Oral Diseases (2004) 10, 99–105

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Periodontal Disease/Pathogenesis

Evidence for local production of antibodies to auto and non-self antigens in periodontal disease

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Autoimmune mechanisms may contribute to periodontal disease (PD) pathogenesis; autoantibody to collagen type 1 is produced at the periodontal site and local levels are found to be higher than in serum.

OBJECTIVES: To find any evidence of autoimmune destruction in diseased periodontal tissues in patients with periodontitis. The study examines the relationship of antibodies to a self antigen collagen Type 1 and antigens from two periodontal pathogens namely *Porphyromonas gingivalis* (Pg), *Actinobacillus actinomycetemcomitans* (Aa) and a non-oral bacterium *Bacteroides fragilis* (Bf) in disease sites and in serum.

MATERIALS AND METHODS: Granulomatous tissues from periodontally diseased sites and serum samples were obtained from 13 patients (15 sites) undergoing surgical therapy. Tissues were homogenized at 4°C on Tris saline buffer [1 g (5 ml)$^{-1}$], homogenate was centrifuged and the resultant supernatants were used in assays. Antibody to collagen and Aa, Pg and Bf in tissue eluates and serum were determined by competitive enzyme linked immunosorbant assay (ELISA) and conventional ELISA respectively using an alkaline phosphatase/p-nitrophenyl phosphate enzyme-substrate system. Sera from age and sex matched healthy subjects and pooled human serum were used as controls. Antibody (Ab) levels in tissues and serum were standardized by concomitant albumin assay.

RESULTS: Level of antibodies to collagen type 1 in tissue was significantly higher than in serum ($P = 0.0001$). Antibody levels in tissue to Pg were significantly higher than in serum ($P = 0.0271$). Ab levels to both Aa and Bf in tissues and serum were not significantly different from each other.

CONCLUSIONS: These findings confirm the process of the local production of antibodies to autoantigen namely collagen type-1 and to bacterial antigens in the granulomatous tissues housed within the periodontal lesions in patients with periodontitis.

Oral Diseases (2004) **10**, 99–105

Keywords: collagen; periodontitis; autoimmunity; pathogens; granulomatous tissue

Introduction

As with any other joint in the body the joint between the tooth and the alveolar bone is dominated by collagen fibers; collagen type 1 being the predominant form in all four tissues of the periodontium. Detection of elevated immunity to type 1 collagen in sera of patients with periodontal disease (Ftis, Singh and Dolby, 1986) led to the suggestion that autoimmunity may play a role in periodontal disease. This was further supported by the report of proliferation of peripheral blood lymphocytes in response to type 1 collagen (Mammo, Singh and Dolby, 1982). Hirsch *et al* (1988) further supported this concept by providing evidence that the number of cells secreting Igs to collagen type 1 in inflamed gingiva was higher than the number of cells secreting immunoglobulins (Igs) to collagen type 1 in peripheral blood mononuclear cells. Anusaksathien *et al* (1992) have also demonstrated that the levels of antibodies to collagen type 1 in periodontal tissues were above the levels detectable in serum from the same patients suggesting auto antibody production occurs predominantly at the diseased sites. However, Anusaksathien *et al* (1992) did use conventional enzyme linked immunosorbant assay (ELISA) to detect anti collagen type 1 antibodies. The preliminary assays carried out in the present study revealed that there were high levels of non-specific binding when conventional ELISA was used to detect anti collagen type 1 antibodies. This threw doubt on the method used to detect anti collagen type 1 antibodies by Anusaksathien *et al* (1992). Hence the present study used competitive ELISA to detect anti collagen type 1

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Received 12 November 2001; revised 24 May 2003; accepted 5 June 2003

antibodies in order to overcome the problem of non-specific binding. We explored the relationship of the tissue and serum levels of antibody to collagen type 1 and to antigens derived from three bacteria: two periodontal pathogens namely, *Actinobacillus actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg) and one non-oral bacteria *Bacteroides fragilis* (Bf). A previously developed competitive enzyme linked immunosorbant assay for collagen and conventional ELISA for the bacterial antigen preparations measured antibody levels to the chosen antigens in granulomatous tissues from diseased sites and autologus sera. Contamination of tissue fluid with antibodies derived from serum was allowed for by assessing the albumin levels in autologous sera and in the tissue cytosol.

Materials and methods

The study group consisted of 13 patients who were attending the periodontology clinic, Dental School UWCM Cardiff, UK. All the patients were destined to undergo surgical periodontal therapy for teeth with periodontal pockets measuring 6 mm or more and all consented to participate in the study. There were three males and 10 females with a mean age of 37 years and a range of 25–57 years. Age- and sex-matched subjects were used as controls for the patient sera assay.

Sampling tissue specimens and blood for sera

Reverse bevel gingivectomy was performed and muco-periosteal flaps were raised at the selected sites and the granulomatous tissues housed within the bony defects were carefully dissected out using scalpel blades. The tissues were then washed in normal saline to remove blood from the surface and stored in preweighed containers in liquid nitrogen.

Three millilitres of blood was obtained by venipuncture immediately prior to surgery and allowed to clot at 37°C, serum separated by centrifugation at 2000 ***g*** and stored at −20°C until used in assays.

The wet weights of tissues were determined and they were then transferred to clean Petri dishes where they were minced into small pieces. The minced tissues (75–475 mg per sample) were placed in glass tissue grinders, Tris saline buffer (pH 7.6) was added [1 g (5 ml)$^{-1}$] and the tissue homogenized at 4°C. The resultant fluid was poured into 1.25 ml capacity micro centrifuge tubes with the tissue homogenate and centrifuged at 18000 ***g*** for 5 min. The resultant supernatant was separated and used in subsequent antibody assays.

Bacterial antigen (Ag) preparation

Bacteroides Fragilis ATCC 25285 (Bf), *P. gingivalils* ATCC 33277 (Pg) and *A. actinomycetemcomitans* Y4 (Aa) were cultured in brain heart infusion broth supplemented with haemin under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) (Dr William Wade in the Dental School Heath Park, Cardiff kindly provided the bacterial strains for the study). Bacteria were harvested after 48–72 h by centrifugation at 10000 ***g*** for 10 min and washed twice in sterile PBS (pH 6.8) and suspended in distilled water. The organisms were then ultrasonicated for 2 min at 4°C and centrifuged at 5000 ***g*** for 30–45 min, supernatants were obtained and freeze dried. Protein concentrations© in the antigen preparations obtained were assayed by using a Kit (Sigma Chemical Co., St Louis, MO, USA) for micro protein assay and the preparations stored at −20°C until used in the ELISA. Preparation of collagen type 1 was carried out by using the Chung & Miller method (Chung and Miller, 1974) with some modifications.

Antibody Assays

Antibodies to bacterial antigens

Polystyrene microtitre plates (Dynatech Laboratories Inc, Chanilly, Virginia. 22027) were coated with 100 μl of the antigen preparation made in PBS; working concentrations of the antigens used were 10, 20 and 20 μg ml^{-1} for Aa, Pg and Bf respectively. These concentrations were determined by preliminary assays carried out using a range of different concentrations of each antigen in PBS. Plates were kept at 4°C overnight, washed with Tris Saline Buffer with Tween 20 (1 ml l^{-1}) (TSBT) three times and 150 μl per well of TSB with 1% BSA added and incubated at 37°C for 60–90 min in order to block non-specific binding sites. The plates were blotted off and 100 μl per well of test fluids were added in appropriate dilutions; with respect to all bacterial antigens, 1/100-serum dilution was used. The dilutions of tissue cytosols used were 1/50 with Aa and 1/20 with Pg and Bf Ags respectively. These dilutions had been determined in previous assays using different dilutions of tissue fluid. Plates were incubated at 37°C for 60–75 min and washed with TSBT ×3 and 100 μl per well of 1/1000 dilution alkaline phosphatase conjugated goat anti human IgG was added and incubated at 37°C for 60–75 min. The plates were washed ×3 with TSBT and 100 μl per well of disodium nitrophenol phosphate (Sigma Chemical Company) added at a [c] of 1 mg/ml in diethanolamine buffer (pH 9.8). After incubation for 30–45 min at 37°C the colour reaction was measured by using an ELISA reader at 410 nm (Dynatech, MR5000, Dynatech Labs Ltd, Guernsey, Channel Islands, UK).

Antibody assay to collagen type 1

As the conventional ELISA described above was found to lead to very high levels of non-specific binding of serum IgG, a competitive ELISA was used for the determination of antibody levels to collagen type 1. Fifty μg ml^{-1} was used as the coating concentration [c] of bovine collagen type 1 and 1/500 and 1/100 dilutions were used as working dilutions for sera and tissue cytosol respectively. Briefly, the plates were coated with 100 μl per well of collagen type 1 and kept at 4°C overnight, washed with TSBT ×3 and blocked with 1% BSA and incubated for 60–90 min at 37°C. The plates were blotted off and rabbit monoclonal antibody (Ab) to collagen type 1 added to detect the total binding of rabbit Ab to collagen. To a second line of wells was added the mixture of rabbit Ab and test fluid, in appropriate dilutions in equal amounts (100 μl per well). The plates were incubated for 1 h at 37°C, washed ×3

with TSBT and alkaline phosphatase conjugated anti rabbit IgG (gamma chain specific) added in 1/8000 dilution in TSB (100 μl per well) and incubated for 60 min. The plates were then washed ×3 with TSB and the substrate buffer added (100 μl per well) and incubated at 37°C, the colour reaction was read at 410 nm wavelength using an ELISA reader.

In all these assays wells with no antigen and no test fluid were used as negative controls.

Albumin assay

As two different body fluids were used, namely, blood and tissue cytosol, the concentration of IgG in the tissue cytosol had to be adjusted to allow for any serum contamination. Therefore the albumin concentration in both fluids was measured using an albumin assay kit (Sigma). The levels of antibody for bacterial antigens in test fluid were expressed as an optical density value per milligram albumin. Antibody levels to collagen in each test fluid were expressed as a percentage inhibition obtained per millilitre per milligram albumin as given below.

$$\frac{\text{OD for total binding} - \text{OD with test fluid}}{\text{OD for total binding} \times [\text{c}]\ \text{albumin in test fluid}} \times 100$$

Albumin levels in fluids were measured using an albumin assay kit (Sigma). The levels of antibody for bacterial antigens in test fluid were expressed as an optical density value per millilitre per milligram albumin. Antibody levels to collagen in each test fluid were expressed as a percentage inhibition obtained per millilitre per milligram albumin.

Statistical analysis

Paired student's *t*-test was used in the analysis of data with regard to antibody levels and non-parametric test (Mann–Whitney *U*-test) was used with regard to ratios.

Results

Antibody (Ab) levels to collagen type 1, whole cell protein of *P. gingivalis* ATCC33277, *A. actinomycetemcomitans* Y4 and *B. Fragilis* ATCC 25285 in serum samples and granulomatous tissues from diseased sites from patients with periodontitis were assessed in this study. Ab levels to collagen type 1 in tissue fluids obtained from patients were significantly ($P < 0.001$) higher than the levels in autologous sera (Figure 1). The levels of antibodies to collagen type 1 in patient sera and age and sex matched control sera were not significantly different ($P = 0.2709$) (Figure 2).

Antibody levels to Ags from a putative pathogen, Pg in tissue cytosol were found to be higher than in sera of 10 of 13 patients (Figure 3). This difference was statistically significant ($P = 0.0271$). Ab levels to Aa in periodontally diseased tissues and autologous sera were not significantly different from each other (Figure 4). However, five of 11 patients had elevated tissue Ab levels to Aa. One of those (pt 9 Figure 4) had very high level of Ab against Aa.

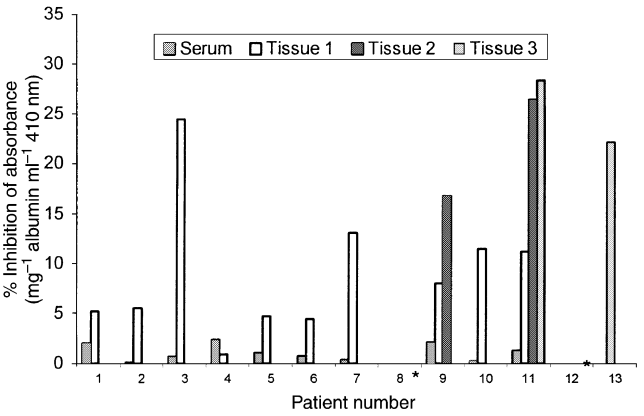

Figure 1 Antibody levels to collagen type 1 in tissue cytosol and sera of patients with periodontitis. *Inhibition = zero for samples 8 and 12

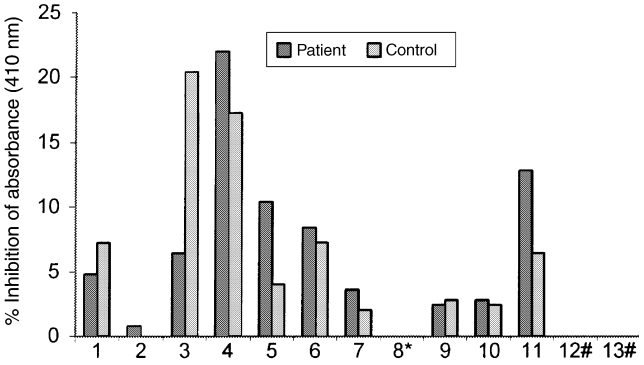

Figure 2 Antibody levels to collagen type 1 in Sera of patients with periodontitis and age and sex matched controls. *Sample not tested, #percentage inhibition = zero)

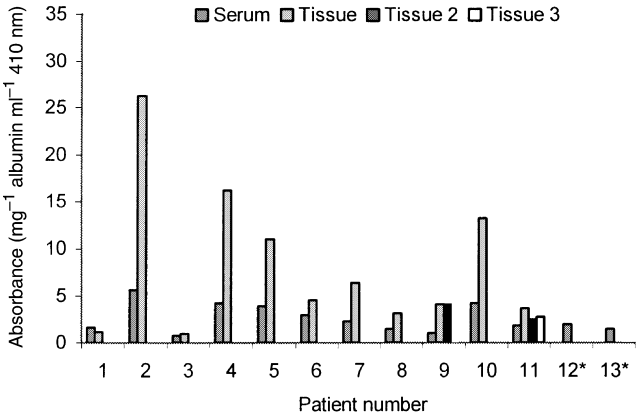

Figure 3 Antibody levels to whole cell protein from *Porphyromonas gingivalis* in tissue cytosol and sera of patients with periodontitis. *Serum levels = zero

Antibody levels to Pg (Figure 5a) and Aa (Figure 5b) in patients sera and age and sex matched control sera were found to be significantly different ($P = 0.0339$, $P = 0.044$ respectively). Ab levels to the non-oral bacterial antigen, Bf, in diseased tissues and autologous

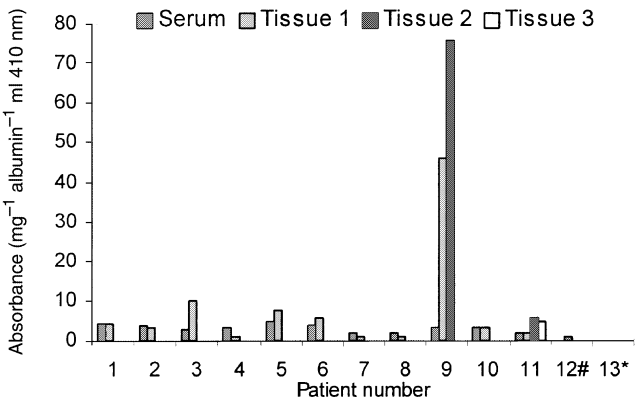

Figure 4 Antibody levels to *Actinobacillus actinomycetemcomitans* in tissue cytosol and sera of patients with periodontitis. #Serum level zero, *tissue and serum levels = zero

sera (Figure 5c), and age and sex matched control sera (Figure 5d) were not significantly different. Ab levels were compared after adjusting the albumin concentration in each test fluid (OD value per milligram albumin per millilitre). Antibody levels to Pg, Aa and Bf in patient 13 were not tested. When comparisons were made with regard to the ratio of specific antibody in tissue to serum for Pg and collagen type 1, ratios for collagen (Figure 6) were significantly higher than that for Pg ($P < 0.01$). However, it should be emphasized that this result relates only to the ratios of specific Ab in

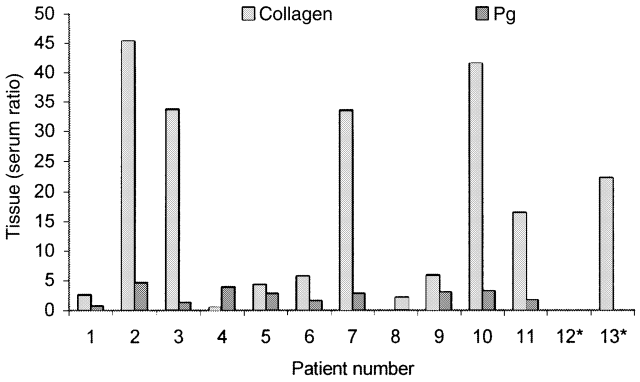

Figure 6 Tissue/serum ratio of antibody levels to collagen type 1 and Pg in patients with periodontitis

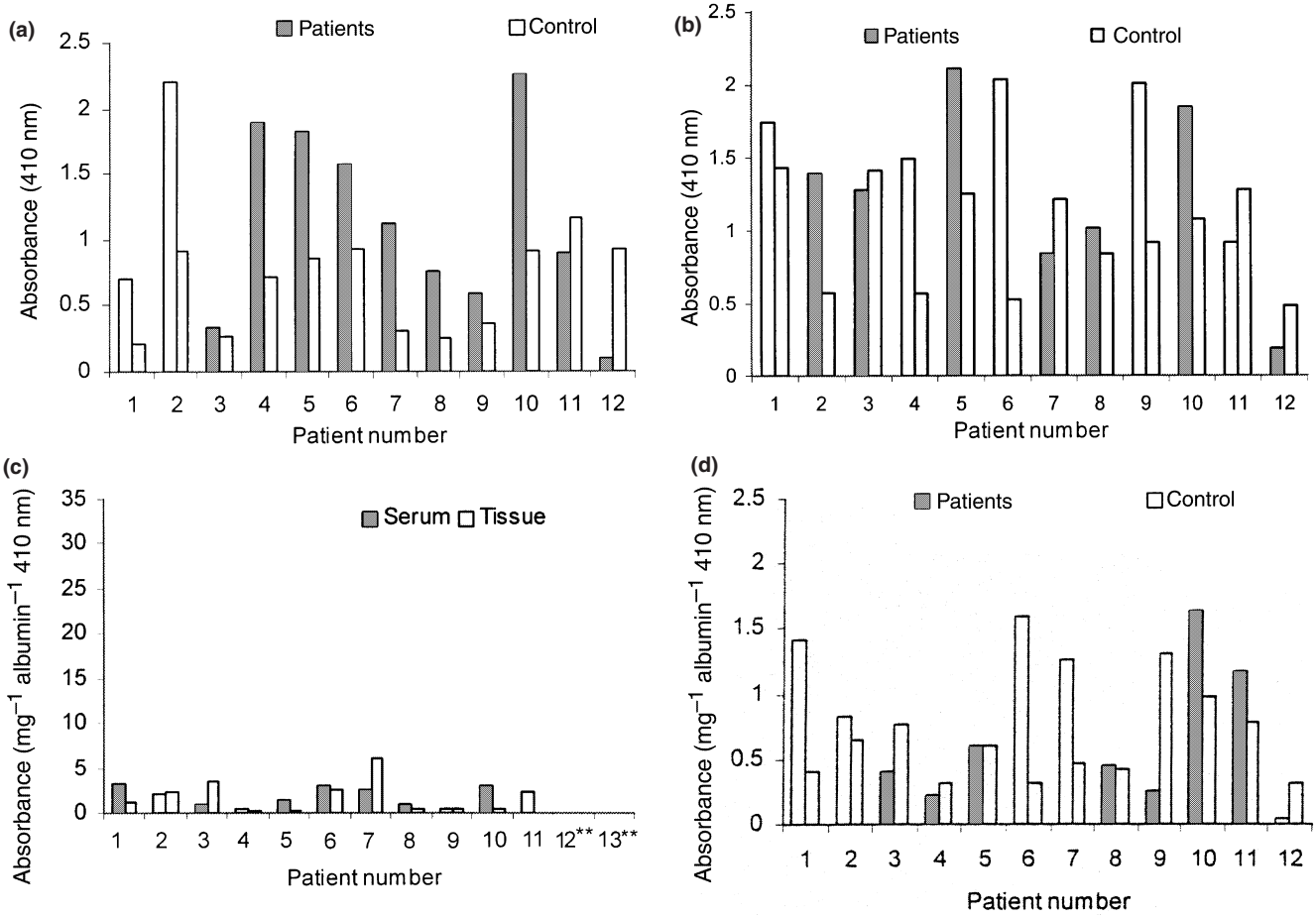

Figure 5 (**a**) Antibody levels to Pg in sera of patients with periodontitis and age and sex matched control subjects. (**b**) Antibody levels to Aa in sera of patients with periodontitis and age and sex matched control subjects. (**c**) Antibody levels to *Bacteroides fragilis* in tissue cytosol and sera of patients with periodontitis. (**d**) Antibody levels to Bf in sera of patients with periodontitis and age and sex matched control subjects.

tissue and serum, the absolute values may reveal a situation in which the ratio imbalance is of little consequence.

Discussion

Antibody levels to collagen type 1 in diseased periodontal tissues were significantly higher ($P < 0.0001$) than the levels in autologus sera suggesting local production of these antibodies. This finding is in accord with Anusaksathien *et al* (1992) and it also supports the findings of Hirsch *et al* (1988) where a high number of cells secreting Abs to collagen type 1 in tissues from diseased periodontal sites, was found whilst in the peripheral blood, cells secreting these Abs were rare. Antibody levels to collagen type 1 were assessed by competitive enzyme linked immunosorbant assay. As it was carried out by measuring the percentage inhibition of binding of rabbit anti bovine collagen type 1 monoclonal antibody to bovine collagen type 1 by test fluid, this technique could be considered as a reliable method in assessing specific anti collagen type 1 antibodies. Ab levels to collagen type 1 in patients' sera and in age and sex matched control sera were not significantly different. However, previous studies using conventional ELISA have detected differences in levels of Ab to collagen type 1 in patient sera and control sera (Hirsch *et al*, 1988). The earlier studies from this laboratory also have shown higher levels of antibodies to collagen in sera from patients with periodontal disease than in sera from disease free subjects (Ftis *et al*, 1986; Refaie *et al*, 1990). The use of conventional ELISA in that study compared with the competitive ELISA used in the present study may have contributed to these differences in results. This is further supported by the observed high level of non-specific binding antibodies when conventional ELISA was used to detect the anti collagen antibodies in the present study (data not shown).

The study of Mammo *et al* (1982) showed that there are more lymphocytes sensitized to type 1 collagen in the peripheral blood of patients with periodontal disease than of control subjects. However, according to the findings of the present study serum Ab levels to collagen type 1 do not significantly differ in patients with periodontitis and age and sex matched controls. A possible explanation for this could be that there are less B cells primed for collagen type 1 in the peripheral lymph nodes than in the diseased periodontal sites so that as a consequence the systemic Ab response to collagen in patients remains as low as noted in the control sera. Findings of previous studies suggest the importance of the existing number of primed B cells specific for a particular antigen in a given site on the level of antibodies associated with that particular site (Reinhardt *et al*, 1988, 1989). Alternatively, anti collagen antibodies found in serum and tissues may have arisen against different epitopes of the collagen molecule (Morgan *et al*, 1993). Thus the anti collagen antibodies detected in the tissues may have been produced by a pathologic clone of B lymphocytes, against a different epitope on the collagen molecule. This explanation would be in accord with the findings of studies in sera of patients with thyroid disorders where the auto Abs to thyroglobulin recognizes mainly the region 11 (Piechaczyk *et al*, 1987) on the thyroglobulin molecule whereas natural auto Abs to thyroglobulin from healthy subjects showed a wide heterogeneity with respect to their recognition of antigenic domains on the thyroglobulin molecule, and there was preference to region v (Bouanani *et al*, 1989) supporting the presence of an oligoclonal proliferation of pathologic B cells. However, further studies are necessary to determine the epitope specificity of anti collagen type 1 Abs in patients with chronic periodontitis. The present findings, that serum levels of Ab to collagen type 1 do not differ between patients and control subjects, may also be due to the low immunogenicity of collagen, which is insoluble in aqueous solutions. The limited release of products of collagen degradation into peripheral circulation may also lead to priming of a limited number of B cells to produce antibodies. Although macrophage processing of antigens undoubtedly takes place, it is claimed that when the macrophages with antigen arrive at the lymph nodes the antigens would have been degraded and cleared by them without contributing significantly to the immune response (Butcher and Weissman, 1989). In contrast the greater solubility of bacterial antigens is in favour of maintaining a more effective immune response in the lymph nodes via antigen antibody complexes (Abe, Hara and Aono, 1991).

Antibody levels to Pg in diseased periodontal tissues of patients were significantly higher than that in sera ($P = 0.0271$) suggests local production of antibodies. This confirms the findings of Smith *et al* (1985) and Spindler *et al* (1986) where there was a high local production of antibodies in the granulomatous tissue. In this study Ab levels to Aa in tissues of patients were not significantly higher than in sera which confirms the findings of Smith *et al* (1985) in which they found tissue Ab levels to Aa in the majority of the subjects fell within the range of Ab levels of control sera. Occasional high responders to Aa in both these studies may be explained by the differences in antibody production in different periodontitis sites (Ebersole *et al*, 1982; Reinhardt *et al*, 1988).

Furthermore, all patients in the study received preliminary prophylaxis and root planing, a procedure which has been shown to reduce the number of Ab secreting cells (Jonsson *et al*, 1991), but it is conceivable that equal efficacy of treatment was not achieved in all cases (Loos *et al*, 1989). Thus antibody production to Aa may have been lowered in some but not all cases to a level, which did not differ significantly from the serum levels. Again the failure to find levels of antibodies to the periodontal pathogen Aa, at significantly higher levels in diseased tissues than in sera, as was found in the studies of Smith *et al* (1985) and Tew *et al* (1985), could be due to differences in the type of periodontal disease experienced by the patients in the study groups. In both these studies patients with juvenile periodontitis were included and Aa is thought to be important in the pathogenesis of

this disease (Murray and Genco, 1980). In particular, in Tew's study all patients were young adults with generalized or localized juvenile periodontitis (Tew *et al*, 1985) and antibody levels were standardized on the basis of serum albumin content. However the patient no. 9 (Figure 4) who was 33 years of age with generalized deep pocketing (6–10 mm) and severe bone loss had a considerably high level of Ab to Aa, which agrees with Tew *et al* (1985). Although the Ab levels to Bf in sera of patients in this study were found to be higher than in tissue, this difference was not statistically significant, a finding which is in accord with Tew *et al* (1985), where there was no significant difference between levels of Ab in tissues and sera to a non-oral antigen (tetanus toxoid). Ab levels to Pg and Aa in control sera were significantly lower ($P < 0.05$) than in patient sera, a finding which is in agreement with previous work (Mouton *et al*, 1981; Ranney *et al*, 1981; Mandell, *et al*, 1987; Dibart *et al*, 1998).

These findings suggest that there is an antigen driven Ab response at the site, to Pg, Aa and collagen rather than a polyclonal response initiated by polyclonal B cell activators. The significantly higher levels of Ab to periodontal pathogens compared with collagen type 1 in the patients sera than that of the control group could be because of the higher number of B cells primed to bacterial antigen which would respond to polyclonal B cell activators or the specific antigen.

The finding in this study that levels of antibodies to collagen type 1 in diseased periodontal sites are higher than those in the autologous sera is similar to the pathological situation in rheumatoid arthritis, where collagen antibody levels are higher in synovial fluid than in autologous sera (Mestecky and Miller, 1975). It is not possible to say which Ab, auto antibody or non-auto antibodies, predominates in the periodontal tissues in this study as two different ELISAs' were used to assess the level of Abs collagen type 1 and bacterial antigens.

However it should be noted that the source of antigen driving the bacterial and collagen immune responses differ with regard to the facility with which each could be removed. Thus removal of the bacterial population of the pocket would have been sufficient to lower the level of antibody to associated antigens whereas scaling and root planing does not significantly lower the number of anti collagen type 1 secreting cells of diseased tissue (Jonsson *et al*, 1991).

Acknowledgement

We deeply appreciate Dr. Sharon Thomas's contribution to this study.

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