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## Immunophenotypic analysis of inflammatory cell infiltrates in four proposed subclasses of apical granulomas

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**IMMUNOPHENOTYPIC ANALYSIS OF INFLAMMATORY  
CELL INFILTRATES IN FOUR PROPOSED SUBCLASSES OF APICAL  
GRANULOMAS**

**BY**

**LEO L. LAZARE, D.D.S.**

**A Thesis Submitted to the Faculty of the Graduate School  
of Loyola University of Chicago in Partial Fulfillment  
of the Requirements for the Degree of  
Master of Science**

**May**

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Especially to my children Darren and Melanie, who have never lost their cool or their love for their Dad.

## VITA

The author, Dr. Leo L. Lazare, was born in Chur, Switzerland, on the twelfth of March, 1945.

The author received his undergraduate training at McGill University in Montreal, majoring in biochemistry and graduating with a Bachelor of Science degree in 1966. This was followed by graduate training in the field of Dentistry at McGill University, where the author received the degree of Doctor of Dental Surgery in May of 1970. The following year was spent in a prosthodontic residency program at the Montreal General Hospital.

Following a six month associateship in general dentistry, the author spent the next three years in private practice in Montreal with a part time appointment in the Faculty of Dentistry at McGill University in the department of removable prosthodontics. He moved to Ottawa, Ontario, Canada in the fall of 1974 where he practiced general dentistry for the next seventeen years. He has been an active member of several regional, national and international dental organizations. As a member of the American Academy of General Dentistry and an ongoing involvement in continuing education, he will receive a Fellowship in the Academy of General Dentistry in July of 1993.

In August of 1991, the author entered the Loyola University School of Dentistry and began what was to become a dual course of study leading to the degree of Master of Science in Oral Biology and a Certificate of Specialty Training in Endodontics.

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# CHAPTER I

## INTRODUCTION

In 1931, sixty-three years ago, Hill (1) stated, "Histologically, the granuloma essentially consists of an edematous moderately collagenous connective tissue, infiltrated with a variable number of lymphocytes, plasma cells, large mononuclear and polymorphonuclear leucocytes." This remarkable observation has stood the test of time until the present day without significant alteration. What has evolved is a clearer understanding of the apical granuloma and the elements that participate and play important roles.

A more contemporary description of the granuloma is considered to be a focal, organized, chronic inflammation, in which macrophages are a major or predominant constituent with the presence of T and B lymphocytes, mast cells, eosinophils, and fibroblasts (2). Generally, it is the result of a type IV hypersensitivity or cell mediated immunological reaction to an insoluble or indigestible antigen. Type III hypersensitivity reaction, involving the formation of complexes between antigen and antibody (3), as well as type I, an anaphylactic response to an antigen have also been reported (4).

The periapical granulomatous lesion is really not a lesion at all, but represents a protective self-limiting cellular and humoral response by the host.

An important step towards understanding the mechanisms involved in the development of periapical granulomas is the identification

of the characteristic cell types and immunophenotypes present based on the expression of cell surface markers or antigens.

Identification of these markers is the core of diagnostic pathology and has brought to prominence the procedures involved in immunohistochemistry. It invokes the use of specific antibodies that will locate the presence of and react with these surface molecules, or more specifically antigenic determinants or epitopes. The cell types involved in the mechanisms under study can thus be recognized and their role evaluated. Immunohistochemistry has become remarkably sensitive and specific, applicable to routinely processed materials, even after long storage. It is compatible with most fixatives currently in use, including buffered formalin, and acetone-fixed fresh-frozen tissues. Like any other relatively new procedure, care must be taken such that results should be assessed within the context of standard histopathological and clinical findings.

In 1989, the International Workshop on Human Leukocyte Differentiation agreed on many CD groups or sub-groups of distinct antigens on leukocyte cell surfaces that were recognized by various available antibodies. Monoclonal antibodies define leukocyte differentiation and these cell surface molecules are assigned a CD designation (5,6). New monoclonal antibodies are constantly being developed and added to the armamentarium of the diagnostic pathologist.

The present research differs from existing studies in its proposal to separate the granulomatous lesions into four distinct subclasses: exudative, granulomatous, granulation tissue, and foreign-body granulomas based on the cellular phenotypic profile within each

subclass. It is suggested that the subclasses proposed, represent a transition in development of the apical granuloma from the exudative to the granulation tissue type with the possible presence of overlapping characteristics.

The objective of this thesis is to utilize several of the most recently developed monoclonal antibodies available in an effort to substantiate and, hopefully, augment current knowledge in the identification of the complex and dynamic periapical immunological mechanisms present.

Apical granulomas, as a distinctive pathological entity, present a fascinating model of the inflammatory process within the human body.

## CHAPTER II

### REVIEW OF THE LITERATURE

#### PERIAPICAL GRANULOMAS AS AN ENTITY:

Interest in periapical granulomas, although indirectly, has captured the interest of mankind since early history if we are to believe the multitude of cartoons depicting the patient with a hand over a swelling in his jaw and a bandage around his head.

It was not until the early twentieth century with the observations by Hill (1) (1931) that the apical granuloma received recognition. Little scientific research was conducted in this area over the next thirty years as other dental issues came to the fore.

Takehashi et al (7) in 1965 published a study linking bacterial contaminants within the pulp, to severe inflammation, necrosis of pulpal tissue and formation of apical lesions. The nature of the inflammatory process was not investigated.

Bhaskar (1966) (8) examined 2,308 apical radiolucencies and classified them into periapical granulomas, apical scar tissue and foreign body granulomas. His report did not elaborate on the etiology or pathogenesis of these lesions.

It was known that antigen-antibody complexes induced severe local inflammatory responses (9) but were thought to be transient in nature. In 1968 Spector and Heesom (10) published a study linking antigen-antibody complexes with the formation of granulomas in rats.

Since the immune complex had now been associated with the formation of granulomas, laboratory procedures were evolving to identify the cellular elements present.

### **CLINICO-LABORATORY DEVELOPMENTS:**

The relationship of plasma cells to antibody production was widely known (11), however the existing methods of plasma cell counting with orthodox histological stains were inadequate. Immunofluorescent staining techniques for the study of immunoglobulins was problematic since fresh-frozen or cold alcohol-fixed tissues were required.

Taylor and Burns (12,13) between 1974 and 1978 had developed a peroxidase-conjugated antibody and an indirect immunoperoxidase sandwich technique on formalin-fixed, paraffin-embedded tissue sections. Using this technique, the authors demonstrated the presence of immunoglobulins and plasma cells in lymphoid tissues. A range of rabbit antihuman immunoglobulin primary antibodies were exposed to sections of tissues, followed by swine anti-rabbit serum IgG conjugated with horseradish peroxidase and diaminobenzidine. The tissue bound peroxidase-conjugated antibody was then counterstained with hematoxylin. The immunoperoxidase technique was found superior to immunofluorescent microscopy because it enabled a detailed histological examination of the tissue sections. Furthermore, it did not require fresh tissues, and in contrast to the rapid fading of

the immunofluorescent technique, immunoperoxidase gave rise to permanence of the tissue sections.

Immunohistochemical procedures have since evolved and expanded through the development of a range of monoclonal primary antibodies. Monoclonal antibodies are the secreted immunoglobulins of the clonal progeny of a single hybrid plasma cell. Having molecular identity, monoclonal antibodies have identical antigen specificity and binding affinity. These antibodies are monospecific and as such react to a single epitope on a cell surface molecule of a particular antigen. Numerous monoclonal antibodies have been developed that define leukocyte differentiation and cell surface molecules (5).

Warnke et al (14) in 1980, published a study using a biotin-avidin-horseradish peroxidase to demonstrate the presence of T and B cell types. Frozen tissues were utilized and reacted with monoclonal antibodies specific for either T or B cells.

Torabinejad (4) in 1981 first used the sensitive peroxidase-antiperoxidase (PAP) method to do studies on plasma cells and immunoglobulins in periapical lesions.

Cymerman et al (15) in 1984 adapted Warnke's method to confirm the presence of T cells, and T cell subpopulations in human apical lesions. The method adopted by the authors for the determination of specific cell types became the standard for the study of cellular inflammatory components in periapical lesions. Essentially frozen tissue specimens were serially sectioned, fixed in acetone, dried, rehydrated and each section was incubated with a monoclonal antibody (primary antibody) prepared so as to be sensitive to an antigen found on a



particular cell or cell subset type. The primary antibody was then exposed to and detected by a secondary antibody, anti-mouse IgG, to which biotin was conjugated. The label was localized to the secondary antibody by avidin-horseradish peroxidase. A chromogen diaminobenzidine reacted with the label to give a brown color reaction with reactive antigens. Hematoxylin was used as a counterstain. This was followed by dehydration clearing and mounting of the sections.

Other laboratory methods were tried but found to be inadequate. For example, hemadsorption was applied to periapical tissue sections by Skaug et al (16) in 1982. Difficulties were encountered due to the loss of histologic detail and masking of indicator cells and to poor tissue preservation. Johannessen et al (17) in 1983, utilized acid alpha-naphthyl acetate esterase (ANAE) on formalin-fixed paraffin-embedded tissues from periapical lesions, however, drawbacks to this system involved the lack of subset recognition for the T cells, and the inconsistencies innate to the ANAE reaction.

#### **ASPECTS OF THE IMMUNOLOGICAL RESPONSE:**

In 1975, Naidorf (18) related the concept of clonal-selection theory of immunity showing antigen specificity and their receptor sites on B cells. The activated B cell acted to produce plasma cells whose function was to produce immunoglobulins specifically able to react with the antigen which activated the B cell in the first place. B cells and humoral antibodies suggested the possibility of clinically evident humoral reactions whereas T cells were associated with cell-mediated immunity

and involved in delayed hypersensitivity. Immunoglobulins were understood to be an integral part of the immune response in apical granulomas. Naidorf felt that the isolation and identification of these immunoglobulins was an essential first step. Suspensions of cells from freeze-thawed apical tissues were isolated and specific antigen/antibody reactions were conducted resulting in the diagnosis of IgA, IgG and IgM immunoglobulin containing cells in apical lesions. One lesion diagnosed as scar tissue was found virtually devoid of immunologically-derived cells. Naidorf's study was a first step in the analysis of periapical pathogenesis and was confirmed by studies of Morse et al (19), Malmstrom (20), Toller (21), and Kuntz et al (22) between 1975 and 1977.

The possibility that the immune response played a key role in periapical lesion formation continued to gain support. Studies showed that although microorganisms were generally not present in chronic periapical lesions (23,24), these areas of inflammatory response were the result of exposure to a continuous egress of irritants from the root canal area. Bacterial products, deteriorating pulp tissue and altered host tissue were found to be potential antigens capable of initiating immunological reactions (25,26).

In 1978 Torabinejad and Bakland (24) reviewed the literature concerning the immunopathogenesis of chronic periapical lesions. Many concepts were assembled from the existing literature to explain the pathological mechanisms involved. An experiment was conducted in which aggregated IgG was introduced into the root canals of cat teeth. Rapidly evolving periapical lesions were observed, characterized by

bone and collagen loss and the accumulation of inflammatory cells. It was postulated the antibody-antigen (aggregated human IgG) complex and triggered the complement system which in turn attracted polymorphonuclear (PMN) leukocytes and the enhancement of phagocytosis. It was further suggested that the chronicity of this process was responsible for the tissue damage found in apical lesions. This propounded the importance of mediators in the development of apical lesions.

Ishizakas (27) in 1971, had demonstrated that an antigen could bridge two molecules of IgE on mast cells with the resultant effect of releasing histamine and other chemical mediators of anaphylactic reaction. The fact that these may have resulted in pathological changes such as bone resorption (28) could be significantly applied to periapical lesions, since Mathiesen et al (29) in 1973 had shown the presence of mast cell in periapical granulomas. Pulver et al (30) in 1978 identified IgE as well as IgG, IgA, and IgM containing cells in periapical tissues in relative concentrations of 10, 70, 14, and 4 percent respectively. Within the vicinity of the IgE positive plasma cells, intact and degranulating mast cells were observed.

#### **T LYMPHOCYTES:**

It was becoming clear that periapical lesions contained most of the necessary components for the development of a host immunologic response. The delayed form of hypersensitivity or cell mediated-immune

reactions, however, were dependent on activated T cell interaction with antigens.

Reinherz et al in 1979 (31) separated human T cells into discrete and functional subsets utilizing monoclonal antibodies. The production of a distinct monoclonal antibody, termed OKT4, was described as being capable of linking with a specific cell surface antigen subpopulation of human peripheral blood T cells and was unreactive with normal B cells, null cells, and macrophages. The OKT4 antibody appeared to define a human T cell subset with OKT4 reactive (OKT4+) and OKT4 unreactive (OKT4-) T cells. Separation of OKT4(+) and OKT4(-) cells showed that the T cell subsets were functionally discrete. The OKT4(+) fraction was found to be T helper/inducer cells (Th/i) while the OKT4(-) fraction was identified as T cytotoxic/suppressor cells (Tc/s).

Reinherz et al (32) in 1980 reviewed evidence to support the development of functionally distinct T cell subsets. Each subset was found to be linked to the expression of a particular cell surface antigen and capable of being singled out by specific monoclonal antibodies. The importance of each subset was evident by their distinctly different regulatory effects on the immune response. Antigen triggering of T cells was governed by the balance between these two subsets. The Th/i cell subset was important in the activation of other T cells, B cells, and macrophages, as well as involvement in hematopoietic differentiation. The inductive influence of the Th/i cells was thought to be regulated by the presence of Tc/s cells which acted to inactivate the inducer subset or the effector population.

## CELLULAR ANALYSIS OF APICAL GRANULOMAS:

In an attempt to further the understanding of the biological nature of the granulomatous response, several studies were conducted to measure the cellular elements in these lesions. Conflicting reports have since plagued the analysis of these apical cellular infiltrates.

Stern et al published three morphometric studies between 1981 and 1982 (33,34,35) on periapical granulomas and cysts. The author found that of the total inflammatory cells observed, 24% were macrophages, 16% were lymphocytic, 7% plasma cells, and 4% were neutrophils with the remainder being fibroblasts. Of the lymphocytes, 81% did not contain immunoglobulins. The authors suggested that these were T cells, null cells, or macrophages/monocytes. The authors indirectly suggested a predominance of T lymphocytic cells within apical lesions. Prior to this study, only tangential data had been presented suggesting the presence of the cell-mediated arm of the immune system in periapical lesions. Of the remaining 19% lymphocytic cells, 6% were B cells, while the remainder were plasma cells. IgG was the most common immunoglobulin (74%) within plasma cells, followed by IgA (20%), IgE (4%), and IgM (2%). The T cells present accounted for 34.5% of the unseparated inflammatory cells while macrophages accounted for 30% confirming that T cells were indeed part of the cellular components of periapical granulomas and that the cell-mediated immune system played a role in the pathogenesis of apical lesions.

Torabinejad et al (1981) (4), using the PAP method, found immunoglobulin IgE to be present in 74% of the periapical tissue specimens which established IgE as an element in the immune complex reaction as well as a mediator in the pathogenesis of periapical lesions.

Skaug in 1982 (16) studied the presence of receptors for the Fc (FcR) region of the IgG molecule in periapical granulomas. He correlated the FcR activity with the degree of lymphoreticular infiltration. Lymphocytes, plasma cells, and macrophages, were present in the majority of granulomas showing intense inflammatory response. This study made use of the fact that T cell receptors for sheep erythrocytes, complement, and the Fc region of IgG had become important markers for the characterization and classification of mononuclear cells. An indirect immunofluorescent study by Skaug et al. (36) in 1984, paralleled the results of these hemadsorption tests.

Bergenholtz et al. (37) in 1983, utilizing electron microscopic techniques together with morphometric methods, assessed the volumetric densities of various tissue components and cells in seventeen chronic periapical lesions (not specified as to granulomas and/or cysts) which included the apical root portion of the tooth. The inflammatory infiltrate contained a majority of plasma cells and lymphocytes with the area closest to the apical foramen having the highest concentration. The areas farthest away from the apex were almost devoid of these inflammatory cells but contained collagen fibers and fibroblasts. Several observations were made in this study. It contradicted the findings of Stern et al (33) who believed that macrophages were a predominant cellular element in the apical infiltrates, while supporting the

observation made by Hill (1) many years earlier that neutrophils were associated primarily in the proliferating epithelium. It was proposed that the most likely cause of the lesions was of infectious origin. A suggestion was also made that since these were chronic apical lesions of at least two years standing following root canal therapy, there was a possibility that the causative agent may have been an irritation caused by the root canal filling. The presence of foreign body granulomas could not be ruled out.

Johannessen et al (17) utilizing acid alpha-naphthyl acetate esterase (ANAE) on formalin-fixed sections of apical lesions recognized T cells as the predominant inflammatory cells present (23%), followed by plasma cells (15%), and monocytes/macrophages (11%). Fifty-one percent of the cells of the inflammatory cell infiltrates were ANAE negative.

The presence of T cytotoxic/suppressor, and T helper/inducer cells were confirmed by Cymmerman et al (15) using specific monoclonal antibodies for the antigens present on the cell surface of those particular cell types. It was suggested that showing the presence of T lymphocytes in periapical infiltrates linked cell mediated immunity or delayed hypersensitivity to the pathogenesis of periapical granulomas.

Nilsen et al. (38) achieved very similar results to Cymerman et al., using cryostat sections, murine monoclonal antibodies, and indirect immunofluorescent microscopy. In addition to the identification of T lymphocytes and their subsets, T helper/inducer and T cytotoxic/suppressor cells, the ratio was found to be 2/1 in favor of T h/i cells. Few natural killer cells were shown. A large number of cells,

probably macrophages, were detected with the OKM 1 and OKla 1 monoclonal antibody. Sheets of small OKla 1 positive cells were detected and diagnosed as B lymphocytes or activated T lymphocytes. Torabinejad et al. (6) in 1985 utilizing almost the same technique, achieved very similar results. They observed that on the average, the total number of cells that tested positive for T cells were greater than those which tested positive for B cells and that one of the specimens which was diagnosed as a scar tissue showed no reactive cells.

The investigations at this juncture implicated humoral and cell-mediated immunological reactions in apical lesions. It also implicated apical granulomas as inflammatory reactions predominated by mononuclear phagocytes and containing other inflammatory cell types as well.

#### **THE ROLE OF MEDIATORS OF INFLAMMATION IN THE PATHOGENESIS OF APICAL GRANULOMAS:**

The presence of excessive amounts of antigens or the persistent exposure of periapical tissues to these antigens, results in periapical bone loss. The antigens were described as being bacterial toxins or denatured host tissue. Gradual destruction of the apical root cementum, periodontal ligament and alveolar bone was replaced by fibro-inflammatory tissue due to both specific and non-specific immunological responses. Vasoactive amines, kinins, complement components, and arachidonic acid metabolites were reported among the non-specific mediators of inflammation. The specific immunological response were thought to be due to the participation of immunoglobulins, and



immunologically competent cells. Torabinejad et al (3) published a comprehensive review of the then current concepts dealing with mediators and mechanisms involved in the pathogenesis of human periapical lesions.

According to the authors (3), trauma to periapical tissues during root canal therapy may have caused degranulation of mast cells present in these tissues. Discharge of vasoactive amines from these mast cells (histamine and serotonin) may then have initiated or aggravated the inflammatory response. The vasoactivity increased vascular permeability which then released Hageman factor.

Activation of the Hageman factor then resulted from activated inflammatory cells which in turn activated kallikrein and the kinin system via the production of bradykinin, the clotting cascade, activated plasminogen to plasmin, and the fibrinolytic system. The kinin system was involved in chemotaxis, contraction of the smooth muscle cells, vasodilation, increased vascular permeability, and direct action on nerve sensory receptor fibers. Pain and swelling may have resulted as a direct consequence (3).

The components of the complement system, the activators of the classical and alternative pathway such as IgM, IgG, bacteria, bacterial byproducts, lysozomal enzymes and clotting factors, have all been found in periapical tissues (39). The complement system is thus involved in periapical tissues by either causing bone resorption directly, or indirectly, by the inhibition of new bone formation (40). The complement system may also have stimulated the metabolism of phospholipids by phospholipases in cell membranes with the resulting

release of arachidonic acid (AA)(41). The metabolism of AA by the cyclo-oxygenase pathway produced prostaglandins. The presence of significant amounts of prostaglandins such as PGE2 have been found in the periapical lesions (42). The presence of high concentrations of complement components and PGE2 suggested that activation of the complement system stimulated bone resorption by increasing PGE2 formation which activates osteoclasts (43).

Specific interactions with invading necrotic pulp tissue derived antigens was said to be another host defence (3). Acquired immunity was the result of learning by the lymphoid tissue. Both B cells, and T cells, had specialized receptors for foreign or antigenic determinant material. B cell differentiation to plasma cells was the source of all soluble immunoglobulins while T cells carried antigen receptors on their cell surfaces. T cells appeared to be the primary regulatory cells for acquired immunity.

There were four main types of immunological reactions as proposed by Gell and Coombs in 1975 (44), any one or more of which may have occurred in periapical granulomas. Type I anaphylactic reactions dealt exclusively with IgE immunoglobulin. Type II, cytotoxic reactions involved the binding of T cells with receptors for Ig complexed to antigens on cell surfaces (antibody-dependent T cell cytotoxicity). Type III reactions were antigen-antibody reactions which often lead to intense inflammatory responses caused by the formation of complexes via the activation of complement. Type IV, cell-mediated or delayed hypersensitivity type responses, involving lymphocytes reacting with an antigen to release lymphokines or participate in cytotoxic

reactions against cells bearing the antigen. An interplay of one or more hypersensitivity reactions seems to be involved in apical lesions.

Monocyte/macrophages have been observed in the periapical granulomas. These acted as antigen presenting cells to specific T or B lymphocytes in the regional lymph nodes. Antigens may have stimulated B lymphocytes to differentiate to plasma cells and produce antibodies. The immunological responses that occurred in the periapical lesions depended on the nature of the antibodies (3).

A shift to the study of the pathogenic mechanisms involved in periapical lesions was underway by 1990. Trowbridge (45) in 1990, suggested that T cells and macrophages played a key role in chronic inflammation. Activated T cells were instrumental in producing lymphokines capable of modifying the behavior of other cells, thus possibly facilitating or suppressing the immune response. Irreversible destruction of parenchymal tissue was replaced with fibrous connective tissue. Cytokines, produced by T cells and macrophages, enhanced the proliferation of fibroblasts, collagen production and neovascularization.

In 1989, Oguntebi had published a study (46) observing first what Stashenko (47) was later to hypothesize. The author induced periapical granulomas in rats that had been injected with indomethacin, a potent inhibitor of prostaglandin (PG) synthesis. The rats demonstrated a much lower level of inflammation and a lesser degree of periapical alveolar bone loss than that of the control group, confirming the involvement of PGs in periapical bone resorption in dental granulomas.

Stashenko (47) in 1990 reviewed the role of resorptive cytokines involved in the development of periapical lesions. He elaborated a hypothesis whereby immune cytokines (cytokines produced by inflammatory cells) were induced by bacterial components including lipopolysaccharides. The presence of these cytokines, including macrophage-derived interleukin-1 beta, and alpha, tumor necrosis factors, and lymphocyte-derived lymphotoxin were said to be involved in stimulating osteoclastic activity with bone resorption and inhibiting bone formation (Fig.1). According to Stashenko, the overall effect was to limit the infection to the apical area and the surrounding tissues thereby preventing direct invasion of bone.

Wang and Stashenko (48) in 1991 published a study based on the hypothesis Stashenko had previously described in dealing with the kinetics of bone resorption. These authors suggested that the highest level of bone resorption correlated temporarily with active lesion development. This was due to osteoclast stimulation by factors other than bacterial lipopolysaccharides (LPS) since these had been artificially blocked by the use of polymyxin-B, an antibiotic that binds to and neutralizes LPS. Cytokines and prostaglandins induced by the immunological response were thought to be directly involved.

Artese et al (49,50) in 1991 investigated the presence and characteristics of interleukin-1 beta, and tissue necrosis factor-alpha in cells from human periapical granulomas. The authors found few of these cells present with staining techniques using monoclonal antibodies. The cells that did react positively were in close proximity to lymphoid or other inflammatory cells, suggesting that these cells may act in a

paracrine fashion to activate lymphoid cells. It was found that the positive reactive cells, capable of producing cytokines, had monocyte/macrophage morphology. These represented only a minor fraction of the total monocyte/macrophages fraction (40%) in inflammatory cells. Most abundant of the inflammatory cells were lymphocytes and plasma cells. Mast cells were also found and said to be part of a negative feedback mechanism. The release of histamine by mast cells, could absorb interleukin 2 and remove it as an immune system stimulant.

Continued research (51,52,53) in 1991 into immune cell cytokines in periapical granulomatous tissues has confirmed the presence of tumor necrosis factor, and prostaglandin E2. McNicholas et al claimed that higher levels of prostaglandin E2 occurred in acute lesions as opposed to chronic lesions.

Later studies by Wang and Stashenko, in 1992 and 1993 (54,55) showed that the mediators involved in resorption were interleukin 1 beta and tumour necrosis factor beta, both being heat labile and protease sensitive, confirming these mediators as distinct from LPS.

Matsuo et al (56) in 1992 have suggested that the CD4(+) T cells in the periapical infiltrate correlated with the presence of IgG containing cells. It was postulated that The CD4(+) T cells have a helper function that promotes B cell proliferation and differentiation. These would also seem to have acted in tandem against antigenic stimuli. The function of CD4(+) T cells would be to surround the site of pathogen invasion so as to defend the host, while the CD8(+) T cells

would prevent an excessive immune response. The ratio of CD4(+) to CD8(+) T cells has varied in the literature and was presented in the present study as a function of the site chosen since CD4 cells tended to be more focal while CD8(+) T cells tended to be scattered. T cells were also thought to be correlated with the size of the lesion involved with these being more numerous in larger lesions than in smaller ones. The observation was made that macrophages played an important role in the inflammatory response and may have been involved in the development of clinical symptoms.

#### **CELL FRACTIONS AND THE DEVELOPEMENT OF APICAL LESIONS:**

The literature continues with the quantitative analysis of the cellular components of periapical infiltrates. The change in relative concentrations of the various cells at particular times in the development of the lesions from acute to chronic stages, or in the transition, if such occurred, from granuloma to cyst formation. The location of these elements was also a topic of investigation. An attempt was made to understand the role that each cell plays in the progression of the periapical lesion.

Johannessen published a study in 1986 (57), relating the relative distribution of ANAE staining cells in inflammatory infiltrates of acute and chronic periapical lesions based on clinical data. Inherent problems in the ANAE staining technique allowed only gross differentiation. Plasma cells were found to be the predominate cells in acute lesions while T cells predominated in chronic lesions. Macrophages

were also present in large numbers in the chronic lesions. The study could not claim that pain correlated with the presence of specific cell types since cysts could also cause pain. Pain was thought by the author, to be the result of increased interstitial fluid pressure caused by the inflammatory exudate or the release of chemical mediators such as bradykinin activated by the inflammatory reaction.

Contos et al (58) conducted a study to determine the presence of Langerhans cells in periapical cysts. Langerhans cells had been shown to express Fc-IgG and C3 receptors (59,60) as well as the synthesis of human leukocyte antigen DR (HLA-DR). Their function was similar to that of mononuclear phagocytes and have been shown to be antigen processing with subsequent presentation of relevant determinants to T cells. Antisera to S-100 protein, muramidase, and monoclonal antibody to HLA-DR were tested on formalin-fixed tissue from apical periodontal cysts. The authors concluded that Langerhans cells were present in the epithelium of apical periodontal cysts. Previous studies had been unable to show the presence of Langerhans cells. Increased numbers were found in areas of intense inflammation suggesting increased challenge and antigen processing activity. The finding of T lymphocytes adjacent to stained Langerhans cells supported the premise that T lymphocytes acted as effector cells after receiving antigenic information from stimulated Langerhans cells.

The observations made by Contos et al were supported in a subsequent study by Gao et al (61), however the use of the anti-S-100 antibody by Contos was criticized claiming that the antibody was not specific for Langerhans cells. Gao claimed that the more specific

T6(CD1) antibody should have been used. His investigations were undertaken to (a) demonstrate the presence and distribution of subpopulations of lymphocytes, monocytes/macrophages and of Langerhans cells in non-inflamed periodontal ligament, periapical granulomata, periapical cysts and dental developmental cysts and (b) to correlate the pattern of immune cell infiltration with the degree of differentiation of associated epithelium.

Gao et al used a panel of monoclonal antibodies with cryostat (frozen) sections to show the presence of the cellular elements. The results indicated that the T cell components outnumbered the B cells. The CD8(+) T suppressor/cytotoxic cells outnumbered the CD4(+) T helper/inducer cells in apical granulomas whereas the CD8(+) cells were equal in number or less than the CD4(+) cells in those cysts examined. This was rationalized by stating that the ratio of T helper/inducer cells to T cytotoxic/suppressor cells may be indicative of the stage of the lesion. This concept would be the subject of several future studies. This study indicated that the distribution of Langerhans cells was associated with the presence of epithelium. The observation was made that dense infiltrates of lymphocytes, HLA-DR positive cells, lysozyme and alpha-1-antitrypsin-positive cells were always closely related to the proliferated epithelium in the periapical granulomas near the epithelial lining of cysts. The CD4(+) T cells were seen more often in the connective tissue around the epithelium while the CD8(+) T cells were located generally within the epithelium. These observations together with the functions of other mediators of inflammation have led the



authors to suggest that there may be a two-way interaction between epithelial cells and the cells of the immune system.

A similar study to that of Gao et al was conducted by Mathews et al (62) with opposite results, indicating that the cells within the cyst capsule were CD4(+) and predominated over the CD8(+) T cell subset. Kopp et al (63) investigated the qualitative and quantitative distribution of T cell subpopulations, macrophages, and HLA-DR controlled cells of chronic granulomatous lesions using cryostat tissue sections and monoclonal antibodies. Their findings suggested that macrophages were the dominant inflammatory cell fraction, followed by the T helper/inducer lymphocytes. Cysts were characterized by a dramatic increase in T helper/inducer cells, T cytotoxic/suppressor cells, macrophages, and HLA-DR(+) cells, with the presence of only a few activated T lymphocytes. The high concentrations of macrophages in cysts is said to be a function of the chronicity of the inflammation due to the continued presence of antigenic material, and predominant T helper chemotactic function. The presence of HLA-DR(+) cells was suggested as an indication that the control of the infection-induced immune response may be genetic. The distinction of high macrophage concentration in periapical lesions may possibly be limited to cysts as the preponderance of reports mentioned have shown. A relatively low concentration of macrophages was observed in granulomas, with a relatively high concentration of plasma cells and lymphocytes. The study by Babal et al (64) is another such example.

Yu and Stashenko conducted a series of studies (65) on periapical granulomas. These authors created a simple model system

which predictably induced apical lesions in rats by exposing the pulps of maxillary and mandibular first and second molars and leaving them open to the environment. Periapical tissues were pooled from all lesions in each animal at different time periods. The pooled cells were then released, stained, and examined by the use of monoclonal antibodies and immunoglucose oxidase. The predominant cell type was found to be lymphocytes, with T lymphocytes outnumbering B lymphocytes at all time frames. The suggestion made was that the T cell rather than the B cell played a more important role in the pathogenesis of periapical lesions. This observation was taken to indicate that the antibody-mediated reaction does not play a critical role in the active phase of periapical lesions. The important role played by the T cell was thought to be through delayed-type hypersensitivity and the secretion of cytokines stimulating the production of bone-resorbing osteoclasts. These include cytokine-activated, macrophage-derived interleukin 1 beta, interleukin 1 alpha, tumor necrosis factor, and T cell-derived lymphotoxin. Subsequent to these observations, T cells received intense examination with respect to the presence of particular subsets during particular time frames in lesion development.

Barkhordar et al (66) in 1988, conducted an investigation into the human T-lymphocyte subpopulation with cryostat tissues and monoclonal antibodies. The presence of T helper/inducer (Th/i) and T cytotoxic/suppressor (Tc/s) cells were confirmed but no statistical quantitative difference was found between the two subsets in the lesions studied.

Stashenko et al (67) in 1989, published a study in which the T cell subsets were enumerated at various time points corresponding to active and chronic phases of lesion development. It was found that the active phase of lesion development occurred between day 1 and day 15 and was thereafter followed by the chronic phase. The significance of this investigation related to changes in relative numbers of Th/i cells and Tc/s cells with the progression of the lesion. The Th/i cells increased in number until day 15 and decreased progressively after that. The Tc/s cells increased steadily after day 15 to day 90. The results indicated a correlation between the Th/i cell and active periapical lesion expansion whereas Ts/c may be involved in lesion stabilization and a chronic state inflammation. This observation was similar to that reached by Babial et al (68) in 1989, who found that the Ts/c lymphocytes together with low numbers of macrophages may be responsible for the chronicity of periapical lesions.

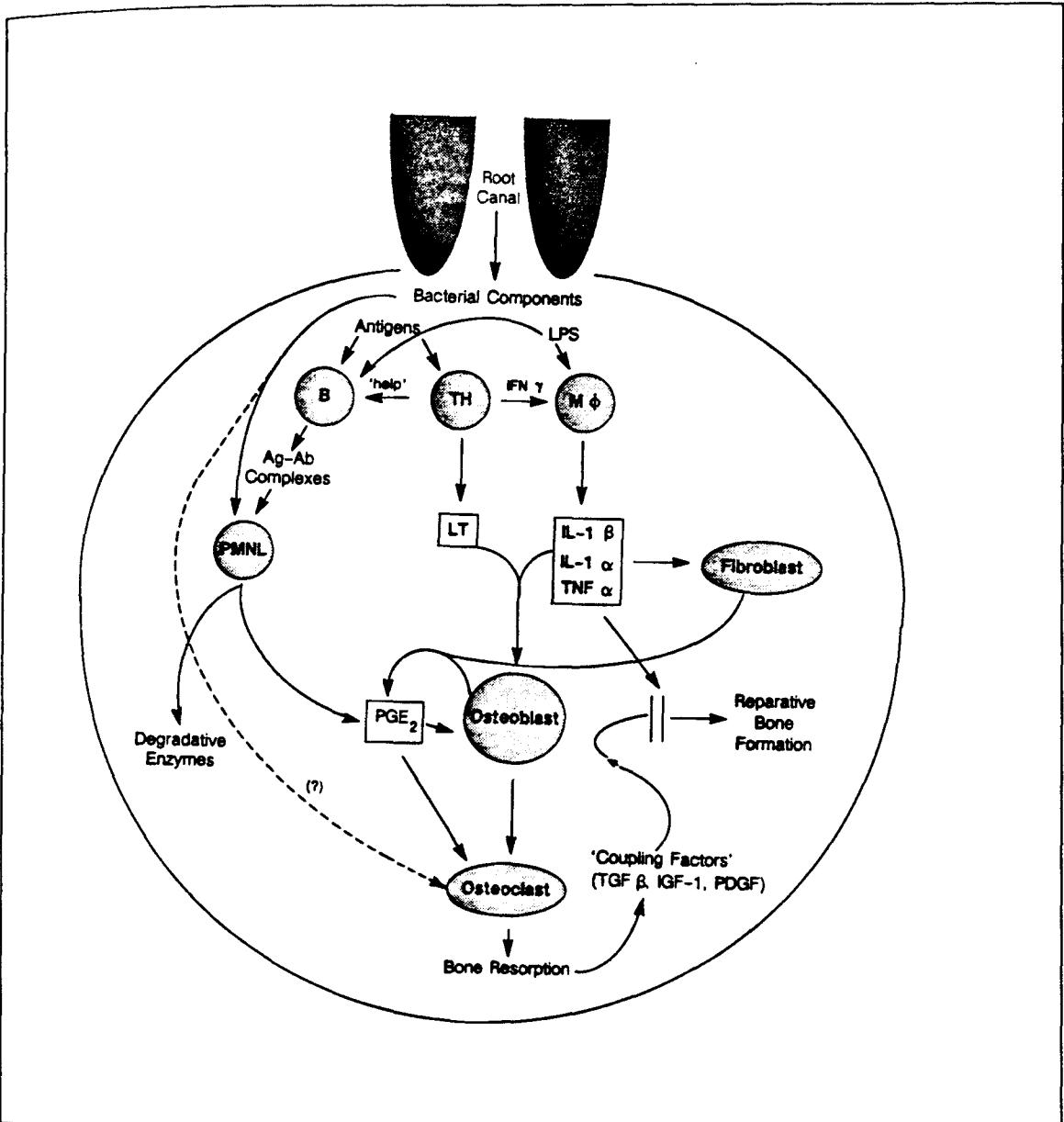
Lukic et al (69) in 1990, studied the prevalence of B and T cells in periapical lesions using CD3, CD4, CD8 and Ig monoclonal antibodies specific for T cells, Th/i cells, Tc/s cells, and subsets of B cell lineage bearing immunoglobulin molecules. In the majority of specimens examined, diffuse infiltrates were present with CD8(+) cells being more numerous than the CD4(+) cells. This was especially true where the granulomas had distinct epithelium. Subsets of the B cell lineage were found in all specimens and were more numerous in those specimens that had focal infiltrates. IgE-positive cells were found to be the most prevalent. The conclusion drawn was that the quantitative difference in subsets of immunocompetent cells correlated with the

development of the granuloma and its possible transformation to a cyst when epithelium is present in the lesion.

**IMMUNOGLOBULINS:**

A new level of sophistication was now present in dealing with the cellular components present in periapical infiltrates. In the case of immunoglobulins, much was known as to their structure and function (5). All normal classes of immunoglobulins are glycoproteins which possess equal numbers of heavy and light polypeptide chains. There is a domain of relatively constant size and one showing more variation in amino acid sequence. Digestion of an immunoglobulin molecule such as IgG by the enzyme papain produces two Fab fragments and one Fc fragment (FIG. 2). The antigen-binding activity is associated with the Fab fragment while most of the secondary biologic activities of immunoglobulins, such as complement fixation, are associated with the Fc fragment. There are five classes of immunoglobulins, designated IgG, IgA, IgM, IgD, and IgE as defined by structural differences. Five classes of H or heavy chains are found in humans; these are gamma, alpha, mu, delta and epsilon. Each class is based on structural differences in the constant region and it is the class of the H chain which determines the class of the immunoglobulin.

**FIG. 1: RESORPTIVE CYTOKINES IN PERIAPICAL LESIONS:**



**Fig.1** Resorptive cytokines in periapical lesion pathogenesis, // indicates inhibition of reparative bone formation by cytokines. **LEGEND:** IL-1: macrophage-derived interleukin-1, TNF alpha: tumor necrosis factor, B: B cell, LT: lymphocyte-product lymphotoxin, PMNL: polymorphonuclear leukocyte, LPS: lipopolysaccharides, PGE2: prostaglandin E2, TH: T helper cell, M: macrophage (From Stashenko P. Endod Dent Traumatol 1990;6:89-96.)

FIG. 2: IMMUNOGLOBULIN GAMMA (IgG):

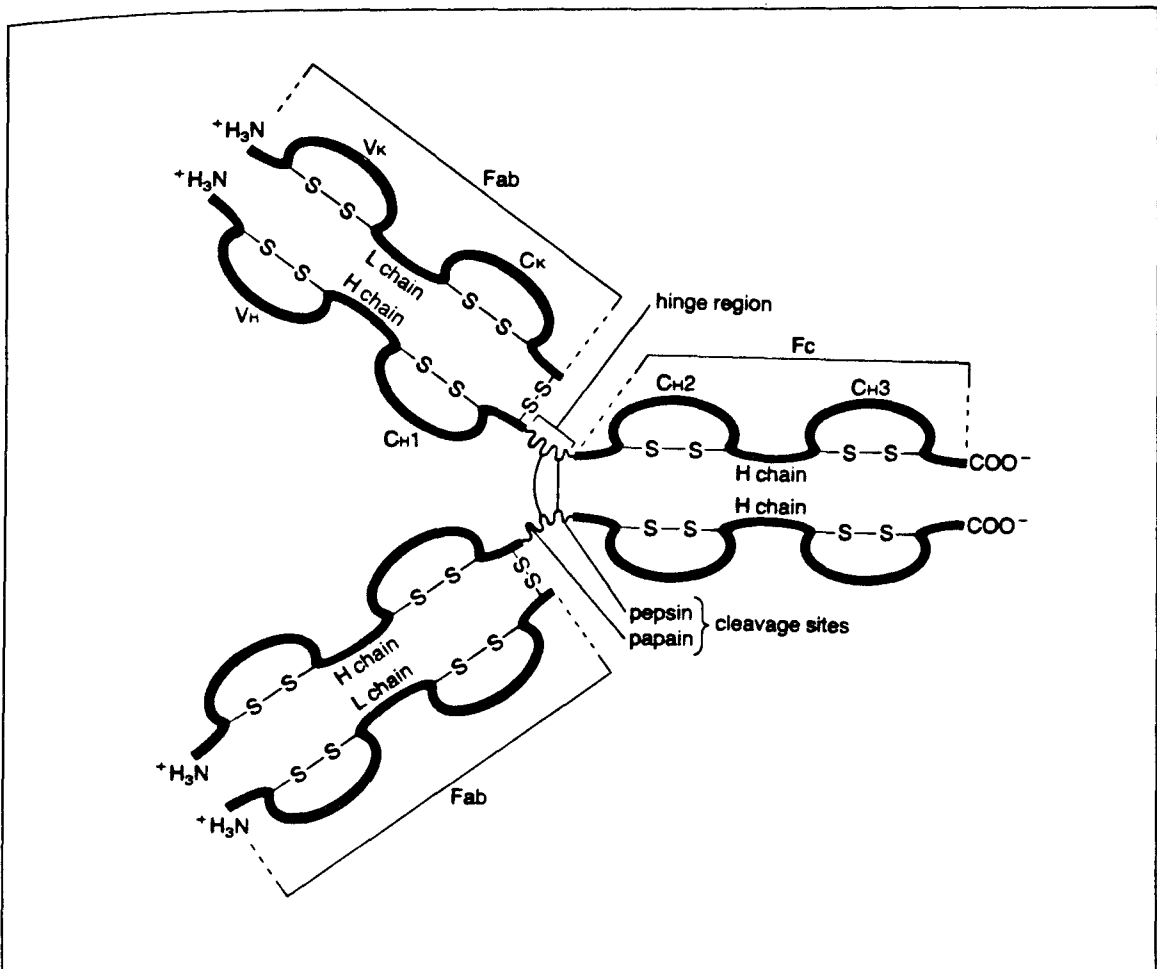


FIG. 2 . Simplified model for an IgG1(k) human antibody molecule showing the basic 4-chain structures and domains (V<sub>H</sub>,C<sub>H</sub>1,etc). V indicates the variable region; C indicates the constant region. Sites of enzyme cleavage by pepsin and papain are shown. Note portion of inter- and intrachain disulphide bonds. (From Stites DP, Terr AI. Basic human immunology, Norwalk, 1991, Appleton and Lange.)

## CHAPTER III

### MATERIALS AND METHODS

Human periapical inflammatory infiltrates possess characteristic cell types and immunophenotypes. The identification of these cell types is based on the expression of surface antigens such as: cluster differentiation (CD), cell surface molecules of human leukocyte antigen class I (HLA-I), human leukocyte antigen class II (HLA-II), specific cell surface receptors on monocytic cell lines including complement, transferrin receptors, and cytoplasmic immunoglobulin sub-classes in B-lineage lymphocytes (Table1). Several different techniques were utilized to determine these cell types as well as the presence of the different surface antigens.

#### MONOCLONAL ANTIBODIES:

The following is a description of a panel of monoclonal antibodies utilized in this study to differentiate groups or subgroups of distinct antigens on leukocyte cell surfaces (Table 2). These were commercially available through the DAKO CORP (California).



### 1. DAKO-CD3:

DAKO-CD3, T3-4B5 is a monoclonal mouse anti-human antibody that reacts with the T cell associated CD3 antigen. CD3 is well characterized in terms of tissue distribution and at the biochemical and DNA level. The molecule consists of five polypeptide chains with MWs ranging from 16-28 kD. The five chains are designated gamma, delta, epsilon, zeta, and eta. Most CD3 antibodies are against the 20 kD epsilon chains. DAKO-CD3, T3-4B5 reacts with the 20 kD CD3 epsilon chain (70).

CD3 is closely associated in the lymphocyte cell surface with the T cell antigen receptor (TCR). It is believed that the CD3 complex transmits activational signals to the T cell's interior upon recognition by TCR. CD45(LCA), a protein tyrosine phosphatase, is a potent regulator of signal transduction by the CD3 complex (71). DAKO-CD3, T3-4B5 reacts with T cells in the thymus, peripheral blood tissue, and blood. No other cells are known to bind antibodies to the CD3 antigen with the exception of Purkinje cells in the cerebellum. DAKO-CD3, T3-4B5 is not suitable for use on formalin-fixed, paraffin-embedded tissue but may be used for labelling acetone-fixed cryostat sections, using the three stage immunoperoxidase technique (12).

### 2. DAKO-CD4, MT310:

DAKO-CD4, MT310 is a monoclonal mouse anti-human antibody that reacts with a glycoprotein (apparent MW around 59 kD)

present on most human T cells of helper/inducer subtype (72). This molecule is designated CD4 in the system for classifying human leukocyte antigens. The antigen recognized by DAKO-CD4, MT310 appears early in intrathymic differentiation of human T cells and is initially co-expressed with CD1 and CD8 antigens on cortical thymocytes (73). T cells in the thymic medulla, which are thought to develop directly from cortical thymocytes, lack the CD1 antigen and express either the CD4 or the CD8 antigen. The CD4-positive medullary thymocytes are very similar in phenotype to helper/inducer cells in peripheral blood.

The antigen recognized by antibodies against CD4 is not only confined to T cells, but is also found on some cells of the monocyte/macrophage origin (74). DAKO-CD4, MT310 is not suitable for use on formalin-fixed, paraffin-embedded sections. It can be used in conjunction with the three stage immunoperoxidase technique for demonstrating T helper/inducer cells in acetone-fixed cryostat sections.

### **3. DAKO-CD8, DK25:**

DAKO-CD8, DK25 is a monoclonal mouse anti-human antibody that reacts with a molecule (MW approximately 32 kD) which is present on human suppressor/cytotoxic T cells (75) and designated CD8 in the international system for classifying human leukocyte antigens.

DAKO-CD8, DK25 labels suppressor/cytotoxic T cells in peripheral human lymphoid tissue. These cells constitute only 15-20% of T cells in T cell regions. In human thymus this antibody labels the

great majority of cortical thymocytes and approximately 30% of medullary thymocytes (76).

DAKO-CD8, DK25 is not suitable for use on formalin-fixed paraffin-embedded sections. It may be used in conjunction with the three stage immunoperoxidase technique when tested on acetone-fixed cryostat sections.

#### 4. DAKO-CD11b, 2LPM19c:

DAKO-CD11b, 2LPM19c is a monoclonal mouse anti-human antibody that reacts with the 165 kD MW alpha-chain (CD11b antigen) of the LFA-1 complex. The molecule functions as a cell surface receptor for the C3bi complement fragment. Two binding sites have now been reported, one recognizing lipopolysaccharide, and the other protein ligands containing the arg-gly-asp sequence (77). This protein belongs to a family of related heterodimeric proteins designated CD11 in the classification system for human leukocyte differentiation antigen, which shares a common beta-chain with a MW of 95kD (CD18).

DAKO-CD11b, 2LPM19c reacts with peripheral blood granulocytes, approximately 80% of monocytes, and with a subpopulation of "null cell" peripheral lymphocytes (CD2,CD3) containing most of the circulating natural killer cells (77). The antibody also labels macrophages in many tissues, although its spectrum of reactivity is less than that of other more specific antibodies.

DAKO-CD11b, 2LPM19c is not suitable for use on formalin-fixed paraffin-embedded tissue sections, however it can be used to label

acetone-fixed cryostat sections using the three stage immunoperoxidase technique.

#### 5. DAKO-CD14, TUK4:

DAKO-CD14, TUK4 is a monoclonal mouse anti-human antibody that react with a 55 kD single chain membrane glycoprotein (CD14, gp55) that is expressed primarily on monocytes and macrophages, but also on granulocytes, dendritic reticulum cells, Langerhans' cells and some tissue macrophages such as alveolar macrophages (78). This antibody was included in the Fourth International Workshop on Human Leukocyte Differentiation Antigens, and studies by a number of laboratories confirmed its reactivity with the CD14 antigen.

DAKO-CD14, TUK4 labels monocytes (>95%) and granulocytes in peripheral blood and bone marrow. This antibody also reacts with perivascular macrophages and Langerhans' cells in the skin. The CD14 antigen is expressed in follicular dendritic cells, sinus histiocytes, some epithelial cells, splenic macrophages, alveolar macrophages and spindle cells of the kidney. The antibody is not suitable for formalin-fixed paraffin-embedded tissue sections but rather may be used for labelling acetone-fixed cryostat tissue sections.

#### 6. DAKO-CD15, C3D-1:

DAKO-CD15, C3D-1 is a monoclonal mouse anti-human antibody that reacts with an antigen found on mature granulocytes. The

specificity of DAKO-CD15, C3D-1 is similar to antibodies Leu-M1, Tu9 and VIM-D5, which reacts with an epitope involving the carbohydrate sequence 3-fucosyl-N-acetyllactosamine, also termed X hapten or CD15 antigen (79). DAKO-CD15, C3D-1 labels all neutrophils in peripheral blood, and dendritic reticulum cells. It does not label monocyte/macrophages, lymphocytes or platelets.

DAKO-CD15, C3D-1 can be used to label formalin-fixed paraffin-embedded sections or acetone-fixed cryostat sections using the three stage immunoperoxidase technique.

#### **7. DAKO-CD16, VIFcRIII:**

DAKO-CD16, VIFcRIII is a monoclonal mouse anti-human antibody that reacts with a 50-7-kD glycoprotein (CD16), low-affinity Fc receptor for complexed IgG = FcgammaRIII). It is expressed on natural killer (NK) cells, granulocytes, activated macrophages and a small subset of T cells (expressing alpha/beta or gamma/delta T cell antigen receptors) (80). The CD16 molecule represents the functional receptor for performing antibody-dependent cellular cytotoxicity. This antibody reacts with K/NK cells and neutrophils in peripheral blood and bone marrow.

DAKO-CD16, VIFcRIII is not suitable for labelling formalin-fixed paraffin-embedded tissue sections but can be used for labelling acetone-fixed cryostat sections using the three stage immunoperoxidase technique.

#### 8. DAKO-CD20, L26:

DAKO-CD20, L26 is a monoclonal mouse anti-human antibody that is directed against an antigen present on the majority of B cells. The antibody reacts primarily with a 33kD polypeptide present in B cells and also with a minor component of 30kD. The epitope recognized by L26 is resistant to formalin fixation. DAKO-CD20, L26 reacts with an intraplasmic epitope localized on the antigen designated CD20. (81,82).

DAKO-CD20, L26 reacts with the majority of B cells present in peripheral blood and lymphoid tissue, germinal center blasts and B immunoblasts are particularly strongly stained.

DAKO-CD20, L26 can be used on routinely formalin-fixed paraffin-embedded or acetone-fixed cryostat tissue sections using the three stage immunoperoxidase technique.

#### 9. DAKO-CD22, To15:

DAKO-CD22, To15 is a monoclonal mouse anti-human antibody that reacts with a glycoprotein (CD22 antigen, MW 130kD) found on the surface of the majority of human B cells. The CD22 antigen appears early in B cell maturation (prior to the pre-B cell stage) as a cytoplasmic constituent, and subsequently (at the small B lymphocytic stage) is expressed on the cell surface (83). It labels primary and secondary lymphoid follicles in cryostat tissue sections of human lymphoid tissue and also scattered extrafollicular B cells.

DAKO-CD22, To15 is not suitable for use on formalin-fixed paraffin-embedded tissue sections but may be used to label acetone-fixed cryostat sections using the three stage immunoperoxidase technique.

#### 10. DAKO-CD45R, 4KB5:

DAKO-CD45R, 4KB5 is a monoclonal mouse anti-human antibody. It belongs to the group of antibodies recognizing a "restricted" form of the leukocyte common antigen (CD45R). It reacts with most B cells in peripheral blood and in tissue sections. It also recognizes a small subpopulation of T cells and monocytes. The epitope recognized by this antibody on B cells is resistant to fixation and paraffin embedding and can be used to stain B cell areas in routine histopathological sections using the three stage immunoperoxidase technique. Acetone-fixed cryostat sections may also be used for labelling.

#### 11. DAKO-CD45RO, UCHL1:

DAKO-45RO, UCHL1 is a monoclonal mouse anti-human antibody which recognizes specifically the 180 kD low molecular weight isoform of CD45 or leukocyte common antigen (LCA/CD45) family (84). The UCHL1 antibody is so far the only reagent with this known specificity. The 180 kD glycoprotein occurs on most thymocytes and activated T cells, but only on a portion of resting T cells. This

antibody and antibodies to the high molecular weight form of CD45 (CD45R) seem to define complementary, largely non-overlapping populations in resting peripheral T cells demonstrating heterogeneity within the CD4 and CD8 subsets. This antibody labels most thymocytes, a subpopulation of resting T cells within both the CD4 and CD8 subsets, and mature activated T cells. Cells of the myelomonocytic series, eg. granulocytes and monocytes, are also labelled by DAKO-CD45RO, UCHL1, whereas most normal B cells and NK cells are consistently negative (85).

DAKO-CD45RO, UCHL1 is used to stain paraffin-embedded tissue sections. Most consistent results are obtained using tissue fixed in neutral buffered formalin using a three stage immunoperoxidase technique. Acetone-fixed cryostat tissue sections can also be used for labelling.

## 12. DAKO-CD68, EBM11:

DAKO-CD68, EBM11 is a monoclonal mouse anti-human antibody that detects a glycoprotein with a molecular weight of approximately 110kD (CD68 antigen). The antigen is expressed primarily as an intracytoplasmic molecule, probably associated with lysosomal granules. This antibody stains macrophages in a wide variety of human tissues. Antigen-presenting cells, eg. Langerhans' cells of skin and interdigitating reticulum cells of T cell zones in tonsil and lymph node are positively stained, but not dendritic reticulum cells. Peripheral



blood monocytes, large lymphocytes, basophils, and mast cells are also positive with a granular staining pattern (86).

DAKO-CD68, EMB11 is not suitable for use on formalin-fixed paraffin-embedded tissue sections but may be used to label aceto-fixed cryostat sections utilizing the three stage immunoperoxidase method.

### 13. DAKO-CD71, Ber-T9:

DAKO-CD71, Ber-T9 is a monoclonal mouse anti-human antibody that reacts with the transferrin receptor, a transmembrane glycoprotein with a MW of approximately 180 kD (87). This antibody reacts with many proliferating cells in both normal and neoplastic cells and most tissue macrophages.

DAKO-CD71, Ber-T9 is not suitable for use on formalin-fixed paraffin-embedded tissue sections but may be used to label acetone-fixed cryostat sections using the three stage immunoperoxidase technique.

### 14. DAKO-HLA-DR, DK22:

DAKO-HLA-DR, DK22 is a monoclonal mouse antihuman antibody. The gene of the human histocompatibility (HLA) complex class II consists of at least four subregions, HLA-DP, -DQ, -DX, and -DR, containing at least one alpha and one beta gene. This antibody reacts with the beta-chain of all DR loci, all DP loci, and DQw1 loci. This antibody labels B cell follicles, interdigitating reticulum cells in T cell

regions, Langerhans' cells in squamous epithelium, many macrophages, endothelial cells, a ubiquitous population of elongated cells found in many tissues, activated T cells, a minority of epithelial cells, and a proportion of fibroblasts. In normal peripheral blood, this antibody stains B cells and most monocytes.

DAKO-CD71, DK22 is not suitable for use on formalin-fixed paraffin-embedded tissue sections but may be used for labelling acetone-fixed cryostat tissue sections utilizing the three stage immunoperoxidase technique.

#### **15. DAKO-MAC 387:**

DAKO-MAC 387 is a monoclonal mouse anti-human antibody that reacts with a human cytoplasmic antigen expressed in granulocytes, blood monocytes, and tissue histiocytes. The human MAC 387 antigen is composed of one alpha and one beta subunit with molecular weight of 12 kD and 14 kD, respectively. This antibody is characterized as belonging to a heterogeneous group of anti-tissue macrophage antibodies. DAKO-MAC 387 labels the cytoplasm of many cells of the monocyte/macrophage series. The antibody reacts with a variety of tissue histiocytes, eg. infiltrating and reactive histiocytes, and alveolar macrophages.

DAKO-MAC 387 is recommended to be used on routinely formalin-fixed paraffin-embedded sections in the three stage immunoperoxidase technique. It may also be used on acetone-fixed cryostat tissue sections.

## **IMMUNOGLOBULINS:**

Three different immunoglobulin antibodies have been utilized in this study in an attempt to reveal the presence of immunoglobulins in plasma cells. These are described as follows:

### **1. DAKO Rabbit Anti-Human IgG:**

DAKO rabbit anti-human IgG is the purified immunoglobulin fraction of rabbit antiserum which reacts with the gamma-chains of human IgG. This antibody may be used on formalin-fixed paraffin-embedded tissue sections which has been treated with a digestive enzyme such as pronase, trypsin or ficin.

### **2. DAKO Rabbit Anti-Human IgM:**

DAKO Rabbit anti-human IgM is the purified immunoglobulin fraction of rabbit antiserum. The antibody reacts with the mu-chains of human IgM. Formalin-fixed paraffin-embedded tissue sections may be used for labelling using the three stage immunoperoxidase technique after treatment with a digestive enzyme such as trypsin or ficin.

### 3. DAKO-F(ab)2 Fragment Of Rabbit Anti-Human IgA:

DAKO-F9(ab)2 fragment of rabbit anti-human IgA is an immunoglobulin isolated from rabbit antiserum where the molecules are degraded with pepsin, and the F(ab)2 fraction is isolated by gel filtration. This antibody reacts with the alpha-chains of human IgA and is suitable for formalin-fixed paraffin embedded tissue sections which have been treated with a digestive enzyme such as trypsin or ficin.

#### LABORATORY PREPARATIONS:

Seventeen fresh periapical tissue specimens were obtained from local endodontic private dental offices in the Chicago metropolitan area where the patients had been scheduled for routine periapical surgical procedures. The tissues were kept in gauze slightly moistened with phosphate buffered saline (PBS) until they reached the laboratory. The tissues were then dissected. One part was placed in Tissue Tek (OCT Compound 4583) embedding medium for frozen tissue specimens, quick frozen and maintained in a freezer at -70 degrees F., to be used for immunohistochemical preparation. The second part was fixed in 10% formaldehyde for a minimum of twelve hours, followed by routine processing and embedded in paraffin. These procedures were accomplished within one hour of the surgery. An additional twenty three specimens were obtained from previous formalin-fixed and paraffin-embedded tissues.

Positive and negative controls were utilized in the testing procedures. These controls were tissue sections of specimens from human tonsil. One positive and one negative control section was included with each reagent tested. These were representative samples, processed according to standard protocols and tested in the procedure before use. Positive control tissue specimens were incubated with the primary antibody and treated as were all other experimental tissues. Negative controls were treated as all other experimental tissue sections except for the fact that they were incubated with a PBS/BSA solution rather than the primary antibody. Non-specific positive reactions in the negative controls caused by endogenous peroxidase would be revealed which might have been confused with the reaction product.

#### **HEMATOXYLIN AND EOSIN STAINING OF FORMALIN-FIXED, PARAFFIN- EMBEDDED TISSUES:**

##### **PROCEDURE 1:**

1. Representative tissue sections which had been formalin-fixed and paraffin-embedded were cut at 3 micrometers on a microtome (AO-American Microtome No.8, Model 820) and placed on slides (Baxter Scientific Products, S/P Micro Slides, Colorfrost Cat. M6148-B) coated with polychloroprene ( Table 3).
2. The sections were air-dried at 20-37 degrees C.
3. Positive and negative control sections, as described, were made and accompanied the prepared sections throughout the procedures.
4. All sections were pre-heated for 20-30 min. at 60 F., then transferred directly to xylene without cooling.

5. Two changes of xylene at 5 min. each followed.
6. The sections were rinsed in two changes of absolute ethyl alcohol for 3 min. each, followed by two changes in 95% ethyl alcohol for 2 min. and one rinse in 80% ethyl alcohol for 2 min.
7. The sections were then stained with Harris hematoxylin (Table 3) for 4 min., rinsed in cold running tap water, differentiated in acid alcohol (1% HCl in 70% ethanol), rinsed in cold running tap water, blued in 0.1% ammonia water, and washed again.
8. This was followed by rinsing in 95% ethyl alcohol, counterstained with eosin for one and a half minutes, dehydrated successively in 95% ethanol twice, and twice in absolute alcohol, cleared in two successive xylene baths and mounted in Accumount.

#### **HISTOCHEMICAL PREPARATION OF FORMALIN FIXED, PARAFFIN EMBEDDED TISSUES:**

The use of antigen-antibody reactions to visualize the location of molecular structures in cells and tissues has evolved into a number of different methodologies for efficiently labelling such structures. The method described and utilized in this study is the avidin-biotin-peroxidase technique for immunohistochemistry. This method is based on that of Hsu et al (88), which uses a primary antibody to react with tissue antigenic sites, a secondary antibody against immunoglobulin of the primary species, covalently labelled with biotin. This is exposed to a freshly prepared complex of biotin-labelled horseradish peroxidase (HRP) and modified egg-white avidin. One mole of avidin binds up to four moles of biotin with a kD of 10(-15). This reaction provides ample labelling of the secondary antibody by peroxidase, which, with dilute

(0.75%) H<sub>2</sub>O<sub>2</sub>, is subsequently visualized in the histochemical peroxidation of diaminobenzidine (Table 3) as a brown stain on reactive tissue.

#### PROCEDURE 2:

1. A tissue section was prepared from each of forty formalin-fixed, paraffin-embedded granulation tissues to which a tonsillar tissue, positive control was added, as previously described.
2. Sections from each tissue and the positive control tissue were reacted with each of the specific monoclonal antibodies from the panel of monoclonal antibodies utilized in this study suitable for labelling formalin-fixed paraffin-embedded tissue sections (TABLE 2).
3. A negative control consisting of tonsil tissue sections was included with each testing situation but was not reacted with the primary antibody.
4. The sections were treated with 3 changes of xylene of 5 min. each.
5. The sections were then rinsed in two changes of absolute alcohol.
6. The endogenous peroxidase and hemoprotein present in the tissue sections was then inactivated by placement of the sections in 0.075% HCl solution in absolute ethyl alcohol for 15 min. (.01 ml of 37% concentrated hydrochloric acid in 50ml. absolute ethanol).
7. The sections were then brought through graded alcohols, 95% twice, 80% once, then rinsed in water.
8. If trypsin was to be used to degrade masking protein, for the detection of immunoglobulins, the sections were placed in the working

trypsin solution (Table 3) at room temperature for 15 min. The reaction was then terminated by washing thoroughly in cold running tap water.

9. The sections were then rinsed in phosphate-buffered saline (PBS), pH 7.4.

10. The sections were covered with a blocking reagent (normal goat serum) and placed in a moist slide container for 30 min. at room temperature.

11. The sections were then blotted quickly on filter paper and covered with an optimal dilution of the primary antibody in phosphate-buffered saline containing bovine serum albumin and 1% normal goat serum-PBS/BSA (Table 3). The section used as a negative control (tonsil) was covered with the PBS/BSA solution free of any antibody. These were placed back in the moistened slide container and incubated in the refrigerator for one hour.

12. The sections were washed in 3 successive changes of PBS (pH 7.4) for 5 min. each time, to remove the unreacted antibody.

13. The sections were then covered with biotinylated antibody against IgG or IgM of the species in which the primary antibody was prepared (goat anti-mouse antibody pre-diluted from concentrated antibody). The sections were allowed to stand for 30 min. at room temperature and then washed again in PBS.

14. The sections were then covered with streptavidin-peroxidase reagent (Table 3), placed in a moist chamber, covered and allowed to stand for 30 min. at room temperature. This was followed once again by rinsing in PBS for 15 min., followed by running tap water and placed in a



solution containing diaminobenzidine (DAB) and hydrogen peroxide (Table 3) and kept for 3 min. in a dark chamber.

15. The sections were then thoroughly washed and counterstained in Harris hematoxylin for 5 min., washed in running tap water, differentiated in acid alcohol (1% HCl in 70% ethanol), washed in running tap water, blued in 0.1% ammonia water, washed again in tap water, dehydrated in successive ethyl alcohol baths (95%x2,100%x2), cleared in two successive xylene baths and mounted in Accumount.

### **HISTOCHEMICAL PREPARATION OF FRESH-FROZEN TISSUES :**

The fixation of tissues by freezing optimizes the retention without altering the native antigenicity and when done rapidly, precisely localizes the antigen on the molecule. This approach generally is used to maximize the possibility of identifying poorly characterized antigens. Frozen tissues do not need to be embedded to obtain sufficient rigidity for sectioning (89). Tissues were kept at -70 degrees F. and cut on a cryostat microtome (2800 Frigocut E, Reichert Jung, Cambridge Instruments Inc.) which kept the tissues at -30 degrees F. while sections were being made.

### **PROCEDURE 3:**

1. A tissue section of 3 micromillimeters was prepared for study from each frozen tissue section as with procedure 2 and fixed in methanol for

a few seconds and allowed to dry at room temperature for a few minutes.

2. The sections were then placed in acetone and kept at -20 degrees C. for 20 min.

3. The sections were then removed from the cooling chamber and placed in a warm forced air oven for a few seconds, only to prevent hydration.

4. The sections were covered with an optimal dilution of the primary monoclonal antibody.

5. The sections were incubated in moist slide containers for an hour.

6. The remainder of this procedure follows the same protocol as for procedure 2: steps 12-15.

#### **ANALYSIS AND STATISTICAL APPLICATIONS:**

Based on phenotype differences observed in periapical granulomas in this study, four subclasses were proposed. These were:

1. exudative 2. granulomatous 3. granulation tissue-type and 4. foreign body apical granulomas. These were evaluated statistically as follows:

1. Sections of each tissue which had been formalin-fixed and paraffin-embedded and stained using Procedure 1, was observed microscopically and identified histologically.

2. Tissue sections for each tissue specimen were observed microscopically after being submitted to either Procedure 2 or 3, depending on whether the monoclonal antibody tested could be used on fresh-frozen or paraffin-embedded tissues.

The negative control tissues were observed for degree of background staining for comparison with the experimental tissues. The positive controls were observed to confirm that a positive reaction had taken place with reactive cells.

The number of reactive cells were counted in 10 microscopic fields (X400) of 5 micron square each for each tissue with each specific reagent. A mean was taken for the number of reactive cells present within the microscopic field for each type of granuloma (TABLE 4). A comparison of the means was then evaluated for significance between the four different groups of granulomas for each of the following monoclonal antibodies and/or reagents: CD4, CD8, CD11b, CD15, CD20, CD45R, CD68, CD74, MAC378, IgG, to locate significant differences between the groups. A comparison was also made between the means of the same groups of granulomas using CD4 and CD8, CD20 and CD45R, as well as CD11b and MAC387 in order to compare T cell subsets, B cells, and macrophages fractions within similar groups using different reagents.

Independent analysis of variance was the statistical test utilized in this study with the location of significance being determined using the Fisher LSD test (TABLES 5-26).

## **CHAPTER IV**

### **RESULTS**

Tissue sections were made from a total of 40 cases of apical granulomas, 17 from fresh-frozen specimens, and 23 tissue sections from formalin-fixed lesions. These were stained using hematoxylin and eosin as well as a panel of monoclonal antibodies and observed microscopically for reactivity.

#### **HEMATOXYLIN AND EOSIN :**

Microscopic examination of hematoxylin and eosin stained paraffin-embedded tissues in this series disclosed four proposed subclasses of apical granulomas.

#### **EXUDATIVE SUBCLASS OF APICAL GRANULOMAS:**

This subclass of apical granulomas showed vascularized, fibrous connective tissue, infiltrated by various inflammatory cells. The inflammatory cells were composed of polymorphonuclear leukocytes, mononuclear cells such as monocytes, histiocytes, macrophages, lymphocytes and plasma cells in various combinations.

Histiocytes and macrophages differed mainly in size. Monocytes were smaller averaging the size of endothelial cells or red blood cells. Macrophages were relatively large cells with varied nuclear morphology; some having prominent nucleoli. Foamy histiocytes were found to be generally larger, mononuclear cells with centrally located nuclei and foamy-appearing cytoplasm.

Plasma cells appeared to be approximately 18-20 microns in diameter with eccentrically located nuclei. Some of these nuclei had cartwheel pattern chromatin. The cytoplasm within the plasma cells was amphiphilic, that is, showed staining with both hematoxylin and eosin.

Lymphocytes were found to be the size of small endothelial cells with dark staining nuclei and inconspicuous cytoplasm. Their distribution varied in different tissues on morphologic grounds alone. The distinction between small monocytes and lymphocytes was found to be impractical. Small monocytes could be identified with nuclei larger than 8-10 microns.

Polymorphonuclear leukocytes are identifiable as leukocytes with segmented nuclei, having 2-5 lobes per individual cells, and being 18-20 micron in diameter. Degenerative neutrophils showed fragmented nuclei and leukocytoclastic or dust cells.

Fibroblasts were spindle shaped cells with an elongated nuclear outline.

Blood vessels were found to be generally small sized capillaries lined by endothelial cells and containing various numbers of red blood vessels.

**GRANULOMATOUS SUB-CLASS OF APICAL GRANULOMAS:**

This subclass of granulomas were characterized by fibrous connective tissue and a preponderance of mononuclear chronic inflammatory cells. These were mainly composed of monocytic cell lines such as small monocytes, macrophages, foamy histiocytes, and lymphocytes with the presence of a varying number of plasma cells.

**GRANULATION TISSUE SUBCLASS OF APICAL GRANULOMAS:**

This subclass was characterized by the presence of a large number of fibroblasts with varying degrees of maturity ranging from young fibroblasts with plump nuclei to more spindle shaped types and various amounts of collagen fibrils. It appeared to be a highly vascularized tissue with blood vessels the size of small capillaries and the presence of a variable number of inflammatory cells.

**FOREIGN BODY SUBCLASS OF APICAL GRANULOMAS:**

This sub-class was characterized by the presence of foreign body particles such as zinc oxide, silver granules, or root canal sealer.

There was a presence of variable numbers of predominantly chronic inflammatory cells such as macrophages, and histiocytes within a dense form of fibrous connective tissue.

## CELLULAR REACTIVITY WITH MONOCLONAL ANTIBODIES:

Although monoclonal antibodies are highly specific for certain antigenic determinants, there still occurs a certain amount of overlap in the reactivity of these antibodies with one or more cell types. The following is a description of the cell types and the monoclonal antibodies utilized in the present investigation that were known to be reactive with these cell types (TABLE 1).

Granulocytes encompass three cell types: eosinophils, basophils and neutrophils. Polymorphonuclear (PMN) leukocytes form part of the neutrophil segment. These cells were reactive with several monoclonal antibodies such as CD11b, CD15, CD16, and CD71.

Monocytes were reactive with several monoclonal antibodies such as CD11b, CD14 (highly specific for monocytes), CD45R, CD45RO, CD68, CD74, MAC387. Macrophages and monocytes were variably reactive with these antibodies (TABLE 1).

T cells as a group comprised of Th/i cell subset, Tc/s cell subset and possibly other T cell subsets. These were reactive with CD3, CD45R, CD45RO, and CD71.

More specifically, the Th/i cell subset was reactive specifically to the CD4 monoclonal antibody. Tc/s cell subset was specifically reactive with the CD8 monoclonal antibody.

B cells were specifically reactive with CD20, and CD22 monoclonal antibodies and less specifically with CD45R, CD45RO, CD71, and CD74.

Plasma cells were variably reactive with immunoglobulin antibodies. In this study, the most successful plasma cell-immunoglobulin antibody reaction occurred with IgG.

#### **STATISTICAL ANALYSIS OF REACTIVE TISSUE CELLS IN IMMUNOHISTOCHEMICALLY STAINED SECTIONS (TABLES 5-26):**

##### **EXUDATIVE LESIONS:**

When staining procedures were carried out with the CD4 and CD8 monoclonal antibodies indicative of the presence of Th/i and Tc/s antigenic determinants, it was found that the exudative subclass had a significantly ( $p < .001$ ) greater number of reactive cells than the three other subclasses.

The exudative type apical granuloma tissue specimens exhibited a significantly ( $p < .01$ ) greater number of reactive cells than the other subclasses when tested with CD11b, CD45R, CD68, CD74, and MAC387 monoclonal antibodies. This was indicative of the presence of monocytic, histiocytic, macrophage, and granulocytic antigenic determinants.

The exudative subclass tissue specimens showed more reactive ( $p < .001$ ) cells than any of the other sub-classes when tested for the presence of cytoplasmic IgG indicating a preponderance of immunoglobulin-producing cells in this group of lesions.

In terms of the presence of B cell antigenic determinants, the exudative type tissue sections showed more reactivity than the granulomatous sub-class, but to a lesser extent ( $p < .05$ ) than it did in



comparison with the foreign body sub-class ( $p < .01$ ) using the CD20 monoclonal antibody. It did not show a significant difference in B cell reactivity in tissue sections between exudative and granulation tissue sub-classes with the CD20 antibody. In reacting these tissues with the CD45R antibody a significant difference was observed, indicating more positive cells for a combination of B cells, monocytes and a subset of T cells.

Within the exudative subclass, it was observed that there was a significantly ( $p < .01$ ) greater Th/i subset presence of reactive cells than Tc/s cells, as demonstrated by testing with CD4 and CD8 monoclonal antibodies.

A comparison made between exudative lesions in terms of the presence of reactive B cells and monocytes yielded no significant difference (CD20 and CD45R antibodies).

#### **GRANULOMATOUS LESIONS:**

The granulomatous subclass specimens had a significantly ( $p < .01$ ) greater number of reactive Th/i cells than the granulation tissue-type subclass sections, as observed when stained with CD4 monoclonal antibody. Similarly it had a greater number ( $p < .05$ ) of reactive Th/i reactive cells than the foreign body subclass.

In terms of reaction with the CD8 monoclonal antibody, indicative of Tc/s reactive cells, the granulomatous specimen contained a significantly ( $p < .01$ ) greater number than the granulation tissue-type

subclass. No significant difference was noted between the granulomatous sub-class and the foreign body subclass for this antigen.

The granulomatous subclass tissue specimens contained a significantly ( $p < .001$ ) greater number of reactive cells when reacted with the CD11b (which turned out morphologically to be mainly monocyte) monoclonal antibody as compared with the granulation tissue and the foreign body sub-classes.

Reaction with the CD15 (granulocytes) monoclonal antibody showed that the granulomatous subclass tissue specimens contained significantly more reactive cells than the granulation tissue-type subclass ( $p < .01$ ), as well as the foreign body subclass ( $p < .05$ ).

A significantly ( $p < .001$ ) greater number of reactive cells occurred in the granulomatous sub-class tissue sections when compared to the granulation tissue-type subclass with the CD68 (monocyte, macrophage) monoclonal antibody. This was slightly less significant ( $p < .05$ ) when compared to the foreign body subclass tissue specimens from the apical lesions.

When the IgG (plasma cells) monoclonal antibody was reacted with the tissue sections, it was found that the granulomatous subclass tissue specimens had a significantly greater number of reactive cells when compared with the granulation tissue subclass ( $p < .001$ ) as well as the foreign body subclass ( $p < .01$ ).

There was no significant difference in the number of reactive cells between the granulomatous sub-class and the other two subclasses when the tissues were reacted with CD20, CD45R, CD74 and the

MAC387 (B cells, monocytes, T cell subset, histiocytes and macrophages) monoclonal antibodies .

A comparison of the means between the granulomatous subclass tissue specimens using the CD4 and CD8 antibodies showed significance at the  $p < .001$  level indicating that there were a greater number of Th/i reactive cells present in these tissues than Tc/s reactive cells.

A similar statistical comparison was made between the mean number of reactive cells in the granulomatous subclass tissue specimens using CD11b (granulocytes, monocytes, natural killer cells) and MAC387 (monocytes, macrophages, histiocytes) with the resulting observation that there were a significantly ( $p < .001$ ) greater number of CD11b positive cells.

A comparison of the means between the reactive cell numbers of different granulomatous subclass tissue specimens when reacted with CD20 (B cells) and CD45R (B cells, monocytes, T cell subset) monoclonal antibodies did not yield any significant differences.

#### **GRANULATION TISSUE TYPE AND FOREIGN BODY TYPE APICAL GRANULOMAS:**

In comparing the means of reactive cells between the granulation tissue subclass specimens with the foreign body subclass specimens, it was noted that for reactions with the CD4, CD8, CD11b, CD15, CD20, CD45R, CD68, CD74, MAC387 and IgG monoclonal antibodies, there was no significant differences in the number of

positive cells (Th/i, Tc/s, granulocytes, monocytes, histiocytes, natural killer cells, B cells, plasma cells) .

When granulation tissue subclass specimens were compared for reactive cells using CD4 and CD8 monoclonal antibodies, it was observed that these tissues specimens contained a significantly greater number of positive Th/i cells than Tc/s cells.

In similar comparisons using CD11b (granulocytes, monocytes, natural killer cells) and MAC387 (monocytes, macrophages, histiocytes) as well as CD20 (B cells) and CD45R (B cells, monocytes, T cell subset) monoclonal antibodies, no significant differences were noted in the number of reactive cells.

**TABLE 1: LEUKOCYTE DIFFERENTIATION NOMENCLATURE:**

No.	Cluster of Differentiation	Primary Antibody (clone)	Epitope Localization
1.	CD 3	T3-4B5	T
2.	CD 4	MT310	T h/i
3.	CD 8	DK25	T s/c
4.	CD 11b	2LPM19c	G,M,NK
5.	CD 14	TUK4	M
6.	CD 15	C3D-1	G
7.	CD 16	VIFcRIII	NK,G,Mac.
8.	CD 20	L26	B
9.	CD 22	To15	B
10.	CD 45R	4KB5	B,M,T(s)
11.	CD 45Ro	UCHL1	T,B(s),M,Mac,UCHL1
12.	CD 68	EMB11	M,Mac.
13.	CD 71	Ber-T9	Act T,Act B,Mac.PMN.
14.	CD 74	DK 22	B,M.
15.	N/A	MAC387	M,Mac,H.
16.	N/A	alpha,gamma,mu chains	Immunoglobulins

**Legend:**

T: T cell lymphocyte, Th/i: T cell lymphocyte subset: helper/inducer, Ts/c: T cell lymphocyte subset: suppressor/cytotoxic, T(s): T cell lymphocyte subset, Act T: activated T cell lymphocyte, B: B cell lymphocyte, B(s): B cell lymphocyte subset, Act B: activated B cell lymphocyte, G: granulocyte, M: monocyte, Mac: macrophage, N/K: "natural killer" cell, H: histiocyte, PMN: polymorphonuclear leukocyte.

**TABLE 2: MONOCLONAL ANTIBODIES (DAKO):**

<b>Code</b>	<b>Products</b>	<b>Antigen Source</b>	<b>Antibody Source</b>
K 355	ABCComplex/HRC	Human	Mouse
M 755	CD20,B-Cell,L26,monoclonal**	Human	Mouse
M 708	CD22,B-cell,To 15,pan B,monoclonal*	Human	Mouse
M 754	CD45 RA,B-cell,4KB5,monoclonal**	Human	Mouse
M 741	CD11b,C3bi receptor,2LPM 19c,monoclonal*	Human	Mouse
M 756	CD3,T-cell,T3-4B5,monoclonal*	Human	Mouse
M 716	CD4,T-cell,MT310,helper/inducer,monoclonal*	Human	Mouse
M 707	CD8,T-cell,DK25,supressor/cytotoxic,monoclonal*	Human	Mouse
M 825	CD14,monocyte,TUK4,monoclonal*	Human	Mouse
M 733	CD15,granulocyte assoc. ag,C3D-1,monoclonal*	Human	Mouse
M 838	CD16,Fc,gamma receptor111, V1FcR111,monoclonal*	Human	Mouse
M 742	CD45RO,UCHL1,monoclonal**	Human	Mouse
M 718	CD68,macrophage,EBM11,monoclonal*	Human	Mouse
M 734	CD71,tranferrin receptor,Ber-T9,monoclonal*	Human	Mouse
M 704	HLA-DR (CD74),DK22,monoclonal*	Human	Mouse
M 747	MAC387,monoclonal**	Human	Mouse
A 408	IgA (alpha chain), F(ab)**	Human	Rabbit
M 793	IgE (epsilom chain),E1,monoclonal**	Human	Mouse
A 423	IgG (gamma chain)**	Human	Rabbit
A 425	IgM (mu chain)**	Human	Rabbit
*	reacts with fresh-frozen tissues		
**	reacts with paraffin-embedded tissues		

**TABLE 3: REAGENTS:**

NO.	TYPE	CONCENTRATION
1.	Phosphate-buffered saline (PBS), 10x concentration:	
	Sodium phosphate monobasic, monohydrate	050 g.
	Sodium phosphate dibasic, anhydrous	250 g.
	Sodium chloride	280 g.
	Tween 20	004 mL.
	Deionized water, to make	004 L
	Dilute 1:10 with water to make working solution	
2.	1% Albumin-saline (PBS/BSA):	
	Bovine serum albumin, fraction V	001.0 g.
	Tween	000.1 mL.
	PBS	100.0 mL.
	Mix and add, as preservative	
	Sodium azide	050.0 mg.
3.	Streptavidin-peroxidase (Kit from)	
4.	Monoclonal Antibody (DAKO Laboratories-Table2)	
5.	Diaminobenzidine tetrahydrochloride (DAB) substrate:	
	A. Buffer:	
	Ammonia acetate	003.85 g.
	Deionized water	900.00 mL.
	Add citric acid, 10% to obtain pH 5.5	
	Add water to make 1 L., store at 4 degrees C.	
	B. Substrate solution:	
	Add two tablets (10 mg. each) DAB (Sigma D-5905) for each 50 mL. of buffer at room temperature. Allow to dissolve. Just before using, add 90 microliters of 3% H <sub>2</sub> O <sub>2</sub> per 50 mL.	
6.	Hematoxylin: obtain Harris-type hematoxylin solution (non-mercuric) from Newcomer Supply (Cat. No. 1201)	

**TABLE 3 Cont.****7. A. Buffer:**

Tris (hydroxymethyl) aminomethane base	006 g.
Deionized water	900 mL.
Add 1N. HCl to obtain pH 7.6	
Add deionized water to make 1 L., store at 4 degrees C.	

**B. Solution:**

Trypsin, pancreatic (Sigma T-8128)	50 mg.
Calcium chloride, dihydrate	50 mg.
Tris buffer, pH 7.6	



TABLE 4:

**MEAN NUMBER OF CD/ANTIGEN REACTIVE PHENOTYPES PER HIGH POWER FIELD IN VARIOUS SUBCLASSES OF APICAL GRANULOMAS:**

CD/Antigen	Exudative	Granulomatous	Granulation-Tissue	Foreign-body
CD3	13.480	07.680	03.770	07.300
CD4	07.375	03.200	00.767	00.300
CD8	03.975	01.533	00.000	00.300
CD11b	08.750	04.511	00.733	00.000*
CD14	01.280	00.340	00.050	00.000*
CD15	04.150	02.633	00.900	02.500
CD16	07.130	06.750	00.030	ND**
CD20	01.760	00.830	00.867	00.000*
CD22	03.600	10.120	01.070	12.300
CD45R	03.867	01.714	00.783	00.567
CD45Ro	15.300	06.800	02.630	03.250
CD68	11.800	05.833	00.333	03.000
CD71	02.750	01.010	00.170	00.200
CD74	04.400	01.430	00.767	00.300
Mac387	07.980	01.838	00.667	00.033
IgG	05.050	02.586	00.167	00.375
IgM	01.470	00.710	00.650	00.000*
IgA	02.980	01.010	00.920	00.130

\* Not detected due to lack of cell type.

\*\* Not detected due to technical difficulty.

**TABLE 5:****CD4: COMPARISON OF MEANS BETWEEN GRANULOMA TYPES USING  
INDEPENDENT ANALYSIS OF VARIANCE**

MEAN FOR EXUDATIVE TYPE = 7.375  
 MEAN FOR GRANULOMATOUS TYPE = 3.200  
 MEAN FOR GRANULATION TISSUE TYPE = 0.767  
 MEAN FOR FOREIGN-BODY TYPE = 0.300

FOR 166 DEGREES OF FREEDOM

T- VALUE AT 0.050- 1.960

T- VALUE AT 0.010- 2.576

T- VALUE AT 0.001- 3.291

\* = P<.05

\*\* = P<.01

\*\*\* = P<.001

		<b>EXUDATIVE.</b>	<b>GRANULOM.</b>	<b>GRANULAT. TISSUE</b>
	<b>MEANS</b>	7.375	3.200	0.767
<b>GRANULOM.</b>	3.200	4.175***		
<b>GRANULAT. TISSUE</b>	0.767	6.608***	2.433**	
<b>FORN-BODY</b>	0.300	7.075***	2.900*	0.467

**TABLE 6:****CD8: COMPARISON OF MEANS BETWEEN GRANULOMA TYPES USING  
INDEPENDENT ANALYSIS OF VARIANCE:**

MEAN FOR EXUDATIVE TYPE = 3.975  
 MEAN FOR GRANULOMATOUS TYPE = 1.533  
 MEAN FOR GRANULATION TISSUE TYPE = 0.000  
 MEAN FOR FOREIGN-BODY TYPE = 0.300

FOR 166 DEGREES OF FREEDOM

T- VALUE AT 0.050- 1.960  
 T- VALUE AT 0.010- 2.576  
 T- VALUE AT 0.001- 3.291

\* = P<.05  
 \*\* = P<.01  
 \*\*\* = P<.001

		<b>EXUDATIVE.</b>	<b>GRANULOM.</b>	<b>GRANULAT. TISSUE</b>
	<b>MEANS</b>	3.975	1.533	0.000
<b>GRANULOM.</b>	1.533	2.441***		
<b>GRANULAT. TISSUE</b>	0.000	3.975***	1.533**	
<b>FORN-BODY</b>	0.300	3.675***	1.233	0.300

TABLE 8:

**CD15: COMPARISON OF MEANS BETWEEN GRANULOMA TYPES USING  
INDEPENDENT ANALYSIS OF VARIANCE:**

MEAN FOR EXUDATIVE TYPE = 4.150  
 MEAN FOR GRANULOMATOUS TYPE = 2.633  
 MEAN FOR GRANULATION TISSUE TYPE = 0.900  
 MEAN FOR FOREIGN-BODY TYPE = 2.500

FOR 166 DEGREES OF FREEDOM

T- VALUE AT 0.050- 1.960  
 T- VALUE AT 0.010- 2.576  
 T- VALUE AT 0.001- 3.291

\* = P<.05  
 \*\* = P<.01  
 \*\*\* = P<.001

		polymorpho	GRANULOM.	GRANULAT. TISSUE
	MEANS	4.150	2.633	0.900
GRANULOM.	2.633	1.517*		
GRANULAT. TISSUE	0.900	3.250***	1.733**	
FORN-BODY	2.500	1.650	0.133*	1.600

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TABLE 9:

**CD20: COMPARISON OF MEANS BETWEEN GRANULOMA TYPES USING  
INDEPENDENT ANALYSIS OF VARIANCE**

MEAN FOR EXUDATIVE TYPE = 1.760  
 MEAN FOR GRANULOMATOUS TYPE = 0.830  
 MEAN FOR GRANULATION TISSUE TYPE = 0.867  
 MEAN FOR FOREIGN-BODY TYPE = 0.000

FOR 376 DEGREES OF FREEDOM

T- VALUE AT 0.050- 1.960

T- VALUE AT 0.010- 2.576

T- VALUE AT 0.001- 3.291

\* = P<.05

\*\* = P<.01

\*\*\* = P<.001

		<b>EXUDATIVE.</b>	<b>GRANULOM.</b>	<b>GRANULAT. TISSUE</b>
	<b>MEANS</b>	1.760	0.830	0.867
<b>GRANULOM.</b>	0.830	0.930*		
<b>GRANULAT. TISSUE</b>	0.867	0.893	0.036	
<b>FORN-BODY</b>	0.000	1.760**	0.830	0.867

**TABLE 10:****CD45R: COMPARISON OF MEANS BETWEEN GRANULOMA TYPES USING  
INDEPENDENT ANALYSIS OF VARIANCE:**

MEAN FOR EXUDATIVE TYPE = 3.860  
 MEAN FOR GRANULOMATOUS TYPE = 1.714  
 MEAN FOR GRANULATION TISSUE TYPE = 0.783  
 MEAN FOR FOREIGN-BODY TYPE = 0.567

FOR 356 DEGREES OF FREEDOM

T- VALUE AT 0.050- 1.960  
 T- VALUE AT 0.010- 2.576  
 T- VALUE AT 0.001- 3.291

\* = P<.05

\*\* = P<.01

\*\*\* = P<.001

		<b>EXUDATIVE.</b>	<b>GRANULOM.</b>	<b>GRANULAT. TISSUE</b>
	<b>MEANS</b>	3.867	1.714	0.783
<b>GRANULOM.</b>	1.714	2.152**		
<b>GRANULAT. TISSUE</b>	0.783	3.083***	0.931	
<b>FORN-BODY</b>	0.567	3.300**	1.148	0.217

**TABLE 11:****CD68: COMPARISON OF MEANS BETWEEN GRANULOMA TYPES USING  
INDEPENDENT ANALYSIS OF VARIANCE:**

MEAN FOR EXUDATIVE TYPE = 11.800  
 MEAN FOR GRANULOMATOUS TYPE = 5.833  
 MEAN FOR GRANULATION TISSUE TYPE = 0.333  
 MEAN FOR FOREIGN-BODY TYPE = 3.000

FOR 166 DEGREES OF FREEDOM

T- VALUE AT 0.050- 1.980  
 T- VALUE AT 0.010- 2.617  
 T- VALUE AT 0.001- 3.373

\* = P<.05

\*\* = P<.01

\*\*\* = P<.001

		<b>EXUDATIVE.</b>	<b>GRANULOM.</b>	<b>GRANULAT. TISSUE</b>
	<b>MEANS</b>	11.800	5.833	0.333
<b>GRANULOM.</b>	5.833	5.967***		
<b>GRANULAT. TISSUE</b>	0.333	11.467***	5.500***	
<b>FORN-BODY</b>	3.000	8.800***	2.833*	2.667

TABLE 12:

**CD74: COMPARISON OF MEANS BETWEEN GRANULOMA TYPES USING  
INDEPENDENT ANALYSIS OF VARIANCE:**

MEAN FOR EXUDATIVE TYPE = 4.400  
 MEAN FOR GRANULOMATOUS TYPE = 1.433  
 MEAN FOR GRANULATION TISSUE TYPE = 0.767  
 MEAN FOR FOREIGN-BODY TYPE = 0.300

FOR 166 DEGREES OF FREEDOM

T- VALUE AT 0.050- 1.960  
 T- VALUE AT 0.010- 2.576  
 T- VALUE AT 0.001- 3.291

\* = P<.05  
 \*\* = P<.01  
 \*\*\* = P<.001

		<b>EXUDATIVE.</b>	<b>GRANULOM.</b>	<b>GRANULAT. TISSUE</b>
	<b>MEANS</b>	4.400	1,433	0.767
<b>GRANULOM.</b>	1,433	2.967***		
<b>GRANULAT. TISSUE</b>	0.767	3.633***	0.667	
<b>FORN-BODY</b>	0.300	4.100***	1.133	0.467



**TABLE 13:****MAC387: COMPARISON OF MEANS BETWEEN GRANULOMA TYPES USING INDEPENDENT ANALYSIS OF VARIANCE:**

MEAN FOR EXUDATIVE TYPE = 7.980  
 MEAN FOR GRANULOMATOUS TYPE = 1.838  
 MEAN FOR GRANULATION TISSUE TYPE = 0.667  
 MEAN FOR FOREIGN-BODY TYPE = 0.033

FOR 346 DEGREES OF FREEDOM

T- VALUE AT 0.050- 1.960  
 T- VALUE AT 0.010- 2.576  
 T- VALUE AT 0.001- 3.291

\* = P<.05  
 \*\* = P<.01  
 \*\*\* = P<.001

		<b>EXUDATIVE.</b>	<b>GRANULOM.</b>	<b>GRANULAT. TISSUE</b>
	<b>MEANS</b>	7.980	1.838	0.667
<b>GRANULOM.</b>	1.838	6.142***		
<b>GRANULAT. TISSUE</b>	0.667	7.313***	1.171	
<b>FORN-BODY</b>	0.033	7.947***	1.805	0.633

**TABLE 14:****IgG: COMPARISON OF MEANS BETWEEN GRANULOMA TYPES USING  
INDEPENDENT ANALYSIS OF VARIANCE**

MEAN FOR EXUDATIVE TYPE = 5.050

MEAN FOR GRANULOMATOUS TYPE = 2.586

MEAN FOR GRANULATION TISSUE TYPE = 0.167

MEAN FOR FOREIGN-BODY TYPE = 0.375

FOR 366 DEGREES OF FREEDOM

T- VALUE AT 0.050- 1.960

T- VALUE AT 0.010- 2.576

T- VALUE AT 0.001- 3.291

\* = P&lt;.05

\*\* = P&lt;.01

\*\*\* = P&lt;.001

		<b>EXUDATIVE.</b>	<b>GRANULOM.</b>	<b>GRANULAT. TISSUE</b>
	<b>MEANS</b>	5.050	2.586	0.167
<b>GRANULOM.</b>	2.586	2.464***		
<b>GRANULAT. TISSUE</b>	0.167	4.883***	2.419***	
<b>FORN-BODY</b>	0.375	4.675***	2.211**	0.208

**TABLE 15:**

**COMPARISON OF MEANS OF EXUDATIVE TYPE GRANULOMAS  
WITH CD4 AND CD8 MONOCLONAL ANTIBODIES USING  
INDEPENDENT ANALYSIS OF VARIANCE:**

Mean of exudative type granuloma (1) using CD4 = 7.375

Mean of exudative type granuloma (1) using CD8 = 3.975

For 78 degrees of freedom.

T- Value at .05- 2.00

T- Value at .01- 2.66

T- Value at .001- 3.460

\* =  $P < .05$

\*\* =  $P < .01$

\*\*\* =  $P < .001$

		<b>EXUDATIVE (1)</b>
	<b>MEANS</b>	7.375
<b>EXUDATIVE (2)</b>	3.975	3.400**

**TABLE 16:**

**COMPARISON OF MEANS OF GRANULOMATOUS TYPE GRANULOMAS  
WITH CD4 AND CD8 MONOCLONAL ANTIBODIES USING INDEPENDENT ANALYSIS OF  
VARIANCE:**

Mean of granulomatous type granuloma (1) using CD4 = 3.189  
Mean of granulomatous type granuloma (2) using CD8 = 1.567

For 178 degrees of freedom.

T- Value at .05- 2.00

T- Value at .01- 2.667

T- Value at .001- 3.460

\* =  $P < .05$

\*\* =  $P < .01$

\*\*\* =  $P < .001$

		<b>GRANULOMATOUS (1)</b>
	<b>MEANS</b>	3.189
<b>GRANULOMATOUS (2)</b>	1.567	1.622***

TABLE 17:

**COMPARISON OF MEANS OF GRANULATION TISSUE TYPE GRANULOMAS WITH CD4 AND CD8 MONOCLONAL ANTIBODIES USING INDEPENDENT ANALYSIS OF VARIANCE:**

Mean of granulation tissue type granuloma (1) using CD4 = 0.767

Mean of granulation tissue type granuloma (2) using CD8 = 0.000

For 58 degrees of freedom.

T- Value at .05- 2.000

T- Value at .01- 2.704

T- Value at .001- 3.551

\* =  $P < .05$

\*\* =  $P < .01$

\*\*\* =  $P < .001$

		<b>GRANULAT. TISSUE (1)</b>
	<b>MEANS</b>	0.767
<b>GRANULATION TISSUE (2)</b>	0.000	0.767**

**TABLE 18:****COMPARISON OF MEANS OF FOREIGN-BODY TYPE GRANULOMAS WITH CD4 AND CD8 MONOCLONAL ANTIBODIES USING INDEPENDENT ANALYSIS OF VARIANCE:**

Mean of foreign-body type granuloma (1) using CD4 = 0.400

Mean of foreign-body type granuloma (2) using CD8 = 0.300

For 18 degrees of freedom.

F ratio found not to be significant

**TABLE 19:****COMPARISON OF THE MEANS OF EXUDATIVE TYPE GRANULOMAS WITH CD20 AND CD45R MONOCLONAL ANTIBODIES USING INDEPENDENT ANALYSIS OF VARIANCE:**

Mean of exudative type granuloma (1) using CD20 = 1.800

Mean of exudative type granuloma (2) using CD45R = 3.867

For 108 degrees of freedom.

F ratio found not to be significant.

TABLE 20:

**COMPARISON OF MEANS OF GRANULOMATOUS TYPE GRANULOMAS WITH CD20 AND CD45R MONOCLONAL ANTIBODIES USING INDEPENDENT ANALYSIS OF VARIANCE:**

Mean of granulomatous type granuloma (1) using CD20 = 0.830

Mean of granulomatous type granuloma (2) using CD45R= 1.752

For 438 degrees of freedom

T- Value at .05- 1.960

T- Value at .01- 2.576

T- Value at .001- 3.291

\* =  $P < .05$

\*\* =  $P < .01$

\*\*\* =  $P < .001$

		<b>GRANULOMATOUS (1)</b>
	<b>MEANS</b>	0.830
<b>GRANULOMATOUS (2)</b>	1.752	0.922**

TABLE 21:

**COMPARISON OF MEANS OF GRANULATION TISSUE TYPE GRANULOMAS WITH CD20 AND CD45R MONOCLONAL ANTIBODIES USING INDEPENDENT ANALYSIS OF VARIANCE:**

Mean of granulation tissue type granuloma (1) using CD20= 0.867

Mean of granulation tissue type granuloma (2) using CD45R= 0.783

For 118 degrees of freedom.

F ratio not found to be significant.

**TABLE 22:****COMPARISON OF MEANS OF FOREIGN-BODY TYPE GRANULOMAS WITH CD20 AND CD45R MONOCLONAL ANTIBODIES USING INDEPENDENT ANALYSIS OF VARIANCE:**

Mean of foreign-body type granuloma (1) using CD20= 0.000  
Mean of foreign-body type granuloma (2) using CD45R= 0.567

For 68 degrees of freedom.

F ratio found not to be significant.

**TABLE 23:****COMPARISON OF MEANS OF EXUDATIVE TYPE GRANULOMAS WITH CD11b AND MAC387 MONOCLONAL ANTIBODIES USING INDEPENDENT ANALYSIS OF VARIANCE:**

Mean of exudative granuloma (1) using CD11b = 8.75  
Mean of exudative granuloma (2) using CD MAC387 = 8.00

For 88 degrees of freedom.

F ratio found not to be significant



**TABLE 24:****COMPARISON OF MEANS OF GRANULOMATOUS TYPE GRANULOMAS WITH CD11b AND MAC387 MONOCLONAL ANTIBODIES USING INDEPENDENT ANALYSIS OF VARIANCE:**

Mean of granulomatous type granuloma (1) using CD11b= 4.511  
 Mean of granulomatous type granuloma (2) using MAC387 = 1.852

For 298 degrees of freedom.

T- Value at .05- 1.960

T- Value at .01- 2.576

T- Value at .001- 3.291

\* =  $P < .05$

\*\* =  $P < .01$

\*\*\* =  $P < .001$

		<b>GRANULOMATOUS (1)</b>
	<b>MEANS</b>	4.511
<b>GRANULOMATOUS (2)</b>	1.852	2.659***

**TABLE 25:****COMPARISON OF MEANS OF GRANULATION TISSUE TYPE GRANULOMAS WITH CD11b AND MAC387 MONOCLONAL ANTIBODIES USING INDEPENDENT ANALYSIS OF VARIANCE:**

Mean of granulation tissue type granuloma (1) using CD11b = 0.733

Mean of granulation tissue type granuloma (2) using MAC387= 0.650

For 88 degrees of freedom.

F ratio found not to be significant.

**TABLE 26:****COMPARISON OF MEANS OF FOREIGN-BODY TYPE GRANULOMAS  
WITH CD11b AND MAC387 MONOCLONAL ANTIBODIES USING INDEPENDENT  
ANALYSIS OF VARIANCE:**

Mean of foreign-body type granuloma (1) using CD11b= 0.000

Mean of foreign-body type granuloma (2) using MAC387= 0.033

For 38 degrees of freedom.

F ratio found not to be significant.

## **CHAPTER V**

### **DISCUSSION**

The term granulomatous inflammation includes an ill-defined group of lesions whose unique feature is their morphologic appearance.

This study attempted to justify the term granulomatous inflammation to apical granulomas. This study has also proposed a classification of apical granulomas into four separate and distinct subclasses: exudative, granulomatous, granulation-tissue type, and foreign body granulomas. Based on the homogeneity in the pattern of the inflammatory cell phenotypes, each subclass was found to satisfy the requirements for sub-classification. The differing characteristics of the subclasses will be presented and discussed according to the findings of this study. It is based on light microscopic observation of 40 apical granulomas routinely and immunohistochemically stained with a panel of relevant, commercially available monoclonal antibodies (TABLE 2).

#### **EXUDATIVE SUBCLASS OF APICAL GRANULOMAS:**

The exudative subclass of apical granulomas was significantly different from the granulomatous, granulation tissue-type, and foreign body type of apical lesions. More reactive cells including polymorphonuclear leukocytes, monocytes, macrophages, histiocytes as well as lymphocytes, and plasma cells were observed in this sub-class than in any of the three other subclasses.

The exudative subclass of apical lesions was consistent with the literature in representing an ongoing acute inflammatory process in reaction to bacterial by-products, toxins and denatured host tissue released from infected or necrotic root canal systems. The presence of large numbers of polymorphonuclear leukocytes highlighted this subclass showing the possibility of a primary non-specific inflammatory response. This, together with all the elements of the humoral induced hypersensitivity reaction, such as B cells, plasma cells as well as the elements of the cell-mediated response, such as monocytes, macrophages and T cells were present in this group of apical lesions. Their presence in significantly larger numbers than that which occurred in any of the three other subclasses was suggestive of the dynamic aspects involved in this form of inflammatory response.

#### **A. POLYMORPHONUCLEAR LEUKOCYTES:**

Exudative type apical lesions were distinct from the other subclasses due to the presence of larger numbers of polymorphonuclear leukocytes (PMN) as observed by light microscope morphology in hematoxylin and eosin stained tissue sections. Granulocytes were subdivided into three basic cell types: basophils, eosinophils and neutrophil polymorphonuclear leukocytes. In this study, the term granulocyte was used interchangeably with polymorphonuclear neutrophil leukocyte (PMN).

## **IMMUNOHISTOCHEMICAL REACTIVITY AND DISTRIBUTION OF POLYMORPHONUCLEAR LEUKOCYTES:**

Immunohistochemically, granulocytes were reactive with several monoclonal antibodies such as CD11b, CD15, CD16, and CD71. The monoclonal antibody CD15 was the most specific for PMN's reacting with receptor sites on the cell membrane as well as the cytoplasm of neutrophilic granules.

Findings in this study, using immunohistochemical staining methods, indicated, as it did with direct microscopic observations, that the exudative subclass tissue specimens had significantly ( $p < .05$ ) higher numbers of PMN's than the granulomatous and granulation tissue-type lesions. It was found not to be significantly greater than the foreign body type (TABLE 8). It was difficult to determine the labelling cell types detected with the other monoclonal antibodies (TABLE 5). This was due mainly to an apparent overlap among immunoreactive cell phenotypes. This was overcome when cell morphology was taken into consideration.

### **SIGNIFICANCE Of PMN's:**

The presence of PMN's in apical granulomas can be explained in terms of a first line of defence of the host against invading microorganisms or the presence of high antigen concentration.

Activation of both the classic complement pathway by antigen-antibody reactions and the alternative complement pathway by certain bacterial products generate complement fragments, able to

amplify the inflammatory response by attracting and activating PMN's. Complement fragments such as C3a, C3b, C5a, and others mediate these effects through neutrophil cell surface complement receptors.

The attraction or chemotaxis of neutrophils to the affected area occurs through a variety of factors derived from cellular as well as plasma sources. In response to minute concentrations of chemotactic factors, neutrophils respond by a series of sequential steps to recognize, adhere to, and phagocytise particles. This is followed by a discharge of cytoplasmic granule contents into phagocytic vacuoles, and the generation of bursts of oxydative metabolism. Phagocytised microorganisms coated with complement and specific antibody (opsonization), are killed by a combination of neutrophil-generated toxic oxygen radicals and cytotoxic cytoplasmic granule-derived proteins (5).

The presence of large numbers of PMN's in the tissue sections of the exudative subclass would justify the designation of this subclass generally referred to as the active or acute phase of apical granulomas (57,65).

The presence of large numbers of PMN's in the foreign body subclass was observed and may suggest that the foreign body apical granuloma may quickly reach a peak in the PMN concentration and then rapidly stabilize (TABLE 8).

#### **B. MONONUCLEAR PHAGOCYTES:**

Monocytes, histiocytes, macrophages, and epithelioid cells are referred to as mononuclear phagocytes. They comprise the

mononuclear phagocytic system derived from myeloid progenitor cells in the bone marrow. Blood monocytes differentiate when stimulated by various substances into macrophages. Tissue monocytes are known as histiocytes.

#### **IMMUNOHISTOCHEMICAL REACTIVITY AND DISTRIBUTION OF MONONUCLEAR PHAGOCYTES:**

Several monoclonal antibodies reacted with monocytes and macrophages. In the present study, these were CD11b, CD14 (highly reactive with monocytes), CD45RO, CD68, CD71, CD74 and MAC387. Various reactions were observed with monocytes and histiocytes and there was considerable overlap in the specific reactivity of the antibodies to one or more mononuclear cell types (TABLE 1).

The present study showed that there was a significantly greater number of reactive monocytes, macrophages, and histiocytes ( $p < .001$ ) in the exudative subclass of apical granuloma tissue specimens, than in any other subclass with the antibodies CD11b, CD68, CD74 and MAC387 (TABLES 7,11,12,13). When reacted with the antibodies CD14, CD16, CD45RO, and CD71 (TABLE 4) there was a greater number of these reactive cells in the exudative tissue specimens than in the other subclasses, however, the degree of significance was not established. Reactivity of the fresh-frozen tissues with CD14 was poor even when tested with several different dilutions of the antibody.

The number of reactive monocytes to antibody CD74 in the exudative granulomas was significantly ( $p < .001$ ) (TABLE 12) greater than in any of the other tissue groups.

## SIGNIFICANCE OF MONONUCLEAR PHAGOCYTES:

The mononuclear phagocytic cells, in this study, composed the most consistent cell types found in all types of subclasses of apical granulomas. This feature confirms these lesions as being chronic granulomatous inflammation (TABLE 4).

Mononuclear phagocytes respond to the same chemotactic factors that attract PMNs (e.g., C5a). The primary chemoattractants to which macrophages respond are soluble factors released from T lymphocytes. Monocytes/macrophages use mechanisms of phagocytosis similar to PMNs. Macrophages appear to be stimulated by the processes involved in phagocytosis and become secretory cells producing acute phase proteins such as IL-1. IL-1 enhances the expression of the antigen on the cell membrane of the ingesting macrophage for presentation to the T cells.

Following an inflammatory stimulus, the macrophages that accumulate constitute exudative macrophages. These cells synthesize and secrete monokines that activate cells of the immune system and influence other aspects of inflammation affecting coagulation, complement activity, and proliferation of cells involved in tissue regeneration and repair.

Macrophages are also responsible for antigen recognition, processing the antigen, and functioning as the antigen presenting cells to B and T cells. Antigen presentation is an important function of a subset of macrophages that have HLA-DR (or Ia) antigens on their surface. The HLA-DR (MHC class II) also known as CD74 molecule



interacts with the antigen and allow the macrophages to communicate with and sensitize antigen-specific T lymphocytes. Therefore, macrophages overlap as critical effectors of both the natural and cell-mediated immunity (90). The presence of CD74(+) macrophages in the exudative type of apical lesions probably modulate a similar immunologic reaction of antigen presentation to T cells. In contrast, CD68 (-) macrophages, monocytes, and histiocytes may not function as antigen presenting cell phenotypes.

### C. T/B CELLS:

Monoclonal antibodies directed against cell surface antigens have demonstrated identifying population heterogeneity among lymphocytes. On the basis of the expression of cell-surface markers, 3 distinct cell lines of lymphocytes have been identified: T cells, B cells, and natural killer (NK) cells. The presence or absence of certain cell surface markers has been used to delineate stages of differentiation, states of cellular activation, and functionally distinct subsets of lymphocytes. Two T cell subsets were of particular importance to this investigation: the T helper/inducer (Th/i) cell subset characterized by the CD4 cell surface marker and T cytotoxic/suppressor (Tc/s) cells characterized by the CD8 cell surface marker.

T cells arise from maturation of stem cells in the thymus, mature T cells are then released into the circulation and peripheral lymphoid tissues.

B cells arise from progenitor cells in the bone marrow. Functional immunoglobulin heavy and light chain genes are synthesized and expressed on the cell surface but have not yet interacted with antigens. They then migrate from the bone marrow to the circulation and home in the peripheral lymphoid tissue. Sensitized B cells, in response to the antigen, migrate to the regional lymph nodes and transform to plasma cells with a subsequent return to the site of activation.

#### **IMMUNOHISTOCHEMICAL REACTIVITY, AND DISTRIBUTION OF T/B CELLS:**

T cells and B cells are distinguished primarily by their antigen receptors and by certain characteristic cell surface markers called clusters of differentiation. T cells recognize antigens by a membrane structure called the CD3/T cell antigen receptor complex, whereas B cells recognize antigens by using surface (fab) immunoglobulin molecules (5).

The commercially available monoclonal antibodies that were reactive with T cells were CD3, CD4, CD8, CD45R, CD45RO and CD71 (TABLE 4). As with other monoclonal antibodies, there was an overlap in reactivity with CD45R, CD45RO, and CD71 (TABLE 1), such that other immunocompetent (expressing the same epitope) cells would also stain positive with these monoclonal antibodies. The monoclonal antibody CD3 was specific for all T cells, CD4 was specific for the Th/i subset, while CD8 was specific for Tc/s subsets.

The B cell antigenic determinants were reactive with CD20, CD22, CD45R, CD45RO (B cell subset), CD71 (activated B cell), and

CD74. Only CD20, and CD22 monoclonal antibodies, however, were specific for B cells. It was observed that among these monoclonal antibodies, CD20, CD45R, CD45RO, reacted with formalin-fixed tissues while the remainder reacted with fresh-frozen tissues.

Results of the immunohistochemical staining procedures suggested that with the CD4 and CD8 monoclonal antibodies, the exudative subclass tissue specimens had significantly ( $p < .001$ ) greater numbers of reactive Th/i cells than any of the other three subclasses. Within the exudative subclasses, it was found that there was a significantly ( $p < .01$ ) greater number of Th/i cells than Tc/s cells (TABLES 5,6,15).

With respect to the number of reactive B cells with the monoclonal antibodies in this study, the exudative subclass tissue sections showed a lesser significance ( $p < .05$ ) when compared with the granulomatous subclass than did the foreign body subclass ( $p < .01$ ). It is to be noted that there was no significant difference between the exudative and granulation tissue subclasses using the CD20 monoclonal antibody (TABLE 9). Results from the tissue section reaction with the CD22 monoclonal antibody was erratic and discarded (TABLE 5). With the use of CD45R, and CD74 significance was present ( $p < .01-.001$ ) (TABLES 10, 12), however, although these were reactive with B cells, there was possible overlap with other reactive cells.

Results of the immunohistochemical staining procedures indicated that with the CD4 and CD8 monoclonal antibodies, the exudative subclass tissue specimens had significantly ( $p < .001$ ) greater numbers of reactive Th/i and Tc/s cells than any of the other three

subclasses. Within the exudative subclasses, it was found that there was a significantly ( $p < .01$ ) greater number of Th/i subset cells than Tc/s cells (TABLES 5,6,15)

#### **SIGNIFICANCE OF T CELLS:**

The presence of significantly larger numbers of Th/i and Tc/s cells in the exudative lesions further defined this subclass within apical granulomas.

Th/i (CD4+) cells augment the B cell responses and amplify the cell-mediated responses of CD8(+) T cells. CD4(+) T cells can also mediate cytotoxicity and immune suppression, hence the name helper/inducer cell. CD4(+) T cells usually recognize peptide antigens that are bound to class II MHC glycoproteins present on the surface of an antigen-presenting cell. T cells become activated and secrete factors such as IL-2 and IL-4 which serve to stimulate cytotoxicity and proliferation of other T and B cells. IL-4 however can also inhibit the activation of B and NK cells by IL-2. The CD4+ T cells can, therefore, provide regulatory factors that either augment or suppress functions mediated by the entire immune system (5).

Tc/s (CD8+) cells mediate most antigen-specific cytotoxicity which is the ability to kill other cells that are perceived to be foreign (cells with altered self antigen), and foreign cells introduced into the host by allogenic transplantation and virally-infected cells. CD8(+) T cells recognize peptide antigens bound to class I MHC molecules on the cell surface of the target as opposed to CD4(+) T cells which recognize

class II MHC molecules. As a consequence of activation CD8(+) T cells also release cytokines such as IL-2 or gamma interferon which can augment immune responses by other B and T lymphocytes. CD8+ T cells can suppress immune responses by the release of soluble factors that interfere with the function of other immune cells, hence the name cytotoxic/suppressor cell (5).

Much has been written in the literature concerning the ratio of Th/i cells to Tc/s cells at different intervals during the development of the apical granuloma (67) indicating that Th/i cells were more numerous during the acute phase with the Tc/s cells becoming more numerous during the chronic phase.

In this series of apical granulomas, it was found that the Th/i cells were more numerous in both the exudative or more acute form of apical granuloma as well as in the granulomatous or more chronic form of the lesion (TABLE 5). Although the ratio stayed the same, the total number of Th/i and Tc/s reactive cells per high power field in the granulomatous sub-class was less than 45% that of the exudative sub-class. This would suggest that although the mechanisms of the immune response were the same in the exudative subclass of lesions as they were in the granulomatous subclass, these mechanisms were greatly diminished. Moreover the number of Th/i and Tc/s cells were rarely observed in the granulation tissue specimens as well as foreign body tissue specimens such as to indicate that there was little cell mediated immune response and pathogenic activity .

## SIGNIFICANCE OF B CELLS:

B cells express immunoglobulin on the cell surface membrane. The immunoglobulins on B cells are able to bind directly and with high affinity to antigens, including the epitope glycoproteins, glycolipids, polysaccharides, peptides, and almost any immunogenic molecule.

Antigen-specific activation of B cells occurs after binding of antigen to cell membrane immunoglobulin independent of the action of the antigen presenting cell. Activation via the phosphatidylinositol pathway, due to soluble factors released from monocytes or T cells, produces clonal expansion as with T cell activation. Two types of antigens have been described : T cell-independent and T cell-dependent antigens. B cell proliferation and immunoglobulin secretion without the presence of Th/i cells occur when B cells encounter T cell-independent antigens.

Response to T cell-dependent antigens requires interaction between B and T cells for subsequent immunoglobulin secretion. Th/i cells recognize the antigen and produce soluble mediators, including IL-4 and -5 and other cytokines that increase or help B cells to respond.

This study concurred with those of others (6) in that the number of B cells were generally fewer than the number of T cells in similar tissues. It was observed that there were more reactive B cells in the exudative tissue sections than in the granulomatous sections. This was indicative of the presence of a humoral response in these tissues but having greater activity in the exudative lesions. It would also seem

to indicate that the dominant activity was the cell mediated immune response.

#### **D. PLASMA CELLS:**

B cells are activated by the antigens and differentiate into plasma cells given the appropriate factors. Once this differentiation has taken place, the plasma cells secrete large amounts of soluble immunoglobulins into the serum or tissue.

#### **IMMUNOHISTOCHEMICAL REACTIVITY AND DISTRIBUTION OF IMMUNOGLOBULINS:**

Five distinct classes of immunoglobulins have been identified: IgG, IgA, IgM, IgD, and IgE. These five classes differ in size, charge, amino acid composition, and carbohydrate content, especially on the heavy chains of the molecule. Monoclonal antibodies utilized in this study were reactive specifically with the gamma-chains of the IgG, the mu-chain of the IgM, the alpha-chain of the IgA, and the epsilon chain of the IgE.

This study showed more B cell reactivity with IgG and IgA antibodies than with IgM and IgE antibodies even through repeated testing with several different dilutions of the antibodies.

There is considerable controversy in the literature regarding immunological staining. Based on the observations of this study for the presence of immunoglobulins, a positive reaction for these antibodies are difficult to measure especially in formalin-fixed tissues.

Torabinejad (4), using similar immunohistochemical procedures on periapical granuloma tissue sections, found the presence of IgE in 74% of the specimens and IgG in 100%. Positive identification consisted of observing at least 1 positive plasma cell in each of 5 microscopic fields at 400x magnification. The validity of this study is questionable since no statistical evaluations were conducted.

Generally higher concentrations of reactive cells were observed in the exudative sub-class of apical granulomas. The greatest concentration of immunoglobulins was in the exudative form of the lesions with IgG. IgA also showed a relatively strong presence in the exudative sub-class. Other tissue sub-classes showed some reactive cells but were few in number and not statistically measured.

#### **SIGNIFICANCE OF PLASMA CELLS:**

Plasma cells secrete immunoglobulins which are bifunctional molecules that bind antigens as well as initiate other biologic phenomena which are independent of antibody specificity (5).

IgG constitutes 75% of total serum immunoglobulins. It is the predominant antibody in the secondary immune response and as such is the majority of the immune response.

IgM is the first immunoglobulin to appear in the immune response and is the predominant antibody in a primary immune response. IgA is the predominant immunoglobulin in secretions. IgD is expressed in association with IgM on the cell surface to produce B cell differentiation. IgE antibodies have several names that reflect their



biologic capabilities. The term cytophilic denotes their propensity to bind firmly to basophilic and mast cells. Skin-sensitizing antibody reflects the effects of IgE in the skin, where mast cells are abundant. It appears that the main function of IgE is to arm mast cells and basophils with antigen-specific receptors. These cells then act as the mediators of allergy and parasitic immunity by releasing potent mediators of inflammation and chemoattractants for a variety of cell types including eosinophils and platelets (91).

In relation to this study and that of others (4), the presence of IgG, IgA, and to a lesser extent IgM antibodies in the exudative subclass type of apical granuloma, is indicative of a type III hypersensitivity response to the presence of abundant antigenic determinants. The reduced numbers in the three other proposed subclasses indicates the reduced reactivity of these mechanisms, probably due to the reduced quantity of antigens present in this subclass.

#### **GRANULOMATOUS TYPE SUBCLASS OF GRANULOMAS:**

The granulomatous subclass type of apical lesions was predominated by the presence of mononuclear phagocytic histiocytes, together with varying numbers of lymphocytes and plasma cells.

**IMMUNOHISTOCHEMICAL REACTIVITY AND DISTRIBUTION OF REACTIVE PHENOTYPES:**

Immunohistochemical staining for this subclass of lesions yielded positive reaction with all monoclonal antibodies but to a lesser degree than that which occurred in the exudative group as has already been noted in characterizing the latter (TABLE 4).

In most cases this subclass showed substantially more reactive phenotypes than both the granulation tissue type and the foreign body subclass. The greatest statistical significance ( $p < .001$ ) occurred with the CD11b monoclonal antibody, showing the presence of a greater number of mononuclear phagocytic monocytes than in either the granulation tissue type or the foreign body type apical granulomas (TABLE 7). Positive reactions occurred with CD4, CD8 (excluding foreign body type), CD15, CD68, and IgG, ranging from  $p < .001$  to  $p < .05$  (TABLES 5,6,8,11,14).

It was also shown that a significantly ( $p < .001$ ) greater number of Th/i subset existed in the granulomatous subclass than Tc/s subset (TABLE 16) as demonstrated by comparing reactive phenotypes with the CD4 and CD8 monoclonal antibodies. When comparing CD20 and CD45R monoclonal antibodies within granulomatous type lesions, it was suggested that there was a greater number of mononuclear phagocytic cells than B cells present (TABLE 20). A comparison between reactivity with CD11b and MAC387 showed significantly ( $p < .001$ ) greater reactive phenotypes present with the CD11b (TABLE 24).

**SIGNIFICANCE:**

The results showed the presence of significant numbers of phagocytic mononuclear monocytes, and to a lesser degree Th/i subset, B cells and IgG when compared to the granulation tissue type and foreign body type apical granulomas. It was suggestive of the continued presence of the elements involved in antibody and cell mediated immunity although less than that occurring in the exudative group.

The presence of the phenotype fractions in this group of lesions, including the predominance of mononuclear phagocytes, and to a lesser extent lymphocytes and plasma cells, fulfills the definition of a granulomatous type inflammation which may be found elsewhere in the body (92), and asserts its subclass description in apical granulomas.

**FOREIGN BODY TYPE SUBCLASS OF GRANULOMAS:**

Foreign body subclass of apical granulomas were composed of monocytes, macrophages, non-digested foreign body particles such as silver, zinc oxide, root canal sealing materials, and varying amounts of fibrosis.

**IMMUNOHISTOCHEMICAL REACTIVITY AND DISTRIBUTION OF REACTIVE PHENOTYPES:**

This subclass of apical granulomas showed signs of acute inflammatory reactions and high cellularity within some of the tissues

observed. It was generally portrayed, however, as an end-stage lesion being walled off by dense fibrous connective tissue with the presence of few reactive inflammatory cells present (TABLE 4).

The presence of reactive cells were observed when the tissues were reacted with CD3, CD45RO, CD68 and CD 15 showing that the possibility exists for an acute inflammatory response to occur within foreign body granulomas.

#### SIGNIFICANCE:

Apical granulomas induced by foreign body dental materials are mostly of a low turnover type, based on the pattern of low cell reactivity, in contrast to the higher turnover demonstrated in other forms of such lesions (92). A low-turnover chronic granulomatous inflammation is separate from the high turnover which exhibits epithelioid cells. Epithelioid cells are the end-stage differentiating of mononuclear phagocytic cell lines. These phenotypes of the mononuclear phagocytic system lose their phagocytic properties and acquire an elaborate system of enzyme secretion as they attempt to encompass but not endocytose foreign body material (92).

Low turnover granulomas also differ from their high turnover counterpart in that the recruitment of new phagocytes from systemic sources or in situ proliferation of already migrating histiocytes is not a prominent feature in the former (92). The number of dead, dying and replacement cells are minimal. Low turnover granulomas may show a rapid onset of development quickly reaching peak size. Once this is

reached, however, growth stops and the lesion remains walled off provided they are not challenged with the presence of new antigens (93).

#### **GRANULATION TISSUE SUBCLASS OF APICAL GRANULOMAS:**

Granulation tissue-type subclass forms of apical granulomas may represent a step in the natural history or transition of these lesions towards repair and replacement of damaged or destroyed tissue by fibrosis. Alternatively, if the antigen is once again presented the inflammatory response may be re-elicited and repair delayed.

Histologically, the transition to this phase of apical granuloma development, demonstrated the presence of granulation tissue composed mainly of fibroblasts, vascularized tissue and the presence of a variable number of inflammatory cells.

#### **IMMUNOHISTOCHEMICAL REACTIVITY AND DISTRIBUTION OF REACTIVE PHENOTYPES:**

It was observed that in general, the granulation tissue specimens showed few reactive cells when exposed to the panel of monoclonal antibodies. No reactive cells were observed when the tissues were exposed to CD8 monoclonal antibody, suggesting the absence of observable reactive Tc/s cells (TABLE 4) however a significant presence of Th/i cells was noted.

**SIGNIFICANCE:**

The described morphologic patterns of this proposed apical granuloma subclass, and the dynamics of these lesions can be interpreted as chronically active and chronically persistent lesions. Based on clinical observations, granulation tissue type apical granulomas may transform into fibrosis, alternatively, they may become reactivated to become chronically active lesions.

The antigenic response like other forms of apical granulomas is not anamnestic. There may be simultaneously contributions from the cell mediated and antibody mediated responses but sensitization does not occur. Secondary challenges by the same antigen would not produce a greater response than that which occurred with the first insult (102).

**ASPECTS OF APICAL GRANULOMAS:****A. DEVELOPMENT AND CLASSIFICATION:**

The granulomatous inflammation appears to be the host's response to a high local concentration of a foreign substance, not destroyed by the acute inflammatory response, but contained and destroyed by mononuclear phagocytes in various stages of maturation or activation .

It is proposed that the life history of apical granulomas evolves through phases of development. Four phases or subclasses are suggested as a result of observations in this study. The primary or

exudative phase is one of acute ongoing inflammatory process followed by the granulomatous phase or chronic stage of development of the lesion. This is then followed by a healing or reparative phase characterized by the granulation tissue type of lesion. The foreign body type of apical granuloma is characterized as a low turnover granuloma in most cases, secondary to the presence of restorative dental materials or other visible non-digestible foreign substance.

This study did not show evidence of epithelium in any of the forty tissues examined despite accounts of its presence in the literature. Findings in this study therefore contrast with earlier observations which indicate that epithelium is uniformly present in all periapical granulomas and it is this epithelium which is responsible for cyst formation (103).

#### **B. IMMUNOLOGIC CONSIDERATIONS:**

The cell phenotype subpopulations in apical granulomas are well described in the literature (4,6,15,17,35,37,50,56). Various studies (33,38) concur that mononuclear phagocytes are a dominant cell type in apical granulomas with the presence, as well, of varying numbers of other inflammatory cells such as lymphocytes, plasma cells and neutrophils. The apical granuloma therefore, would fulfill the definition of granulomatous inflammation. However, the description of apical granulomas have been based purely on the basis of morphological criteria.

Warren (102) in 1976 proposed a functional classification for the granulomatous inflammation. According to the author, the granulomatous inflammation can be divided into immunologic and non-immunologic granulomas based on the presence or absence of a secondary anamnestic response. This study proposes to adopt this functional classification of granulomatous inflammation in the description of apical granulomas. It is felt that this approach can provide a means for a better understanding of apical granulomas. The concept is based entirely on the presence or absence of the anamnestic response and specificity of reaction. The anamnestic response is determined by the presence of an immunizing antigen. Any class of antigens, immunizing or non-immunizing, may induce the development of antibodies and/or cell mediated responses as well as memory cell production. For non-immunizing antigens, however, the immunity to infection and specificity of response to the antigen may not develop. This may be due to the antigen variety and non-specificity encountered in apical granulomas.

It is suggested that apical granulomas fulfill the criteria of a non-immunogenic granuloma although all the elements of the immunologic reaction are present in these lesions. Based on clinical observations, these granulomatous inflammatory reactions show little or no anamnestic pattern of immunologic reactivity, they demonstrate no specificity of reaction and do not show a protective ability on re-infection. This feature disagrees with previous descriptions in the literature qualifying apical granulomas as immune-type based on relative numbers of lymphocytes and plasma cells (104).



To a large extent, this pattern of non-immune response reflects the nature and source of the antigen commonly encountered in these lesions. Antigens such as degraded or necrotic pulp and periapical tissue, lipopolysaccharides from gram (-) bacteria as well as cell wall antigens from streptococcal bacteria, denatured host tissue and various other debris seem to elicit this reactivity pattern in apical granulomas.

As observed in this study, apical lesions cannot be assigned purely to cell-mediated or antibody mediated inflammatory reactions. The general pattern of non-specific reactions to antigenic stimuli implies the simultaneous occurrence anaphylactic, cell-mediated, and antibody-mediated reactions.

Although this study did not show the presence of IgE and mast cells in apical granulomas, their presence has been widely demonstrated in the literature (24,27,29). This would indicate all the elements necessary for the anaphylactic reaction to occur. It has been postulated that the IgE-mediated anaphylactic response plays a role in the early development of the apical granuloma (4). The direct effect of this reaction is the liberation of chemical mediators from basophils and mast cells. Although their primary role is defence against injury, pathologic changes such as bone resorption are evidenced in the apical granuloma.

The type III antigen-antibody or arthus reaction could only be observed indirectly through the presence of immunoglobulins, antibody-antigen complexes, PMN's, and complement. The presence of an excess of antigens present from a partially viable or necrotic root

canal system reacts with high titer specific antibodies produced in periapical granulomas against these antigens forming antibody-antigen complexes. These complexes in apical lesions in excess of antibodies give rise to an arthus-like reaction, fix complement and consequently attract PMN's causing degranulation and the elaboration of lysosomal enzymes and free radicals (93,94,95). The presence of aggregated IgG has been shown to possess many of the immunologic properties of the antigen-antibody complexes (96). Aggregated IgG induced a rapidly evolving periapical lesion characterized by bone and collagen loss and an accumulation of inflammatory cells. The continuing challenge by root-canal system derived antigens produces a chronic process leading to tissue changes such as those that would be found in the granulomatous sub-class apical granuloma (3).

The cell-mediated or type IV hypersensitivity reaction is based on the presence of lymphocytes and their ability to react with antigens and either produce lymphokines or participate in cytotoxic reactions against cells bearing the antigens. This study demonstrated the presence of the type IV sensitivity reaction.

The type II or cytotoxic hypersensitivity reactions involves binding of antigens to antibodies on the cell surfaces (97). This form of antibody-dependent cell-mediated cytotoxicity (ADCC) or complement mediated-lysis may not be operational in apical granulomas due to the nature of the antigen which must be cell bound.

## MEDIATORS OF INFLAMMATION:

Chemical mediators of inflammation play an important role in the development of apical granulomas. Several biochemical systems are implicated as mediators of signs and symptoms of inflammation. These include the vasoactive amines, arachidonic acid metabolites, the kinin system, the complement system, and cytokines of innate immunity (3,5).

There are two major vasoactive amines involved in inflammatory reactions: histamine and serotonin. Histamine, the most important of the two, is released from mast cells by a number of stimuli including physical injury (97), products of complement activation (98), activated T lymphocytes (99) and bridging of membrane bound IgE by allergens (96). The net result is to react with three separate target cell receptors: H1, H2, H3 which affect the development of apical lesions by enhanced or inhibiting leukocyte chemokinesis, the production of prostaglandins, and stimulating Tc/s subsets.

Two major families of inflammatory mediators include prostaglandins (PG) and leukotrienes. These are the products of enzymatic cyclo-oxygenation and lipoxygenation of arachidonic acid derived from cell membrane phospholipid (5). PGE2 and PGE1 have been shown to be associated with vascular permeability and pain via the other chemical mediators of pain such as histamine and bradykinins. Leukotrienes produce chemotactic effects for neutrophils, eosinophils, and macrophages as well as increased permeability and release of lysozomal enzymes from PMN's and macrophages (100). The actions of these mediators are enhanced by the vasoactive amines and kinins.

The kinins are potent mediators of inflammation producing chemotaxis of inflammatory cells and capillary permeability in addition to causing pain by direct action on nerve fibers. Proteolytic cleavage of kinninogen by trypsin-like serine proteases, the kallikreins, produces the kinins (101). Bradykinin and lys-bradykinin, the two major kinins, may also be able to interrelate with the complement system and further affect the inflammatory reaction.

The complement system is a term which designates a group of plasma and cell membrane proteins, approximately twenty five in number, involved in the host defence process. There are two main pathways of complement activation; the first being the classic complement pathway is initiated by antigen-antibody complexes, the second being alternative complement pathway, does not require antibody-antigen complexing. These function through the interaction of proteins called components involving complement. Both pathways proceed by means of sequential activation and assembly of a series of proteins leading to the formation of a complex protease enzyme capable of binding and cleaving a key protein, C3, which is common to both pathways. The two pathways then proceed together through binding of the terminal components to form a membrane attack complex, which ultimately causes lysis of the cell (5).

The functions of the complement system are of critical importance in the development of the apical granuloma from the exudative to the granulation tissue type phases. The primary function is to cause cell lysis. The second involves the coating of foreign particles with specific complement protein fragments which can then be

recognized by receptors for these fragments on phagocytic cells (opsonization). This enables the phagocytes to work much more effectively. The third function allows the generation of peptide fragments that regulate features of the inflammatory and immune response. These proteins are involved in 1. vasodilation at the site of inflammation 2. adherence of the phagocytes to blood vessel endothelium 3, egress of the phagocytes from blood vessels 4. in directed migration of phagocytic cells to areas of inflammation and 5. ultimately, in clearing infectious agents from the area (5).

A host of cytokines are produced which mediate innate immunity. These involve interferons alpha/beta, interleukins, tumor necrosis factors, growth factors and several others (91).

Interferon alpha/beta increases cytotoxic T lymphocyte activity and can augment mitogenesis of B lymphocytes to promote B cell antibody production. In addition, this cytokine can increase both the antigen-presenting cell function and bacteriocidal functions of macrophages. Interferon alpha/beta can also promote phagocytosis of immune complexes as well as enhancing macrophage production of IL-1 and tissue necrosis factor which augments lymphocyte activation. Interferon alpha/beta can either augment or suppress cellular and humoral immunity. Stimulation occurs by enhancing the activity of T h/i subsets or inhibiting Tc/s subsets (5).

IL-1 is a major reactive cytokine of innate immunity. It has both local and systemic effects on cell metabolism and immune and inflammatory reactions. Based on its presence at inflammatory sites and

its ability to induce many effects of the inflammatory response, it is considered an important mediator of inflammation.

During antigen activation, IL-1 synergizes with T helper factors (such as IL-2), resulting in increased B cell proliferation and immunoglobulin production. IL-1 also augments the production of other cytokines by T helper cells, including IL-2, IL-4, and IL-5 which control various stages of B cell activation, proliferation and secretion (5).

Tumor necrosis factor is considered a major inflammatory mediator. It induces IL-1 and PGE<sub>2</sub> production by macrophages as well as tissue destruction resembling IL-1 activity with bone resorption via the activation of osteoclasts and the inhibition of bone synthesis. Tumor necrosis factor accelerates induced resorption of proteoglycan and inhibited proteoglycan synthesis, and induces proliferation of fibroblasts for wound repair or with pathological effects as noted in fibrosis induced by chronic inflammation. In addition, tumor necrosis factor has multiple stimulatory activities on activated T cells (5).

Transforming growth factor beta affects cells involved in immunity and inflammation. Although it is produced by T cells and released after T cell activation, it acts as a negative or inhibitory feedback role. Despite the inhibitory effects on lymphocytes, transforming growth factor beta is a potential mediator of inflammation because it is a product of activated macrophages, a chemoattractant for macrophages, and can activate macrophages to produce IL-1. Other growth factors may also play key roles by enhancing human T

lymphocyte proliferation responses to antigens by increased expression of MHC class II antigens on macrophages (5).

## CHAPTER VI

### CONCLUSIONS

It is proposed that apical granulomas, based on light microscopic observations and expressed immunophenotype, may be classified into four different subclasses: 1. exudative, 2. granulomatous, 3. granulation tissue type, and 4. foreign body. The classification presented is reproducible and fulfills all the requirements for classification of lesions.

These subclasses suggest a continuum of the inflammatory reaction process that may show overlap features as the development progresses from an acute, to chronic, to repair phases with the possibility of regression due to the added influx of new antigens.

Apical granulomas may be categorized as non-immunologic entities according to established criteria. Based on clinical observations, there appears to be little or no anamnestic response, no specificity of reaction or protective ability when re-exposed to the same antigen. These features being directly related to the nature of the presenting antigens.

Monoclonal antibodies are significantly useful in the designation of the subclasses of apical granulomas. They have, with the possible exception of immunoglobulins, provided an adequate means of identifying the immunophenotypes involved in these inflammatory processes in both fresh-frozen and formalin-fixed tissues.



**FIG. 3:**

A. Formalin-fixed, paraffin-embedded positive control (tonsil) with monoclonal antibody CD4 (DAKO). Positively staining Th/i cells appear as brown rings around cells with lymphocyte morphology. B. Negative control, primary antibody not applied. (Original magnification, x 400).

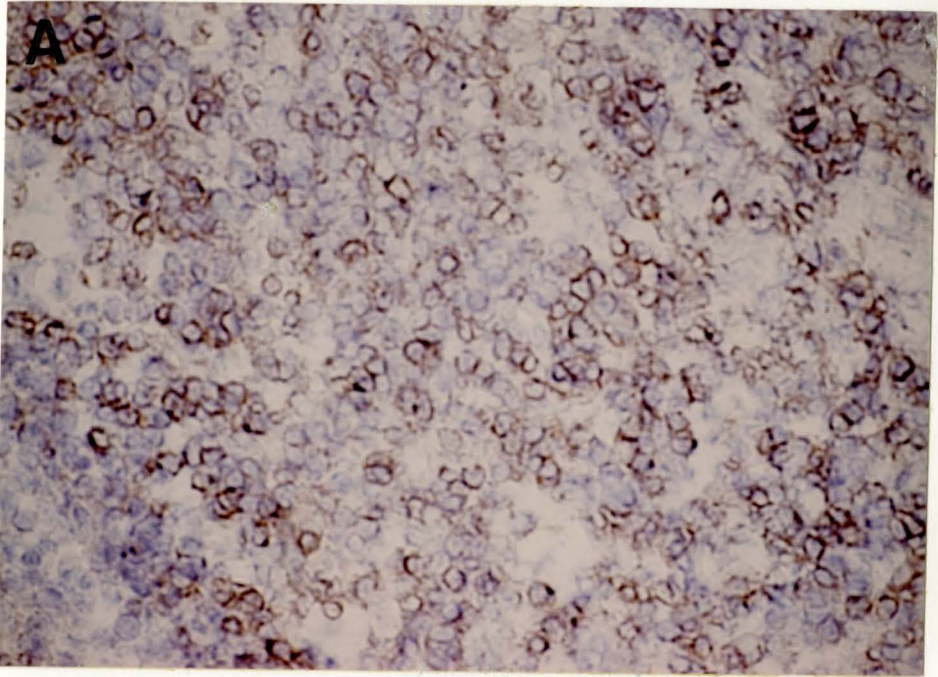


FIG.

A and B. Hematoxylin and eosin stained, paraffin-embedded, serially cut sections of the same tissue. (Figure magnification: A x100, B x400).

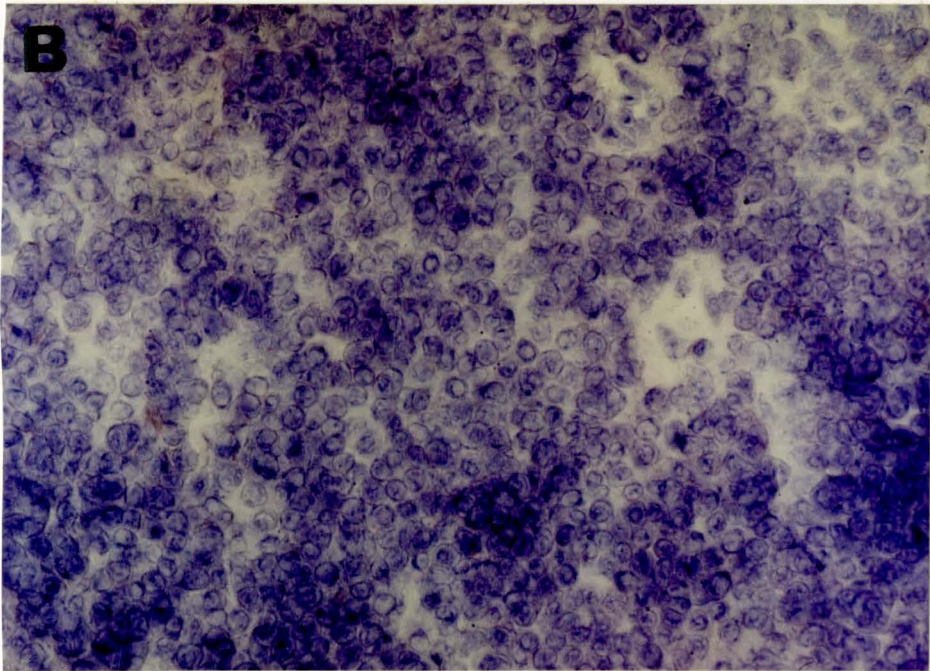


FIG. 4:

A and B. Hematoxylin and eosin stained, formalin-fixed, paraffin-embedded exudative subclass apical granuloma. (Original magnification A x100, B x400).

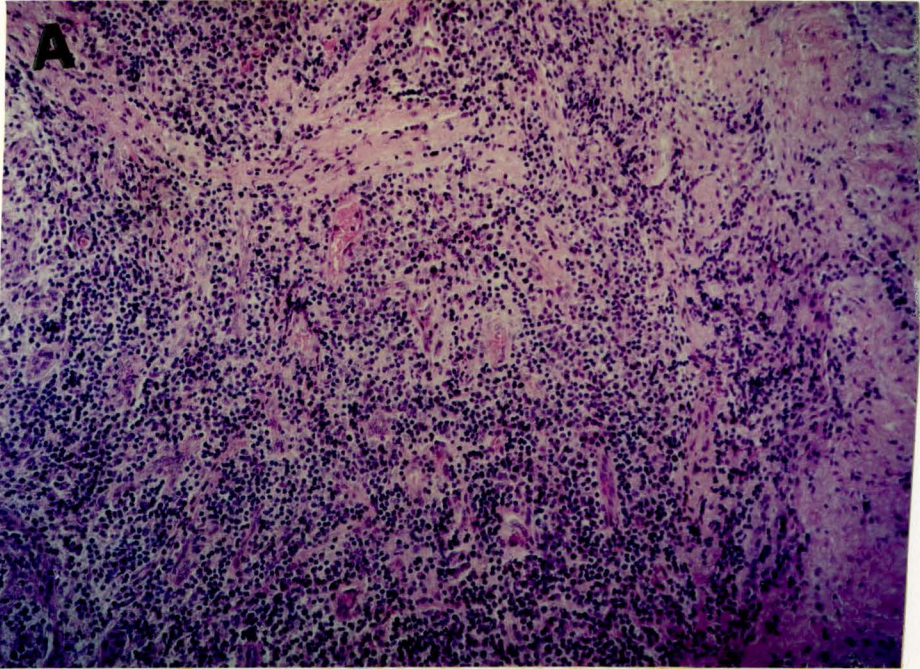


FIG.

A. and B. Eosinophilia and cellular reaction, eosinophilic, granular, embedded granulocytous cellular reaction. (Original magnification  $\times 200$ ,  $\times 400$ )

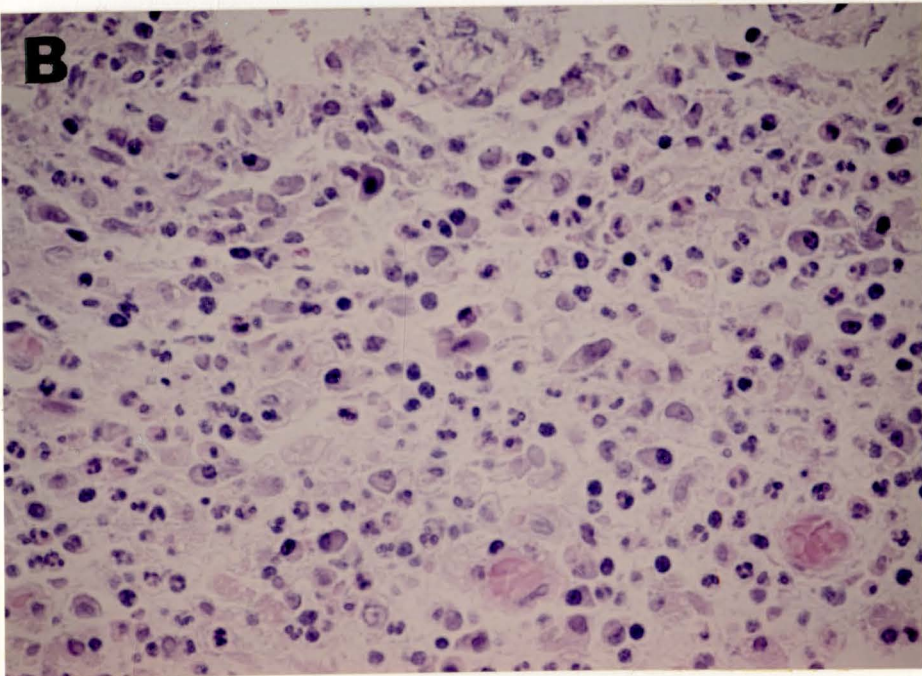


FIG. 5:

A. and B. Hematoxylin and eosin stained, formalin-fixed, paraffin-embedded granulomatous subclass apical granuloma. (Original magnification A x100, B x400)

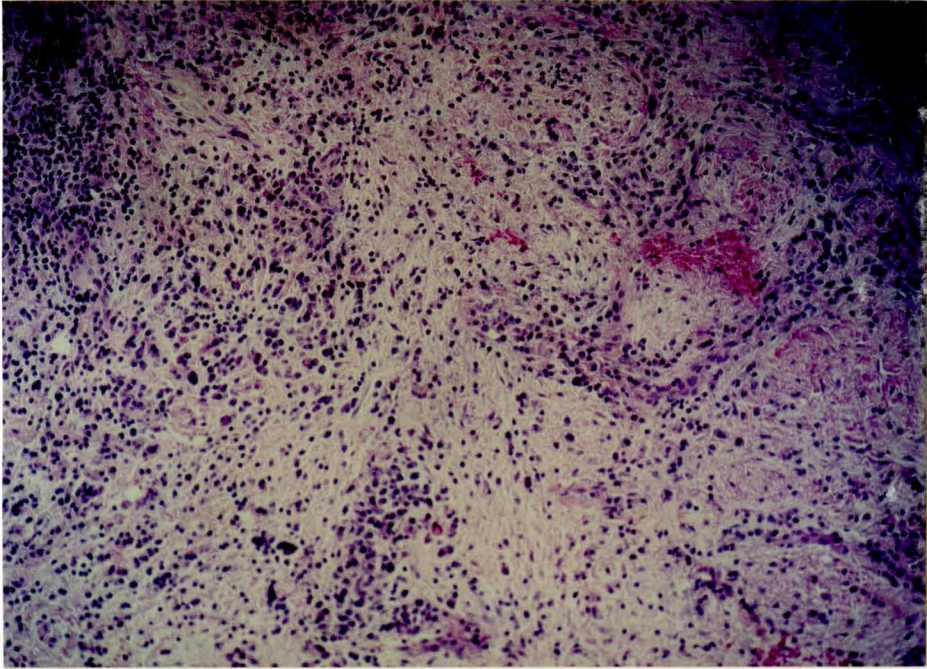


FIG. 1. A and B. Hematoxylin and eosin stained, formalin-fixed, paraffin-embedded granuloma tissue type obtained from guinea pig. (Magnification: A 100x, B 400x).

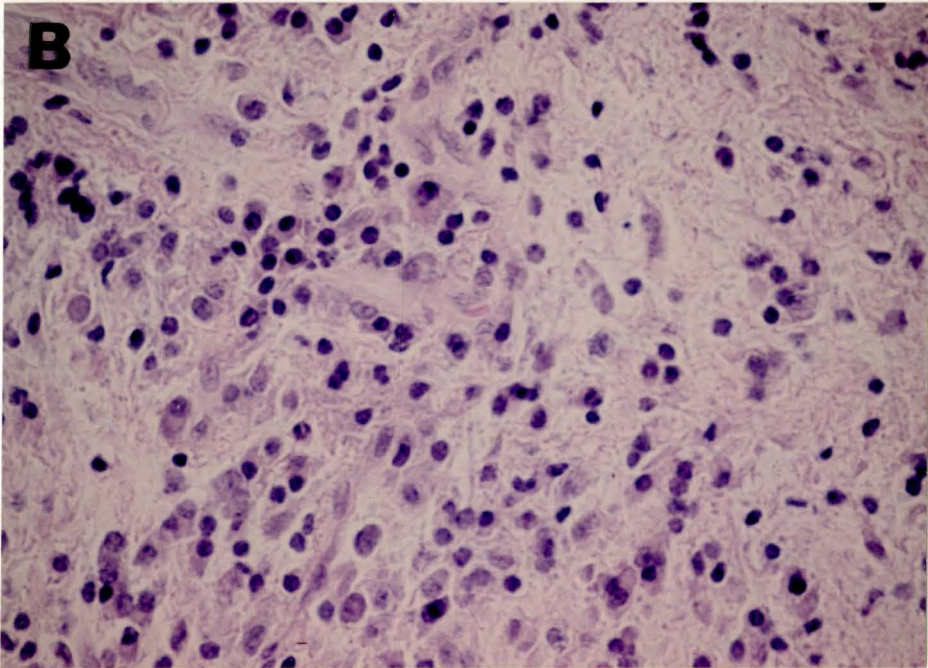


FIG. 6:

A. and B. Hematoxylin and eosin stained, formalin-fixed, paraffin embedded granulation tissue type subclass apical granuloma. (Original magnification A x100, B x400).

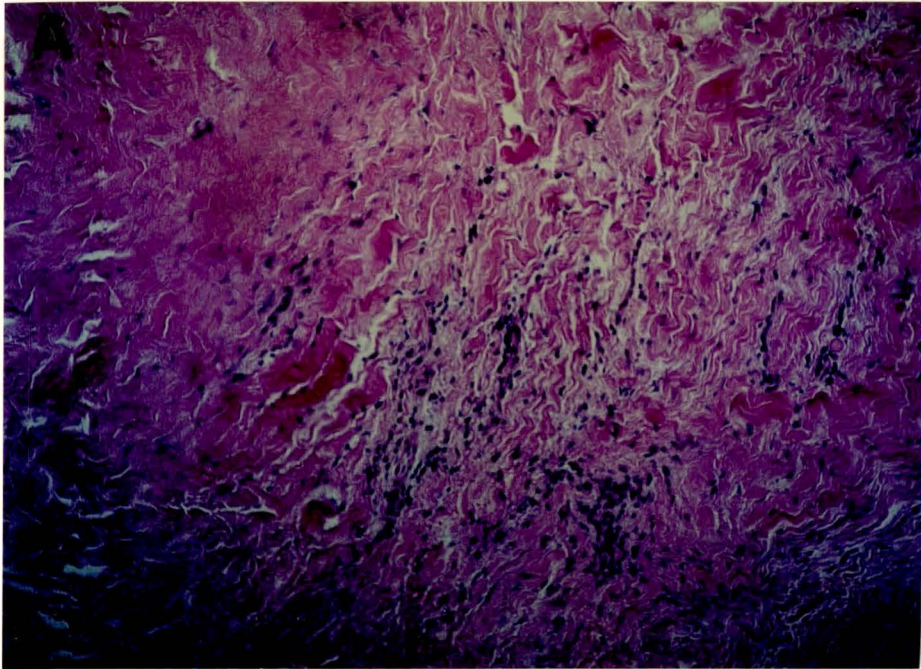


FIG.

A. and B. *Ranunculus* root made into a paste, powdered, and embedded in paraffin. (A) 100x, (B) 400x.

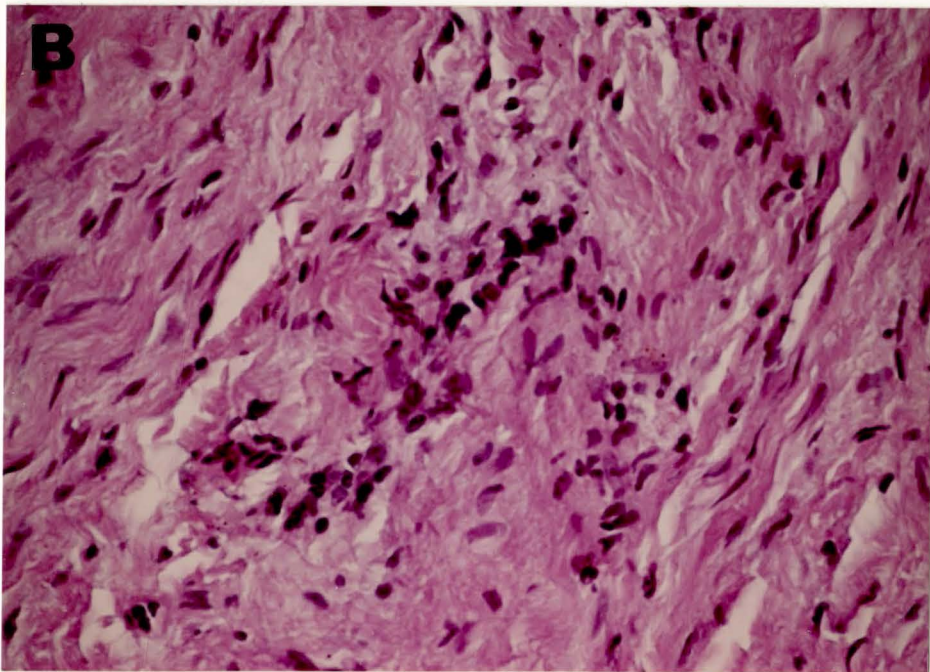
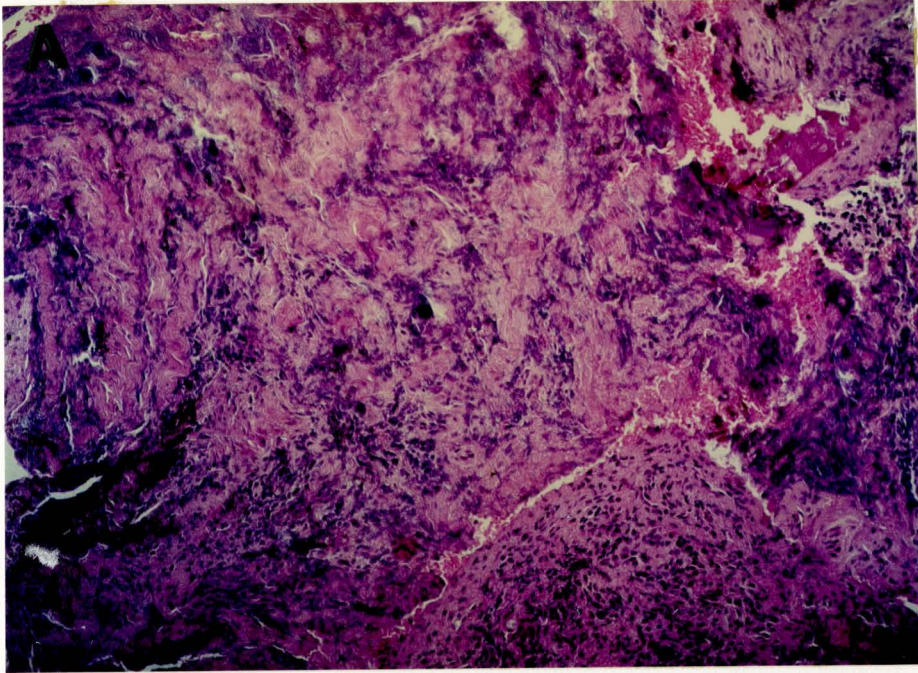




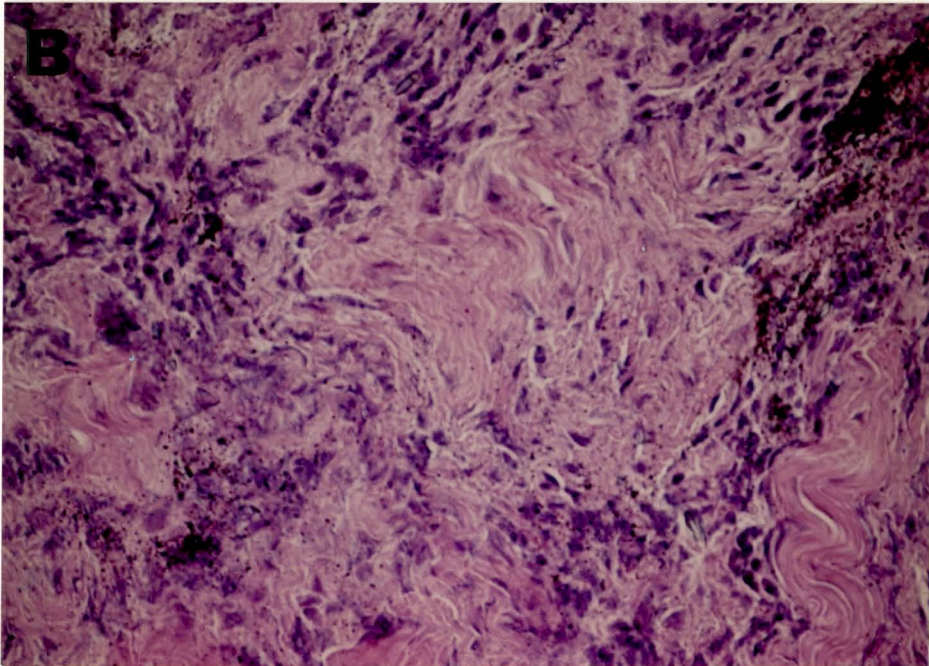
FIG. 7:

A. and B. Hematoxylin and eosin stained, formalin-fixed, paraffin-embedded foreign body subclass apical granuloma. (Original magnification A x100, B x400).



FIG

Fresh-Groza sections fixed section of spinal granuloma stained with CD4 monoclonal antibody (DAPI). Positively staining T<sub>H</sub>1 cells appear as brown rings around cells with lymphocyte morphology. A. Exudation type subclass. B. Granulomatous type subclass. (Original magnification, x400).



## FIG. 8:

Fresh-frozen acetone fixed section of apical granuloma stained with CD4 monoclonal antibody (DAKO). Positively staining Th/i cells appear as brown rings around cells with lymphocyte morphology. A. Exudative type subclass. B. Granulomatous type subclass. (Original magnification, x400).

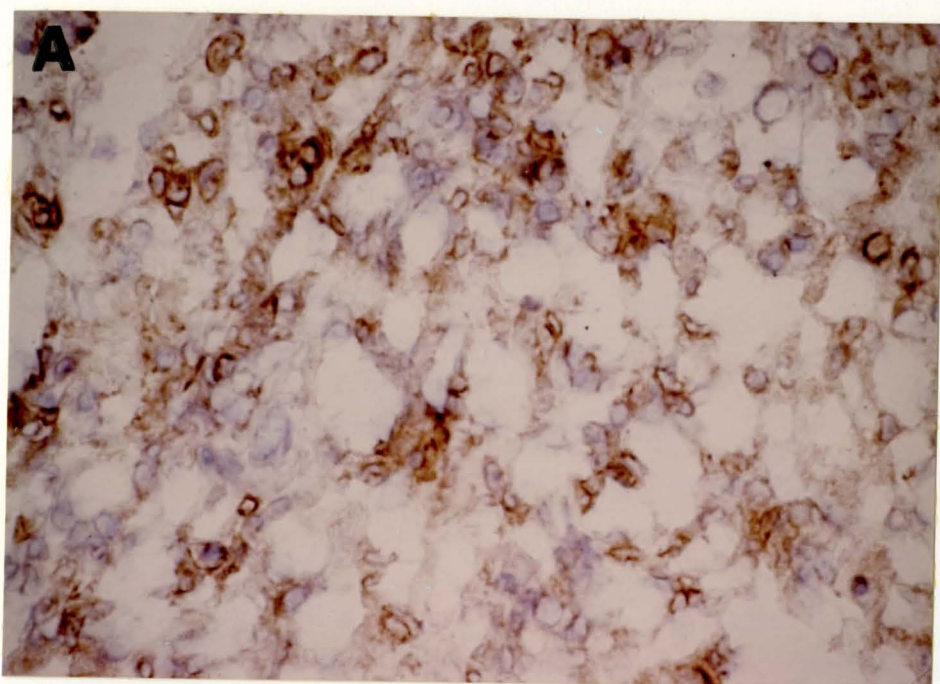
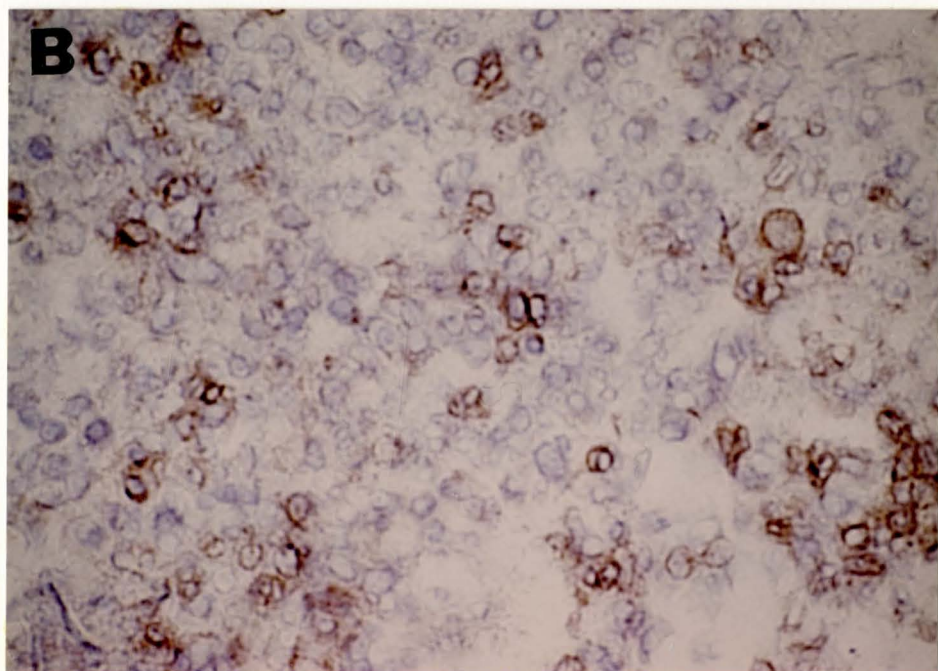


FIG.

Fresh-frozen, acetone fixed section of spinal granulosa stained with CD4 monoclonal antibody (DAKO). Positively staining T4/3 cells appear as brown rings around cells with lymphocyte morphology. A. Granulosa type subunit. B. Foreign body type subunit. (Glycolic infiltration, x400).



**FIG. 9:**

Fresh-frozen, acetone fixed section of apical granuloma stained with CD4 monoclonal antibody (DAKO). Positively staining Th/i cells appear as brown rings around cells with lymphocyte morphology. A. Granulation type subclass. B. Foreign body type subclass. (Original magnification, x400).

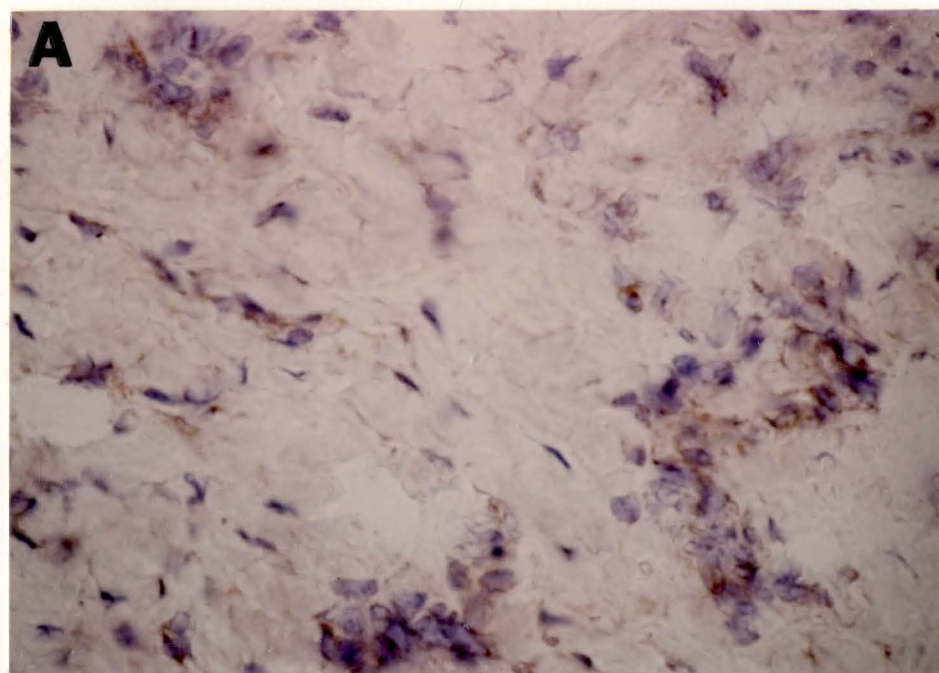
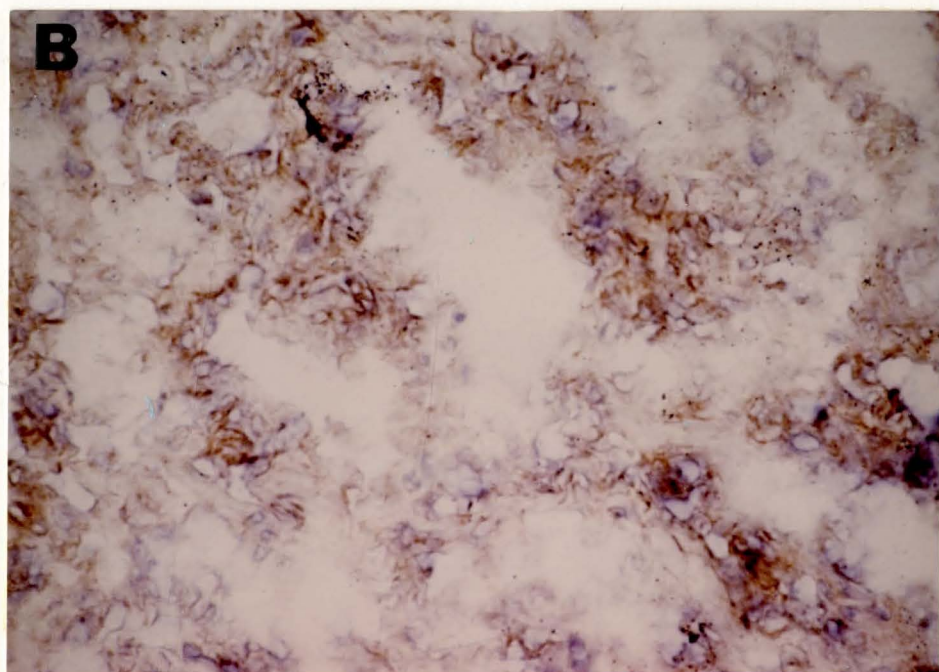


FIG.

*Proteinase, acetone fixed section of spleen stained with CD8 monoclonal antibody (DAKO). Positively staining T8 cells appear as brown rings around cells with lymphocyte morphology. A. Endothelial type subclass. B. Granulomatous type subclass. (Original magnification, x400).*



**FIG. 10:**

Fresh-frozen, acetone fixed section of apical granuloma stained with CD8 monoclonal antibody (DAKO). Positively staining Thc/s cells appear as brown rings around cells with lymphocyte morphology. A. Exudative type subclass. B. Granulomatous type subclass. (Original magnification, x400).

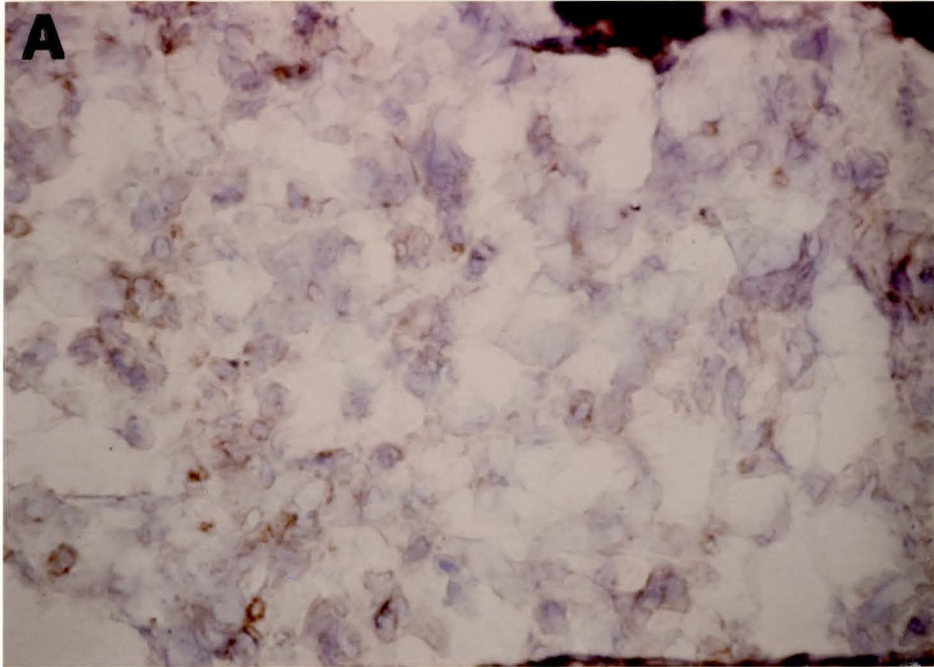
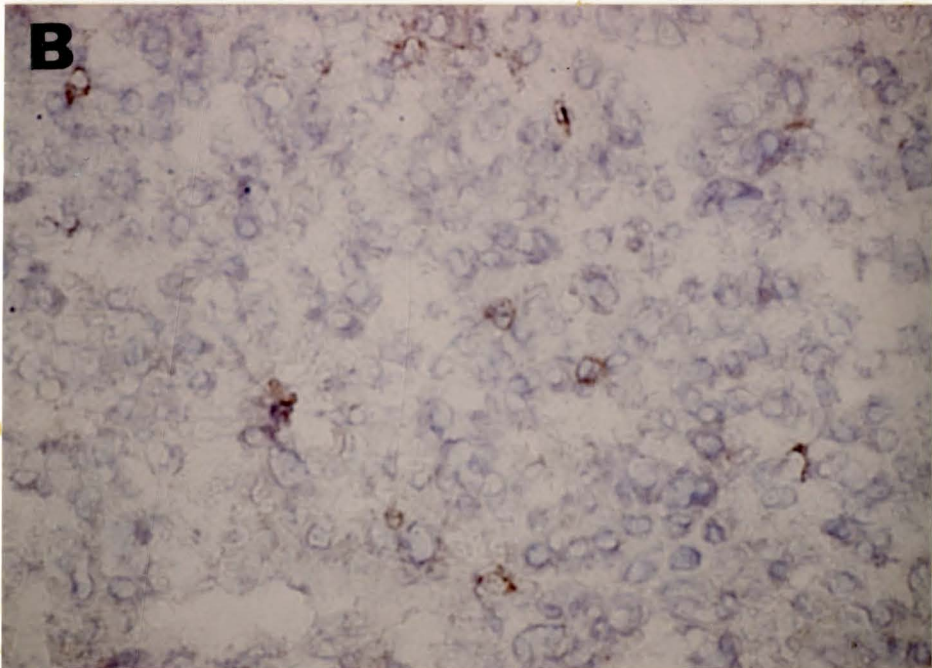


FIG.

Fresh-frozen human placenta, section of spiral structure stained with CD8 monoclonal antibody (DAKO). Positive staining for CD8 appears as brown rings around cells with hematoxylin counterstain. A: Granulation type placenta. B: Fetal type placenta. (H&E stain, magnification,  $\times 400$ ).





**FIG. 11:**

Fresh-frozen, acetone fixed, section of apical granuloma stained with CD8 monoclonal antibody (DAKO). Positively staining Th/i cells appear as brown rings around cells with lymphocyte morphology. A. Granulation type subclass. B. Foreign body type subclass. (Original magnification, x400).

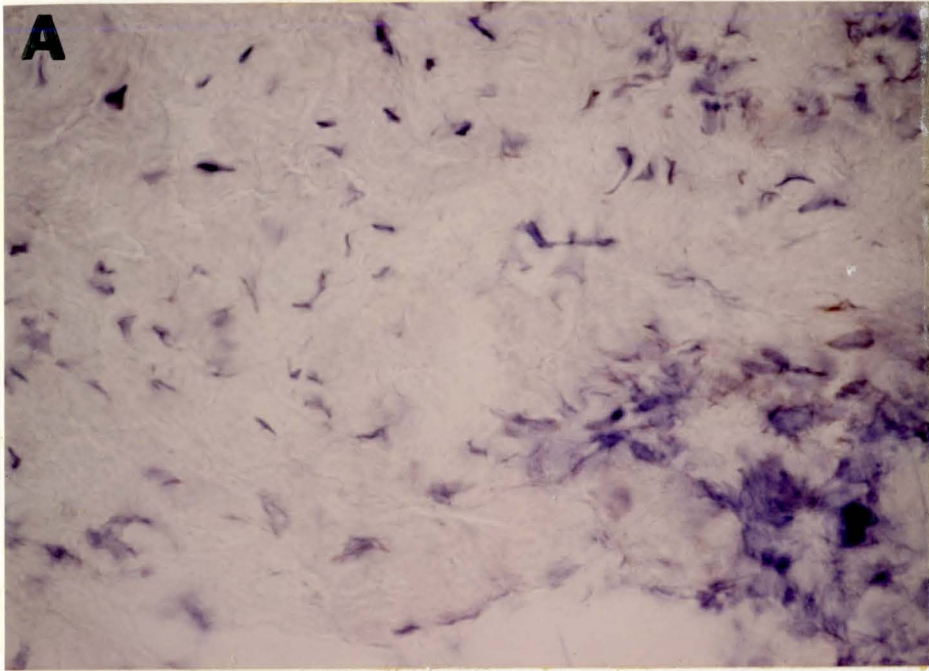
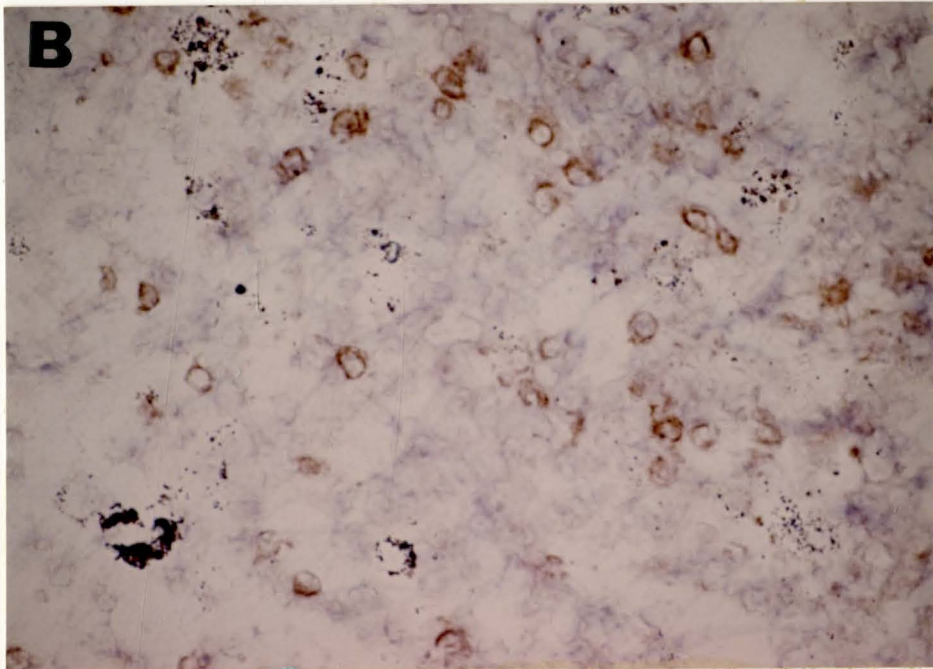


FIG.

Fresh-frozen rat brain tissue stained for CD11b monoclonal antibody (10A10). Positively stained neurons appear as brown ring-shaped neurons cells. A. Frontal rat subcortex. B. Cerebellar cortex. (Original magnification  $\times 400$ ).



## FIG. 12:

Fresh-frozen acetone fixed section of apical granuloma stained with CD11b monoclonal antibody (DAKO). Positively staining monocytes, appear as brown rings around reactive cells A. Exudative type subclass. B. Granulomatous type subclass. (Original magnification, x400).

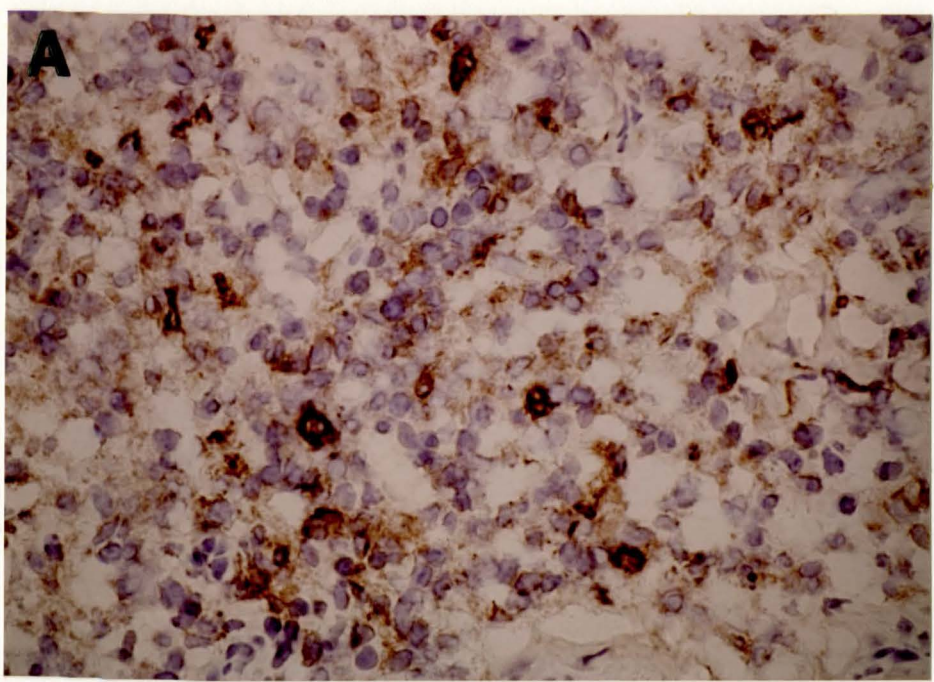
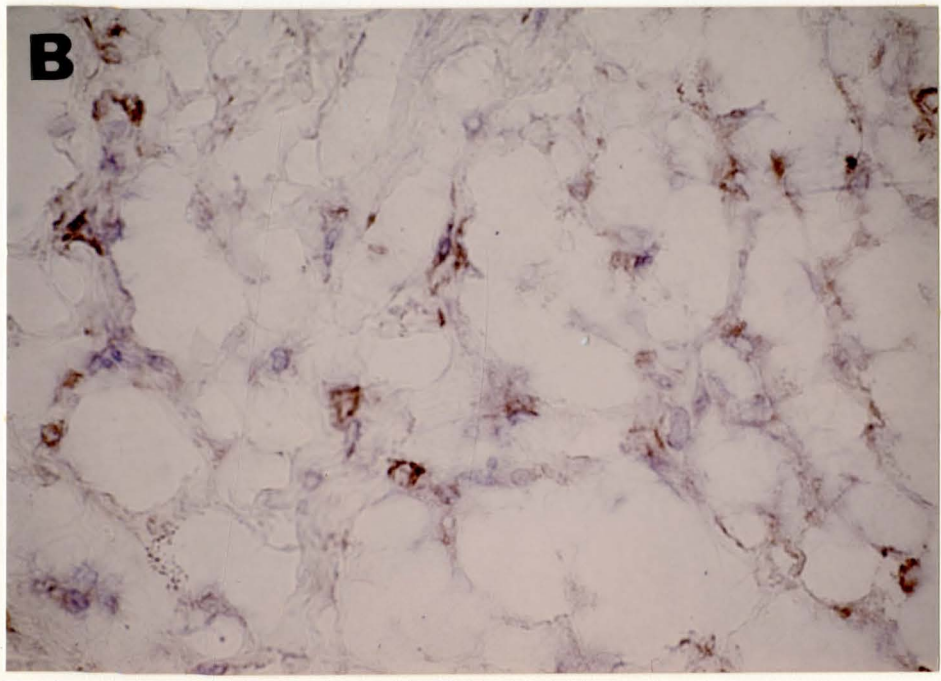


FIG.

Fresh-frozen, acetone fixed section of spinal ganglion stained with anti-CD11b monoclonal antibody (DAKO). Positively staining granulocytes and subtypes appear at lower magnification stained reactive cells. A. Granulation tissue type reaction. B. Foreign body type reaction. (Original magnification, 200x).



## FIG. 13:

Fresh-frozen, acetone fixed section of apical granuloma stained with CD11b monoclonal antibody (DAKO). Positively staining granulocytes, and monocytes appear as brown rings around reactive cells. A. Granulation tissue type subclass. B. Foreign body type subclass. (Original magnification, x400).

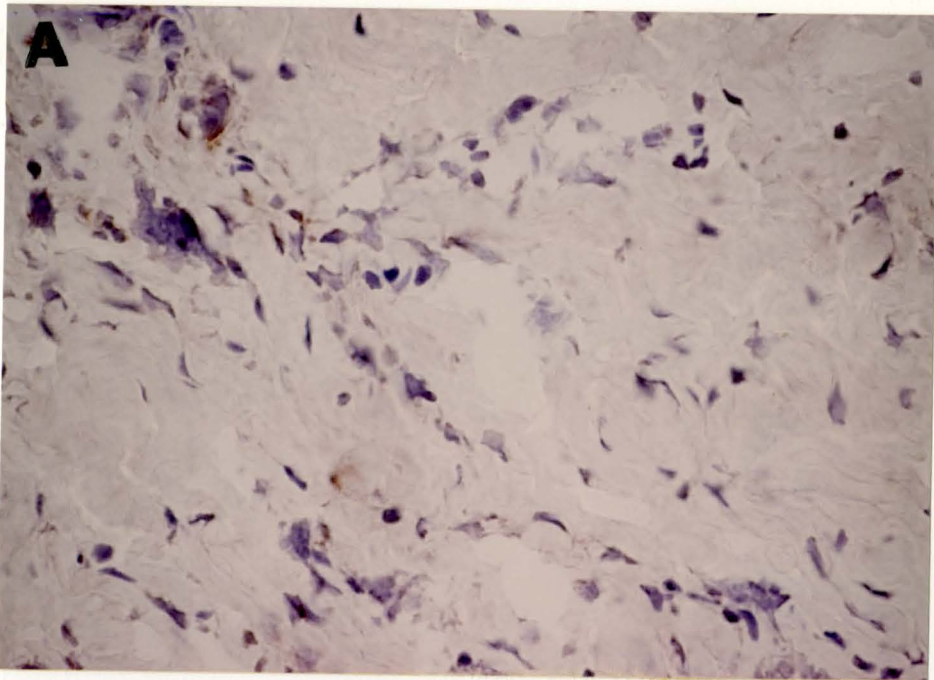
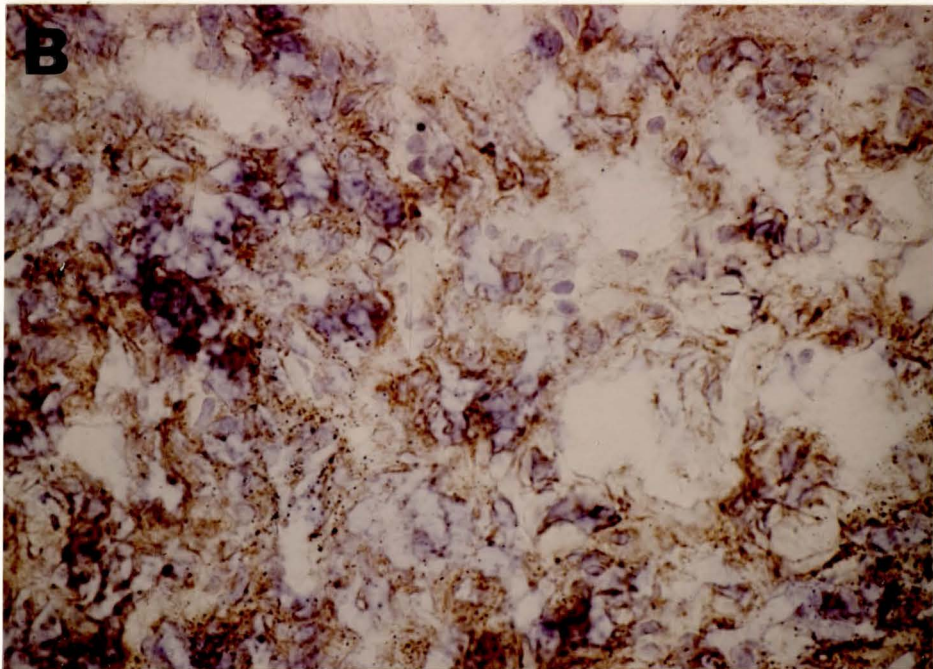


FIG.

Fresh-frozen, acetone fixed sections of spinal granulosa stained with CD15 immunohistochemical antibody (D1303). Granulocytes staining granulocytes appear as brown rings around purple cells. In this section, they subclass. B. Granulocytosis type subunit. (Original magnification, x400).



**FIG. 14:**

Fresh-frozen, acetone fixed section of apical granuloma stained with CD15 monoclonal antibody (DAKO). Positively staining granulocytes appear as brown rings around reactive cells. A. Exudative type subclass. B. Granulomatous type subclass. (Original magnification, x400).

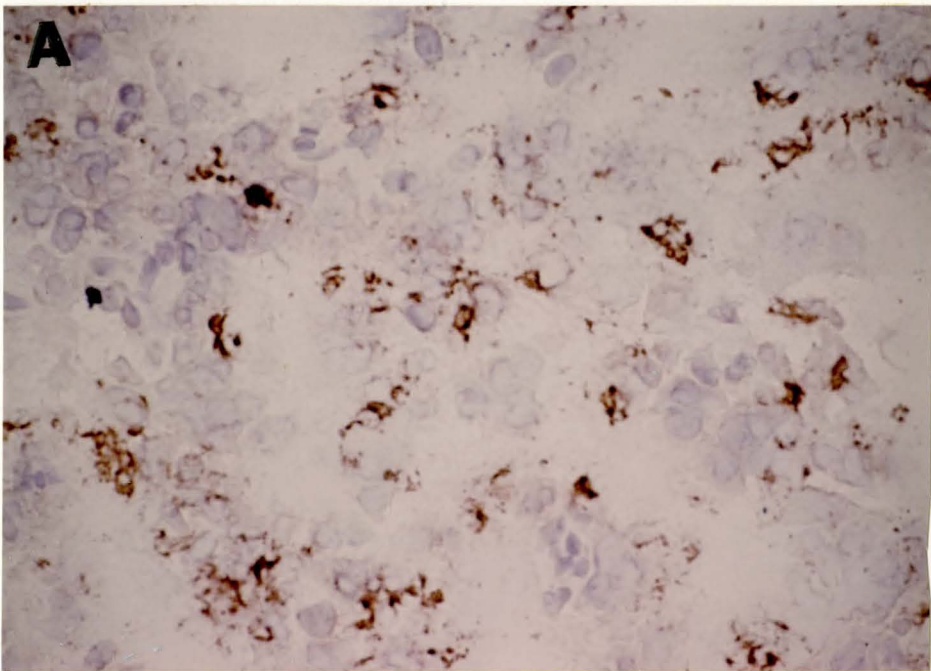


FIG.

Fresh-frozen, autologous chondrocyte culture of a patient with osteoarthritis stained with CD13 monoclonal antibody (1A-2A). Positively staining granulocytes appear as brown rings around reactive cells. A. Granulocyte ring type subclass. B. Foreign body type subclass. (Original magnification, x400).

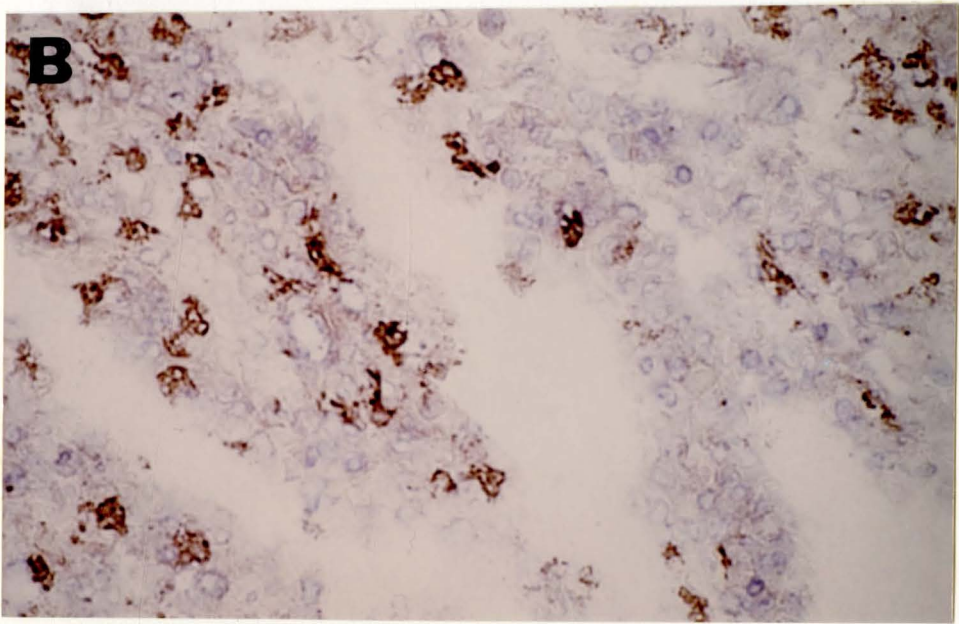




FIG. 15:

Fresh-frozen, acetone fixed section of apical granuloma stained with CD15 monoclonal antibody (DAKO). Positively staining granulocytes appear as brown rings around reactive cells. A. Granulation tissue type subclass. B. Foreign body type subclass. (Original magnification, x400).

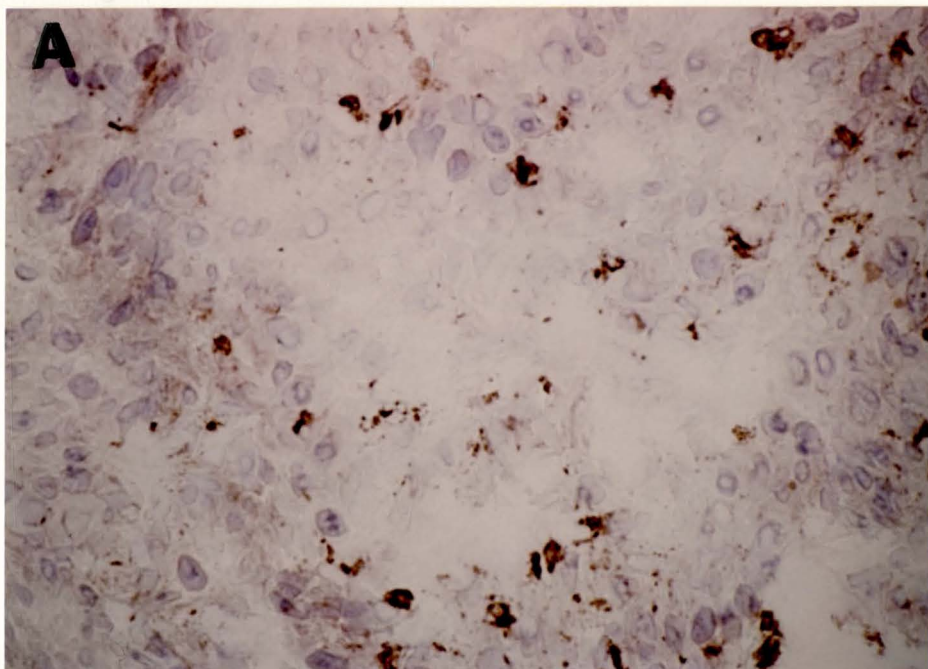
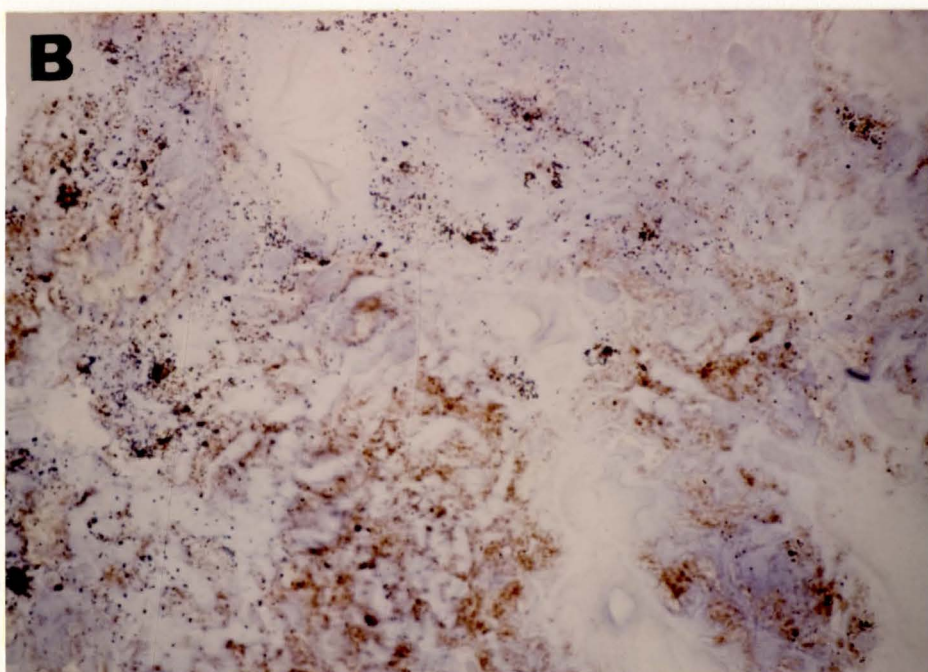


FIG.

Paraffin-fixed, paraffin-embedded sections of spinal granulosa stained with CD45 monoclonal antibody (DAKO). Positively staining B cells, monocytes, and a subset of T cells appear as brown rings around reactive cells. A. Excitatory type subclass. B. Granulocytous type subclass. (Original magnification,  $\times 400$ ).



**FIG. 16:**

Formalin-fixed, paraffin-embedded section of apical granuloma stained with CD45 monoclonal antibody (DAKO). Positively staining B cells, monocytes, and a subset of T cells appear as brown rings around reactive cells. A. Exudative type subclass. B. Granulomatous type subclass. (Original magnification, x400).

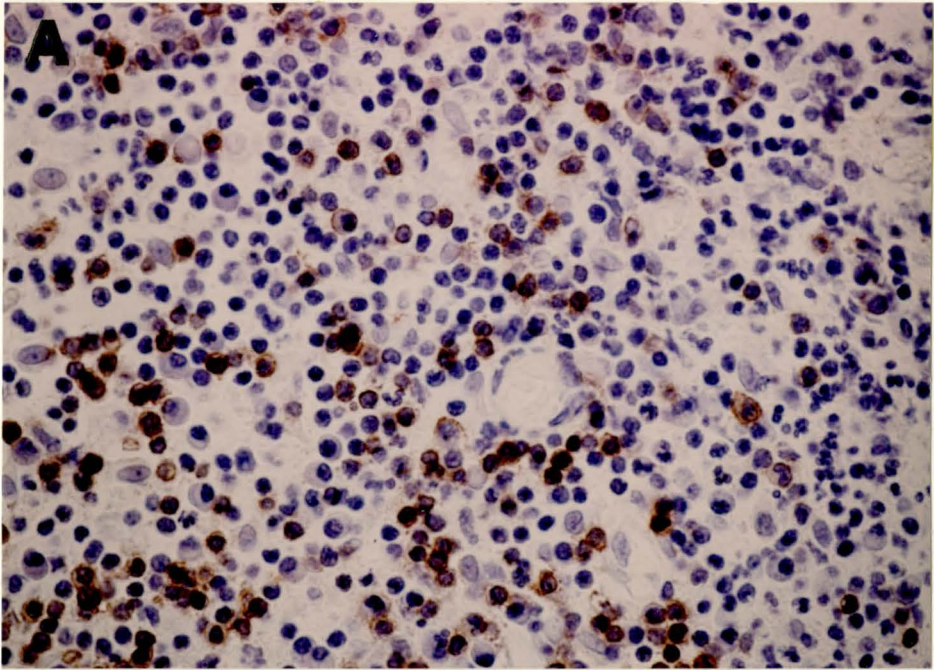
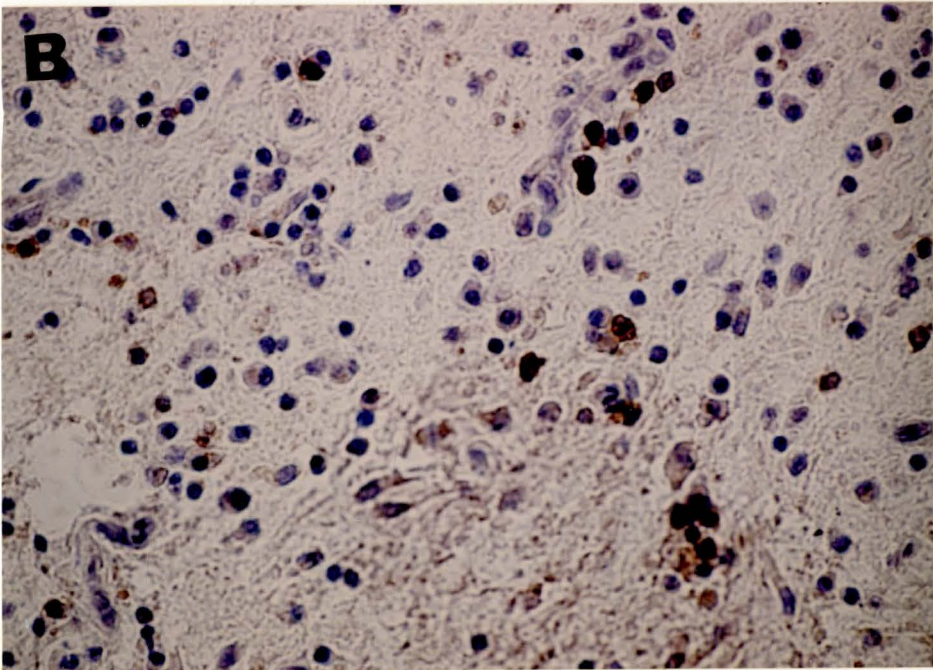
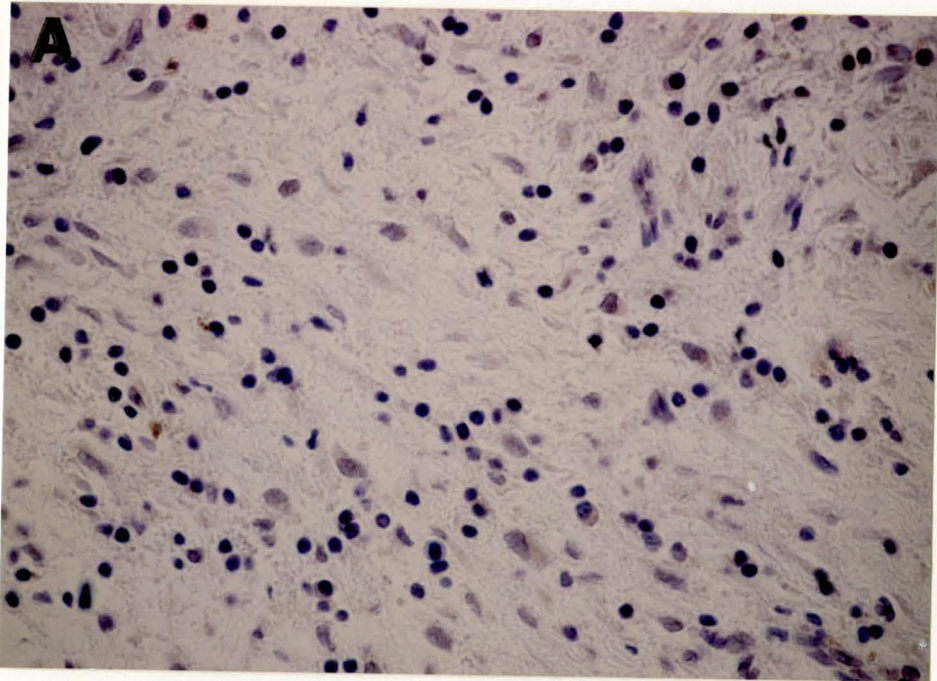


FIG. 1. Formalin-fixed, paraffin embedded sections of splenic granulosa stained with CD45 monoclonal antibody (DAKO). Positively staining B cells, monocytes, and a subset of T cells appear as brown rings around reactive cells. A. granulation tissue type subclass. B. Foreign body type subclass. (Original magnification, x400).



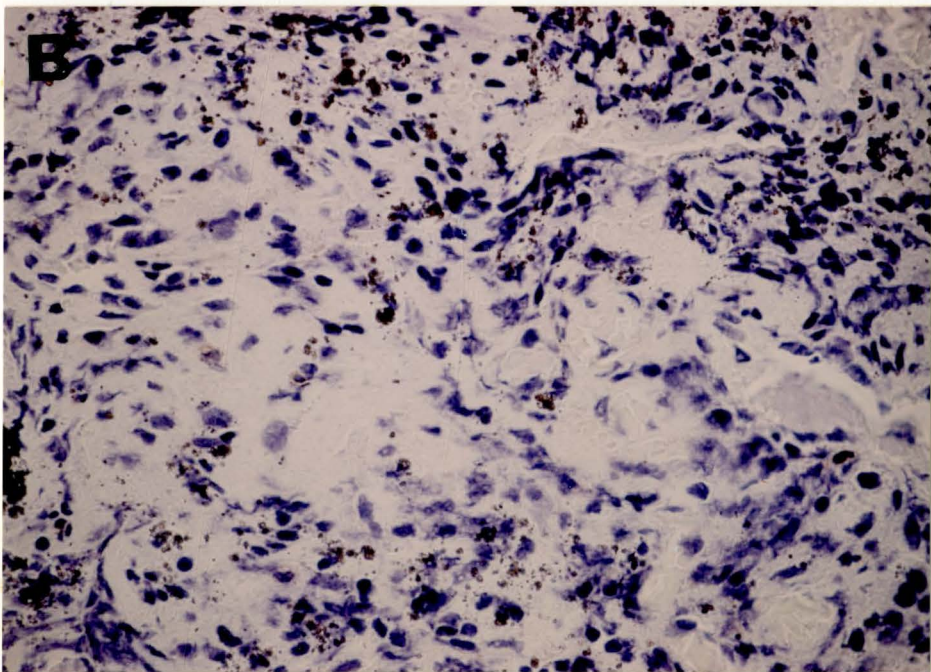
**FIG. 17:**

Formalin-fixed, paraffin embedded section of apical granuloma stained with CD45R monoclonal antibody (DAKO). Positively staining B cells, monocytes, and a subset of T cells appear as brown rings around reactive cells. A. granulation tissue type subclass. B. Foreign body type subclass. (Original magnification, x400).



FIG

Fresh-frozen, section fixed section of spiral granulosa stained with CD74 monoclonal antibody (DAKO). Positively staining B cells, and monocytes appear as brown ring around invasive cells. A. Exudative type subchorion. B. Granulomatous type subchorion. (Original magnification, x400).



**FIG. 18:**

Fresh-frozen, acetone fixed section of apical granuloma stained with CD74 monoclonal antibody (DAKO). Positively staining B cells, and monocytes appear as brown rings around reactive cells. A. Exudative type subclass. B. Granulomatous type subclass. (Original magnification, x400).

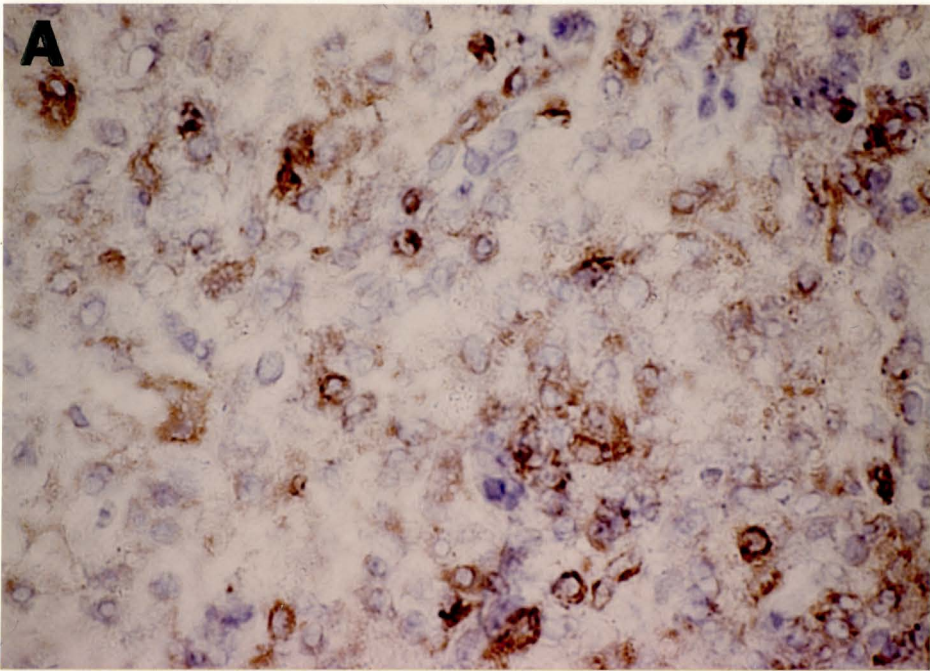
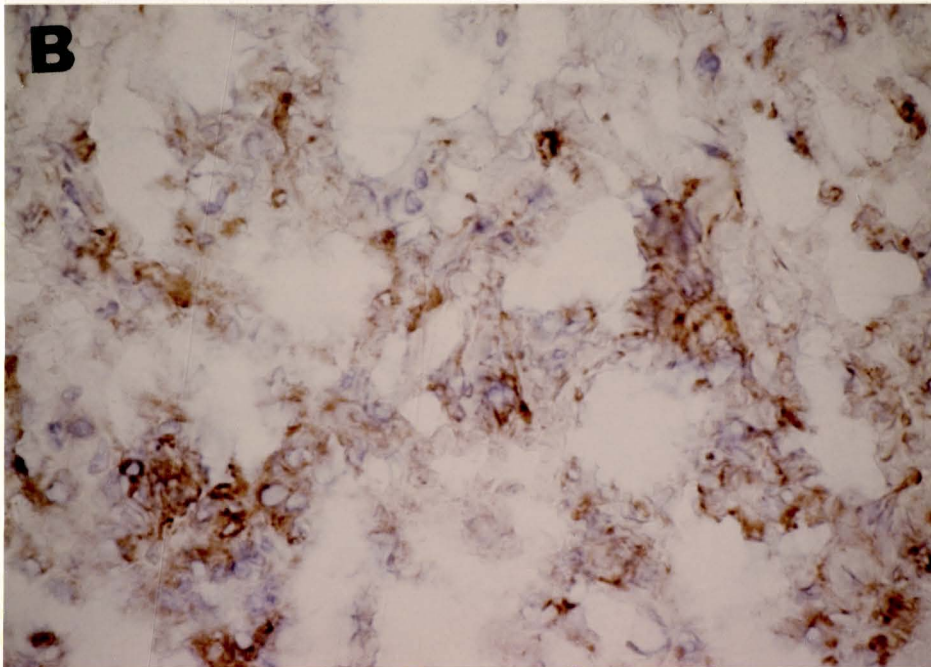


FIG.

Fresh-frozen, acetone-fixed section of spinal granuloma stained with CD74 monoclonal antibody (DAKO). Positively staining B cells, and monocytes appear as brown rings around reactive cells. A. Gossulium tissue type subclass. B. Foreign body type subclass. (Original magnification, x400).





**FIG. 19:**

Fresh-frozen, acetone-fixed section of apical granuloma stained with CD74 monoclonal antibody (DAKO). Positively staining B cells, and monocytes appear as brown rings around reactive cells. A. Granulation tissue type subclass. B. Foreign body type subclass. (Original magnification, x400).

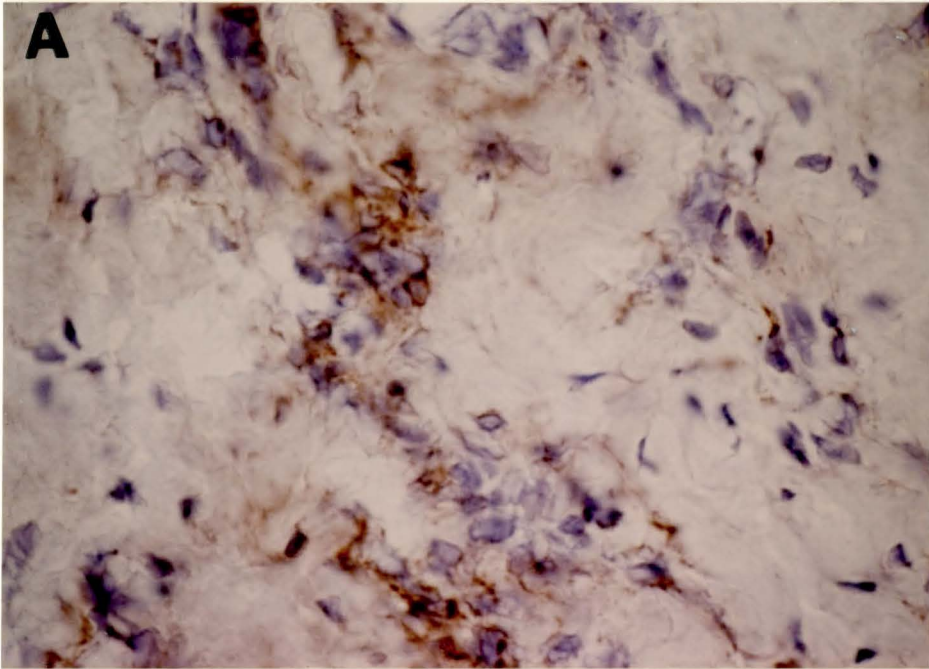
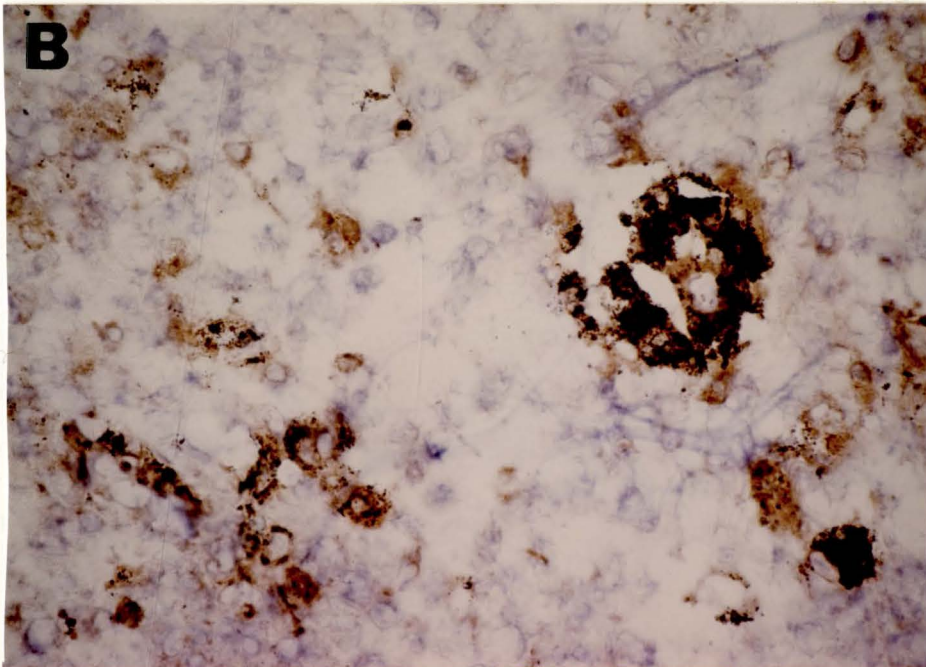


FIG.

Formalin-fixed, paraffin-embedded sections of epithelioid granulomas stained with M6C387 monoclonal antibody (D&KCO). Positively staining monocytes, macrophages, and histiocytes appear as brown rings around reactive cells. A. Exudative type subclass. B. Granulomatous type subclass. (Original magnification, x400).



**FIG. 20:**

Formalin-fixed, paraffin-embedded section of apical granuloma stained with MAC387 monoclonal antibody (DAKO). Positively staining monocytes, macrophages, and histiocytes appear as brown rings around reactive cells. A. Exudative type subclass. B. Granulomatous type subclass. (Original magnification, x400).

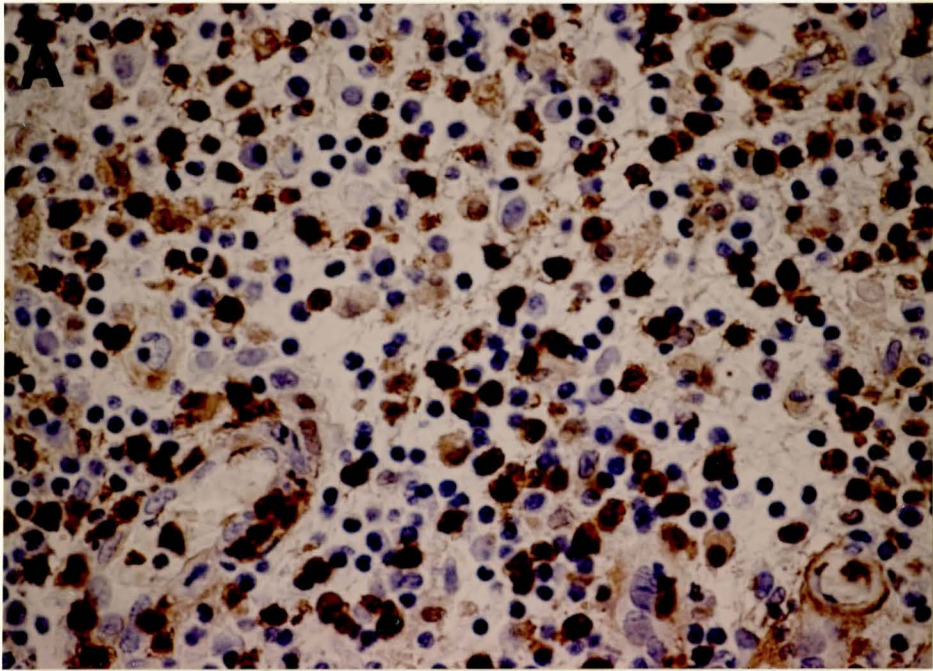
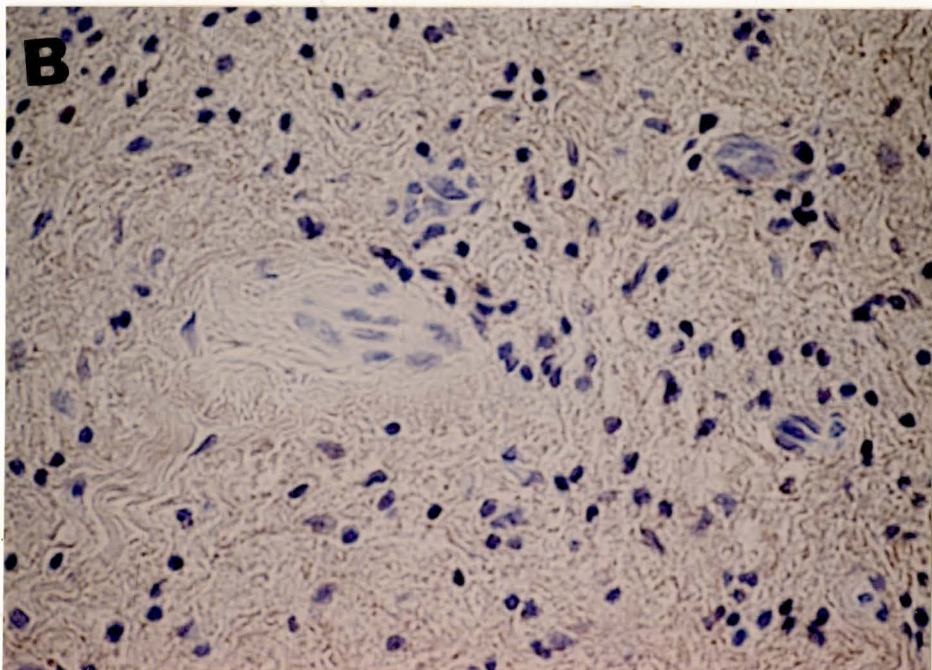


FIG.

Formalin-fixed, paraffin-embedded section of apical granules stained with MAC387 monoclonal antibody (DAPO). Positively staining monocytes, macrophages, and histiocytes appear as brown rings (reactive cells). A. Granulation tissue type subclass. B. Foreign body type subclass. (Original magnification,  $\times 400$ ).



**FIG. 21:**

Formalin-fixed, paraffin-embedded section of apical granuloma stained with MAC387 monoclonal antibody (DAKO). Positively staining monocytes, macrophages, and histiocytes appear as brown rings around reactive cells. A. Granulation tissue type subclass. B. Foreign body type subclass. (Original magnification, x400).

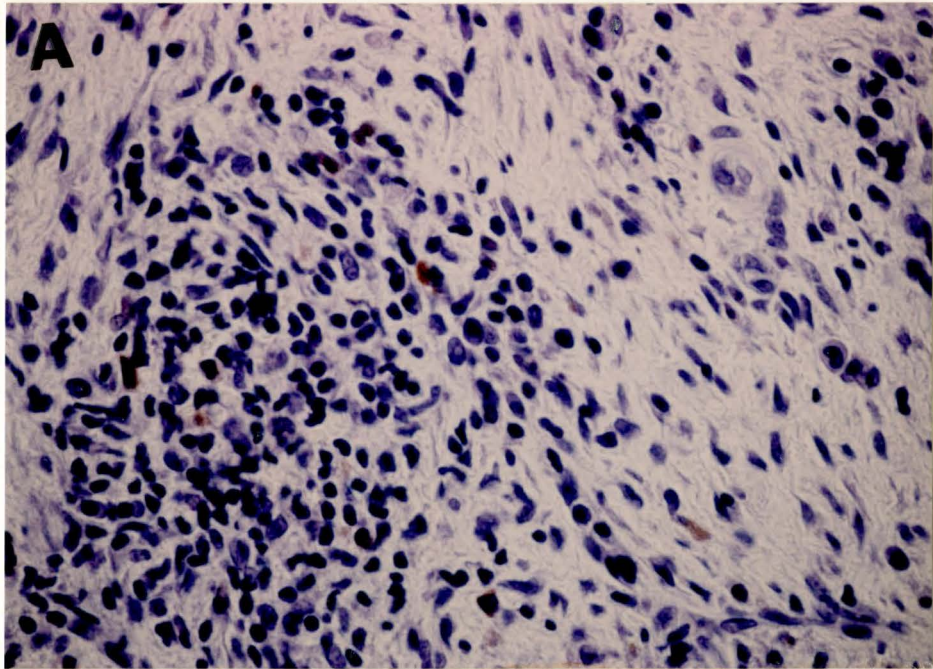
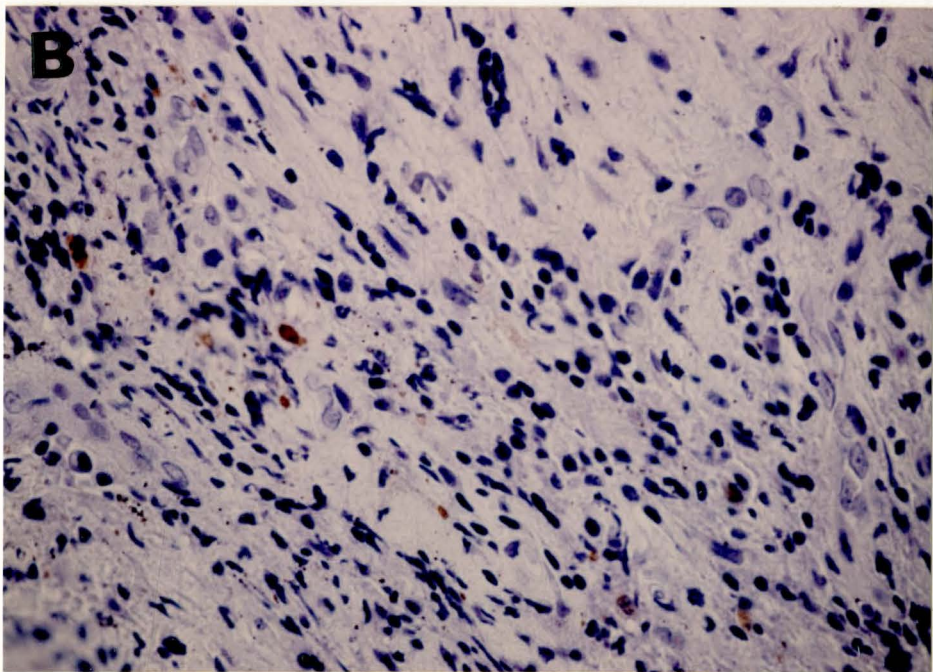


FIG.

Formalin-fixed, paraffin-embedded section of spinal granuloma stained with IgA antibody (BA50). Positively staining plasma cells appear with dark brown cytoplasm within reactive cells. A. Exudative type subclass. B. Granulomatous type subclass. (Original magnification, x400).



**FIG. 22:**

Formalin-fixed, paraffin-embedded section of apical granuloma stained with IgA antibody (DAKO). Positively staining plasma cells appear with dark brown cytoplasm within reactive cells. A. Exudative type subclass. B. Granulomatous type subclass. (Original magnification, x400).

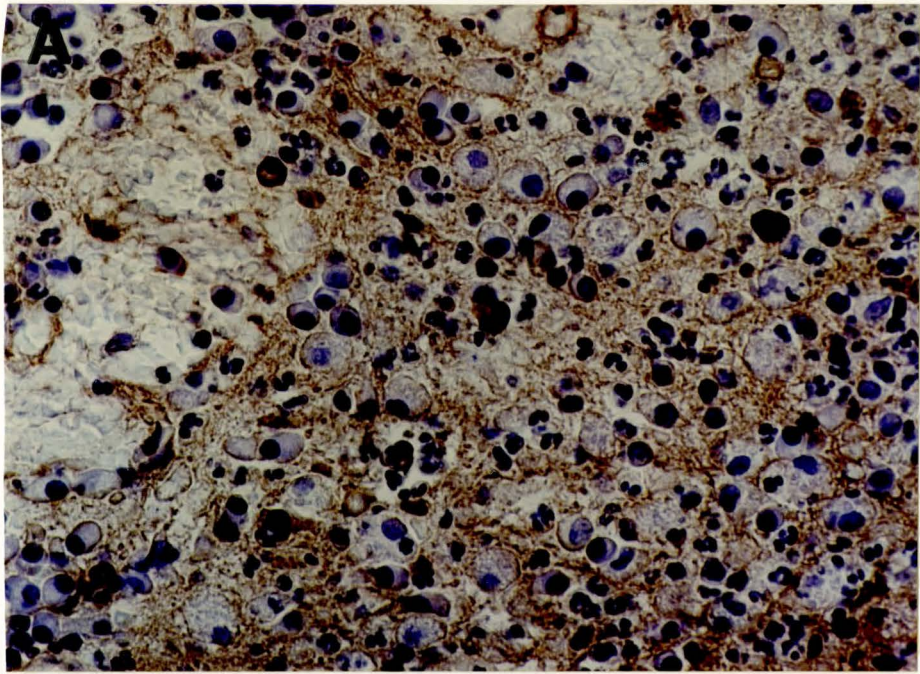
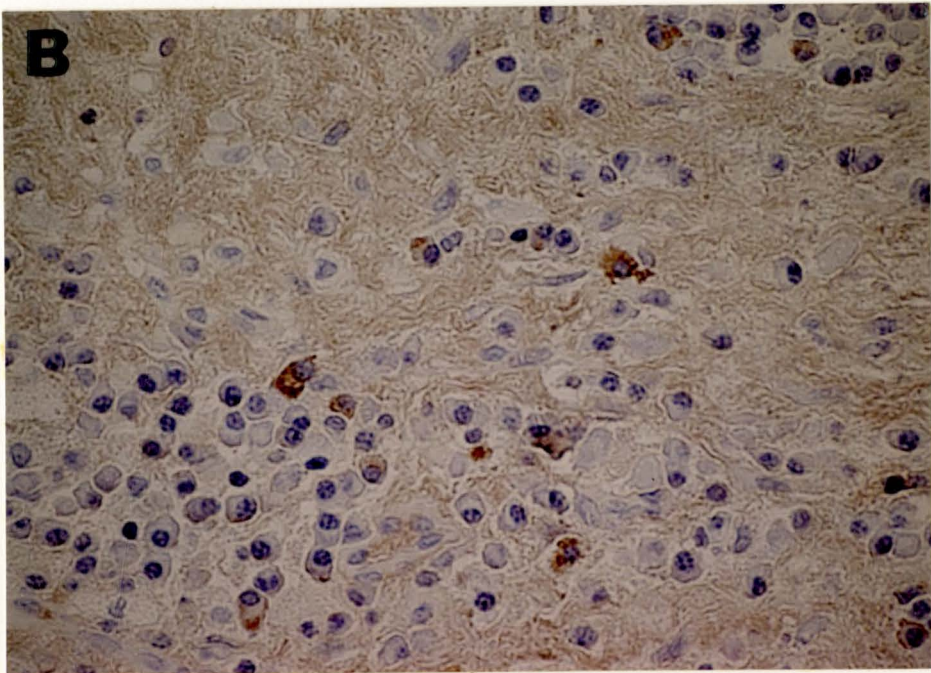


FIG.

Formalin-fixed, paraffin-embedded section of splenic granuloma stained with IgA antibody (24X0). Positively staining plasma cells appear with dark brown cytoplasm within reactive cell. A. Granulation tissue type subclass. B. Foreign body type subclass. (Original magnification, x400).





**FIG. 23:**

Formalin-fixed, paraffin-embedded section of apical granuloma stained with IgA antibody (DAKO). Positively staining plasma cells appear with dark brown cytoplasm within reactive cell. A. Granulation tissue type subclass. B. Foreign body type subclass. (Original magnification, x400).

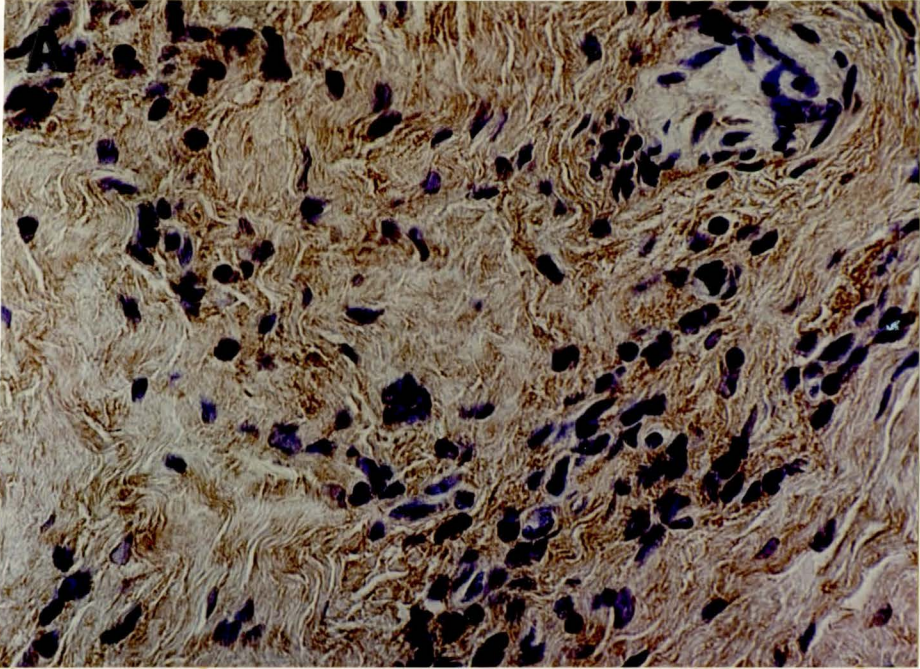
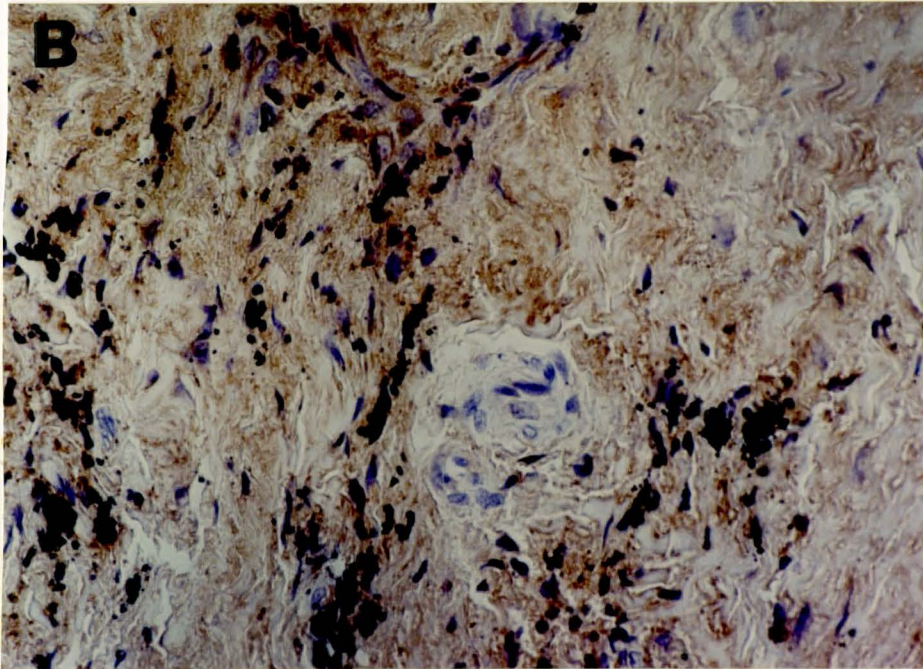


FIG.

Formalin-fixed, paraffin-embedded section of spinal ganglion stained with IgG antibody (DARD). Positively staining plasma cells appear with dark brown cytoplasm within reactive cells. A. Eosinophilic type subclass. B. Granulomatous type subclass. (Original magnification, x400).



**FIG. 24:**

Formalin-fixed, paraffin-embedded section of apical granuloma stained with IgG antibody (DAKO). Positively staining plasma cells appear with dark brown cytoplasm within reactive cells. A. Exudative type subclass. B. Granulomatous type subclass. (Original magnification, x400).

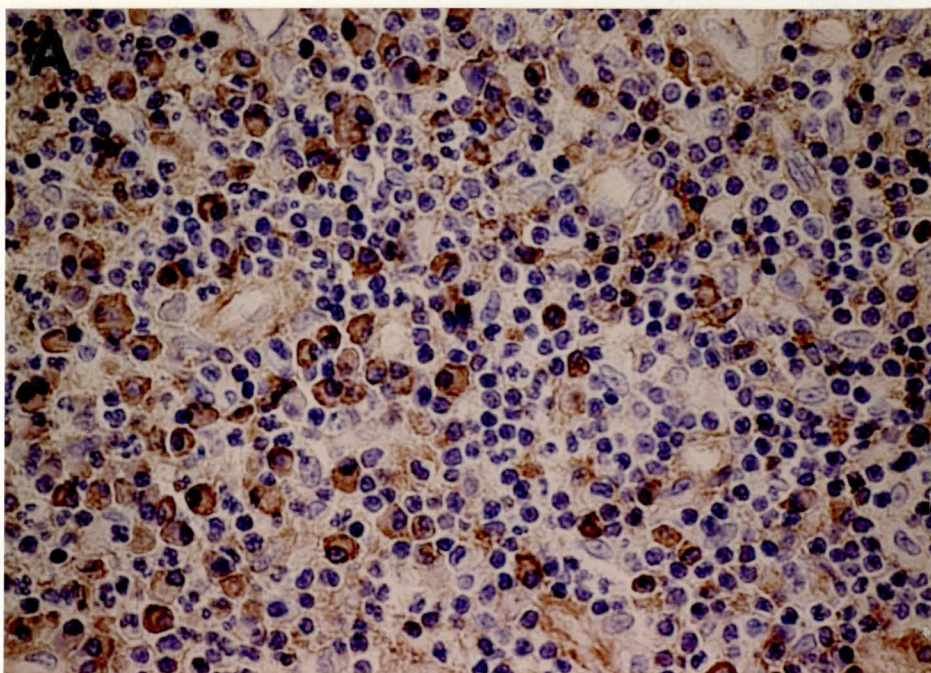


FIG.

Formalin-fixed, paraffin-embedded sections of spinal ganglia stained with IgG antibody (DAKO). Positively staining plasma cells appear with dark brown cytoplasm within reactive cells. A. Granulation tissue type subclass. B. Foreign body type subclass. (Original magnification,  $\times 400$ ).

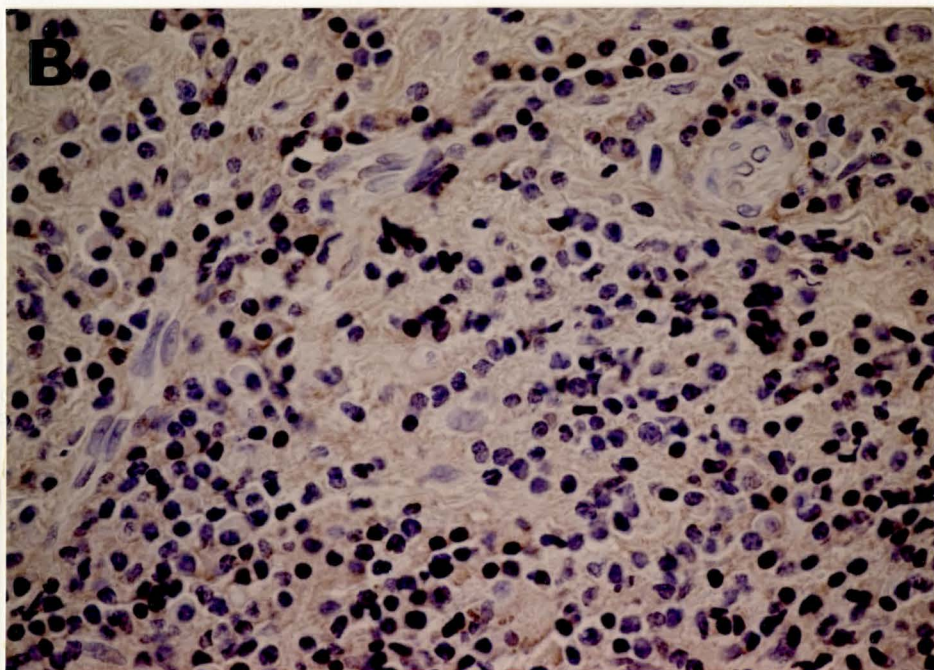
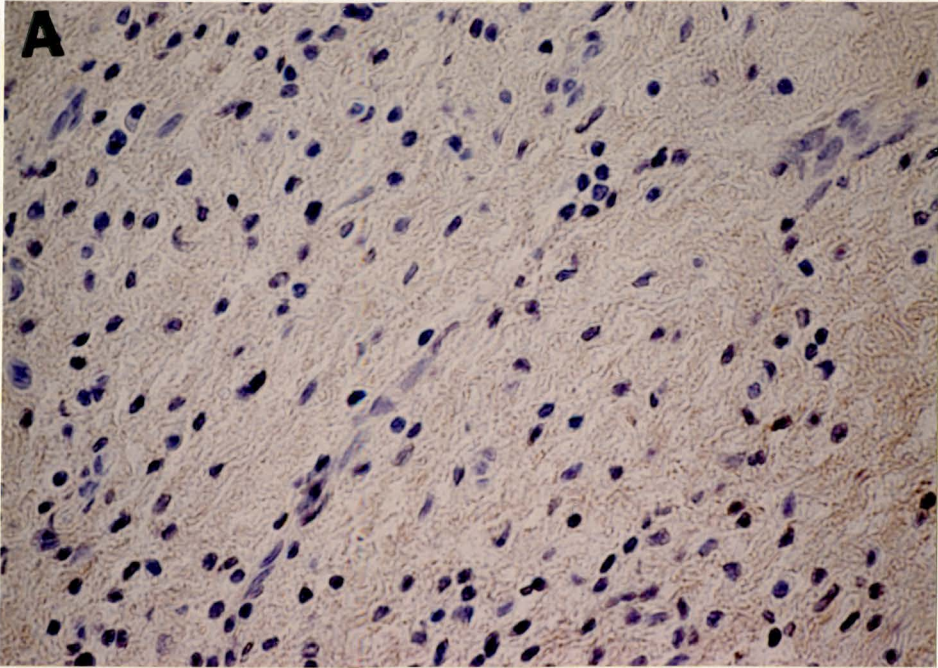


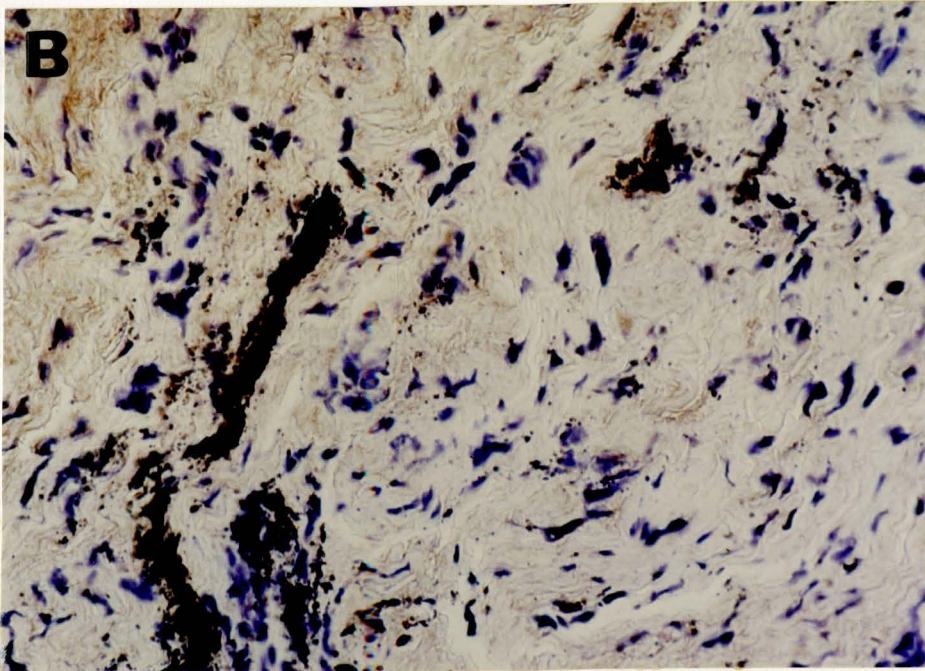
FIG. 25:

Formalin-fixed, paraffin-embedded section of apical granuloma stained with IgG antibody (DAKO). Positively staining plasma cells appear with dark brown cytoplasm within reactive cells. A. Granulation tissue type subclass. B. Foreign body type subclass. (Original magnification, x400).



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## CHAPTER VII

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## APPROVAL SHEET

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

April 15, 1993  
Date

Hasan Nadimi  
Director's Signature