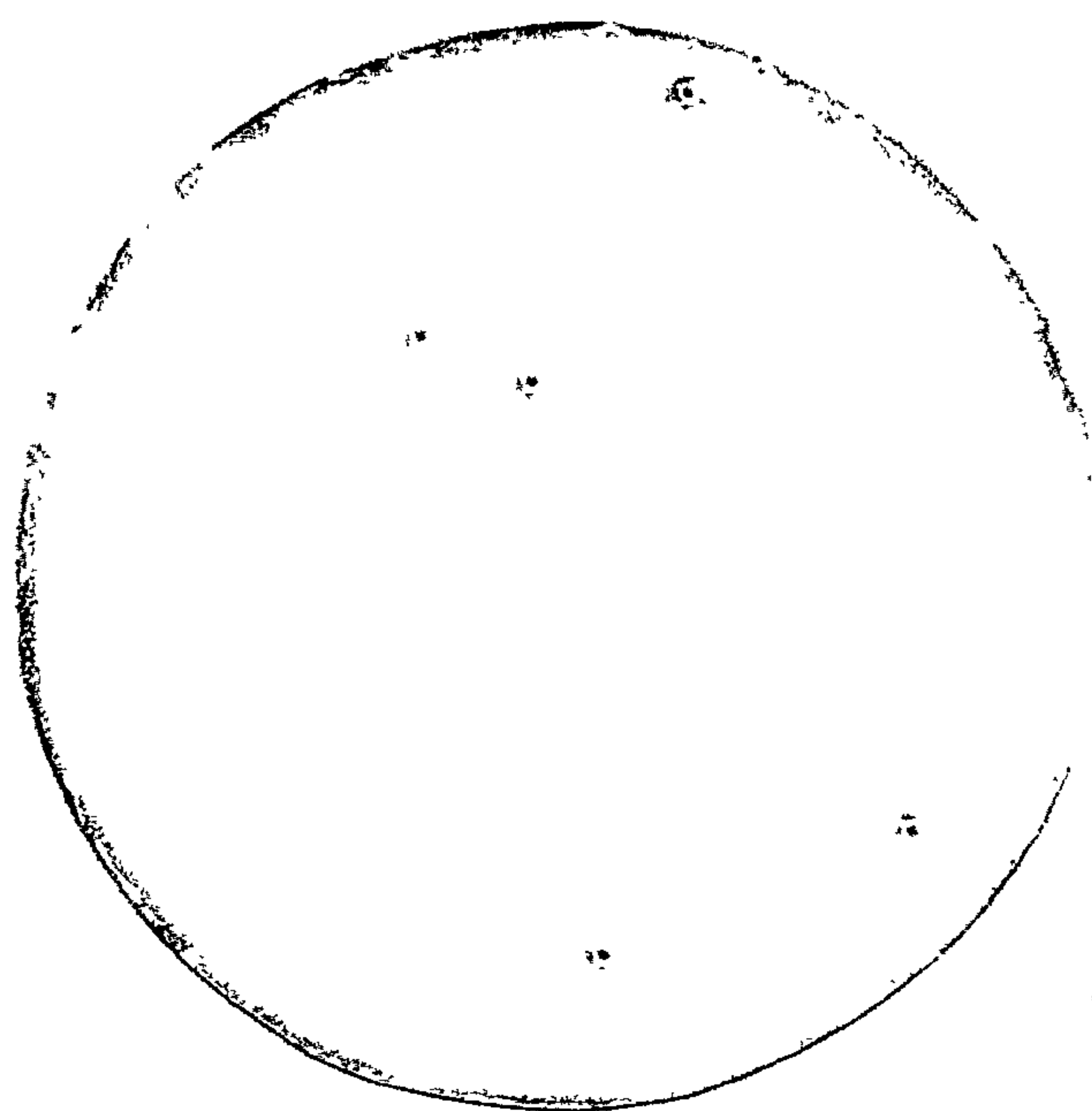


Fig. 6.3a Positive plaques screened by pooled patient sera.

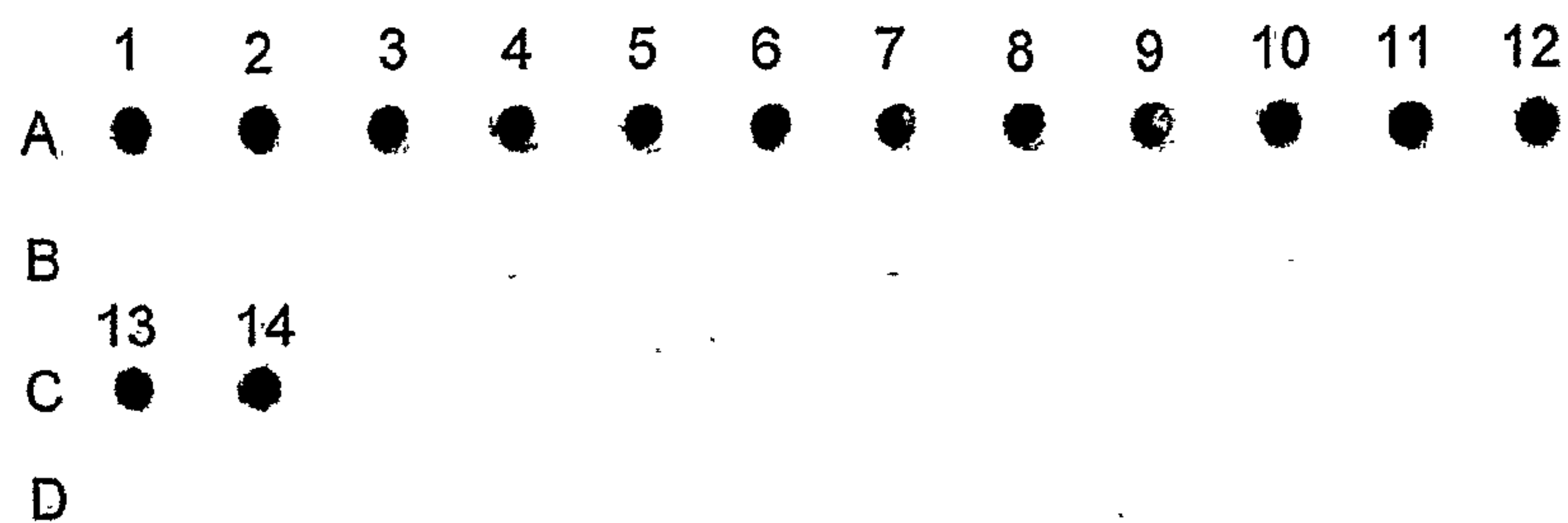


Fig. 6.3 b Selected positive plaques were tested by dot blot using pooled sera.

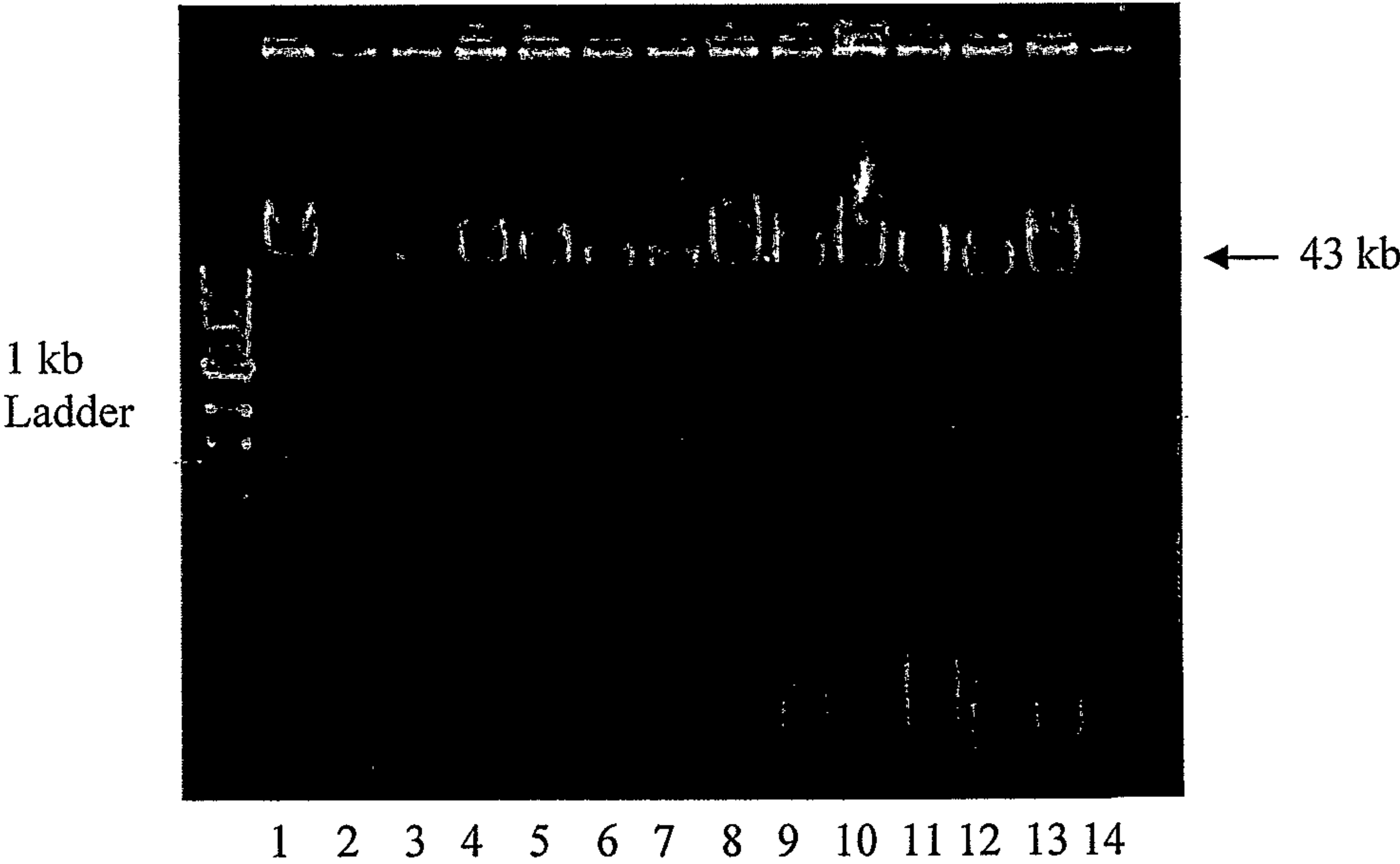


Fig. 6.4 The DNA extraction (around 43 kb) was shown on an agarose gel with some contaminating RNA from 14 plaque samples before insert excision.

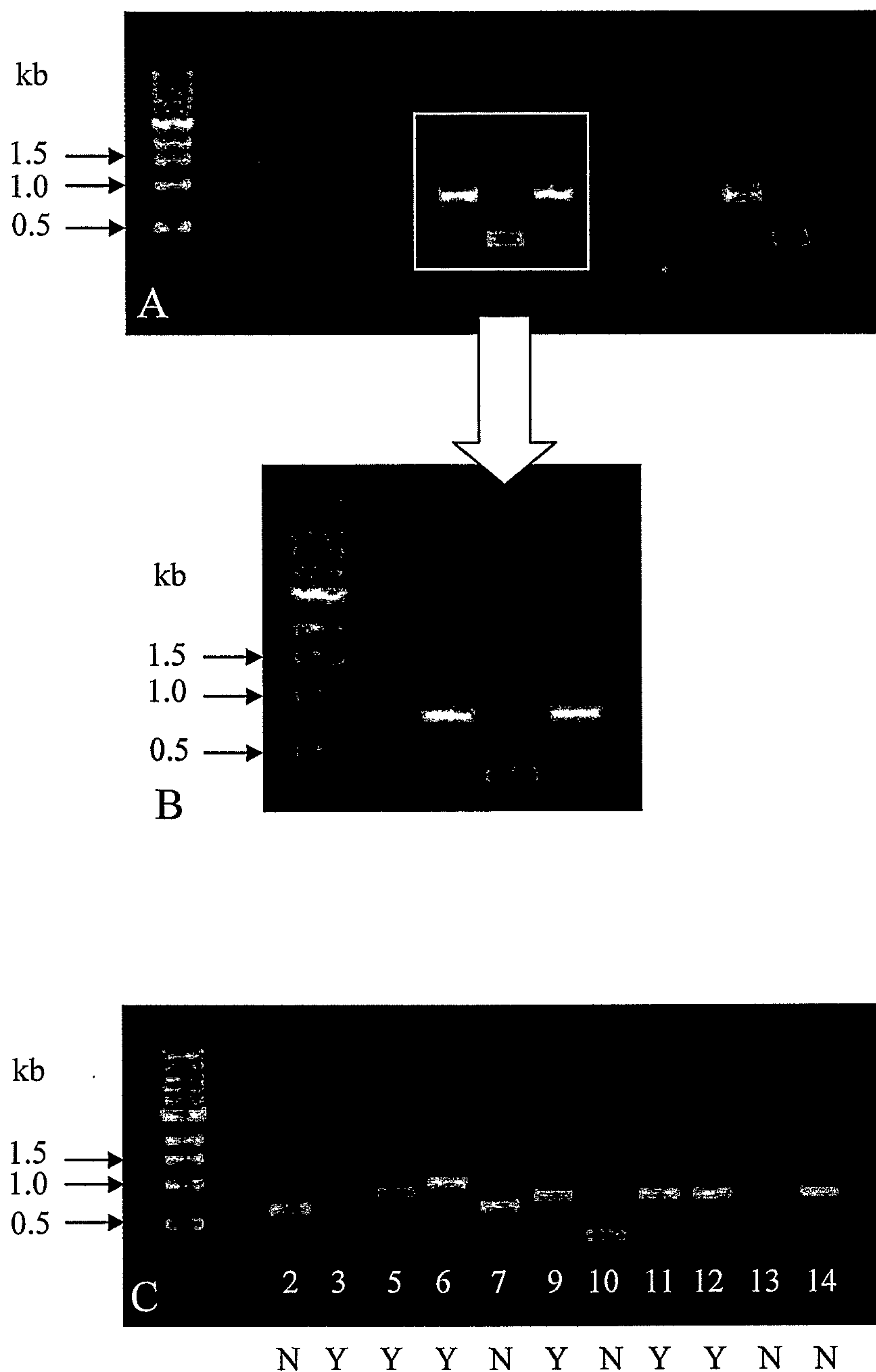


Fig. 6.5 Insert size determination by PCR and DNA sequencing analysis.

A: Showing insert size (0.3–1.0 kb) determination by PCR products.

B: Purification of the insert.

C: Eleven selections were sequenced, and 6 positive clones (0.6–1.2 kb) were found to encode for protein fragments corresponding to polypeptides expressed by the library. Lane 3: gene in chromosome 1, lane 5: AOP2, lane 6 gene in chromosome 18, lane 9: NFAT5, lane 11: CD24 and lane 12: LDH-A.

Table 6.1 Identification of auto-antigens by cDNA library screening from periodontitis patient sera IgG (n =22)

Analyzed insert DNA sequences from positive plaques				Recognition of serum
Antigens/genes	Clone	Accession No.	Identity (%)	samples' no (% , n =22)
CD24	KAT09618	AK026603	100	12, 16, 18 (14)
Lactate dehydrogenase A (LDH-A)	MGC: 4065	BC001829	100	2, 16, 18, 19 (18)
Anti-oxidant protein 2 (AOP2)		NM_004905	100	2, 12, 16, 18 (18)
Nuclear factor of activated T-cells 5 (NFAT5)		NM_173215	94	2, 16, 18, 19 (18)
Two novel genes on chromosome 1p35	465N24	AL031432	97	2, 8, 10, 11, 12, 16, 18, 21 (36)
Chromosome 18	RP11-13N13	AC106037	100	7, 8, 11, 16, 18 (23)

Table 6.2 Identified auto-antigens contain shared structure/ epitopes with cross-reactive oral bacteria

Cross-reactive bacteria	Antigens/genes																												
	CD24				LDH-A ³				AOP2 ⁴				NFAT5 ⁵				Chromosome 1p35				Chromosome 18								
	P# ⁶	12	16	18	2	16	18	19	2	12	16	18	2	16	18	19	2	8	10	11	12	16	18	21	7	8	11	16	18
<i>S¹. mitis</i> (NCTC 12261)	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-
<i>S. constellatus</i> (206)	+	-	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-	-	-	+	-	+	+	-	-	-	-	-	-
<i>S. intermedius</i> (ATCC 27335)	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+
<i>S. intermedius</i> (488)	+	+	+	+	-	+	+	+	+	-	-	+	+	-	-	+	+	+	-	-	+	-	+	-	+	+	+	-	-
<i>A². georgiae</i> (DSM 6843)	+	+	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-	+	-	+	-
<i>A. sp oral clone</i> (BL005)	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-

¹ *Streptococcus* and ² *Actinomyces*, reference strains with closest homology are shown in parentheses. ³ Lactate dehydrogenase A. ⁴ Anti-oxidant protein 2.

⁵ Nuclear factor of activated T-cells 5. ⁶ patient number.

6.5 DISCUSSION

In this study, 4 auto-antigens (CD24, AOP2, LDH-A and NFAT5), 2 novel genes encoding on chromosome 1 and one novel gene on chromosome 18 that contain shared structure/peptides cross-reactive with streptococci and *Actinomyces* were detected. Although these antigens have not yet been reported in periodontitis, investigations have shown that CD24, AOP2 and LDH-A play an important role in epithelial cell responses related to cancer and infectious diseases.

CD24 (nectadrin, heat stable antigen), a cell surface glycoprotein thought to be involved in cell-cell adhesion and signaling, is highly expressed in keratinocytes (Redondo *et al.*, 1998; Magnaldo & Barrandon, 1996; Kobayashi *et al.*, 1993), and also expressed in pre-B lymphocytes (Nielsen *et al.*, 1997; Karran *et al.*, 1995; Taguchi *et al.*, 2003), T lymphocytes (Williams *et al.*, 1996), neurons (Rougon *et al.*, 1991), muscle cells (Figarella-Branger *et al.*, 1993) and carcinoma cells (Akashi *et al.*, 1994; Kriatiansen *et al.*, 2002, 2003; Schindelmann *et al.*, 2002; Welsh *et al.*, 2001). Its function is not completely known. CD24 has recently been identified as the human molecule homologous to the murine heat-stable antigen (HSA). HSA is expressed by murine keratinocytes and delivers co-stimulatory signals in T-cell activation (Williams *et al.*, 1996). CD24 antigen is expressed in a wide range of epithelial cells and carcinoma cell lines (Akashi *et al.*, 1994; Kriatiansen *et al.*, 2002; Schindelmann *et al.*, 2002); however, has not been reported in inflamed gingiva.

The function of CD24 in keratinocytes has not yet been clearly demonstrated, but it has been suggested that it is involved in cell adhesion and signalling. For instance, heavily glycosylated CD24 may act as a receptor for lectin-like molecules (Kay *et al.*, 1991; Shirasawa *et al.*, 1993) such as P-selectin (Sammar *et al.*, 1994). CD24 also participates in cell-cell and cell-matrix interactions of neural cells (Kadmon *et al.*, 1992, 1995 Hubbe & Altvogt, 1994). Some observations indicate that CD24 may also be involved in signal transduction (Stefanova *et al.*, 1991). Also, intracellular calcium concentrations are significantly increased when lymphocytes are treated with an anti-CD24 antibody (Fischer *et al.*, 1990). In this context, calcium modulates significantly the proliferation and differentiation of keratinocytes. In work in progress, CD24 was shown to be expressed in the pocket epithelium.

Also the core molecule of CD24 antigen (3kb) is expressed in a wide range of epithelial cells and carcinoma cell lines, eg: lung, gastric, ductal, bronchiolar, renal tubular and oviduct. This is evidence that CD24 is transiently expressed on the surface of epithelial cells in the process of cellular maturation (Akashi *et al.*, 1994).

Reactive oxygen species, especially hydrogen peroxide, are important in cellular signal transduction. However, excessive amounts of these species damage tissues and cells by oxidizing virtually all important bio-molecules. Peroxiredoxin 6 (PRDX6) (also called antioxidant protein 2, or AOP2) is a novel peroxiredoxin family member whose function *in vivo* is unknown. But some data strongly suggest that AOP2 is a novel thiol-dependent antioxidant that functions to scavenge particular hydroperoxides in the cell and mediate specific signals (Phelan, 1999). PRDX6 protein is widely expressed in most tissues, being particularly abundant in epithelial cells (Wang *et al.*, 2003; Fatma *et al.*, 2001). The PRDX6s knockout mouse model demonstrates the importance of this molecule in protection against damaging oxidizing species (Wang *et al.*, 2003).

Lactate dehydrogenase A (LDH A), a tetrameric gene product, is expressed in human retinoblastoma cell lines (Maekawa *et al.*, 2002), human corneal endothelial cells (Sakai *et al.*, 2002), breast cancer cell lines (Forti *et al.*, 2002) and cultured porcine sertoli cells (Boussouar & Benahmed, 1999; Nehar *et al.*, 1997). Lactate dehydrogenase A (LDH A) activity is a key point in the stimulatory effect of epidermal growth factor (EGF) on lactate production in cultured Sertoli cells (Boussouar & Benahmed, 1999). This gene has not previously been reported to be expressed in keratinocytes. LDH A also exists in some bacteria, including streptococci, *Actinomyces* and *Lactobacillus* spp (Sommer *et al.*, 1985).

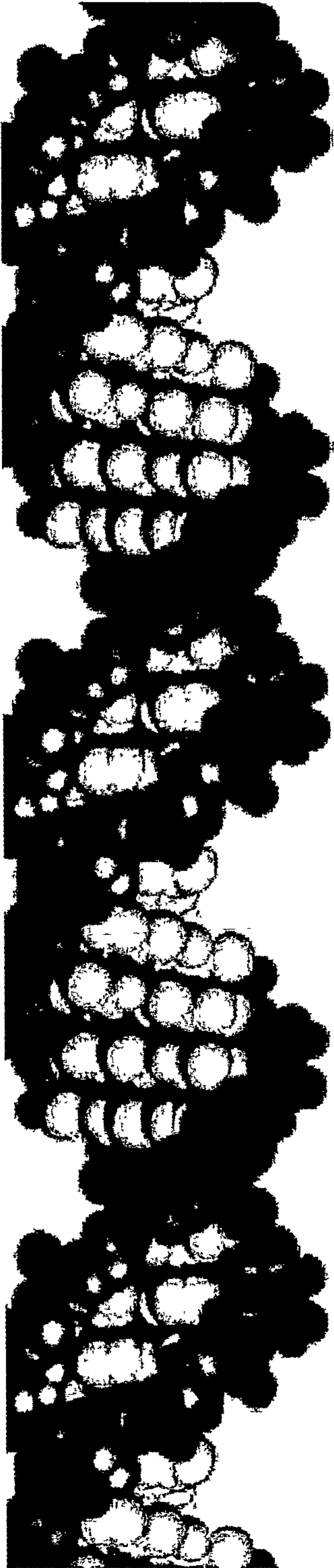
Members of the NFAT (nuclear factors of activated T cells) gene family have been investigated in numerous organisms, including man and mouse. All NFATs may be synthesized in several isoforms differing in amino or carboxy termini due to 5' and 3' alternative splicing of the corresponding mRNA (Dalski *et al.*, 2002).

The functions of NFAT5 under isosmotic conditions present *in vivo* remain unknown. Investigations have demonstrated that NFAT5 is necessary for optimal cell development *in vivo* and allows for optimal cell growth *ex vivo* under conditions associated with osmotic stress (Trama *et al.*, 2002). The cells are protected from the osmotic effect of high levels of sodium ion and urea by accumulating compatible osmolytes such as sorbitol, betaine, and myo-inositol. These osmolytes are involved in maintaining cell volume and electrolyte contents because they do not perturb the protein structure and function over a wide range of concentrations. Sorbitol is produced via the reduction of glucose by aldose reductase (AR), while betaine and myo-inositol are transported into the cells through specific transporters. Under hyperosmotic stress, transcription of genes encoding these proteins is strongly induced (Ko *et al.*, 2000).

Integrins, receptors for extracellular matrix ligands, are critical regulators of the invasive phenotype. Specifically, the alpha(6)beta(4) integrin has been linked with epithelial cell motility, cellular survival and carcinoma invasion, hallmarks of metastatic tumours. The NFAT (nuclear factor of activated T-cells) family of transcription factors exhibit strong anti-tumour-promoting activity. NFAT1 and NFAT5 activity correlate with the expression of the alpha(6)beta(4) integrin. In addition, the transcriptional activity of NFAT5 is induced by alpha(6)beta(4) clustering in the presence of chemo-attractants, resulting in enhanced cell migration. These observations show that NFATs are targets of alpha(6)beta(4) integrin signalling and are involved in promoting carcinoma invasion, highlighting a novel function for this family of transcription factors in human cancer (Jauliac *et al.*, 2002).

NFAT5, also known as tonicity enhancer binding protein (TonEBP) or NFATL1, is a new member of the immunologically important NFAT protein family. Despite its obvious relationship to this transcription factor family, NFAT5 shows distinct pathways of regulation and function.

Thus CD24, AOP2, LDH-A and NFAT5 appear to be very important in the function of epithelial cells. In conclusion, antibodies reactive with auto-antigen targets cross-react with components of oral bacteria, suggesting microbial involvement in an autoimmune process of pathogenesis.



CHAPTER 7

General

Discussion

AUTOIMMUNITY

IN CHRONIC

PERIODONTITIS

CHAPTER 7: GENERAL DISCUSSION

7.1 THE SPECTRUM OF AUTOIMMUNE DISEASE RESEARCH

A basic understanding of the human immune system is central to the understanding of the development of autoimmune disease. Scientists searching for ways to prevent and treat autoimmune disease are studying disease pathogenesis and investigating new ways to modify the immune system. Specifically, investigators are focusing on: 1) studies of the immune system during the progression of an autoimmune disease; 2) analysis of genetic influence on autoimmune disease expression and progression; 3) the role of infectious agents in autoimmune diseases; 4) studies of animal models of autoimmune diseases; and 5) the effects of therapeutic intervention on the immune system.

7.2 SUMMARY: key findings from thesis study

7.2.1 Epithelial integrity in inflamed gingival tissues

Immunohistochemical analysis indicated that in both clinically healthy gingiva and lesions of advanced periodontitis, expression patterns of E-cadherin, involucrin and connexins 26 and 43 were similar, with statistically significant reduction in staining intensity from the external oral epithelium through the gingival sulcus to the junctional epithelium or pocket epithelium respectively. Further, there was a pronounced reduction in staining for E-cadherin, involucrin and both connexins (26 and 43) in the pathological lining epithelium of the periodontal pocket. These changes were associated with marked alterations of filamentous actin (F-actin) expression, collectively indicating profound perturbation of epithelial structure. The data supported the concept that the ability of the pathological lining epithelium to function as an effective barrier against the ingress of microbial products into the tissues is severely compromised.

7.2.2 The distribution of the potent immuno-modulators, TGF- β s and their receptors, in inflamed gingival epithelia

TGF- β 1 was not detected in gingival epithelial cells. TGF- β 2 indicated a progressive reduction of staining from the external oral epithelium through to gingival sulcus and the gingival attachment or pocket epithelium. TGF- β 3 showed intense staining in all domains of both minimally inflamed gingiva and advanced periodontitis tissues. TGF- β RI showed focal staining of the spinous layer in the external oral epithelium of both

periodontitis lesions and minimally inflamed tissues. TGF- β RII was present throughout the strata but with progressive reduction in intensity from the oral epithelium to gingival attachment or pocket epithelium respectively while, conversely, TGF- β RIII showed an increase in diffuse staining intensity from external oral epithelium to pocket epithelium.

7.2.3 Antibody responses to CCL-4 epithelial components

The protein profile of extracted CCL-4 antigens indicated a wide distribution of bands between 21–200 kDa. Western blots displayed distinct patterns for individual patient sera with CCL-4 antigens; however, there existed a common recognition of a 30 kDa band. Trace recognition of epithelial antigens in only a few periodontally healthy sera was observed.

7.2.4 Identity of cross-reactive colonies

Identification of those colonies both reacting with patient serum IgG and affinity isolated (CCL-4-specific) IgG was by colony morphology, Gram-stain (under anaerobic conditions), conventional Biochemical test and 16S rDNA PCR. Six potentially cross-reactive bacterial strains were isolated from the plaque samples and the reference organisms identified by closest homology were *Streptococcus mitis* NCTC 12261, *Streptococcus constellatus* 206, *Streptococcus intermedius* ATCC 27335, *Streptococcus intermedius* 488, *Actinomyces georgiae* 6843 DSM and *Actinomyces* sp. oral clone BL008, respectively.

7.2.5 Cross-reactivity between affinity-isolated (CCL-4-specific) antibodies and streptococci / *Actinomyces* antigens

Different profiles for the 6 bacteria were detected by patient serum IgG and affinity-isolated (CCL-4-specific) IgG. Patterns of reactivity for affinity-isolated (CCL-4-specific) IgG's varied for different bacterial cells; however, there were apparently shared cross-reacting antigens at 27, 30, 35, 38 and 50 kDa among the potentially cross-reactive bacterial strains. In contrast to the relatively strong recognition of multiple antigens by serum antibodies in the majority of periodontitis subjects, sera from periodontally healthy subjects showed only weak recognition of this group of organisms.

7.2.6 Confirmation of removal of cross-reactive autoantibodies by serum absorption to *Streptococcus*/ *Actinomyces* sp.

To investigate whether absorption would remove all cross-reactivity with epithelial (CCL-4) components, Dot blots and Western blots were employed. Absorption of sera by four different streptococcal and two different *Actinomyces* strains partially removed cross-reactivity as demonstrated by quantitative analysis of Dot blots. An overall $P < 0.05$ (Kruskal-Wallis test) was observed among medians of these groups. Also this reactivity was confirmed using qualitative Western blot analysis with the indication that autoreactive antibodies were variably removed by absorption with the target species. Absorption of sera with the combined streptococci and *Actinomyces* species removed essentially all reactivity for epithelial components, indicating the potential origin of immunological reactivity to self-epitopes. The responses were specific and were not observed with other Gram-positive bacteria, *Lactobacillus acidophilus* ATCC 4356 or *Lactobacillus casei subsp. rhamnosus* ATCC 7469.

7.2.7 Recognition of autoantigens by individual subjects

Four known antigens (CD24, NFAT5, LDH-A and AOP2) and one gene in chromosome 18 encoding a novel protein and two genes in chromosome 1 also encoding novel peptides, were identified by the pool of patient sera, using a human keratinocyte cDNA library. Among the identified autoantigens, CD24 antigen was strongly recognized by sera from 3 patients, AOP2, NFAT5 and LDH-A antigens were recognized by 4 sera respectively. Chromosome 18 and chromosome 1 antigens were recognized by 5 and 8 sera respectively. The six different antigens were recognised by one control subject. The six bacteria previously identified (Chapter 5), especially *S. intermedius* were demonstrated to absorb antibodies reacting with identified auto-antigens in patterns varying between individuals.

In conclusion, reactivity against epithelial antigens derived from a rat source indicated the potential to develop animal models. Similar patterns of reactivity were observed in preliminary studies using the human oral epithelial line H413 (Prime *et al.*, 1990) with reactivity for human antigens confirmed by probing an expression library. A focus of this study was the possible contribution of bacteria from the predominantly anaerobic flora to the induction of epithelial-reactive antibodies. The method employed to test this was biased only by the limitation of detecting cultivable organisms. By

testing a number of sera against a diverse collection of plaque samples, a small group of bacteria were consistently recognised by affinity isolated (epithelial-specific) antibodies. These bacteria were provisionally identified on the basis of 16S rDNA sequences as members of the genus *Streptococcus* (*S. mitis*, *S. constellatus* and two *S. intermedius*) and *Actinomyces* (with homology to *A. georgiae* and *A. sp. oral clone*).

The findings support a mechanism of molecular mimicry during the progression of periodontitis. In addition, cross-reactive antigens at common apparent molecular weights of 27, 30, 35, 38 and 50 kDa that were shared between two or more bacterial strains, were found.

7.3 LIMITATION OF THE WORK

Thesis studies were commenced in an environment of poor understanding of the origin and biology of the reactive pocket epithelium. It was therefore necessary to examine the biology of this tissue in some detail before proceeding to investigate possible pathogenic mechanisms. Interpretation of the findings is necessarily limited by the cross-sectional nature of the analysis and the available patient cohort. This investigation did, however, establish that recognition of epithelial antigens is a consistent feature of patients with periodontal disease. A mechanism for the generation of these antibodies was discovered although the range of auto-antigens recognised by patient sera requires further definition as a basis for establishing links to cross-reactive bacterial peptides. There has not been the opportunity to proceed with the key aspect of the pathogenic potential of these antibodies.

7.4 FUTURE STUDIES

It is encouraging that the antigens identified by probing an expression library of epithelial products could be related back to the bacterial species originally identified as the so-called cross-reactive consortium. This provides two key opportunities. In relation to the limited quantities of sera available from this study (that is frozen stocks of the samples described) it would not be possible to prepare useful quantities of affinity-purified antibodies with specificity for epithelial antigens suitable for experimentation. It would therefore be necessary to proceed using the approach of specific depletion of reactivity for particular antigens to evaluate direct pathogenic potential of antibodies against cultured epithelial cells. That is loss of function in depleted sera would indicate the importance of antibodies reactive with particular antigens. This would be possible by the preparation of recombinant epithelial peptides. The second aspect is the capacity

to establish immunological relationships at the polypeptide level which would enable the identification of corresponding bacterial proteins and therefore the production of recombinant forms suitable to establish animal models of immunopathological reactions.

Specific objective 1. The keratinocyte expression library would be screened exhaustively to a stage where the majority of antigens are repeats of previously identified clones. The inserted sequences could then be prepared for expression in a Qiagen vector system with attached C-terminal 6-His tag to facilitate isolation by binding to a nickel-affinity column, a method used at the IDR (Institute of Dental Research) (DeCarlo *et al.*, 1999). The expressed peptides would then be used to prepare specifically-depleted sera as tools for studies on cultured cells.

Specific objective 2. In comparison with unabsorbed sera, specifically depleted sera from Aim 1 above could be used to probe extracts of the implicated bacterial species. This would allow the direct antigenic relationship between bacterial and epithelial antigens to be established by subtraction and further, could facilitate the identification of potential common tissue cross-reactive antigens that might exist in the identified bacterial species.

Specific objective 3. A human epithelial cell line H413 (derived from an oral squamous cell carcinoma) could be used as the target to screen for structure/function changes. As the profile of key surface antigens is a reliable indicator of cell behavior, novel cluster of differentiation (CD) antibody microarrays developed at The University of Sydney could be employed (Belov *et al.*, 2001). To avoid possible degradation of surface antigens by proteinase action, cultured cells would be treated with the metabolic inhibitor NaN_3 , chilled and harvested by scraping into EDTA solution. This yields a mixture of single cells and small clusters, suitable for the arrays which detect the density of cells bound to the array grid by dark field microscopy and densitometry. The role of antibodies to specific cross-reactive antigens would be inferred with reference to the depleted sera. Changes in CD expression will provide an indicator of modulation of structure/function. To provide a comprehensive overview of altered gene expression patterns for those antibody-antigen reactions demonstrated to be potent, mRNA profiles could be studied by separate microarray analysis.

Specific objective 4. For members of the bacterial consortium known to be transformable, peptides containing antigens of prime interest (from 2 and 3 above) could be identified in Western blot and amino acid sequence data obtained by mass spectrometry (MS/MS analysis). This could be utilised either to identify the corresponding gene from the published sequences or to probe a library. Considerable progress has been made in the genomic analysis of oral streptococci with the Institute for Genomic Research (TIGR) about to complete the primary sequencing of the *S. mitis* genome. The objective is to prepare recombinant peptides for subsequent animal studies.

Specific objective 5. The rat keratinocyte line CCL-4 would be used to confirm the pathogenic effects of serum antibodies against specific antigens in a model system. Panels of inbred rats could be immunised with recombinant bacterial proteins of interest and titres of cross-reactive serum antibodies related to tissue pathology induced.

Significance: Evidence has been obtained to indicate that a consistent feature of chronic periodontitis is the production of auto-reactive antibodies to epithelial antigens including recently characterised and novel proteins. Planned experiments will enable the linkage of the epithelial antigens to defined bacterial proteins and the preparation of recombinant bacterial polypeptides for analysis in an animal model. Epithelial antigens of prime interest would be determined by broad spectrum microarray analysis of the response of cultured epithelial cells by exploiting the capacity to specifically deplete patient sera with recombinant epithelial antigens. The novelty of the cross-reacting epithelial antigens identified to date does not allow ready extrapolation to pathogenic potential. CD24 does, however, serve as a template. Increased expression of this adhesion molecule has been reported to indicate invasive potential of carcinomas (Kristiansen *et al.*, 2002) while ligation of this receptor on pre-B cells induces apoptosis (Taguchi *et al.*, 2003). Therefore there is a strong basis for proposing that anti-CD24 antibodies could mediate or contribute to a variety of mucosal and other disease processes. In tissue environments of inflammation and infection, recognition of intracellular antigens could promote inflammation by complement fixation. The proposed studies would therefore provide a basis for evaluating the contribution of chronic periodontitis to immunopathological disease.

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APPENDICES

A: ABBREVIATIONS

.....A – (A1-4)

B: SEQUENCING OF IDENTIFIED BACTERIA

.....A – (B1-6)

C: SEQUENCING OF IDENTIFIED AUTO-ANTIGENS

.....A – (C1-9)

D: POSTER

.....A – D1

2-D	2-dimension
3-D	3-dimension
16S	16 subunits
A ₂₈₀	absorbance at 280 nm
A ₂₆₀	absorbance at 260 nm
<i>A. georgia</i>	<i>Actinomyces georgiae</i>
<i>A. sp oral clone</i>	<i>Actinomyces sp oral clone</i>
Adv	advanced periodontitis
AOP2	Antioxidant protein 2, or Peroxiredoxin 6 (PRDX6)
AP	alkaline phosphatase
AP	advanced periodontitis
BCIP	5-bromo-4chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
CCL-4	rat oral epithelial cell line
CD24	cluster of differentiation 24, or nectadrin, or heat stable antigen
CDC	Centre for Disease Control
cDNA	complementary deoxyribonucleic acid
CHG	clinically healthy gingiva
CO ₂	carbon dioxide
DAB	3,3'-diamidinobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNTP	deoxyribonucleoside triphosphate
DPBS	Dulbecco's phosphate buffered saline
DTT	dithiothreitol
E-cadherin	epithelial cadherin
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid

F-actin	Filamentous actin
FCS	foetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
γ	gamma
GA	gingival attachment
GC	gingival crest
GS	gingival sulcus
h	hour
H413	human oral epithelial cell line
HLA	human leukocyte antigen
HLA-DR	human leukocyte antigen-class II
ICAM-1	intercellular adhesion molecule-1
IEF	Isoelectric Focusing
Ig G	immunoglobulin G
IL-1	interleukin-1
IPTG	isopropyl β -D-thiogalactopyranoside
JE	junctional epithelium
kDa	kilodalton
kb	kilobase
kbp	kilobase pair
λ	lambda
L	litre
LB media	Luria Bertani media
LDH-A	lactate dehydrogenase-A
M	molar
mA	milli-ampere
MHC	major histocompatibility complex
MHC-II	major histocompatibility complex-class two
min	minute
Min	minimally inflamed gingiva
ml	millilitre
mM	millimolar
mm	millimetre

mRNA	messenger ribonucleic acid
<i>n</i>	simple numbers
NCBI	National Centre for Biotechnology Information
NFAT5	nuclear factors of activated T cells, or Tonicity-responsive enhancer (TonE) binding protein (TonEBP)
ng	nanogram
nm	nanometre
no	number
OE	oral epithelium
PAGE	polyacrylamide gel electrophoresis
PE	pocket epithelium
pfu	plaque forming units
<i>P. gingivalis</i>	<i>porphyromonas gingivalis</i>
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	propidium iodide
rDNA	ribosomal deoxyribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RTF	reduced transport fluid
SLCM	scanning laser confocal microscope
SDS	sodium dodecyl sulfate
<i>S. mitis</i>	<i>Streptococcus mitis</i>
<i>S. constellatus</i>	<i>Streptococcus constellatus</i>
<i>S. intermedius</i>	<i>Streptococcus intermedius</i>
<i>S. milleri</i>	<i>Streptococcus milleri</i>
<i>S. anginosus</i>	<i>Streptococcus anginosus</i>
TAE	Tris/acetate/EDTA (buffer)
TBS	Tris-buffered saline
TGF- β	transforming growth factor-beta
TGF- β R	transforming growth factor-beta receptor

Tris-HCl	Tris-hydrochloride
V	voltage
vs.	against
°C	degree celsius
µg	microgram
µl	microlitre
× <i>g</i>	gravities
WebANGIS	Australian National Genomic Information Service

1) *Streptococcus mitis* NCTC 12261

SM16SRR1 emb|:-Streptococcus mitis 16S rRNA gene (1403 nt)
initn: 2210 initl: 2210 opt: 2210 Z-score: 1607.4 expect() 2.5e-82
100.000% identity in 442 nt overlap

SEQ1				10	20	30
				GGGAATCTTCGGCAATGGACGGAAGTCTGA		
				::::::::::::::::::::::::::::::::::::		
SM16SR	CGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGGAAGTCTGA					
	330	340	350	360	370	380
SEQ1		40	50	60	70	80
	CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTAAGAGAA					
	::					
SM16SR	CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTAAGAGAA					
	390	400	410	420	430	440
SEQ1		100	110	120	130	140
	GAACGAGTGTGAGAGTGGAAAGTTCACACTGTGACGGTATCTTACCAGAAAGGGACGGCT					
	::					
SM16SR	GAACGAGTGTGAGAGTGGAAAGTTCACACTGTGACGGTATCTTACCAGAAAGGGACGGCT					
	450	460	470	480	490	500
SEQ1		160	170	180	190	200
	AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGG					
	::					
SM16SR	AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGG					
	510	520	530	540	550	560
SEQ1		220	230	240	250	260
	CGTAAAGCGAGCGCAGGCGGTTAGATAAGTCTGAAGTTAAAGGCTGTGGCTTAACCATAG					
	::					
SM16SR	CGTAAAGCGAGCGCAGGCGGTTAGATAAGTCTGAAGTTAAAGGCTGTGGCTTAACCATAG					
	570	580	590	600	610	620
SEQ1		280	290	300	310	320
	TACGCTTTGGAAACTGTTTAACTTGAGTGCAAGAGGGGAGAGTGGAAATTCATGTGTAGC					
	::					
SM16SR	TACGCTTTGGAAACTGTTTAACTTGAGTGCAAGAGGGGAGAGTGGAAATTCATGTGTAGC					
	630	640	650	660	670	680
SEQ1		340	350	360	370	380
	GGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCTTGTA					
	::					
SM16SR	GGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCTTGTA					
	690	700	710	720	730	740
SEQ1		400	410	420	430	440
	CTGACGCTGAAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTTAGNNCCA					
	::					
SM16SR	CTGACGCTGAAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG					
	750	760	770	780	790	800

2) Streptococcus constellatus 206

AF104677 emb -:Streptococcus constellatus strain 206 16 (1485 nt)	
initn: 2204 init1: 2204 opt: 2204 Z-score: 1749.2 expect() 3e-90	
99.774% identity in 442 nt overlap	
SEQ2	GGGAATCTTCGGCAATGGGGGGAACCCCTGN
AF1046	CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGGAACCCCTGA
	10 20 30
	300 310 320 330 340 350
SEQ2	CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTAAGGAA
AF1046	CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTAAGGAA
	40 50 60 70 80 90
	360 370 380 390 400 410
SEQ2	GAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTACTTAACCAGAAAGGGACGGCT
AF1046	GAACGTGTGTGAGAGTGGAAAGTTCACACAGTGACGGTACTTAACCAGAAAGGGACGGCT
	100 110 120 130 140 150
	420 430 440 450 460 470
SEQ2	AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCAGCGTTGTCCGGATTTATTGGG
AF1046	AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCAGCGTTGTCCGGATTTATTGGG
	160 170 180 190 200 210
	480 490 500 510 520 530
SEQ2	CGTAAAGCGAGCGCAGGCGGTTAGATAAGTCTGAAGTGAAAGGCAGTGGCTCAACCATTG
AF1046	CGTAAAGCGAGCGCAGGCGGTTAGATAAGTCTGAAGTGAAAGGCAGTGGCTCAACCATTG
	220 230 240 250 260 270
	540 550 560 570 580 590
SEQ2	TAGGCTTTGGAAACTGTTTAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGC
AF1046	TAGGCTTTGGAAACTGTTTAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGC
	280 290 300 310 320 330
	600 610 620 630 640 650
SEQ2	GGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAA
AF1046	GGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAA
	340 350 360 370 380 390
	660 670 680 690 700 710
SEQ2	CTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTTAGTTCCA
AF1046	CTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTTAGTCCACG
	400 410 420 430 440 450
	720 730 740 750 760 770

3) *Streptococcus intermedius* ATCC 27335

AF104671 emb| -:Streptococcus intermedius strain ATCC273 (1558 nt)
initn: 2180 initl: 1251 opt: 2193 Z-score: 1846.0 expect() 1.2e-95
99.111% identity in 450 nt overlap

				10	20	30
SEQ3				GGGAATCTTCGGCAATGGGGGGAACCCTGN		
						
AF1046	CGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGGAACCCTGA					
	340	350	360	370	380	390
		40	50	60	70	80
SEQ3	CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAAGGAA					
						
AF1046	CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAAGGAA					
	400	410	420	430	440	450
		100	110	120	130	140
SEQ3	GAACGAGTGTGAGAATGGAAAGTTCATACTGTGACGGTACTTAACCAGAAAGGGACGGCT					
						
AF1046	GAACGAGTGTGAGAATGGAAAGTTCATACTGTGACGGTACTTAACCAGAAAGGGACGGCT					
	460	470	480	490	500	510
		160	170	180	190	200
SEQ3	AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCAGTTGTCCGGATTTATTGG					
						
AF1046	AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGC-GTTGTCCGGATTTATTGG					
	520	530	540	550	560	570
		220	230	240	250	260
SEQ3	GCGTAAAGCGAGCGCAGGCGGTTAGATAAGTCTGAAGTTAAAGGCAGTGGCTCAACCATT					
						
AF1046	GCGTAAAGCGAGCGCAGGCGGTTAGATAAGTCTGAAGTTAAAGGCAGTGGCTCAACCATT					
	580	590	600	610	620	630
		280	290	300	310	320
SEQ3	GTAGGCTTTGGAAACTGTTTAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAG					
						
AF1046	GTAGGCTTTGGAAACTGTTTAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAG					
	640	650	660	670	680	690
		340	350	360	370	380
SEQ3	CGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTA					
						
AF1046	CGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTA					
	700	710	720	730	740	750
		400	410	420	430	440
SEQ3	ACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGTTAAGTCCA					
						
AF1046	ACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGT-AGTCCA					
	760	770	780	790	800	810

4) *Streptococcus intermedius* 488

AF104673 emb| -:Streptococcus intermedius strain 488 16S (1521 nt)
initn: 2154 initl: 1245 opt: 2171 z-score: 1629.0 expect() 1.5e-83
98.670% identity in 451 nt overlap

				10	20	30
SEQ4				GGGAATCTTCGGCAATGGGGGGAACCTGN		
				::::::::::::::::::::::::::::::::		
AF1046	CGGCCCAGACNNCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGGAACCTGA					
	330	340	350	360	370	380
		40	50	60	70	80
SEQ4	CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAAGGAA					
	::::::::::::::::::::::::::::::::					
AF1046	CCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAAGGAA					
	390	400	410	420	430	440
		100	110	120	130	140
SEQ4	GAACGAGTGTGAGAATGGAAAGTTCATACTGTGACGGTACTTAACCAGAAAGGGACGGCT					
	::::::::::::::::::::::::::::::::					
AF1046	GAACGAGTGTGAGAATGGAAAGTTCATACTGTGACGGTACTTAACCAGAAAGGGACGGCT					
	450	460	470	480	490	500
		160	170	180	190	200
SEQ4	AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCNGAGCNGTTGTCCGGATTTATTG					
	::::::::::::::::::::::::::::::::					
AF1046	AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCC-GAGC-GTTGTCCGGATTTATTG					
	510	520	530	540	550	560
		220	230	240	250	260
SEQ4	GGCGTAAAGCGAGCGCAGGCGGTTAGATAAGTCTGAAGTTAAAGGCAGTGGCTCAACCAT					
	::::::::::::::::::::::::::::::::					
AF1046	GGCGTAAAGCGAGCGCAGGCGGTTAGATAAGTCTGAAGTTAAAGGCAGTGGCTCAACCAT					
	570	580	590	600	610	620
		280	290	300	310	320
SEQ4	TGTAGGCTTTGGAAACTGTTTAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTA					
	::::::::::::::::::::::::::::::::					
AF1046	TGTAGGCTTTGGAAACTGTTTAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTA					
	630	640	650	660	670	680
		340	350	360	370	380
SEQ4	GCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGT					
	::::::::::::::::::::::::::::::::					
AF1046	GCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGT					
	690	700	710	720	730	740
		400	410	420	430	440
SEQ4	AACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGTCTANTCC					
	::::::::::::::::::::::::::::::::					
AF1046	AACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTG-GTAGTCC					
	750	760	770	780	790	800

5) *Actinomyces georgiae* 6843 DSM

AG16SRRNA emb| -:A.georgiae 16S rDNA (1422 nt)
initn: 1084 initl: 1084 opt: 2088 Z-score: 1801.3 expect() 4.1e-93
97.539% identity in 447 nt overlap

				10	20	30
SEQ5				GGGTNAATATTAGCACAAATGGGCGCAAGCCT		
				::	:::::	:::::
AG16SR	ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT-GCACAATGGGCGAAAGCCT					
	320	330	340	350	360	370

	40	50	60	70	80	90
SEQ5	GATGCAGCGACGCCGCGTGAGGGATGGAGGCCTTCGGGTTGTGAACCTCTTTCGCCCCGTG					
	:::::	:::::	:::::	:::::	:::::	:::::
AG16SR	GATGCAGCGACGCCGCGTGAGGGATGGAGGCCTTCGGGTTGTGAACCTCTTTCGCCCCGTG					
	380	390	400	410	420	430

	100	110	120	130	140	150
SEQ5	GTCAAGCCGCAACTGTGGGTTGTGGTGAGGGTAGTGGGTAAAGAAGCGCCGGCTAACTAC					
	:::::	:::::	:::::	:::::	:::::	:::::
AG16SR	GTCAAGCCGCAACTGTGGGTTGTGGTGAGGGTAGTGGGTAAAGAAGCGCCGGCTAACTAC					
	440	450	460	470	480	490

	160	170	180	190	200	210
SEQ5	GTGCCAGCAGCCGCGGTAATACNGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAA					
	:::::	:::::	:::::	:::::	:::::	:::::
AG16SR	GTGCCAGCAGCCGCGGTAATAC-GTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAA					
	500	510	520	530	540	550

	220	230	240	250	260	270
SEQ5	AGGGCTTGTAGGCGGCTGGTCGCGTCTGCCGTG-AAATCCTCTGGCTCAACTGGGGGCGT					
	:::::	:::::	:::::	:::::	:::::	:::::
AG16SR	AGGGCTTGTAGGCGGCTGGTCGCGTCTGCCGTGAAAATCCTCTGGCTCAACTGGGGGCGT					
	560	570	580	590	600	610

	280	290	300	310	320	330
SEQ5	GCGGTGGGTACGGGCCTGGCTTGAGTGCGGTAGGGGAGGCTGGAACCTCCTGGTGTAGCGG					
	:::::	:::::	:::::	:::::	:::::	:::::
AG16SR	GCGGTGGGTACGGG-CTGGCTTGAGTGCGGTAGGGGAGGCTGGAACCTCCTGGTGTAGCGG					
	620	630	640	650	660	670

	340	350	360	370	380	390
SEQ5	TGGAATGCGCAGATATCAGGAAGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGTTACT					
	:::::	:::::	:::::	:::::	:::::	:::::
AG16SR	TGGAATGCGCAGATATCAGGAAGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGTTACT					
	680	690	700	710	720	730

	400	410	420	430	440	450
SEQ5	GACGCTGAGGANCGAAAGCGTGGGGAGCGAACAGGATTANATACCCTGGTTANTCCANCT					
	:::::	:::::	:::::	:::::	:::::	:::::
AG16SR	GACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGG-TAGTCCACGC					
	740	750	760	770	780	790

6) *Actinomyces* sp. oral clone BL008

AF385553 emb| -:Actinomyces sp. oral clone BL008 16S rib (1487 nt)
initn: 2135 initl: 1251 opt: 2151 Z-score: 1324.3 expect() 1.4e-66
98.658% identity in 447 nt overlap

SEQ6				10	20	30
				GGGGAATATTGCACAATGGGCGCAAGCCTG		
						
AF3855	ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTG					
	340	350	360	370	380	390
SEQ6		40	50	60	70	80
	NTGCAGCGACGCCGCGTGAGGGATGGAGGCCTTCGGGTTGTGAACCTCTTTCGCCAGTGA					90
						
AF3855	ATGCAGCGACGCCGCGTGAGGGATGGAGGCCTTCGGGTTGTGAACCTCTTTCGCCAGTGA					
	400	410	420	430	440	450
SEQ6		100	110	120	130	140
	AGCAGGCCTGCTCCTTGTGGGTGGGTTGACGGTAGCTGGATAAGAAGCGCCGGCTAACTA					150
						
AF3855	AGCAGGCCTGCTCCTTGTGGGTGGGTTGACGGTAGCTGGATAAGAAGCGCCGGCTAACTA					
	460	470	480	490	500	510
SEQ6		160	170	180	190	200
	CGTGCCAGCAGCCGCGGTAATACGTAGGGCGCNGAGCNGTTGTCCGGAATTATTGGGCGT					210
						
AF3855	CGTGCCAGCAGCCGCGGTAATACGTAGGGCGC-GAGC-GTTGTCCGGAATTATTGGGCGT					
	520	530	540	550	560	570
SEQ6		220	230	240	250	260
	AAAGAGCTCGTAGGCGGCTGGTCGCGTCTGTCTGAAATCCTCTGGCTTAACTGGGGGCT					270
						
AF3855	AAAGAGCTCGTAGGCGGCTGGTCGCGTCTGTCTGAAATCCTCTGGCTTAACTGGGGGCT					
	580	590	600	610	620	630
SEQ6		280	290	300	310	320
	TGCGGTGGGTACGGGCCGGCTTGAGTGCGGTAGGGGAGGCTGGAACTCCTGGTGTAGCGG					330
						
AF3855	TGCGGTGGGTACGGGCCGGCTTGAGTGCGGTAGGGGAGACTGGAACTCCTGGTGTAGCGG					
	640	650	660	670	680	690
SEQ6		340	350	360	370	380
	TGGAATGCGCAGATATCAGGAAGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGTTACT					390
						
AF3855	TGGAATGCGCAGATATCAGGAAGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGTTACT					
	700	710	720	730	740	750
SEQ6		400	410	420	430	440
	GACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTTAGTNCAANN					450
						
AF3855	GACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGG-TAGTCCACGC					
	760	770	780	790	800	810

1) Homo sapiens CD24 antigen (CD24), mRNA

Length = 2116

Score = 1197 bits (604), Expect = 0.0

Identities = 619/623 (100%), Gaps = 1/623 (0%)

Strand = Plus / Plus

Query: 15	cggttctccaagcaccagcatcctgctagacgcgccgcacccgacggaggacatgg	74
Sbjct: 1	cggttctccaagcaccagcatcctgctagacgcgccgcacccgacggaggacatgg	60
Query: 75	gcagagcaatggtggccaggctcgggctgggctgctgctggcactgctcctaccca	134
Sbjct: 61	gcagagcaatggtggccaggctcgggctgggctgctgctggcactgctcctaccca	120
Query: 135	cgcagatttattccagtgaaacaacaactggaacttcaagtaactcctcccagagtactt	194
Sbjct: 121	cgcagatttattccagtgaaacaacaactggaacttcaagtaactcctcccagagtactt	180
Query: 195	ccaactctgggttgcccccaaatccaactaatgccaccaccaaggcggctggtggtgcc	254
Sbjct: 181	ccaactctgggttgcccccaaatccaactaatgccaccaccaaggcggctggtggtgcc	240
Query: 255	tgcagtcaacagccagtccttctcggtctcactctctcttctgcatactctcttaag	314
Sbjct: 241	tgcagtcaacagccagtccttctcggtctcactctctcttctgcatactctcttaag	300

Appendix C: Sequencing of identified auto-antigens

```
Query: 315 agactcaggccaaagacgtcttctctaaatttcccacatcttctaaacccaatccaaatggc 374
|||||
Sbjct: 301 agactcaggccaaagacgtcttctctaaatttcccacatcttctaaacccaatccaaatggc 360

Query: 375 gtctggaaagtccaatgtggcaaggaaaaaacagggtctctcatcgaatctactaatccacac 434
|||||
Sbjct: 361 gtctggaaagtccaatgtggcaaggaaaaaacagggtctctcatcgaatctactaatccacac 420

Query: 435 cttttattgacacagaaaaatgttgagaatcccaaatttgattgatttgaagaacatgtga 494
|||||
Sbjct: 421 cttttattgacacagaaaaatgttgagaatcccaaatttgattgatttgaagaacatgtga 480

Query: 495 gaggtttgactananatgatggatgccaaatatataaatctgctggagtttcatgtacaagatg 554
|||||
Sbjct: 481 gaggtttgactagatgatgaatgccaaatatataaatctgctggagtttcatgtacaagatg 540

Query: 555 aaggagaggcaacatccaaaaatagtttaagacatgatttccttgaaatgtggcttgagaaa 614
|||||
Sbjct: 541 aaggagaggcaacatccaaaaatagtttaagacatgatttccttg-aatgtggcttgagaaa 599

Query: 615 tatggacacttaatactaccttg 637
|||||
Sbjct: 600 tatggacacttaatactaccttg 622
```

2) Homo sapiens anti-oxidant protein 2 (AOP2), mRNA

Length = 1653

Score = 398 bits (201), Expect = e-108

Identities = 201/201 (100%)

Strand = Plus / Minus

Query: 3 gccattttcatttaataagccatttggtatagcaacctaataaaccttggctgtgatgacac 62
|||||
Sbjct: 863 gccattttcatttaataagccatttggtatagcaacctaataaaccttggctgtgatgacac 804

Query: 63 caggatgtgtttatggaattgctgcaggaaaacacaattggcagctgacatcctctggct 122
|||||
Sbjct: 803 caggatgtgtttatggaattgctgcaggaaaacacaattggcagctgacatcctctggct 744

Query: 123 cacagcaccaacttctccaaagagacttaaggctgggggtgtgtagcggaggatttcttgc 182
|||||
Sbjct: 743 cacagcaccaacttctccaaagagacttaaggctgggggtgtgtagcggaggatttcttgc 684

Query: 183 cagatggggagctcttttggtga 203
|||||
Sbjct: 683 cagatggggagctcttttggtga 663

3) Homo sapiens, lactate dehydrogenase A, clone MGC:4065 IMAGE:2960999, mRNA

Length = 1984

Score = 1132 bits (571), Expect = 0.0

Identities = 599/599 (100%)

Strand = Plus / Minus

Appendix C: Sequencing of identified auto-antigens

Query: 3	cctaaatctgggtgcagagtcttcagagagacaccagcaacattcattccactccataca	62
Sbjct: 1027	cctaaatctgggtgcagagtcttcagagagacaccagcaacattcattccactccataca	968
Query: 63	ggcacactgggaatctccatgttccccaaaggaccccatgacagcttaatgggtgaact	122
Sbjct: 967	ggcacactgggaatctccatgttccccaaaggaccccatgacagcttaatgggtgaact	908
Query: 123	cccagcctttcccccatcaggtaacgggaatcgggtgaatccagattgcaaccacttcca	182
Sbjct: 907	cccagcctttcccccatcaggtaacgggaatcgggtgaatccagattgcaaccacttcca	848
Query: 183	ataacacgggtttttgggaaaaaccacttatcttccaagccacgtaggtcaagatatccact	242
Sbjct: 847	ataacacgggtttttgggaaaaaccacttatcttccaagccacgtaggtcaagatatccact	788
Query: 243	ggatttgaaaacaataagcaacttgcagttcggggctgtattttacaacattaggaatgatg	302
Sbjct: 787	ggatttgaaaacaataagcaacttgcagttcggggctgtattttacaacattaggaatgatg	728
Query: 303	aattttaaagatgttcacgttacgctggaccacaaattaagacggccttctccctcttgctga	362
Sbjct: 727	aattttaaagatgttcacgttacgctggaccacaaattaagacggccttctccctcttgctga	668
Query: 363	cgtgccccagccgtgataatgaccagcttgaggtttgcagttacattatagctcttgcca	422
Sbjct: 667	cgtgccccagccgtgataatgaccagcttgaggtttgcagttacattatagctcttgcca	608

Appendix C: Sequencing of identified auto-antigens

```

Query: 423 gagacaatctttggtgttctaaggaaaaggctgccatgttgagatncatcatctctccc 482
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct: 607 gagacaatctttggtgttctaaggaaaaggctgccatgttgagatccatcatctctccc 548

Query: 483 ttcaatttgtcttcgatgacatcaacaagagcaagttcatctgccaaagnccttcattaaa 542
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct: 547 ttcaatttgtcttcgatgacatcaacaagagcaagttcatctgccaaagtccttcattaaag 488

Query: 543 atactgatggnacaggncatgccnacancaccnaccncccaactgtantctttattctg 601
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct: 487 atactgatggcacaggccatgccaaacagcaccaaccccaactgtaattctttattctg 429

```

4) Homo sapiens nuclear factor of activated T-cells 5, tonicity-responsive (NFAT5),
transcript variant 5, mRNA

Length = 5304

Score = 268 bits (135), Expect = 5e-69

Identities = 259/276 (94%), Gaps = 4/276 (1%)

Strand = Plus / Minus

```

Query: 14  tgctgctgctgtgatgcttcagttactgtgacaaagttaccactgtat--tctgactgt 70
          ||||| || ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct: 2603 tgctgttgtgtgatgcctcagtcagttgtgacaaaattaccactgtaccatctgactgt 2544

Query: 71  atttgtctctctggactgagcttctcttctgtctgaaactgtgtgcctgctgcagtagtgca 130
          | | | ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct: 2543 aatatctctctagactgagtttctcttctgtctgaaactgtgtgtagcctgctgcaatagtgca 2484

```

Query: 315 agactcaggccaagaaacgtcttcttaaatttcccccatcttctaaacccaatccaaatggc 374
|||||
Sbjct: 301 agactcaggccaagaaacgtcttcttaaatttcccccatcttctaaacccaatccaaatggc 360

Query: 375 gtctggaaagtccaatgtggcaaggaaaaaacagggtcttcatcgaatctactaattccacac 434
|||||
Sbjct: 361 gtctggaaagtccaatgtggcaaggaaaaaacagggtcttcatcgaatctactaattccacac 420

Query: 435 cttttattgacacagaaaaatgttgagaatccccaaatttgattgatttgaaagaacatgtga 494
|||||
Sbjct: 421 cttttattgacacagaaaaatgttgagaatccccaaatttgattgatttgaaagaacatgtga 480

Query: 495 gaggtttgactanatgatggatgccaatattaaatctgctggagtttcatgtacaagatg 554
|||||
Sbjct: 481 gaggtttgactagatgatgaatgccaatattaaatctgctggagtttcatgtacaagatg 540

Query: 555 aaggagaggcaacatccaaaatagttaagacatgatttccttgaaatgtggcttgagaaa 614
|||||
Sbjct: 541 aaggagaggcaacatccaaaatagttaagacatgatttccttg-aatgtggcttgagaaa 599

Query: 615 tatggacacttaatactaccttg 637
|||||
Sbjct: 600 tatggacacttaatactaccttg 622

Query: 131 tcgctgctgggcagctgactagcagagaaactgcgggaaagggtcccggtgtgagatg 190
|||||
Sbjct: 2483 tcgctgttgggcagctgactagaagcagagaaactgctggaaaaagtaccaggctgtgagatg 2424

Appendix C: Sequencing of identified auto-antigens

Query: 183 gttacagtagcaccaatacatattc aaattaagagtttatttagaataactttggccgggtg 242
 |||||
Sbjct: 102847 gttacagtagcaccaatacatattc aaattaagagtttatttagaataactttggccgggtg 102906

Query: 243 cagtggtcacacgtgtaatcccagctctttgggagggccaaggcgggtggatcacaaaggt 302
 |||||
Sbjct: 102907 cagtggtcacacgtgtaatcccagctctttgggagggccaaggcgggtggatcacaaaggt 102966

Query: 303 caggagatcgagaccatcctaacaacggtgaaaccccatctctactataaaaataca 356
 |||||
Sbjct: 102967 caggagatcgagaccatcctaacaacggtgaaaccccatctctactataaaaataca 103020

6) Homo sapiens chromosome 18, clone RP11-13N13, complete sequence

Length = 1780

Score = 581 bits (293), Expect = e-163

Identities = 293/293 (100%)

Strand = Plus / Plus

Query: 3 ctgttcagcttccttttagacatttatggcagcacgtgtaagattcacacaaaaactcaga 62
 |||||
Sbjct: 117742 ctgttcagcttccttttagacatttatggcagcacgtgtaagattcacacaaaaactcaga 117801

Query: 63 gggtaaattaaaagggtgtattaaagtattactttaatgtagtggttctcaaaactttttgg 122
 |||||
Sbjct: 117802 gggtaaattaaaagggtgtattaaagtattactttaatgtagtggttctcaaaactttttgg 117861

Appendix C: Sequencing of identified auto-antigens

Query: 123	tcttaggatcccccttacactcttaaaaaattatgtaggactgcaagtagcttttggtttata	182
Sbjct: 117862	tcttaggatcccccttacactcttaaaaaattatgtaggactgcaagtagcttttggtttata	117921
Query: 183	taggtttatatctatctgatagttactgtattaggaattaattccatatataattaattcat	242
Sbjct: 117922	taggtttatatctatctgatagttactgtattaggaattaattccatatataattaattcat	117981
Query: 243	ttgaaaataattaaactacagcttaataataaaataacatttttaattttaataaa	295
Sbjct: 117982	ttgaaaataattaaactacagcttaataataaaataacatttttaattttaataaa	118034

DO ORAL BACTERIA CONTRIBUTE TO AUTOIMMUNE DISEASES?

Ping Ye^{*1}, Derek Harty¹, Cheryl Chapple¹, Mangala Nadkarni¹, Arthur DeCarlo² and Neil Hunter¹

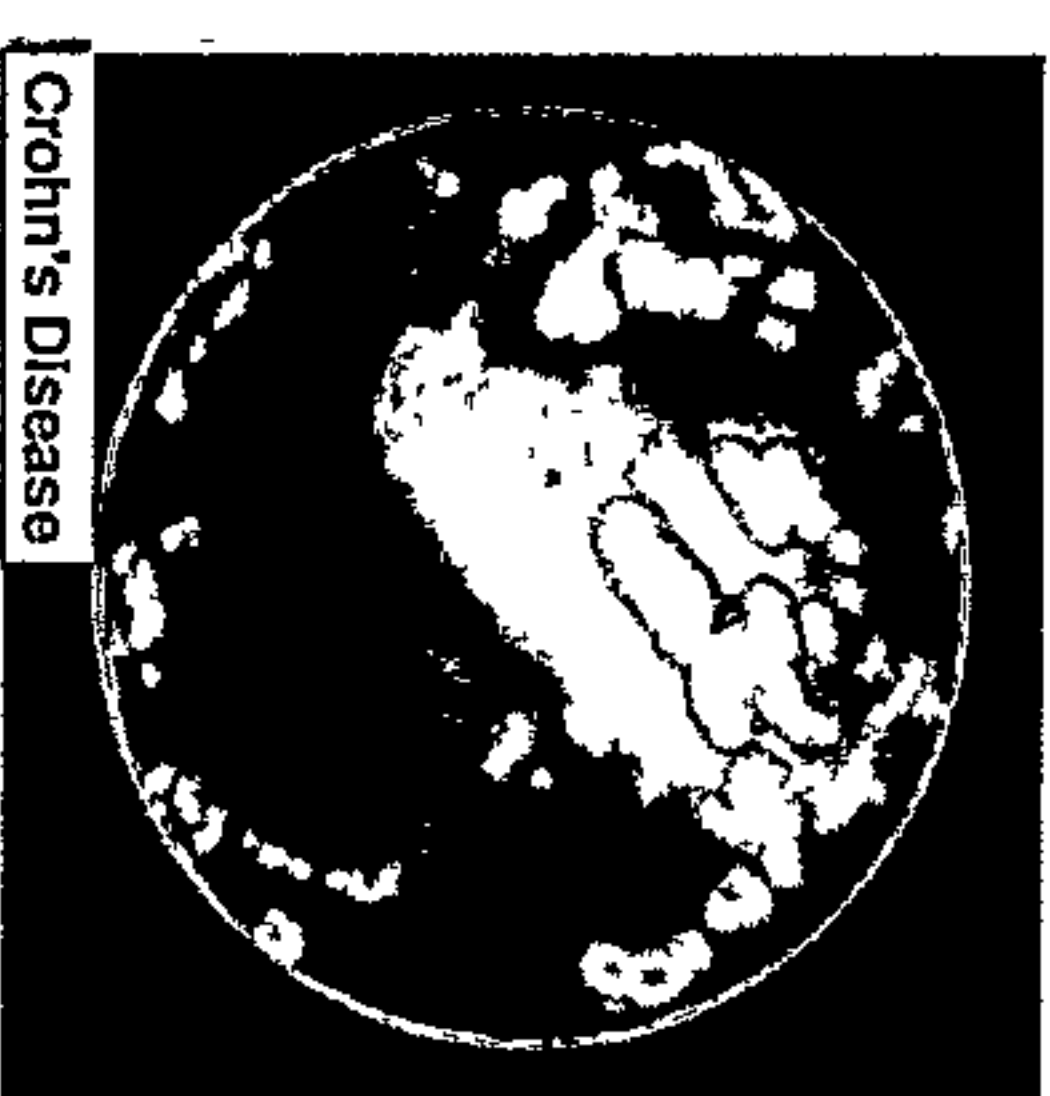
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Attack by the immune system against the host is the basis for the pathology of a diverse group of diseases. Of particular importance is the inappropriate recognition of the epithelium of the skin, mucous membranes and glands.



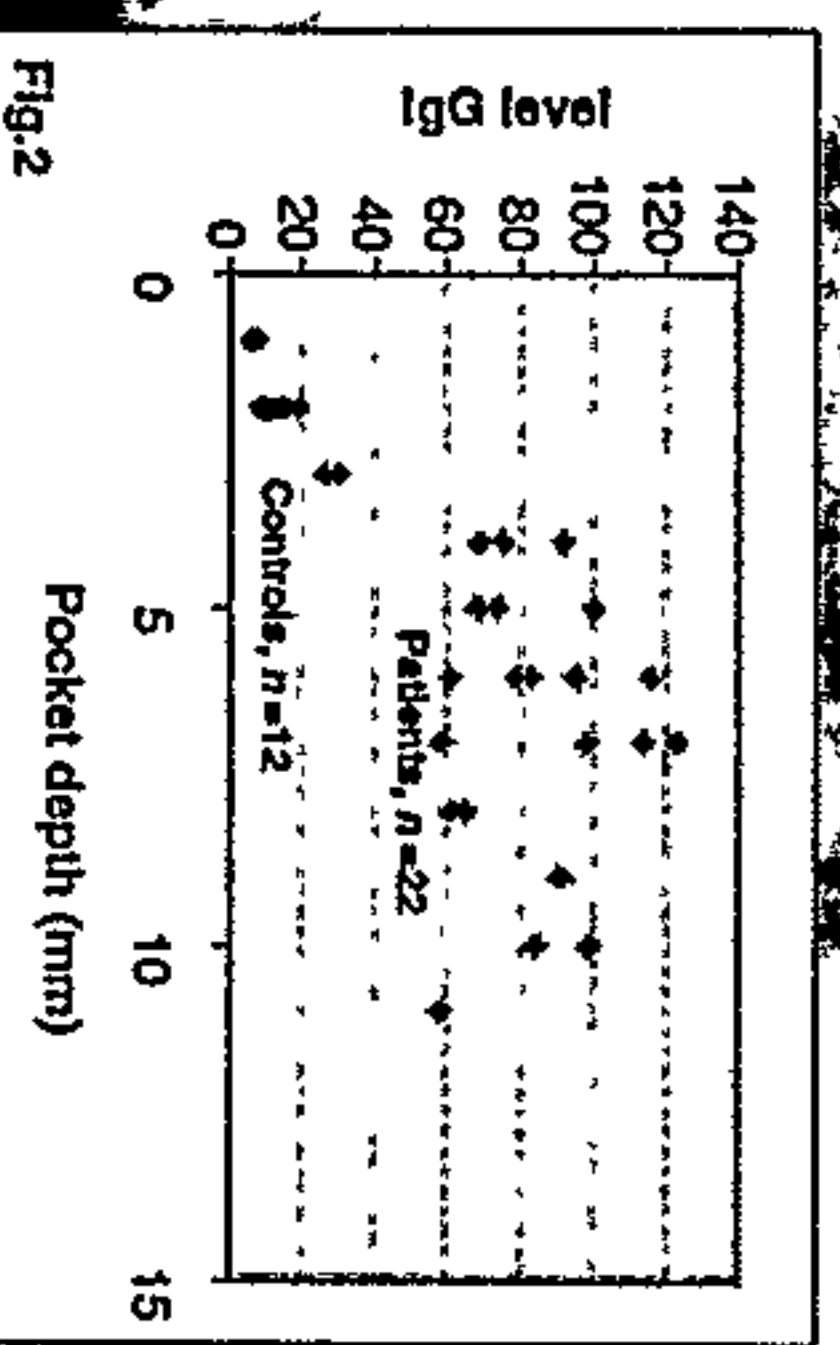
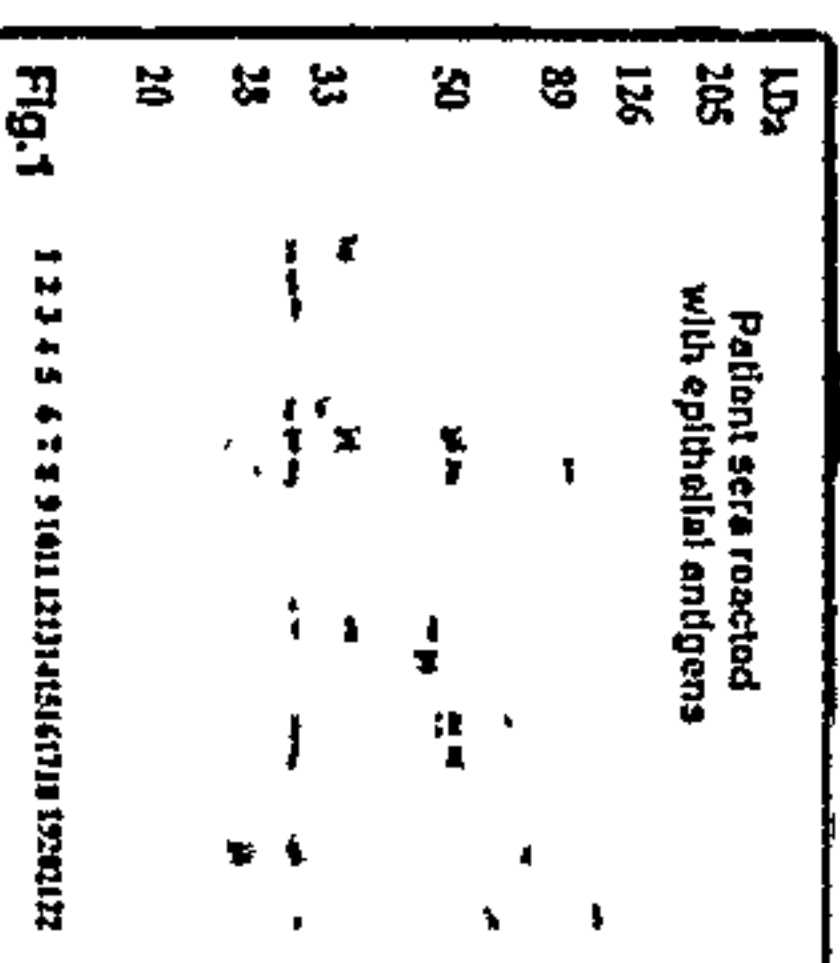
Pempigus vulgaris

Critical in the generation of autoimmune disease is the escape from normal regulatory mechanisms that prevent recognition of self components. In this context, there is much speculation but limited evidence for the role of infection. One mechanism is the property of microorganisms of mimicry of the structure of tissue components. Recognition of the microbial structures by the immune system could lead inadvertently to attack against self.



Crohn's Disease

Patients with chronic gum diseases (periodontitis) uniformly had antibodies that recognised epithelial components whereas a control group of periodontally healthy subjects did not. Levels of antibodies reactive with epithelial components (Fig. 1) were positively related to the severity of disease (Fig.2).



Epithelium-specific antibodies from the periodontal disease group were employed to screen for recognition of the pathogenic microbial flora of dental plaque associated with periodontitis (Fig.3). Reactive bacteria were cultured and identified using DNA sequence information (Table 1) and shown to comprise commonly isolated members of the oral flora.

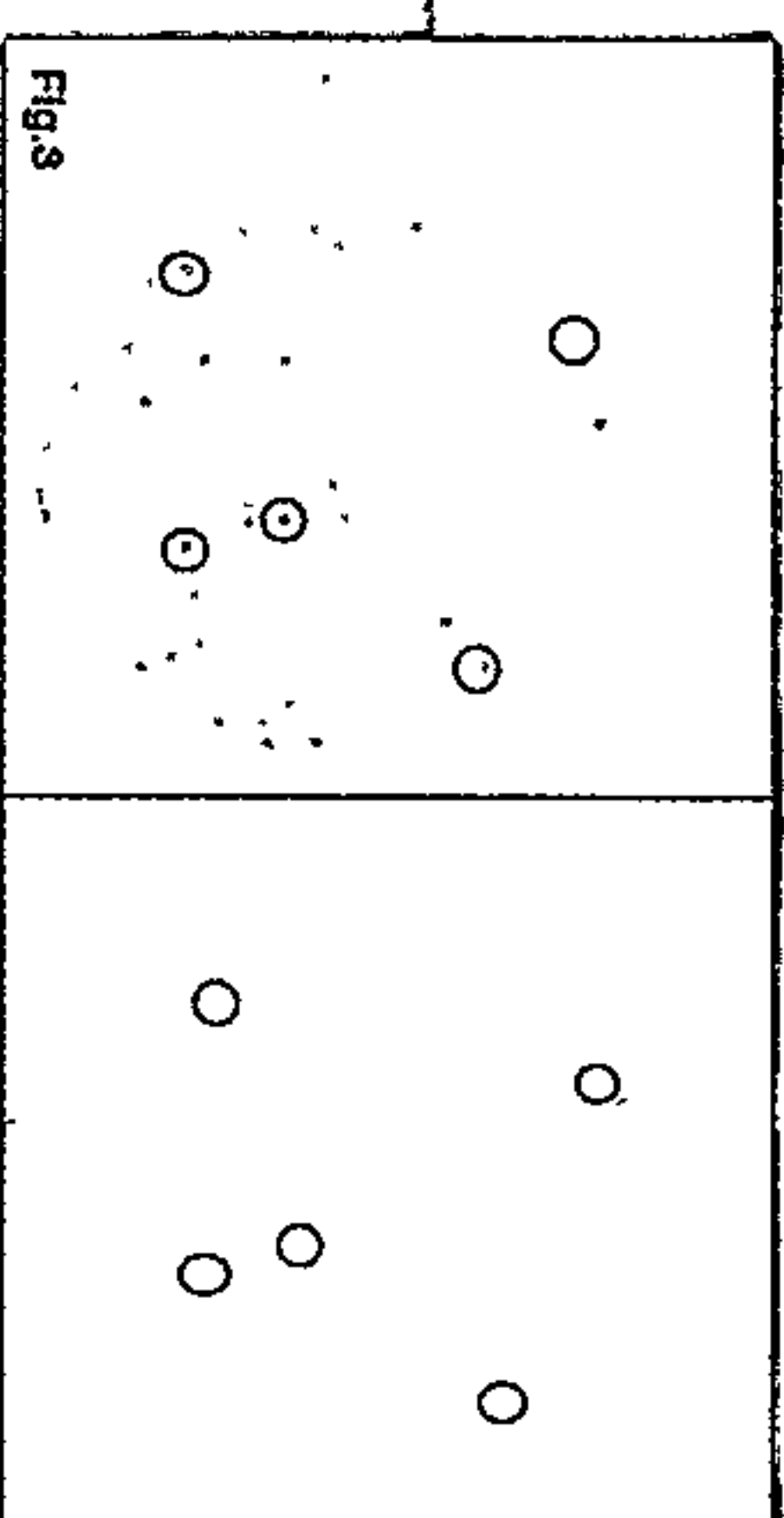


Fig.3

Table 1. The frequencies of detection of cross-reactive bacteria from 16 plaque samples

Bacterial strains/clone	Anaerobes n (%)
<i>Streptococcus mitis</i> (NCTC 12261)	2 (12.5)
<i>Streptococcus constellatus</i> (206)	3 (18.6)
<i>Streptococcus intermedius</i> (ATCC 27335)	4 (25.0)
<i>Streptococcus intermedius</i> (488)	3 (18.6)
<i>Actinomyces georgiae</i> (DSM 6843)	1 (6.0)
<i>Actinomyces</i> sp. oral clone (BL005)	1 (6.0)

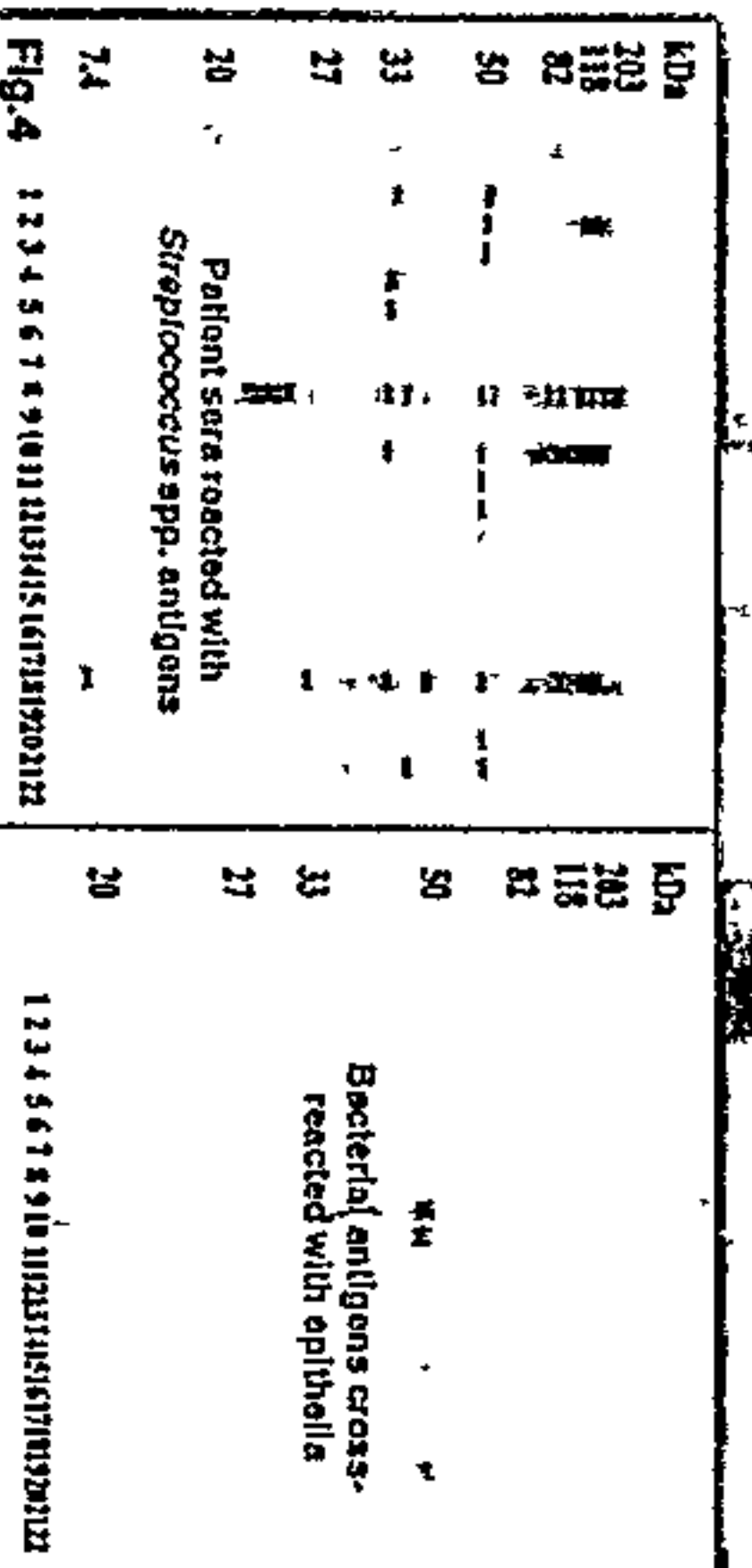


Fig.4

As predicted, serum antibodies from periodontitis patients recognised many of the antigens of the target bacteria, including a restricted number of epithelial cross-reactive antigens (Fig.4) while sera from the periodontally healthy controls showed only trace recognition of these common bacteria. Conversely, absorption of the sera with the target bacteria specifically removed reactivity for some of the epithelial antigens (Fig.5).

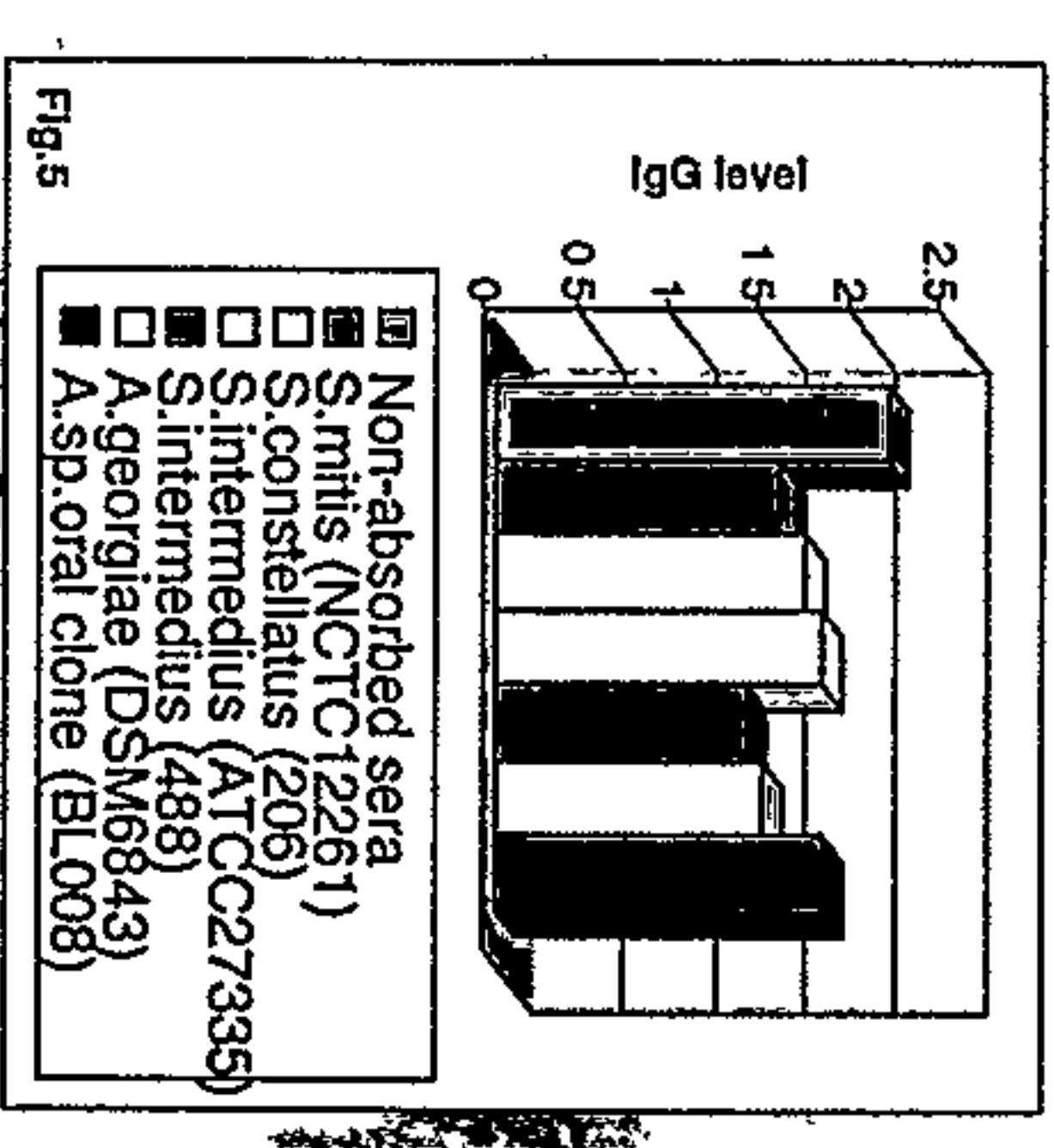
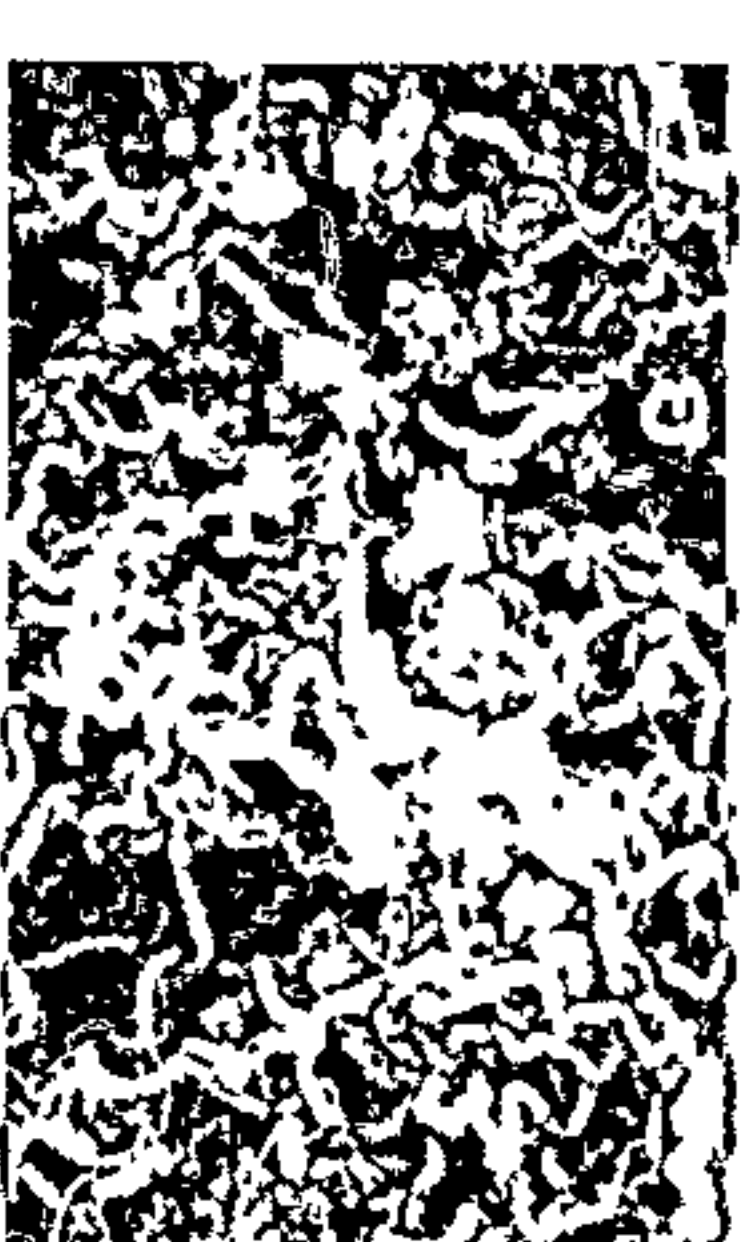


Fig.5



Periodontal disease & Psoriasis



CONCLUSIONS

During the course of development of chronic periodontitis there is increased immune reactivity against antigens of the bacterial plaque, including those antigens that mimic epithelial components. This has potential implications for the progression of periodontal disease but also in sensitising the individual for more widespread autoimmune attack against epithelial tissues.