EXPRESSION OF AUTOREACTIVE B CELL (B-1a) IN PERIODONTAL HEALTH AND DISEASE –AN IMMUNOHISTOCHEMICAL STUDY

Dissertation submitted to

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BRANCH II

PERIODONTOLOGY

APRIL 2013

CERTIFICATE

This is to certify that this dissertation titled "**Expression of Autoreactive B Cell (B-1a) in Periodontal Health and Disease –An Immunohistochemical Study**" is a bonafide record of work done by **Dr. Keerthana Kumar .N** under my guidance during the study period of **2010-2013.**

This dissertation is submitted to **THE TAMILNADU Dr. MGR MEDICAL UNIVERSITY** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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LIST OF ABBREVIATIONS

1. Aa	–	Aggregatibacter actinomycetemcomitans
2. Ab	–	Antibody
3. Ag	–	Antigen
4. AgP	–	Aggressive Periodontitis
5. APC	–	Antigen Presenting Cell
6. APES	–	3 amino propyl tri ethoxy silane
7. B1-DMP	–	B-1 cell Derived Mononuclear Phagocyte
8. BCR	–	B Cell Receptor
9. BOP	–	Bleeding on Probing
10. C I	–	Collagen 1
11. CAL	–	Clinical Attachment Loss
12. CD	–	Cluster of Differentiation
13. DAB	–	diamino benzidine
14. DNA	–	Deoxy Ribonucleic Acid
15. DPX	–	di-n-butyl phthalate in Xylene
16. EDTA	–	ethylene diamino tetraacetic acid
17. ELISA	–	Enzyme Linked Immuno Sorbent Assay
18. GCF	–	Gingival Crevicular Fluid
19. HSP	–	Heat Shock Protein
20. HSS-HRP	–	High Sensitivity Streptavidin conjugated to Horse Radish Peroxidase

21. IFN	–	Interferon
22. Ig	–	immunoglobulin
23. IHC	–	Immunohistochemistry
24. IL	–	Interleukin
25. LI	–	Labelling Index
26. LPS	–	Lipopolysaccharide
27. MHC	–	Major Histocompatibility Complex
28. mRNA	–	messenger Ribonucleic Acid
29. PBMC	–	Peripheral Blood Mononuclear Cells
30. PBS	–	Phosphate Buffered Saline
31. PD	–	Probing Depth
32. Pg	–	Porphyromonas gingivalis
33. RF	–	Rheumatoid Factor
34. TGF	–	Transforming Growth Factor
35. Th	–	Helper T cells
36. TNF	–	Tumour Necrosis Factor
37. Treg	–	T regulatory

ABSTRACT

BACKGROUND

Autoreactive B cell (B-1a) may play a role in the pathogenesis of periodontal disease and this study was undertaken to elicit the autoimmune nature of B-1a cell in periodontal health and disease.

MATERIALS AND METHODS

Thirty gingival tissue samples were collected from two groups of patients (periodontal health-Group A and periodontal disease- Group B). The tissue sections were paraffanised and processed. B-1a cell expression in connective tissue was determined using CD5+ as a marker by immunohistochemical analysis. Intensity of staining was assessed and interexaminer error was calculated by kappa analysis. The mean labeling index was statistically analysed using independent ‘t’ test.

RESULTS

A good interexaminer agreement was observed between two examiners while assessing intensity. Comparison of mean labeling index between the two study groups was not statistically significant since p value was >0.01 (p value = 0.276).

CONCLUSION

An increase in the number of autoreactive B-1a cells (CD5+) has been found in periodontitis as compared to healthy gingival specimens, which was found to be statistically insignificant.

Keywords:

B-1a cells, Periodontitis, Immunohistochemistry

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INTRODUCTION

Periodontitis is a common multifactorial disease that leads to irreversible attachment loss, bone destruction and eventually tooth loss. Although the initiation of periodontal disease is thought to be of microbial origin[36] further progression and tissue destruction is largely modulated by host related factors.[78] Periodontal disease activity is generally accepted to be a result of an imbalance between periodontal pathogens and immune response of the host.[50]

The definitive host response in periodontal disease is thought to be provided by adaptive immune responses as the disease process has largely been correlated to a few putative periodontopathogens.[89] Page and Schroeder 1976[77] reported that during the early stage of inflammation, the cellular infiltrate is mostly T cells, whereas in the established lesions, B cells predominate.

In response to exogenous antigenic stimuli, the B-lymphocyte initiates humoral immune response. Initial exposure of immunocompetent B-lymphocytes to foreign antigen at the site of entry or in peripheral lymphoid tissue results in sensitization of these B-cells and they respond by undergoing blastogenic transformation and replication to become a plasma cell that is capable of producing biologically active immunoglobulins.[25] B-cells express the low affinity IgM constitutively which then undergo isotype switching to a

high affinity IgG. IgM and IgG2a activate the complement cascade to generate C3b and iC3b, which opsonise bacteria for phagocytosis. IgG1, IgG2b, and IgA bind to different Fc-R isoforms on macrophages, monocytes, and neutrophils *via* a separate internalization process to eliminate the pathogens.[65,71]

B cells have been classified into conventional B cells (B-2 cells) and unconventional B cells (B-1 cells) which are further subdivided into B-1a cells & B-1b cells.

B-1 cells, first characterized by Hayakawa et al in 1985,[40] express the phenotype `IgD low IgM high CD23- CD19+ CD11b+´ whilst conventional B cells are identified by the phenotype `IgD high IgM low CD23+ CD19+ CD11b- CD5-´. Further B-1a cells also referred to as autoreactive B cells; differ from B-1b cells by the expression of CD5 molecules in the former.[46] B-1a cells are predominantly found in pleural and peritoneal cavities, being few in the spleen and almost absent in lymph nodes and persist as self replenishing population.[12] In adults they represent 10-25% of B cells in blood and about 15-30% in tonsil. Contrary to the bone marrow origin of conventional B cells, B-1 cells are long lived and auto renewing.[53]

The concept of autoimmunity has in recent years, been expanded to include not only the typically autoimmune diseases, but also to chronic inflammatory lesions. Chronic inflammatory process that is characterized by

proteolytic breakdown of the extracellular matrix may result in formation of peptide/protein forms that may be perceived as foreign by the immune cells. These breakdown products may act as `foreign´, resulting in production of autoantibodies and/or evoking tolerogenic responses from the host. The presence of such autoimmune responses to collagen type 1 is common in chronic periodontitis, implying an interplay between periodontal infection and autoimmunity.[45]

The concept of autoimmune reactions involved in periodontitis was introduced by Brandzaeg and Kraus,[16] but there is no direct evidence linking them to tissue destruction. B-1a cells with autoreactive properties have been previously identified in periodontal disease.[10,23,94,5] B-1a cells have been suggested to play a role in host immune response in periodontitis as plasma cells may develop from both conventional B cells (B2) and B-1a cells.

However the physiology of B-1a cells and their functions on the immune system as a whole is yet to be fully understood. This is most probably due to their paucity and localization and to the difficulties in their culture characteristics.

This study was undertaken to identify the presence of B-1a cells in gingival tissue with and without periodontal disease, so as to have an insight into their pathophysiological role.

AIMS AND OBJECTIVES

The aim and objective of the present study was

- To identify the presence of B-1a cells in gingival tissues
- To evaluate the number of B-1a cells in gingival tissues in periodontal disease and compare it with the healthy samples using immunohistochemistry.

REVIEW OF LITERATURE

Periodontitis is a chronic inflammatory disease of multifactorial origin, where there is a complex interplay between host immune complexes.[36]

PATHOGENESIS OF PERIODONTITIS

Changes in the biofilm mainly an increase in gram-negative micro-organisms,[92] with early vascular changes occuring in the periodontium, along with exudation and migration of phagocytic cells (neutrophils and monocytes/macrophages), into the junctional epithelium and gingival sulcus, resulting in initial gingival inflammation. These changes are accompanied by increase in the size of the connective tissue infiltrated by leukocytes and loss of perivascular collagen fibers. The resulting cellular and fluid exudates cause further breakdown of the adjacent connective tissue and epithelium, followed by proliferation, apical migration, and lateral extension of the junctional epithelium. All of these alterations contribute to periodontal pocket formation.

As the disease proceeds to more advanced stages, tissue destruction involves significant alveolar bone resorption and loss of the collagen needed for tissue attachment. Widespread manifestations of inflammatory and pathological responses associated with periods of quiescence and active exacerbation become evident.[70,79] Much of the damage that occurs in periodontitis is the result of host immune and inflammatory responses to the invading pathogens. The early contact and interactions between the acquired

immunity and different innate immune cells[7] may have a drastic impact on the development of periodontal disease and/or its progression. It has been shown that both humoral and cell-mediated immune responses play important roles in the host defence against microbial infectious disease.[25]

B-CELL-MEDIATED HUMORAL IMMUNITY AND PERIODONTITIS

Page and Schroeder 1976[77] reported that during the early stage, the inflammatory infiltrate is mostly T-cells, whereas in the established lesions, B-cells become the most common inflammatory cells.

Seymour et al 1979[88] have shown in his study that in the pathogenesis of chronic inflammatory periodontitis, there is a conversion from a stable T cell lesion to a progressive B cell lesion.

Janeway and Travers 1997[44] stated that, after encountering invading pathogens, in particular extracellular micro-organisms, antigen (Ag)-specific naïve B-cells undergo affinity maturation via clonal selection, somatic hypermutation, and Ig receptor editing.

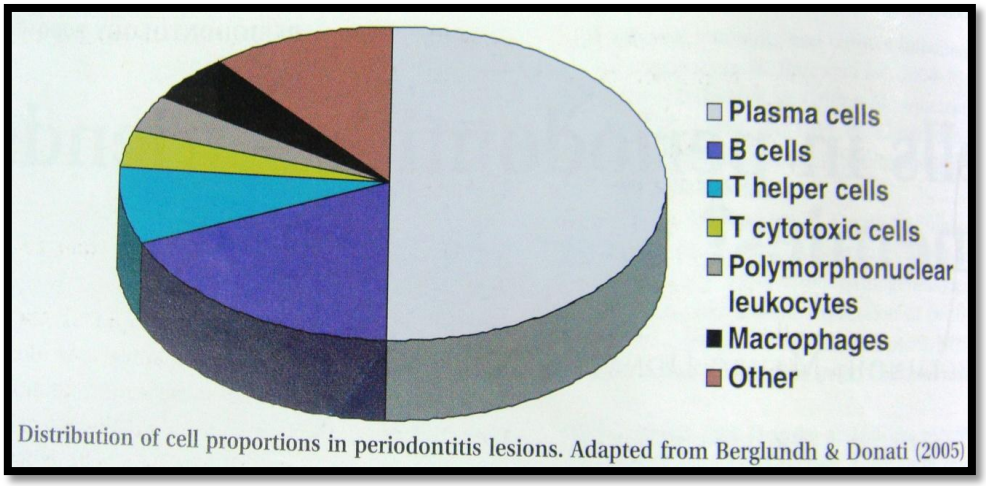
McGhee et al 1999,[65] **Nahm et al 1999**[71] suggested that certain cytokines such as interferon-γ (IFN-γ), interleukin- 4 (IL-4), IL-5, IL-10, IL-12, transforming growth factor-β (TGF-β) and bacterial lipopolysaccharide (LPS) are potent stimulators of Ab class switching i.e., IgG. Both IgM and

IgG2a (an IgG isotype) activate the complement cascade to generate C3b and iC3b, which opsonise bacteria for phagocytosis.

An early Ab-mediated control of periodontal infection through this process may limit the bacterial spread and/or associated tissue damage, thereby reducing the bacterial load for the subsequent acquired immune responses. Thus they are able to opsonise the invading pathogens, promote the FcR-mediated phagocytosis, neutralize the bacterial toxins, trigger complement-mediated lysis and Ab-dependent cytotoxicity and prevent bacterial entry at the mucosal surfaces, all of which enable the effective immune responses to provide the host protection from developing undesirable diseases.[71]

DISTRIBUTION OF INFLAMMATORY CELLS IN PERIODONTITIS

Berglundh T et al 2005[9] carried out a metaanalysis with regard to the cell composition in periodontitis lesions. It was reported that plasma cells represent about 50% of the cells present in lesions, while B cells comprise about 18%. The proportion of B cells was larger than that of all T cells and T helper cells were present in larger numbers than cytotoxic T cells. Polymorphonuclear leukocytes and macrophages were present at a proportion that represented about 5% of all cells.



Distribution of cell proportions in periodontitis lesions. Adapted from Berglundh & Donati (2005)

Mackler et al 1977[59] collected gingival tissues from chronic periodontitis subjects and healthy/gingivitis subjects and concluded that periodontitis group contained higher concentration of lymphocytes and plasma cells (IgG, IgM).

Seymour & Greenspan 1979[88] analysed 12 biopsies from chronic periodontitis patients and reported that majority of lymphocytes in the lesions had B cell phenotype and were positive for IgM and IgG.

Lindhe et al 1980[57] assessed the composition of inflammatory infiltrates in gingival tissues obtained from 22 subjects with advanced periodontitis and reported that plasma cells occupied 31% of the lesion volume, while proportion of lymphocytes varied between 5% and 10%. The study concluded that volume of plasma cells was three times larger than the proportion of lymphocytes.

Charon et al 1981[19] analysed gingival biopsies from patients with advanced periodontitis and reported that plasma cells dominated the lesions.

B CELLS AS ANTIGEN PRESENTING CELLS

The function of B cells in antigen presentation differs to some extent from that of other, so called professional antigen-presenting cells (APCs), e.g. Langerhans cells, macrophages and dendritic cells. Professional APCs take up antigens through pinocytosis or internalization of receptors for immune complexes, while B cells internalize antigens by an immunoglobulin-receptor (BCR) in the cell membrane. The antigen is degraded into peptides and subsequently attached to class II molecules of the major histocompatibility complex (MHC II). Finally, the processed antigen is transported to the B cell membrane for presentation to helper T cells (CD4+). B-1a cells may also serve as APCs in the development of an immune response to self-antigens.[56,81] Thus, B cells express class-II antigens upon stimulation and use the capacity of their memory systems in antigen presentation in periodontitis lesions.

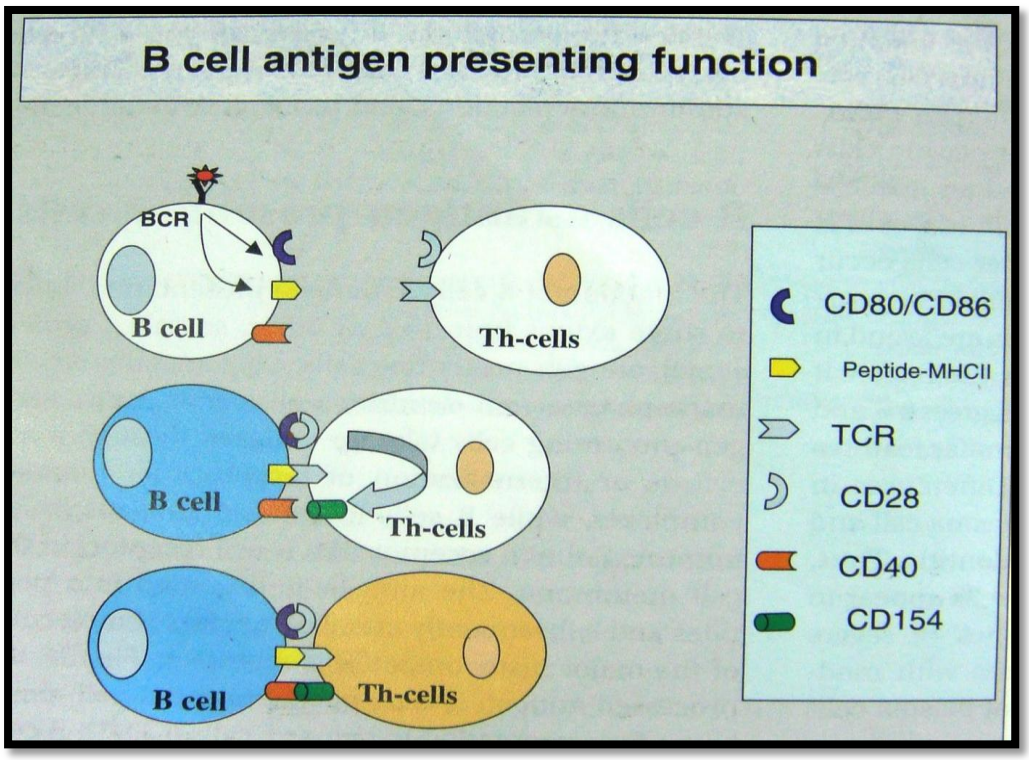
Orima et al 1999[75] studied 14 patients with moderate to advanced adult periodontitis by taking gingival biopsy samples and an immunohistochemistry was done to detect the expression of CD 40, CD40L, CD80, and CD86 on lymphocytes. It was concluded that most T cells and B cells expressed CD28 and CD80 and CD86 respectively in gingival tissues. The distribution of CD40 cells was similar to that of CD19 cells and the

percentage of CD40 cells in CD19 cells was nearly 100, suggesting a role of B cell in antigen presentation.

Gemmel et al 2001[30] studied 21 healthy individuals and 26 periodontitis subjects by taking gingival biopsy samples. An immune peroxidase technique was used to examine CD28, CD152, CD80, and CD86 in T and B-lymphocytes and macrophages. This study concluded that there were a higher percentage of CD86 cells which suggests a predominance of Th2 responses in both healthy and periodontitis tissues. The analysis of these cells in relation to the increasing numbers of B cells in proportion to T cells and also to macrophages, suggested that CD80 was expressed predominantly by macrophages while CD86 was expressed by both macrophages and B cells.

Mahanonda et al 2002[61] who observed a significant upregulation of CD86 and the dendritic cell-marker CD83 on B cells in periodontitis lesions, suggested that B cells may serve as potent APCs in the host response of periodontal disease.

Gemmel et al 2002[29] researched on the role of B cells as APCs in periodontitis in a study on gingival biopsies obtained from 26 subjects with periodontitis. The analysis revealed that the B cells were the predominant type of APCs in the biopsies containing the largest lesions.



B LYMPHOCYTE SUBSETS

B cells develop from hematopoietic stem cells initially in the fetal liver before birth and subsequently in bone marrow.[39] Among peripheral mature B cells there are 3 recognized subsets as identified in mouse models and to some extent in humans. First, B-1 cells, or unconventional B-lineage cells, are divided into B-1a and B-1b. The B-1a cells express the surface marker CD5, while B-1b cells do not. Second, B-2 cells, i.e. conventional B cells, are the traditional and representative B cells of the adaptive immune system. Third, marginal zone (MZ) B cells which are noncirculating mature B cells that segregate anatomically in the marginal zone of the spleen.[64,58]

The B-2 cells participate in T-dependent germinal center (GC) reactions and yield isotype-switched, high-affinity memory cells and long lived plasma cells. On the other hand, B-1 and MZ B cells are residing in specific anatomical compartments (e.g. peritoneal or pleural cavities and splenic MZ respectively) and are responsible for early antibody responses, which may be T cell independent as well as frequently polyreactive and with low-affinity.

DEVELOPMENT AND FUNCTION OF B-1a CELLS

B-1 cells, first characterized by Hayakawa et al in 1985, express the phenotype `IgDlow IgMhigh CD23-CD19+CD11b+´ whilst conventional B cells are identified by the phenotype `IgDhigh IgMlow CD23+CD19+CD11b–CD5–´. Further, B-1a cells differ from B-1b cells by the expression of CD5 molecules in the former.

B-1a cells are distinguished from conventional B cells (B-2) by their developmental origin, their surface marker expression and their functions. Compared to B-2 cells, B-1a are long-lived and self-renewing, with reduced immunoglobulin receptor (BCR) diversity and affinity.[46] B-1a cells also differ from B-2 by the expression levels of several surface markers, including IgM, IgD, CD5, CD43 and B220.[12]

In a recent study it was demonstrated that the expression of the marker CD5 makes B-1a cells survive following the immunoglobulin receptor

interaction.[28] It was suggested that CD5 promotes downregulation of the early immunoglobulin receptor reactions and hence, prevents B-1a cells from immunoglobulin receptor-mediated cell death.

B-1a cells, following activation by T cells, undergo class switching and somatic mutation which result in changes from production of low-affinity autoantibodies to IgG auto-antibodies with high affinity.[97,24] Recent studies have also shown that CpG microbial DNA released during infections may exacerbate autoimmunity by stimulating autoreactive B cells to switch from IgM to a more pathogenic IgG isotype independent from T cells.[41] In addition, other characteristics, such as the production of high level of IL-10[74,93, 28, 67] and enhanced antigen presentation capacities,[68,56,81] have implicated B-1a cells in autoimmunity.

IL-10 AND PERIODONTITIS – ROLE IN B1-A CELL PROLIFERATION

Interleukin-10 is a multifunctional cytokine with diverse effect on most hemopoietic cell types. It is produced by several cell sources such as monocytes/macrophages, dendritic cells (DC), B-lymphocytes (particularly the CD5+ B cells), various subsets of CD4+ and CD8+ T cells[69,67] and also by human keratinocytes.[83] The biological activities of IL-10 were confined to the suppression of pro-inflammatory cytokines produced by Th-1 cells[26] (IL-2, IFNγ, TNFα), but today it is known that IL-10 has several effects in immunoregulation and inflammation. Thus, IL-10 inhibits MHC class II and

co-stimulatory molecule expression on monocytes and macrophages and inhibits macrophage microbicidal activity by downregulating the production of reactive oxygen and reactive nitrogen intermediates, which are involved in macrophage killing mechanisms.[69] Other reports suggest that IL-10 may exhibit inhibitory and anti-inflammatory properties,[52,14]

The expression of CD5 on B1a cells has a dual regulatory role. Down-modulating the B1a cell responsiveness to BCR mediated signaling[13] and by this way controlling the production of a potentially autoreactive antibody repertoire, CD5 favours the secretion of interleukin-10 (IL10) by this cell subset.[28] B-1a cells are major producers of IL10,[74] a cytokine that contributes to the balance of type 1 and type 2 helper T cell (Th1/Th2) responses, that inhibit proinflammatory cytokine production by monocytes and macrophages and that controls the proliferation of antigen-specific CD4+ T cells by suppressing the antigen presenting capacity and decreasing co-stimulatory molecule expression by professional antigen-presenting-cells.[22] Thus, through the secretion of IL10, B-1a cells contribute to the orchestration of cellular immune responses involved in inflammation and autoimmunity.

Levy & Brouet 1994[55] showed that IL-10 enhances survival of normal human B cells (depending on their activation state), which correlates with increased expression of the anti-apoptotic protein bcl-2. Furthermore, IL-10 promotes proliferation of autoreactive B cells and autoantibody production. Indeed, several autoimmune diseases have been associated with increased

levels of IL-10. The presence of IL-10 in periodontitis lesions has been reported previously.

Yamazaki and co-workers 1997[99] applied a reverse transcription polymerase chain reaction technique to determine the level of mRNA for interferon gamma (IFNγ), interleukin 4 (IL-4), IL-10, IL-12 and IL-13 in gingival biopsies and peripheral blood mononuclear cells (PBMC) obtained from patients with chronic periodontitis. The mean expression of IFNγ was significantly higher in PBMC than in gingival tissues, while the expression of IL-10 mRNA was higher in gingival tissues than in PBMC.

Stein et al 1997[93] showed an increased level of IL-10 together with large proportions of B-1a cells in type 1 diabetic patients. It was suggested that periodontal pathogens induce a hyperactive IL-10 response leading to proliferation of B-1a cells and autoantibody production.

Aramaki and co-workers 1998[5] analyzed gingival biopsies from 10 periodontitis patients. It was observed that the proportion of autoreactive B lymphocytes (B-1a cells) and the amount of IL-6 and IL-10 were significantly higher in the inflamed gingival tissues than in peripheral blood from the healthy subjects.

Lappin and co-workers 2001[54] analyzed the occurrence of different cytokines in periodontitis lesions. Tissue biopsies were retrieved from 10

patients with chronic periodontitis and 10 subjects with aggressive forms of periodontal disease. Polyclonal nonspecific antibodies and oligonucleotide probes were used to detect cells expressing IL-2, IL-4, IL-6, IL-10, IL-15, TNFα and IFNγ and they reported that the most widely expressed cytokine in gingival tissues was IL-10.

Beebe et al 2002[8] stated that IL- 10 stimulates B cell proliferation, differentiation, antibody production and isotype switching.

Nakajima et al 2005[72] reported that the expression of IL-10 was higher in periodontitis than in gingivitis lesions. Thus, IL-10 expression is associated with severe periodontitis and appears to be an important cytokine in the regulation of periodontitis lesions.

ROLE OF SELF ANTIGENS

Autoantibodies against a wide range of self-antigens, such as collagen type I (CI), host DNA, laminin, fibronectin, and desmosin, have been described.[4]

De-Gennaro and co-workers 2006[21] showed increased autoantibodies to fibronectin and laminin, but not to CI, in patients with AgP.

Govze & Herzberg 1993[33] analysed blood samples and GCF of 10 periodontitis patients and showed increased reactions of IgG with desmoplakins and desmogleins, when compared to controls in sera. In GCF,

antidesmosomal IgG from diseased sites showed greater reactivity than healthy sites.

Schenkein et al 2003[87] in his study reported that the prevalence of patients with chronic periodontitis and generalized periodontitis positive for anti cardiolipin was greater than that in healthy controls. Analysis of data indicates that generalized periodontitis subjects have elevated levels of autoantibodies reactive with phospholipids.

Auto-Antibody to Collagen

Autoantibody to collagen, detected in periodontal disease, may represent either a response to local tissue damage or be the manifestation of a disturbance of the host immune response induced by the periodontopathogens and its products.

Although B-cell hyper-responsiveness has been reported in periodontal disease,[3] there is no evidence that patients who may possess an autoimmune predilection are also those who have high levels of antibody to collagen.

Grabar P 1975[34] reported that the immune response to collagen may be a physiologic event, reflecting an attempt to remove damaged components from the inflamed site.

Smith S 1980,[91] **Suzuki JB 1984**[95] stated that the reason for the raised level of collagen auto-antibody in periodontal disease is the action of the polyclonal B cell activators present in dental plaque.

Postlethwaite1984[82] concluded in his study that the activation of cellular immune responses by collagen, acting as an autoantigen, would lead to participation of aggressive lymphocytes and to the release of lymphokines which may modify behaviour of adjacent cells.

Ftis et al 1986[27] studied blood samples of 97 patients with periodontal disease by using ELISA and concluded that levels of antibody to type 1 collagen detected in patients were higher (p value <0.001) than in control subjects.

Hirsch et al 1988[43] in his study revealed that among the cells producing antibody to collagens I-VI sought in the diseased tissue, only cells producing antibody to collagen Type I, III, IV, V, and VI were increased in number, not those producing antibody directed against Type II collagen.

Peng TK et al 1988[80] demonstrated the presence of elevated levels of antibody to collagen in the sera or cells producing antibody to collagen in tissue of patients with periodontal disease.

Guilbert B 1982,[35] **Avrameas S 1988**[6] stated that collagen is included in the list of self-antigens and is present at low levels in apparently healthy individuals.

Rajapakse & Dolby et al 2004[85] studied by taking gingival biopsy samples from 13 periodontitis patients and periodontally healthy patients subjects by ELISA method for detection of antibody to collagen and Pg in tissue elutes and serum. They concluded that level of antibodies to collagen type 1 and porphyromonas gingivalis in tissue were significantly higher than in serum. Antibody levels to Aa in tissue and serum were not significantly different from each other.

Koutouzis and co-workers 2009[51] in a small pilot study have shown increased tissue autoreactivity and anti-CI antibody titers specifically in patients with localized AgP.

Autoantibody to HSP60

Heat shock protein 60 (HSP60) has also been suggested as another important candidate antigen. HSP60 belongs to a family of related proteins which have been conserved during evolution. Despite being highly homologous between prokaryotic and eukaryotic cells, HSP60 is strongly immunogenic, and immune responses to microbial HSP60 is speculated to initiate chronic inflammatory diseases in which autoimmune responses to human HSP60 may be central to pathogenesis.[47] Major periodontopathic bacteria such as Porphyromonas gingivalis,[60] Aggregatibacter actinomycetemcomitans,[73] Fusobacterium nucleatum, Prevotella intermedia, Taneralla Forsythia[98] and Campylobacter rectus[42] are reported to produce HSP's homologous to Escherichia coli GroEL.

Tabeta K 2000[96] demonstrated that the frequency of seropositivity and titers of antibodies to human HSP60 and P. Gingivalis GroEL were significantly higher in periodontitis patients than in periodontally healthy control subjects. Furthermore, affinity-purified serum antibodies to human HSP60 and P. Gingivalis GroEL cross-reacted with each other. These results suggest that an immune response based on the molecular mimicry between P. Gingivalis GroEL and human HSP60 may play a role in periodontitis.

Yamazaki et al 2004[100] studied blood samples of 21 patients with moderate to advanced chronic periodontitis to evaluate levels of antibody to human HSP60 before and after periodontal treatment by using ELISA and concluded that the mean level of anti human HSP60 antibody remained unchanged although individual levels of antibody either increased or decreased after periodontal treatment, suggesting that synthesis of these antibodies might be regulated independently during the course of periodontal infection.

Finally, the reason for the higher reactivity to HSP60 in periodontitis patients remains unknown. Further studies are required to elucidate the role of HSP60-specific T cells in chronic inflammatory conditions such as periodontitis.

ROLE OF B-1a CELL IN OTHER CHRONIC INFLAMMATORY LESIONS

An increase in the numbers of CD5+ B-cells or B-1a cells has been reported in patients with several autoimmune diseases, including rheumatoid arthritis,[101] Sjogren's syndrome,[103] systemic lupus erythematosus[90] etc.

Diabetes Mellitus

In a study by **Rebecca A. Smerdon et al 1994**[86] the percentage levels of total B-cells (CD20+) and the proportion expressing CD5 were increased in patients with recent-onset ($P < 0.001$ for both) but not long-standing type I diabetes compared with control subjects. Percentage levels of CD20+ B-cells were increased in prediabetic twins throughout the prediabetic period ($P < 0.05$) and there was an increased proportion of CD5-expressing B-cells that failed to reach statistical significance ($P = 0.08$). Percentage levels of CD20+ B-cells and the proportion expressing CD5 were normal throughout the study in twins remaining nondiabetic. These results showed an increase in B-cell percentage levels at the time of diagnosis of type I diabetes, which is because of an expansion of the CD5+ subset. These changes are also evident in twins throughout the prediabetic period, which suggests that they are related to the processes that lead to diabetes mellitus.

The role of CD5+ B-cells in the pathogenesis of autoimmune diseases, including type I diabetes, is unclear. The role of such an increase in B-cells in the pathogenesis of type I diabetes remains to be established.

Rheumatoid Arthritis

Chung E 1974,[20] **Gosslau B 1979,**[32] **Michaeli D 1974,**[66] **Mammo W 1982**[62] concluded in their study that both humoral and cell-mediated immunity to native and denatured Type I collagen is seen in the sera and synovial fluids of patients with rheumatoid arthritis, and it has been proposed that the autoimmunity may contribute to progression of the disease.

Hardy RR 1987[38] stated that rheumatoid arthritis is characterized by an increase in the number of CD5+ B lymphocytes.

Kipps TJ 1987[48] corroborates the findings of a study in monozygotic twins discordant for rheumatoid arthritis. In each case, the healthy twin had a level of CD5+ B cells similar to that of the twin with rheumatoid arthritis.

Burastero SE 1988[17] did a study to determine the state of activation of fluorescence-activated cell sorted CD5+ and CD5- B cells in patients with active rheumatoid arthritis, investigated light-scattering properties and incorporation of [3H] thymidine. It was found that most CD5+ B cells, but very few CD5- B cells, from rheumatoid arthritis patients were low density, large-sized, spontaneously proliferating cells. Moreover, these CD5+ B lymphocytes, but not their CD5- counterparts, spontaneously secreted high

levels of IgM, IgG, and IgA RF. The fact that CD5+ B cells are activated, proliferating, and producing RF in rheumatoid patients may be consistent with a role for CD5+ B cells in the disease process. Thus, unlike CD5+ B cell frequency, the CD5+ B cell activation state may be an indicator of disease activity.

Burastero SE 1989[18] with studies of rheumatoid arthritis patients demonstrated that CD5+ B cell levels did not correlate with RF levels in the serum. These findings suggest that CD5+ B cell frequency may not be directly related to disease activity, but rather to genetic background.

Youinou P 1990[101] in their study on rheumatoid arthritis patients and their healthy relatives suggested that family members may also have elevated proportions of circulating CD5+ B cells.

Chronic Lymphocytic Leukemia

Hall et al 2005[37] studied patients with autoimmune haemolytic anaemia with chronic lymphocytic leukaemia. The study concluded that fractions of putative antigen presenting cell population from peripheral blood of patients by negative selection showed that CD5+ chronic lymphocytic leukaemia B cells are the most effective cell type in processing and presenting purified rhesus protein to autoreactive T helper cells. Thus it provided evidence for malignant cells driving an autoimmune response by acting as aberrant antigen presenting cells.

Systemic Lupus Erythematosus (SLE)

Smith HR et al 1990[90] reported that in systemic lupus erythematosus the percentage of peripheral blood CD5+ B lymphocytes were increased to 68 % when compared to normal.

B-1a CELLS AND PERIODONTITIS

The presence of B-1a cells in periodontitis patients has been demonstrated in several reports.

Jonsson et al 1991[45] reported that plasma cells in periodontitis may develop from both B-1a and B-2 cells and antibodies produced by plasma cells may also be autoantibodies.

Afar et al 1992[1] collected blood samples from 18 patients with varying severity of periodontitis and from 16 healthy control subjects. Flow cytometry analysis revealed that B-1a cells occurred in significantly larger amounts in periodontitis patients than in controls.

Sugawara et al 1992[94] studied blood samples, GCF and tissue biopsies from 20 periodontitis and 10 healthy controls and concluded that the percentage of B cells bearing CD5 surface marker was statistically significant in gingival than in peripheral blood obtained from both the patients and healthy subjects. Anticollagen IgG antibody level in patients GCF was higher than that in sera from healthy subjects and slightly higher than in autologous

sera. CD5+ B cells produced considerably more IgM and IgG antibody to collagen than CD5- B cells.

Aramaki et al 1998[5] evaluated 34 adult periodontitis patients and 10 periodontally healthy controls. Blood samples and tissue biopsy samples were considered for ELISA and two colour flow cytometry for the analysis of immunoglobulins, cytokines, CD20+ and CD5+. The study concluded that the proportion of B-1a cells and the amount of IL-6 and IL-10 were significantly higher in the inflamed gingival tissues than in peripheral blood from healthy subjects.

Berglundh et al 1999[11] in a study on local and systemic features of host response in chronic periodontitis before and after non-surgical periodontal therapy found that the elevated numbers of B-1a cells did not decline after therapy despite the sufficient clinical signs of healing. Large amounts of B-1a cells were detected in the periodontitis lesions of chronic periodontitis patients in the study.

Berglundh et al 2002[10] analyzed B-1a cells in peripheral blood of 3 different groups of subjects; 22 subjects with severe generalized chronic periodontitis, 7 children with localized aggressive periodontitis and 26 healthy controls. The proportions of B-1a cells were 5-6 times greater in the periodontitis groups than in the controls and it was stated that up to 40-50 % of all circulating B (CD19+) cells were positive to the additional marker of CD5, i.e. the characteristic of B-1a cells.

Donati et al 2009[23] concluded in their study that B-1a cells constitute a significant part of the host response in periodontitis lesions and that plasma cells may develop from both B-2 and B-1a cells.

MATERIALS AND METHODS

STUDY POPULATION

The study population comprised of randomly selected patients from those attending the Department of Periodontics, Ragas Dental College and Hospital, Chennai. The study protocol was approved by the Institutional Review Board of Ragas Dental College. Prior to conducting the study, informed consent was obtained after explaining the study protocol to the patients. A total of 30 patients were randomly selected for the study.

The selected patients were divided into two groups:

Group A: 15 periodontally healthy subjects (5 males & 10 females of age 20-50 yrs (mean 35.6 years)) exhibiting no signs of periodontal disease as determined by the absence of bleeding on probing (BOP) and clinical attachment loss (CAL) , with probing depth (PD)≤ 3mm.

Group B: 15 subjects with chronic periodontitis (7 males & 8 females of age 20-65 yrs (mean 45.7 years)) exhibiting PD ≥5 mm, CAL ≥ 3 mm and presence of BOP in at least six sites.

Inclusion criteria:

1. Patients who were systemically healthy.

2. Patients who were non-smokers.

3. No history of anti-microbial therapy for the past three months.

Exclusion criteria:

1. Patients who had received periodontal treatment within the previous 6 months.

2. Patients with other dental problems such as pulpal diseases.

GINGIVAL TISSUE SAMPLE COLLECTION

Gingival tissue samples were obtained from periodontally healthy patients (Group A) during crown lengthening procedure, by placing an external bevel incision 1mm from gingival margin (1*3 mm tissue).

In patients with chronic periodontitis (Group B) gingival biopsy was obtained, one week after scaling and root planning (SRP). An internal bevel incision was placed 1mm from gingival margin (1*3 mm tissue) and tissue was excised during the modified Widman flap procedure.

The obtained gingival tissue specimens were fixed with 10% neutral buffered formalin. Prompt fixation was carried out to ensure the preservation of tissue architecture and cell morphology. The tissues were paraffin embedded within 48 hours, to avoid degradation of the antigens.

ARMAMENTARIUM FOR IMMUNOHISTOCHEMISTRY PROCEDURE

Instruments/Equipments

1. Aluminium foil
2. Microscopic slides
3. Detergent solution (Laxbro)
4. Autoclave
5. Beakers
6. Coplin jars
7. Cover slips
8. Electronic timer
9. Slide warming table
10. Light microscope
11. Measuring jar
12. Micropipettes
13. Rectangular steel trays with glass rods
14. Refrigerator
15. Sterile gauze
16. Tooth forceps
17. Diamond pencil
18. Coulter´s chamber

19. Weighing machine (Sartorius)

Reagents:

1. Distilled water
2. Acetone
3. Xylene
4. Absolute alcohol
5. Alcohol 70%
6. Alcohol 50%
7. Hydrogen peroxide 3%
8. EDTA buffer (pH 8.0)
9. Phosphate buffered saline (pH 7.0)
10. Harris's Hematoxylin
11. DPX

ANTIBODIES:

Primary Antibody:

R& D systems, Human CD5 Affinity Purified Polyclonal Ab (100 UG); Catalog Number AF1636, Minneapolis, USA.

Immunohistochemistry Kit:

- R & D systems Anti-Goat HRP-DAB Cell & Tissue Staining Kit; Catalog Number: CTS008; Minneapolis, USA.
    - **Peroxidase Blocking Reagent** - 6 mL of 3% Hydrogen Peroxide (H_2O_2).
    - **Avidin Blocking Reagent** - 6 mL of Avidin solution, containing 0.1% Sodium Azide (NaN_3).
    - **Biotin Blocking Reagent** - 6 mL of Biotin solution, containing 0.1% NaN_3.
    - **"Vial A" Secondary Biotinylated Antibodies** - 6 mL of either anti-mouse, anti-rabbit, anti-goat, anti-rat, or anti-sheep secondary antibodies, respectively, in 0.01 M PBS containing 0.1% NaN_3.
    - **"Vial B" High Sensitivity Streptavidin-HRP Conjugate (HSS-HRP)** - 6 mL of Streptavidin conjugated to HRP in 0.01M PBS containing 1% carrier protein, with preservatives and stabilizer.
    - **DAB Chromogen** - 2 mL of 2.5% 3, 3- diamino benzidine (DAB) in stabilizing buffer.
    - **DAB Chromogen Buffer** - 2 vials (15 mL/vial) of 0.1% H_2O_2 in Tris HCl Buffer.

SLIDE PREPARATION

Precoating procedure

Before taking the sections onto the slide, all the slides were coated with APES (*3 amino- propyl tri- ethoxysilane*). Pre coating procedure of the slides was as follows:

- Slides were first washed in tap water for few minutes.
- They were then soaked in detergent solution (Laxbro) for an hour.
- After 1 hour each slide was brushed individually using the detergent solution and was transferred to distilled water.
- Slides were washed in two changes of distilled water.
- Later slides were washed in autoclaved distilled water.
- The slides were then immersed in 1 N HCl overnight.
- The following day the slides were washed in two changes of autoclaved distilled water.
- All the slides were then transferred to slide trays, wrapped in aluminum foil and baked in hot air oven for 4 hours at 180° c.

APES coating procedure:

The slides were allowed to cool down and were then coated with APES using the following procedure.

- Slides were dipped in a Coplin jar containing acetone for 2 minutes.
- The slides were then dipped in a Coplin jar containing APES for 5 minutes.
- Following this, the slides were dipped in two changes of distilled water for 2 minutes each to remove excess APES and were left to dry.

TISSUE SECTIONING

- The paraffin embedded tissues were sectioned approximately 4 microns thick using a manual rotary microtome.
- The sections were placed in a water bath containing deionized distilled water preheated to 40-44º C to remove micro-wrinkles.
- Two tissue sections were placed flat, wrinkle and fold free on the APES coated microscope slides.
- Tissue sections were dried by placing on a slide warming table at 100° C for 60 minutes.

IMMUNOHISTOCHEMISTRY (IHC) PROCEDURE

Positive control

Tonsil specimens were used as positive control.

Negative control

Gingival tissue to which primary antibody was not added, was used as negative control.

Immunohistochemistry procedure was carried out according to the R&D systems IHC protocol.

Deparaffinisation of tissues sections

The slides with tissue sections were heated on a slide warming table at 100˚C for 2 minutes. The slides were treated with two changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with Phosphate Buffered Saline (PBS). Circles were drawn around the tissues, so that the antibodies added later did not spread and were restricted to the circle.

Antigen Retrieval

The slides were then transferred to coplin jars containing EDTA buffer and antigen retrieval was done in an autoclave at a temperature of 121°C and

pressure of 20psi for 15 minutes. Then the slides were allowed to cool till it reached room temperature.

Staining procedure

The slides were treated with 3% hydrogen peroxide for 15 minutes to quench endogenous peroxidase activity of cells that would otherwise result in non – specific staining. The slides were put in one change of PBS. Slides were then treated with avidin blocking agent (R&D systems, CTS008, Minneapolis, USA) for 15 minutess. Slides were rinsed in PBS following which the slides were treated with biotin blocking agent for 15 minutes. Avidin and biotin blocking agents were used to suppress endogenous avidin binding activity (EABA). Slides were again rinsed in PBS.

The primary antibody CD5 (R&D systems, AF1636, Minneapolis, USA) was added to the tissue on the slides. The stainless steel tray with glass rods containing the slides was incubated overnight at 2-8◦C. The slides taken out were washed in three changes of cold PBS for 5 minutes each, to remove the excess antibody. Then the slides were wiped carefully without touching the tissue section to remove excess PBS. Then 1-3 drops of biotinylated secondary antibody (R&D systems, CTS008, Minneapolis, USA) was added onto the sections and the slides were incubated for 1 hour. Later the slides were washed in three changes of cold PBS for 15 minutes each. The slides were again wiped carefully without touching the tissue section to remove excess PBS.

Then 1-3 drops of HSS- HRP from the secondary antibody kit (R&D systems, CTS008, Minneapolis, USA) was added onto the sections and the slides were incubated for 30 minutes. The sections were then washed in three changes of cold PBS for 2 minutes each. Then the slides were again wiped carefully to remove excess PBS. Then a drop of freshly prepared DAB (diamino benzidine – a substrate Chromogen) (R&D systems, CTS008, Minneapolis, USA) was added onto the sections and incubated for 15 minutes. Slides were then washed in three changes of PBS for 10 minutes each and then rinsed in distilled water to remove excess DAB and counter stained with Harris's Haematoxylin. They were then washed with alcohol and xylene. The tissue sections were mounted with DPX. Throughout the procedure, care was taken not to dry the tissues.

Observation:

All the slides were viewed under the light microscope at magnification 10x and 40x. The slides were checked for positive staining in the tissue sections. The strong brown immunostaining exhibited by the gingival tissue was taken to be positive. Thus the intensity of staining and the number of cells in the connective tissue was recorded.

Positively stained cells seen in the inflammatory infiltrate of connective tissue in all the slides were examined. The intensity of staining was observed by two examiners and graded as Negative (-), mild (+), Moderate

(++) and Intense (+++). The inter-examiner variation was determined by kappa analysis.

The number of positive cells in the connective tissue was manually counted using Coulter`s chamber and the mean labelling index was calculated.

$$LI = \frac{\text{Number of positive cells}}{1000} \times 100$$

IHC PROCEDURE FLOW CHART:

APES coated slides with sections preheated on slide warming table (100°C;2mins)

↓

Placed in xylene I (10 min)

↓

Placed in xylene II (10 min)

↓

Placed in 100% isopropanol (10 min)

↓

Placed in 70% isopropanol (5 min)

↓

Placed in 50% isopropanol (5 min)

↓

Washed in PBS (10 mins)

↓

Kept in EDTA buffer and autoclaved (20 psi ;15 mins)

↓

Cooled to room temperature

↓

Placed in 3 % hydrogen peroxide (15 min)

↓

Washed in PBS once (5 min)

↓

1-3 drops avidin blocking agent added (15 min)

↓

Rinsed in PBS

↓

1-3 drops biotin blocking agent added (15 min)

↓

Rinsed in PBS

↓

Primary antibody added & incubated for overnight at 2-8° C

↓

Washed thrice in PBS (5 mins)

↓

Incubated with 1-3 drops of biotinylated secondary antibody (1 hour)

↓

Washed thrice in PBS (15 min)

↓

Incubated with 1-3 drops of HSS- HRP (30 mins)

↓

Washed thrice in PBS (2 min)

↓

DAB was added and incubated (15 min)

↓

Washed with PBS thrice (10 min)

↓

Rinsed in distilled water (5 min)

↓

Stained with haematoxylin (30 seconds)

↓

Washed in distilled water

↓

Placed in 70% isopropanol (5 min)

↓

Placed in 100% isopropanol (5 min)

↓

Placed in xylene (5 min)

↓

Slides were mounted using DPX

↓

Slides were observed under the microscope

STATISTICAL ANALYSIS

Data entry and descriptive analysis was performed using SPSS version 10.0.5. Mean LI and standard deviation was calculated to assess B-1a (CD5+) expression.

- The intensity of staining was determined by two examiners and the inter-examiner variation was determined by kappa analysis.

- Independent `t´ test was used to compare the two groups, (Healthy and Chronic Periodontitis) with regard to the CD5 expression.

RAGAS DENTAL COLLEGE AND HOSPITALS, CHENNAI.

DEPARTMENT OF PERIODONTICS

PROFORMA

NAME: AGE: SEX: DATE:

ADDRESS: OCCUPATION:

OP.NO:

CHIEF COMPLAINT:

PAST DENTAL HISTORY:

MEDICAL HISTORY:

PERSONAL HABITS:

INTRAORAL EXAMINATION:

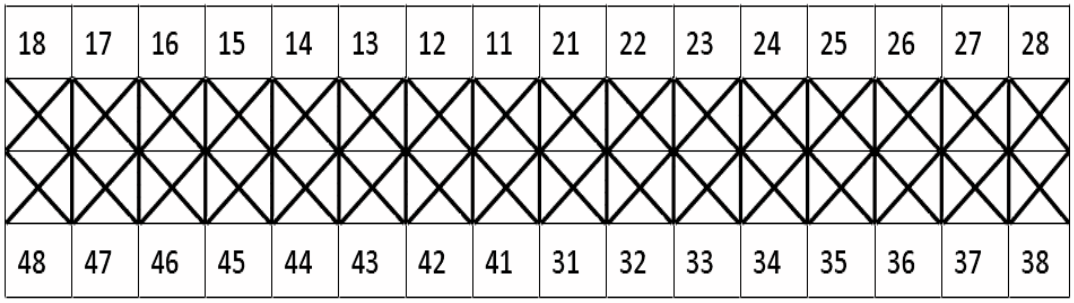
A.HARD TISSUE EXAMINATION:

B.SOFT TISSUE EXAMINTAION:

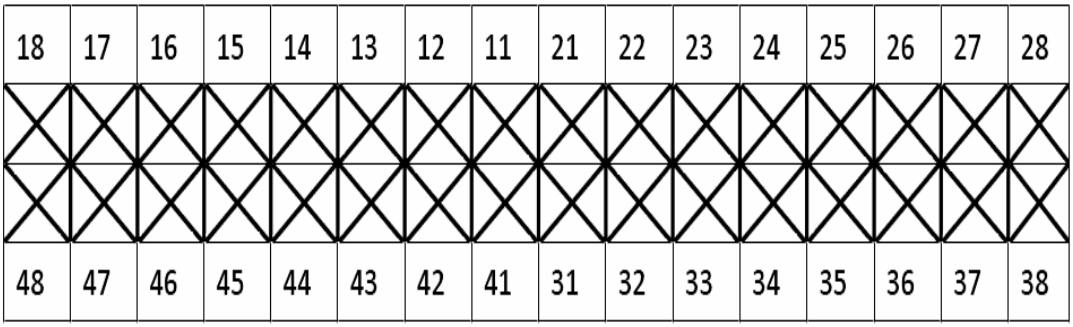
- GINGIVAL FINDINGS

- DENUDED ROOTS (MILLERS CLASSIFICATION)

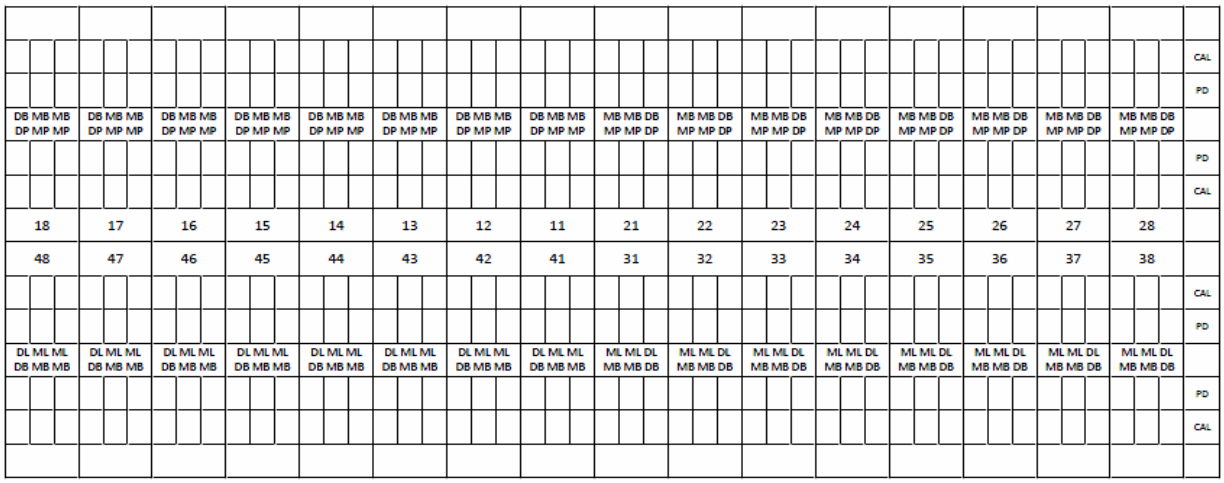
PLAQUE SCORE:



BLEEDING SCORE:



Measurement of Probing Depth and Clinical Attachment Loss



PROVISIONAL DIAGNOSIS:

TREATMENT PLAN:

BIOPSY DETAILS:

CONSENT FORM

I,_____s|o,w|o,d|o_____ _____ aged about _____ years Hindu/Christian/Muslim/ _____ residing at _____do hereby solemnly and state as follows.

I am the deponent herein; as such I am aware of the facts stated here under.

I state that I came to Ragas Dental College Hospital, Chennai for my treatment for ……

I was examined by Dr. _____ and I was requested to do the following tests.

1.

2.

3.

I was also informed and explained about the pros and cons of the treatment / test in the _____ (language) known to me.

I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full

consequences of the dissertation/ thesis /study and I undertake to cooperate with the doctor for the study.

I also assure that I shall come for each and every sitting without fail.

I also authorize the doctor to proceed with further treatment or any other/suitable/alternative method for the study.

I have given voluntary consent to undergo treatment without any individual pressure.

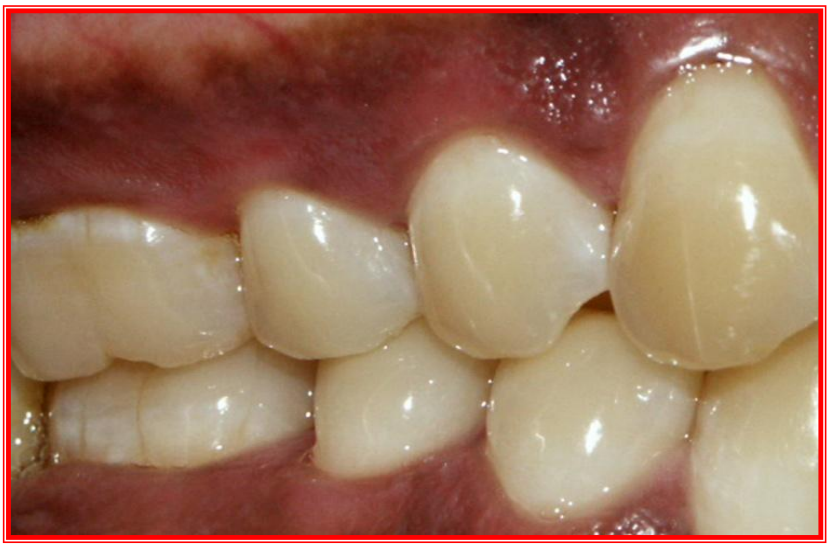
I am also aware that I am free to withdraw the consent at any time during the study in writing.

Signature of the Patient/Attendant

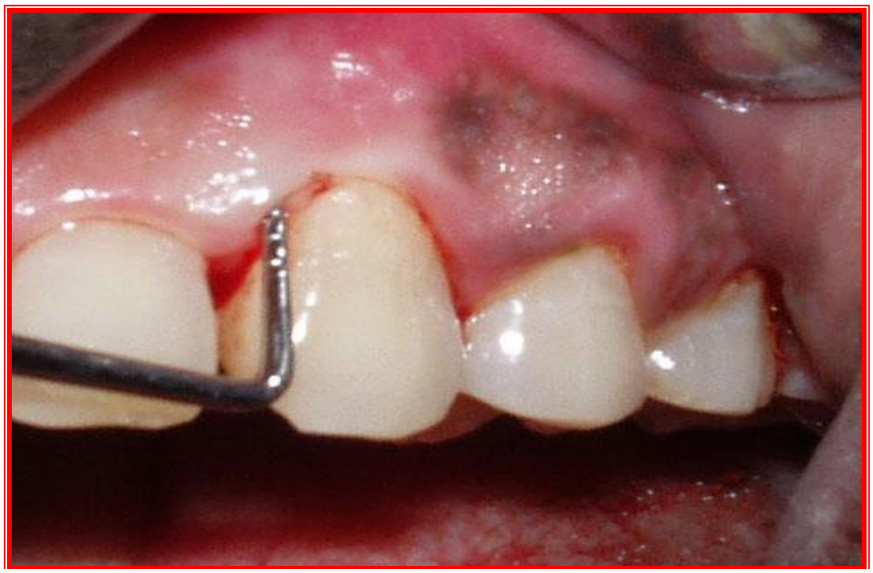
The patient was explained the procedure by me and he has understood the same and with full consent signed in (English/ Tamil/ Hindi/ Telugu/ _____) before me.

Signature of the Doctor

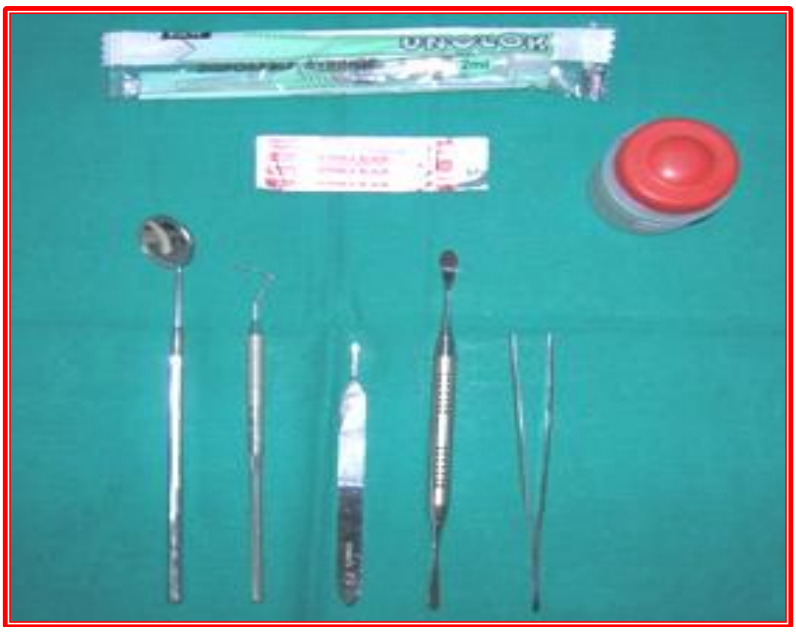
HEALTH GROUP (GROUP A)



DISEASE GROUP (GROUP B)



ARMAMENTARIUM



REAGENTS


ACETONE
APES
PBS
Hematoxylin
1%
AMMONIA


Xylene
I
Xylene
II
Alcohol
I 100%
Alcohol
II 70%
3% H_2O_2

PRIMARY ANTIBODY

SECONDARY ANTIBODY KIT

RESULTS

A total of 30 gingival tissue samples (15 health and 15 diseased) were evaluated for expression of CD5. (The results are summarized in Table 1 & Table 2)

Assessment of intensity

All the tissues were assessed for intensity of staining in gingival connective tissue. Intense staining was observed in 3 specimens in group A and 4 specimens in group B. Moderate staining was observed in 3 specimens in group A and 6 specimens in group B. Mild staining was seen in 3 specimens in group A and 4 specimen in group B. Brown staining was not seen in 1 specimen in group A. Therefore only 14 specimens in group A expressed CD5 in connective tissue while all 15 specimens in group B were positive for CD5 in gingival connective tissue. A good inter examiner agreement was observed between the two examiners (H-0.796, D-0.797).

Cell counting

Immunostaining of gingival connective tissue showed localization of CD5 to inflammatory infiltrate in connective tissue. The number of positive cells in lamina propria was evaluated using mean labeling index. The results of the mean labeling index were as follows: in group A, the mean labeling index was 39.72±9.49; in group B, 44.19±11.44. There was no statistically

significant difference in the mean labeling index between the two study groups, since p value is >0.01 (p =0.276).

Figure 1, 2,3 & 4 describes B-1a cells (CD5+) expression in health and disease when viewed under light microscope at 10x and 40x.

Table 1.

Tabulation of Intensity and Labelling Index in Gingival Tissue Samples

S. NO.	AGE/SEX	SITE	PPD	CAL	Procedure	Intensity 1	Intensity 2	LI	SLIDE NO.
1.	39/M	27-B	222	-	C L*	++	+++	39.77	4585
2.	20/F	17-B	2 3 3	-	CL	+	+	22.58	4519
3.	41/M	37-L	3 3 2	-	CL	+	++	32.32	4380
4.	49/F	36-B	3 3 3	-	CL	+++	+++	46.45	4521
5.	24/F	37-L	2 3 3	-	CL	+	+	34.44	4464
6.	25/F	25-P	2 2 2	-	CL	+	+	44.23	4694
7.	37/F	46-B	3 2 3	-	CL	+	+	32.45	4561
8.	24/F	36-L	3 3 3	-	CL	++	++	34.53	4234
9.	21/F	13-P	2 2 2	-	CL	+++	+++	58.36	4555
10.	25/F	25-B	3 2 3	-	CL	++	++	43.16	4592
11.	44/M	36-L	3 3 3	-	CL	+	+	39.69	4509
12.	32/F	21-P	2 3 3	-	CL	+++	+++	53.14	4627
13.	22/M	46-L	3 2 3	-	CL	+	+	35.22	4437
14.	22/F	13-P	2 2 2	-	CL	+	+	-ve (>10 cells)	4345
15.	24/F	36-L	3 3 3	-	CL	-ve	-ve *	-ve	4560
16.	60/F	11- P	6 6 6	7 7 7	MWF*	++	++	27.88	4359
17.	43/M	16-P	9 9 7	10 11 8	MWF	+	+	61.96	4499
18.	39/M	46- B	10 6 8	12 7 8	MWF	+++	+++	57.40	4327
19.	48/M	47-B	10 8 8	10 9 9	MWF	+++	+++	57.34	4488
20.	36/F	17-P	7 6 8	8 7 9	MWF	+	+	41.95	4443
21.	40/F	46-L	8 6 8	9 8 9	MWF	++	++	40.70	4554
22.	48/F	34-B	9 7 9	10 9 10	MWF	++	++	50.64	4540
23.	38/F	45 -L	6 5 6	777	MWF	+	++	24.44	4612
24.	37/F	46 -B	5 6 8	7 7 9	MWF	++	++	34.93	4379
25.	37/F	46 -L	6 4 6	7 5 7	MWF	++	++	31.91	4344
26.	37/F	16 -B	8 8 8	10 9 9	MWF	+	+	55.98	4247
27.	43/M	27-B	8 6 8	9 8 9	MWF	+	+	43.32	4674
28.	45/M	25-B	5 7 7	7 8 8	MWF	++	+	39.68	4720
29.	58/M	16-B	7 10 8	9 10 9	MWF	+++	+++	51.76	4711
30.	55/M	23-B	6 8 8	7 10 9	MWF	+++	+++	42.92	4719

*B- Buccal, P- Palatal, L- Lingual, CL- crown lengthening, MWF- modified Widman flap, -ve - Negative

Table 2.

Comparison of Intensity Scoring and Mean Labelling Index Between Health and Disease

	Group A(health)	**Group B (periodontal disease)**	**p value**
intensity	Intense - 3 Moderate - 3 mild – 8 Negative-1	Intense – 5 Moderate – 6 mild – 4	-
mean LI± SD	39.72+-9.49	44.19+-11.44	.276

[p value: <0.001- statistically significant; p value >0.001- not statistically significant]

Fig.1: EXPRESSION OF B-1a CELLS (CD5+) IN HEALTH (10x)

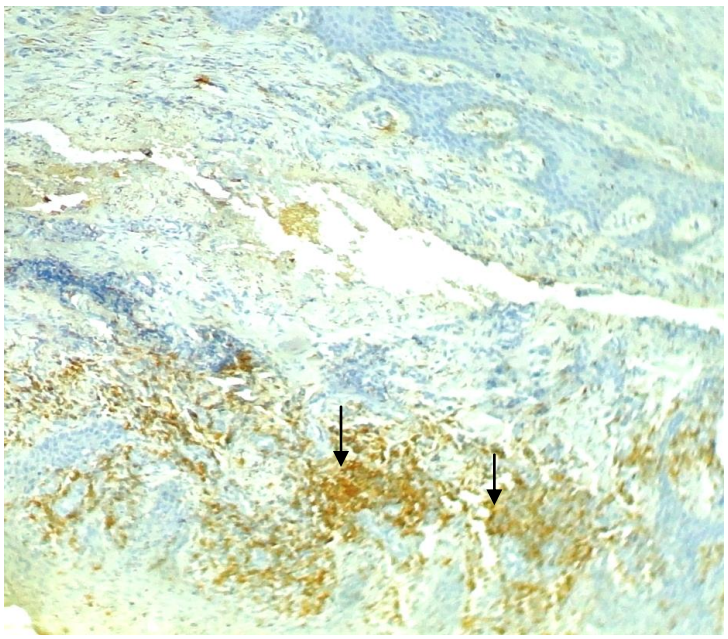
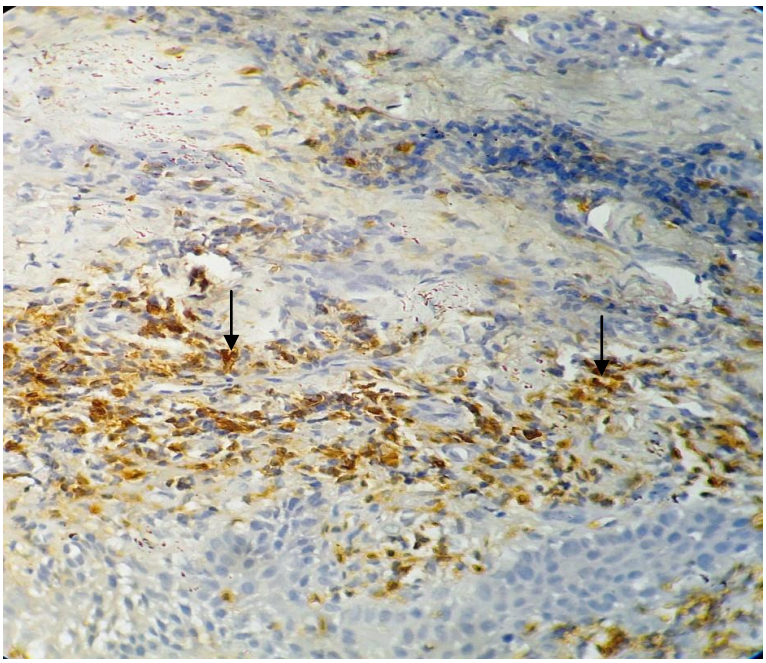


FIG.2: EXPRESSION OF B-1a CELLS (CD5+) IN HEALTH (40x)



↓ - indicates positive staining

Fig.3: EXPRESSION OF B-1a CELLS (CD5+) IN DISEASE (10x)

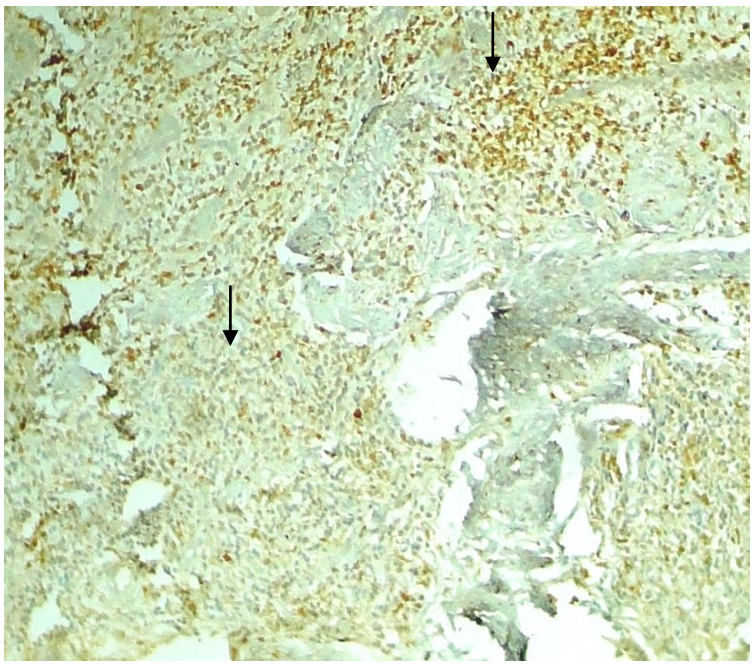
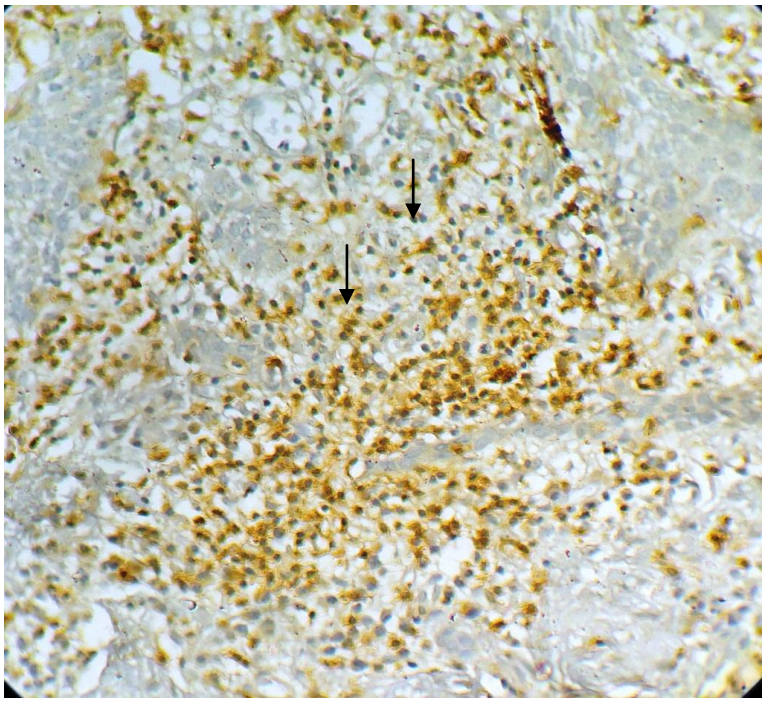


Fig.4: EXPRESSION OF B-1a CELLS (CD5+) IN DISEASE (40x)



↓ - indicates positive staining

DISCUSSION

Periodontal disease is a chronic inflammatory disease of multifactorial origin in which the normal balance between microbial plaque and host response is disrupted.[78] The pathogenesis of periodontal disease process involves both innate and adaptive responses which are specifically tailored against the microbial challenge. The host response is largely directed against exogenous antigens that are derived from periodontopathogenic bacteria such as Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola.[92]

Seymour et al 1979[88] have shown in their study that in the pathogenesis of chronic inflammatory periodontitis, there is a conversion from a stable T cell lesion to a progressive B cell lesion. Evidence for the importance of B cells in periodontal disease has come from the following studies. Lindhe et al 1980[57] assessed the composition of inflammatory infiltrates and reported that the volume of plasma cells was three times larger than the proportion of lymphocytes. Gmur et al[31] reported that IgG antibody levels to Porphyromonas gingivalis were highly correlated with the extent of periodontal destruction. Zambon et al[102] stated that there was a significantly increased antibody response to A.actinomycetemcomitans in localized early onset periodontitis.

B cells also serve as APCs and express class II antigens upon stimulation and use the capacity of their memory systems in antigen

presentation. Mahanonda et al 2002[61] showed a significant increase in CD86 and dendritic cell marker CD83 on B cells in periodontitis lesions and thus concluding that B cells may serve as potent APCs in immune response of periodontal disease.

B-1 cells, or unconventional B-lineage cells, are divided into B-1a and B-1b. B1a cells have the property of autoreactivity and self renewal. B-1 cells form a dominant population of B lineage cells in the peritoneal cavity.[48] The B-1a cells express the surface marker CD5, while B-1b cells do not.[64] The CD5 B cells (B-1a) and their phenotypic CD5 "twins" (B-1b cells) differ from conventional peripheral B cells (B-2) by anatomical location, developmental origin, surface marker expression, antibody repertoire and growth properties.[40,63] B-1a cells also differ from B-2 by the expression levels of several surface markers, including IgM, IgD, CD5, CD43 and B220.[12] Brandzaeg and Kraus 1965[16] showed the presence of autoantibody producing plasma cells in periodontitis lesions.

Autoimmunity is a condition characterized by a specific humoral or cell-mediated immune response against the constituents of the body's own tissues (autoantigens). An increase in the numbers of CD5+ B-cells or B-1a cells has been reported in patients with several autoimmune diseases, including rheumatoid arthritis[101] systemic lupus erythematosus[90] and type 1 diabetes mellitus.[86] Autoantigenicity may also be found in other chronic inflammatory disorders. Autoantibodies against a wide range of self-antigens,

such as collagen type I (CI), host DNA, laminin, fibronectin, and desmosin, have been described.[4] Ftis et al[27] have demonstrated the presence of elevated levels of antibody to collagen in the sera or cells producing antibody to collagen in tissue of patients with periodontal disease.

The present study was carried out to evaluate the expression of B-1a cell (CD5+) in gingival tissue in health and periodontal disease. Previous studies in this department have been carried out with other aspects of autoimmunity such as HSP60 and T regulatory cells. As sequel of previous studies, this study was conducted to have a better understanding of the autoreactivity of B-1a cells in periodontal disease.

Health samples were collected from subjects with PD <3mm, absence of CAL and absence of BOP (indicative of lack of inflammation) during crown lengthening by gingivectomy. Disease samples were collected from subjects with PD ≥5mm and CAL ≥ 3mm, during internal bevel incision given during flap surgery. All biopsy specimens obtained were fixed in formalin and paraffinised within 48 hours to prevent any loss of detail of the antigen structure. From each tissue portion, thin sections were prepared using microtome and exposed to immunohistochemical staining.

CD5 was chosen as a marker for the immunohistochemical analysis because.

- CD5 is the distinguishable feature between B1 and B2 cells as well as B-1a and B-1b cells
- CD5 has its role in B1a clonal expansion

The inter-examiner variation for intensity was evaluated to remove bias and was analysed by kappa analysis, which showed a good interexaminer agreement (health - 0.796; disease - 0.797). The cells were manually counted under 40x magnification and the mean labelling index for the total number of cells was calculated and a value of 0.276 was obtained.

The comparative results showed that there was an increase in the number of B-1a cells in periodontitis group compared to health, but not statistically significant.

Previous studies have validated the presence of B-1a cells in periodontal disease. The results of our study are in partial agreement with the studies of Donati et al[23] who showed that two thirds of B lymphocytes were positive for CD5 marker (B-1a) in tissue samples with periodontitis, Aramaki et al[5] and Berglundh et al[10] showed that 48.9% & 30-60% of B cells in periodontitis lesions were B-1a cells respectively. In contrast to the above studies, no comparative evaluation of the B-1a/B cell ratio was undertaken in our study. Therefore, the population of these cells in relation to total B cell infiltrate could not be evaluated. The significant difference between health and

disease previously reported could be related to the use of different, presumably more sensitive protein detection methods.

Our results are also in partial agreement with Afar et al[1] who reported significant increase in CD5+ B cells in peripheral blood of patients with chronic periodontitis. The study was however carried out in circulatory levels, unlike ours on gingival tissue samples.

The results of our study suggest that there may be a putative role for B-1a in the pathogenic process in chronic periodontitis. A previous study in this department on a tolerogenic immune cell (Treg cells), yielded similar results. Taken together, these results suggest that autoimmune destructive process may play a small but an important role in pathogenesis of periodontal disease.

The increase in number of B-1a cells (CD5+) in periodontal disease may be explained as follows:

- The conversion of autoreactive B cells to B1 cell derived mononuclear phagocyte (B1-DMP). B-1 cells migrate from the peritoneal cavity to a site of non-specific inflammation where they become morphologically similar to monocytes derived macrophages and have a high phagocytic ability.[2,15]

- B-1a cells undergo class switching and somatic mutation which result in changes from production of low-affinity autoantibodies to IgG auto-antibodies with high affinity.[24,97]
- Periodontal pathogens may induce a hyperactive IL-10 response leading to proliferation of CD5 positive B cells and to auto-antibody production.[28,67,74,93] Indirect evidence for this is derived from autoantibodies directed against collagen[51] and HSP60.[100]

The clinical implications of B-1a cells are yet to be fully elucidated. However, it is clear that the role of B cells is not unidimensional; it may play both destructive and protective roles. Therefore, treatment strategies in periodontal disease need to be directed not only against exogenous antigens, but also to host modulatory components.

SUMMARY AND CONCLUSION

The present study was undertaken to evaluate the expression of B-1a cell in gingival health and disease tissue samples. The study population comprised of 30 patients and were divided into two groups, Group A (healthy gingiva) and Group B (chronic periodontitis).

Gingival biopsies were taken and all tissue samples were paraffinised and processed. B-1a (CD5+) expression was determined using Human CD5 Affinity Purified Polyclonal Ab employing immunohistochemical procedures. Brown staining observed in the connective tissue infiltrate was considered positive. Samples were analyzed for intensity of staining and cell counting was done to calculate the number of positively stained cells.

Assessing the intensity using kappa analysis, good inter examiner agreement was observed between the two examiners (H-.796, D-.797). The mean labeling index between the two study groups using independent ‘t’ test yielded a p value of 0.276.

From the results of this study we may conclude that:

There was an increase in the number of autoreactive B-1a cells (CD5+) in periodontitis as compared to health, which was found to be statistically insignificant. Further studies with greater

sample size and statistically significant data are needed to confirm the role played by B-1a cells in the etiopathogenesis of periodontal disease.

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