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INVESTIGATION OF THE LYMPHATIC SYSTEM IN HUMAN DENTAL PULP TISSUE IN VARIOUS STATES OF HEALTH AND DISEASE

A THESIS SUBMITTED TO

THE FACULTY OF THE DEPARTMENT OF ORAL BIOLOGY

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

BY

BETH A. SHERIDAN, D.D.S.

SAVANNAH, GEORGIA

JUNE, 1993

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INTRODUCTION

Pulpal dynamics have been examined in detail by many investigators (1-3). It is generally conceded that pulpal irritations have regional effect, with spread throughout the system being the exception rather than the rule. Usually, the pulp tissue possesses the defenses necessary to dispel harmful stimuli, thus preserving its vitality. When the pulp tissue is exposed to destructive forces such as heat, cold, or bacteria, the defense mechanisms are summoned. Such phenomena as blood vessel dilation and constriction, cellular influx, and cytokine activation play roles in preserving pulp vitality. This activity within the closed confines of the dental pulp chamber results in increased intrapulpal pressure. This pressure, in itself, causes subsequent pain. In some cases, the tooth has the resources to dispel the harmful effects of fighting off a foreign stimulus. In other situations, the increased pressure and collection of noxious byproducts in one area of the dental pulp spread throughout the tissue with resultant pulp necrosis.

In correlation with other body tissues, there appears to be a need for drainage within the pulp in order to remove toxins and to relieve situations of increased pressure. Thus, not every confrontation with excess heat or cold or with bacteria results in death of the entire pulp tissue of the tooth. Nonetheless, it has remained difficult to ascertain and identify positively the intrapulpal lymphatic vessels.

Under light or electron microscope, investigators have isolated alleged lymphatic vessels by morphology alone (4-17). This technique is open for debate due to the variable size and structure of lymphatic vessels. Furthermore, alterations in sample preservation and in sectioning and staining techniques can affect the specimen with resultant inaccurate interpretations.

The advent of immunohistochemistry has opened up new avenues of discovery in science. In this study, tissue specific immunohistochemical markers will be employed in identifying lymphatic vessels in human dental pulp tissue.

Normal Pulp Tissue

The histological profile of dental pulp tissue is consistent with easily recognizable and well-defined layers present. Morphologically, four zones are identified (18). These are the odontoblastic zones at the edge of the pulp tissue, the cell-free zone, an adjacent cell-rich zone, and the pulp core. It is useful to refer to Torneck's description of pulp tissue constituents when embarking on an analysis of this structure. Odontoblasts are found at the periphery of the pulp tissue in a single layer. They appear columnar in shape in the crown of the tooth. The cells flatten in the apical regions. The odontoblast experiences several phases of activity ranging from times of high metabolism to periods of rest. Secretory odontoblasts are plump cells containing the organelles required for synthesis and secretion. As odontoblastic output slows, there is a shrinking of the cell and fewer organelles are identifiable.

Encompassing the cell-rich zone are the fibroblasts. These are the most numerous cells in the pulp tissue. In a manner similar to that of odontoblasts, the fibroblasts experience active and passive stages. The young fibroblasts produce collagen and ground substance for the pulp matrix. The size and shape of the fibroblasts at this point reflect their function. The cell has a plump cytoplasm with many organelles. As the call for pulp matrix diminishes, the fibroblasts become smaller and take on a flattened, spindle-shaped appearance (18).

Undifferentiated mesenchymal cells form the pool from which new odontoblasts and fibroblasts are formed. These cells are located in the cell-rich area and in the pulp core. The cells are microscopically large and polyhedral, with a light staining, centrally located

nucleus. As the pulp tissue ages, the number of undifferentiated mesenchymal cells decreases, and a consequent loss in the regenerative ability of the pulp occurs. Macrophages and lymphocytes are found in dental pulp tissue. The macrophages are oval or spindle-shaped, with dark staining cytoplasms and nuclei. They are most often found in conjunction with vessels. As in other tissue, the macrophages remove foreign debris and are involved in the inflammatory process of the pulp tissue. Lymphocytes isolated from dental pulp tissue have been determined to be of the T-type. They can be involved in immune defense (18).

Additionally, the most recent literature reports the identification of mast cells in both inflamed and normal pulp tissue (19). The investigator in this study identified heavily granulated mast cells near blood vessels of healthy pulp tissue. Inflamed pulp tissue showed degranulated mast cells with granules found outside the cell membrane. This finding is in contrast to Zachrisson's earlier work in which the author did not isolate mast cells in healthy pulp tissue (20).

The extracellular matrix of dental pulp tissue is composed of collagen and ground substance. An appearance similar to that of loose connective tissue is evidenced. The ground substance with its delicate mesh-like structure aids in transport of nutrients from the pulpal vasculature to the cells. Breakdown of the ground substance as a result of aging or outside irritation affects this communication, and the vitality of the tooth is compromised (18).

Pulp tissue collagen exists in Type I and Type II composition with Type I collagen composing fifty-five percent of the total. As the collagen content of the pulp increases with age, previously scattered fibrils become organized into fiber bundles. Most of the collagen is found at the apical aspect of the tooth (21). Dental pulp tissue is nourished by an abundant blood flow introduced by arterioles through the apical and accessory foramina of the tooth. As the vessels travel to the central portion of the pulp cavity, the lumen size

increases as muscular content is lost. The arterioles give off many branches culminating in a vast network at the tooth's coronal aspect (18).

Efferent return is accomplished by venules of a diameter similar to that of arterioles. Completely lacking vessel wall musculature, venules present with larger lumens in relation to the afferent vessels (18).

Capillary networking in the pulp tissue is extensive, as seen with electron microscopic, perfusion, and microangiographic techniques. The bulk of the capillary network terminates under the odontoblastic layer. These microvessels are not visible under light microscopy unless the tooth is in an active phase of dentinogenesis. Capillary fenestrations, enabling nutrient exchange, are covered with a continuous basement membrane. Pericytes are dispersed at intervals on the capillary periphery. These cells are believed to regulate vessel diameter (18).

Torneck's discussion includes pulpal lymphatic vasculature. With reference to Bernick (9), he describes small, blind, thin-walled vessels distinguished from venules by the lack of red blood cells in their lumens and by a discontinuous wall and basement membrane.

The pattern of innervation of the dental pulp mirrors that of the blood vasculature. The extensive branching of nerve fibers reaches to the cell-free zone forming the subodontoblastic plexus. The plexus is best visualized in silver stained sections (8).

Bernick also defines anatomical characteristics of pulp tissue lymphatic vessels relative to their place within the pulp chamber (8). Origin of the vessels is near the pulp-odontoblast interface. The author also notes that lymphatic vessels form a vast network much like that of the capillaries.

The aforementioned pulpal components are considered normal and form a framework for subsequent observations. As dental pulp tissue undergoes irritation from outside sources, its defense mechanisms initiate processes which cause structural and

cellular changes. Dependent upon the surrounding environment and stimuli encountered, the pulp tissue presents a variety of configurations. Consequently, it can be expected that the architecture of the lymphatic vasculature will change as the enclave is altered. Identification of lymphatic vessels in the pulp may help explain their significance in various stages of health and disease.

As stated, the dental pulp tissue exists in a variety of forms. Simple classifications, such as "healthy" and "diseased," have proved to be inadequate as investigators isolate many different stages of pulp tissue vitality (2). In terms of this analysis, it will be necessary to characterize consistently the state of health or disease of each pulp tissue examined in order to assess accurately the differing roles of the lymphatic vasculature.

From a clinical standpoint, histological labeling of pulpal states is noncontributory. Oftentimes, clinical symptoms do not correspond with cellular activity. For instance, histologically chronically inflamed pulps are often asymptomatic; whereas, very minimally inflamed pulp tissue can cause severe pain in some individuals. Nonetheless, Seltzer's descriptions of pulp tissue in various stages of pathosis organize a cluttered subject.

Structural Variations of Normal Pulp Tissue

Intact, uninflamed pulp tissue exhibits structurally intact, normal cells. There are minimal numbers of collagen fibers. The fibroblastic nuclei and cytoplasm are distinct entities. Chromatin staining is intensely blue. Blood vessels and nerve tissue are regularly distributed (2).

Pulpal Fibrosis

Another state of pulpal vitality is exhibited by a condition of atrophic, hypocellular pulp tissue. Evidenced by an actual decrease in size of the pulp chamber and root canal, the pulpal contents are more fibrotic in nature. Blood vessel caliber increases in this condition.

Also, the odontoblastic layer is more narrow because of the flattening of inactive odontoblasts. Typically associated with aging teeth, this condition is created by an increasing deposition of reparative secondary dentin (2).

Pulp Tissue Calcification

Pulp tissue at different stages of life often can show varying degrees of mineralization. Pulpal atrophy in response to deep caries often evolves into calcific change. As the underlying cells seem to shut off nutrient production, mineralized deposits can be seen in the walls of blood vessels and perineural sheaths (2).

Simple aging can be a factor in the production of calcific changes. Alterations in the ground substance seem to trigger mineralization through decreased reactivity and an accumulation of less soluble macromolecules (2). Bernick found the collagen bundles of the vascular and neural sheaths in aging pulps to be nidi of calcification (22). Two forms of pulpal calcifications have been identified. The discrete type are found in the coronal aspect of the tooth; whereas, those termed diffuse are located in the radicular area. Coronal calcifications or stones are classified as laminated or nonlaminated, based on surface smoothness. Laminated stones grow by the opposition of collagen fibers to their surface. On the other hand, nonlaminated calcifications develop via mineralization of preformed collagen fibers. A fuzzy outer layer is thus formed (23).

Dystrophic mineralization develops in areas of previous liquefaction necrosis. Occurring most often in teeth with chronic inflammation, this change is seen as both cells and fibers are affected. Cells look smooth and folded. Degeneration of the cell begins with initial effects appearing within the mitochondria. There is a subsequent membrane permeability allowing for calcium ingress. A retrogressive, degenerative change of the pulp tissue develops. Fibers appear beaded, with eventual merger. Most often, the roots of the teeth are affected in this age related phenomenon (2).

Diffuse calcification occurs as mineralization originating in the connective tissue walls of thrombosed blood vessels and nerves. The calcified area appears diffuse, with an uneven border and a discernible longitudinal pattern (24).

Pulpal Denticles

When large calcifications develop, they are termed denticles and are characterized as free or attached and true or false. The classification as to free or attached refers to the position of the denticle in the tooth. Those stones deemed attached are fixed to the walls of the pulp chamber or the root of the tooth. Free denticles are not attached to tooth structure and are usually found in the pulp chamber. True denticles, with a dentinal composition, are not results of dystrophic calcification. They are made of detached odontoblasts or fragments of Herwig's sheath. A false denticle originates with a degenerating cell serving as a nidus for the deposition of concentric layers of calcified tissue (24).

Pulpal Inflammation

A situation of an intact pulp with scattered chronic inflammatory cells is described by Seltzer. In this case, there are low numbers of inflammatory cells present. These cells are located in proximity to deep caries and in teeth which have undergone other stresses such as previous restorations, bruxism, and periodontal disease. Dilated blood vessels are identified near the inflammatory cells. This type of inflammation does not produce adequate numbers of cells to be termed exudative and mainly gives evidence of the tooth's ability to ward off noxious stimuli (2).

Acute partial pulpitis is often a regional entity occurring adjacent to an area of pulpal exposure. Histological evidence includes dilated, congested blood vessels, edema, and an accumulation of polymorphonuclear leukocytes, macrophages, and red blood cells in association with the odontoblastic layer. Odontoblastic variations are evidenced, including

displaced nuclei. Results of damage to the odontoblastic layer show up after the inflammation has cleared in the form of reparative tertiary dentin (2).

Pulpal abscess describes an irreversible process with a histological picture of edema, necrosis, polymorphonuclear neutrophil influx, and vasodilation. Congestion is evident by the visualization of engorged blood vessels. As the inflammation persists, macrophages appear to engulf dead and dying cells. As polymorphonuclear leukocytes are degraded, the byproducts of this process form pus. Additionally, some cells release self-digesting proteins which contribute to suppurative accumulation. These noxious products cause destruction of the odontoblastic layer (24).

Chronic pulpitis arises from a condition of irreparable damage. Varying amounts of the pulp tissue may be involved in increasing numbers of fibroblasts and their fibers. Blood vessels are dilated and quite visible. The cellular profile is one of macrophages, polymorphonuclear leukocytes, lymphocytes, and plasma cells. These species are often surrounded by dense collagen fibers (2).

Chronic partial pulpitis can occur with accompanying areas of liquefaction or coagulation necrosis (2). Similarly, chronic ulcerative pulpitis is defined by a situation of surface ulceration at a carious site. The base of the ulcer is separated by granulation tissue from the rest of the pulp tissue (23).

Finally, chronic total pulpitis describes the situation presenting when the entire pulp shows cellular signs of inflammation. Without fail, an area of liquefaction or coagulation necrosis is evidenced in the coronal pulp tissue. Granulomatous tissue growth permeates the rest of the root canal and can extend to the periapical tissues (2).

If the entire pulp tissue becomes subject to coagulation or liquefaction, total necrosis is diagnosed. Cellular evidence of coagulation necrosis includes a fixed protoplasm, with no intracellular detail. In contrast, liquefaction necrosis is visualized by the complete lack of cellular outline. This liquefied area is enveloped by a dense zone of

dead and dying polymorphonuclear leukocytes in conjunction with other typical inflammatory cells (2).

Seltzer chose to simply, yet accurately, classify the realms of pulpal histology. Nonetheless, he explained that pulp tissue from the same tooth can exhibit different signs due to its location within the root canal system and to the dynamic nature of viable dental pulp tissue.

REVIEW OF RELATED LITERATURE

Characterization of the developmental pathway of the human lymphatic system has been difficult and the source of much controversy. This problem has been addressed since the early twentieth century with many theories gaining, and eventually losing, acceptance.

In 1901, Florence Sabin established herself in the debate by proposing the centrifugal theory of lymphatic development (25). According to this investigator, lymph vessels developed from "centrally placed lymph sacs" spreading outward through the body as epithelial "sprouts" (26). Sabin states that lymphatics come from preexisting veins. Many other scientists, including Ranvier and Lewis, espoused this theory (27-28).

Concurrently, another school of thought was being touted by some investigators (29-31). In 1915, McClure published a paper explaining the "centripetal theory" of lymphatic formation (30). Proponents of this concept believed that lymph vessels developed in the periphery of the embryo by arising from the coalescence of mesenchymal spaces (32).

McClure explained that peripheral lymph collected in vessels which, through concresence, became continuous and established a back flow to the venous circulation (30).

The centripetal and centrifugal theories were the most widely accepted proposals establishing the two opposing sides. Additionally, Kampmeier had interest in the topic and suggested a slight variation to the centripetal hypothesis. This investigator suggested that lymph vessels form when preexisting blood vessels degenerate into the perivascular space. The lymph vasculature was purported to arise from this situation and subsequent anastomoses with the subclavian veins (33). He stresses that his theory follows the concept

of the ever-evolving and developing embryo, and likes the turnover of blood vasculature to that of developing bone (34).

Finally, there was a school of thought purporting that lymph vasculature evolved from both preexisting and mesenchymal spaces (35-36).

By the early 1940s, interest in the topic of lymphatic vessel origin had waned. Gray and Skandalakis reported in their text, Embryology for Surgeons, in 1972, that no notable research had been conducted in recent years. They recommended Kampmeier's 1960 bibliography as the most recent source of information. Additionally, the authors mentioned the need for innovative investigative techniques in order to shed new light on the subject (32). Such suggestions were heeded with the study by Van der Putte and Van Limborgh in 1980. These scientists believe that much of the lymphatic controversy is due to errant research methodology. In contrast to earlier studies, Van der Putte and Van Limborgh were able to examine human embryos in good condition. The authors point out that few of the previous investigations involved human embryos and those analyzed were not of good quality (37).

Results of Van der Putte's and Van Limborgh's research of forty human embryos established some credible concepts on lymphatic development. Based on their findings, several questions have been reliably answered. The authors hold to the centrifugal theory of origin of lymph vessels. All lymphatic primordia, with the exception of the thoracic duct, was found to arise from sprouting of the endothelial lining of venous walls. The authors did not definitely observe thoracic duct primordia in the human embryo, but hypothesize its development from sprouting intercostal veins (37).

Van der Putte and Van Limborgh vehemently disregard the centripetal theory of lymphatic development. They state, "spaces in the mesenchyme ... do not play any role whatever" (37). Explained further, the investigators say that lymphatic primordia rapidly

increase in size, merge together, and spread to the periphery. They never identified peripheral primordia of lymphatic vessels (37).

Van der Putte and Van Limborgh isolated the lymphatic primordia as paired jugular and axillary lymph sacs and paratracheal, internal thoracic, lumbar and iliac lymph plexuses, and single subtracheal and mesenteric lymph plexuses. They believe the thoracic ducts come from additional small primordia (37).

The authors point out that communication between venous and lymphatic systems in the periphery of the body represent developmental abnormalities (37).

At this juncture, the preponderance of evidence has indicated a centrifugal process of lymphatic vessel development. Modern research has been lacking on this subject with Van der Putte's and Van Limborgh's paper providing the most current research.

A continuing controversy exists as to the presence of a lymphatic vessel system in human dental pulp tissue. Numerous studies use morphologic criteria to identify and discern lymphatics from other vessels (4-17). More recently, there have been attempts to locate lymphatic vasculature through immunohistochemistry (38).

On the other hand, there have also been papers decrying the existence of pulp tissue lymphatics (17, 39, 40).

Lacking solid evidence as to their existence, it is often assumed that pulpal lymphatic vessels are present in human teeth. Ogilvie and Ingle state in their text that "logic dictates that the pulp should possess a lymphatic network as elaborate as that of blood capillaries" (41).

Additionally, in Weine's text, Endodontic Therapy, the controversy is acknowledged, but again the authors emphasize the physiologic need for lymphatic drainage in the tooth. Therefore, they accept, without absolute proof, that lymph vasculature must exist in human dental pulp tissue (24).

As early as 1917, Dewey and Noyes were among the pioneers who sought to investigate the lymphatic system in the dental pulp. Using direct and indirect staining methods, they injected a staining solution into the pulp chambers of the teeth of recently sacrificed animals. Dewey and Noyes encountered mixed results. Some teeth exhibited lymphatics, whereas others did not. The discrepancy might explain methodologic staining procedures which influenced outcome. While employing direct injection techniques, it was incumbent that blood vessels not be mistaken for lymphatics. In most of their sampling procedures, the investigators first injected the blood vasculature with carmine gelatin. In these cases, distention of blood vasculature within the closed confines of the tooth might have precluded filling of lymphatic vessels when they were subsequently injected. Contrastingly, with a smaller number of samples, the authors injected the lymph vasculature first followed by the blood vessel injection. This technique allowed for the visualization of lymphatic vessels (4).

MacGregor (5) introduced some modifications into the staining techniques of Dewey and Noyes. This investigator used lead acetate as a staining medium and conducted his experiments on live animals. MacGregor employed direct and indirect injection methods. He also implanted staining solution-saturated cotton into the sulcus and into the gingival tissue of the animals. MacGregor found evidence of lymph vessels in the pulp tissue. He reported a communication of pulpal and periodontal ligament vessels. Also, the author purported a system of lymphatic channels from pulp through dentin and cementum through bone to gingival tissues.

Kukletova presented electron microscopic evidence of lymphatic vessels in the dental pulp of calves (6). The author addressed several characteristics of the alleged lymphatic vessels, thus distinguishing them from blood vasculature. These included irregular, relatively large lumens, thin endothelium, lack of red blood cells in the lumen,

absence of a basal lamina, albuminal cytoplasmic projections, and a discontinuous endothelium with intercellular clefts.

Dahl and Mjor (7) investigated pulpal tissue through light and electron microscopic studies. Sections from pulp tissue specimens of extracted molars of 10- to 13-year-old children were used. No differences between lymphatic tissue and blood vasculature were found under light microscopy. Electron microscopic investigations, however, showed morphological characteristics of suspected lymphatic vessels. The latter were found to be only intermittently lined with endothelial cells. Accordingly, the purported lymph vessels were found to have no basement membrane.

Bernick (8) performed a study using calcified tooth sections. He stained thick sections (50-150um) with iron hematoxylin. This technique reportedly allowed for better visualization of both blood and lymphatic vessels. The author positively identified lymph vessels in all 75 of his specimens. Morphologically, lymph vessels were identified by their characteristic absence of a basement membrane, their tendency to anastomose with each other, and their variability in caliber and shape. Bernick objected to injection studies, citing the possibility of infiltrating blood vessels with the injection medium. He also supported the use of thick sections which allow for more complete visualization of the course of the vessels.

Bernick also noted that the lymphatic vessels formed a vast network with origin near the pulp-odontoblast interface (8).

In a followup study, Bernick (9) reported on changes in lymph vasculature in differing states of pulpal health. He noted that at the onset of inflammation, lymphatic vessels dilated and facilitated drainage. There was a subsequent release in pressure. As the pulp tissue became more profoundly inflamed, lymphatic vessels at the center of the disturbance were closed, fluids accumulated, pulp pressure increased, and pulp death ensued.

Frank and associates (11) attempted to enlarge lymphatic lumens before injecting them with colloidal carbon. They felt this would aid uptake of the material. Consequently, the authors induced an inflammatory reaction in the experimental teeth before injecting the pulp tissue. Colloidal carbon was then injected into the pulp tissue of the vital teeth. After extraction, the pulp tissues were stained and examined under electron microscopy. These investigators were convinced as to the existence of pulpal lymphatics. They cited the enlarged lumens of the lymphatics as being instrumental in their ability to identify the vessels. Morphological characteristics including a thin endothelial lining with occasional large clefts, absence of basement membrane, and absence of luminal red blood cells were evidenced in their study.

Baratieri and colleagues conducted several studies of the lymphatic tissues of human dental pulp tissue (12-13). They were able to visualize morphologic changes between lymph vessels in normal and inflamed pulp tissue. Using paraffin-fixed, periodic acid Schiff stained sections, the investigators found increased numbers of lymph vessels -- many with dilated lumens -- in cases of inflammation (12). While examining calcified pulp tissue, Baratieri's group found a decreased distribution of lymphatic vessels (13).

Bishop and Malhotora (16) identified lymphatic vessels in feline dental pulp tissue. The vessels were noted in the coronal, middle, and apical areas of the pulp chambers, although not all teeth exhibited lymphatic circulation in all areas. In some samples, no lymphatic vessels were located in the coronal and middle thirds. Ultrastructure of the pulpal lymph vessels was consistent with that found in other tissues. The endothelium was irregular and attenuated with little or no basement membrane.

Marchetti and associates examined lymphatic pulpal vessels in states of health and inflammation (14-15). First, the investigators looked at healthy pulps via electron and light microscopy (14). The authors mentioned morphological characteristics similar to other studies. They were concerned with variations in endothelial lining and noted end-to-end

cellular contacts, overlapping cells, and interdigitations among protrusions of the cells. The authors found that overlapping cells help to create intraparietal channels in the vessels for communication between the interstitium and vessel lumen.

In a followup investigation, Marchetti and his colleagues examined lymphatics of inflamed pulp tissue (15). Under light and electron microscopy, the authors noted dilated vessels with "open junctions" between endothelial cells. They commented on the absence of intraparietal channels previously identified in healthy pulpal lymphatic vessels. According to these investigators, in the inflamed instance, "open junctions" allow more rapid drainage. Marchetti and his cohorts recognized the dynamic nature of the lymphatic vasculature and made an important contribution by studying teeth in various states of health.

A more recent investigation by Green and associates (38) attempted to identify lymphatics using immunohistochemistry. These authors were unsuccessful in differentiating between blood vessels and lymphatics. In their experiment, specimens were fixed and embedded in paraffin prior to labeling. Lymphatic specific antibodies to ATPase, 5' nucleotidase, and alkaline phosphatase did not yield positive results. The authors proposed further studies using fresh frozen rather than fixed specimens.

In contrast to these investigations, several other examiners have been unsuccessful in isolating lymphatic vessels in human dental pulp tissue (17, 39-40).

Balogh and Boros (17) attempted to identify lymphatic vessels by using an India ink diffusion method. The ink was placed on exposed pulps with hopes of it following the entire path of the lymph vessels from the pulp chamber to the apex of the tooth. The authors were able to identify lymphatic vessels only in the roots of the teeth. There was no evidence of lymphatic vasculature in the crown portion while viewed under light microscopy.

Using a similar technique, Isokawa noted lymphatic vessels at the apex of the tooth root and labeled these periodontal lymphatic vessels (39).

Takada studied pulp tissue vessels with light and electron microscopy and could not isolate lymphatic vessels (40).

Also, Avery, in Orban's Oral Histology and Embryology, gives no definitive statement on pulpal lymphatics, saying, "the presence of lymph vessels in the dental pulp is questioned by some and agreed upon by other investigators" (42).

Finally, Chien, in his report on the state of research in the field of pulp hemodynamics (43), called for further study in the area of pulpal lymphatics. He stressed the importance of determining whether or not lymphatic vessels are present in dental pulp tissue and added that determining whether or not lymphatic vessels are present in dental pulp tissue is vital to the future of pulpal research.

This study will apply immunohistochemistry to fresh frozen sections, as well as Formalin-fixed pulp tissue specimens. Employing monoclonal antibodies and tissue reactive lectins, an immunoprofile will be established for lymph vessels. The monoclonal antibodies PAL-E, EN-4, Type IV collagen, and Factor VIII-related antigen (FVIII-RA), plus the lectin UEA-1 will be used. Previous investigations have identified lymph and blood vasculature with these techniques (14-19, 44-55).

Employing an immunoperoxidase method, Mukai and colleagues reported on the successful characterization of FVIII-RA in the endothelial lining of blood vessel walls (44). They were unable to achieve significant staining of lymphatic vessels.

Several investigators have determined that endothelial cells synthesize FVIII-RA (56-57). Visualization of this antigen has been used to diagnose tumors with a vascular origin (45).

Following up on Mukai's study, Schested and Hou-Jensen used the unlabeled antibody peroxidase-antiperoxidase technique, with slight modifications, to identify

vasculature in normal and malignant tissue. The authors reported more enhanced staining following trypsinization of the specimens. Additionally, endothelial cells were visualized even in cases of vasculitis with accompanying necrosis of the vessel wall (45).

Holthofer and cohorts studied the use of UEA-1 for vascular labeling (46). UEA-1 stained all sizes of vessels, including lymphatics. UEA-1 was found to be a more sensitive marker than FVIII-RA. Contrary to previous ideas (58), the stain is not blood-group specific.

Crocker and Smith used an unlabeled antibody peroxidase-antiperoxidase method to identify FVIII-RA in Hodgkin's disease (47). Their results were similar to previous studies with positively staining blood vasculature. The sinuses of lymph nodes were negative for FVIII-RA, and cells were positively stained.

Similarly, Ordonez and Batsakis compared UEA-1 and FVIII-RA in vascular lesions (48). Notably, while using an immunoperoxidase staining method, UEA-1-treated blood vessels and lymphatics exhibited strong immunoreactivity. In contrast, FVIII-RA treated vessels lost reactivity as their size increased. The authors confirm the hypothesis that FVIII-RA and UEA-1 do not share binding sites.

Svanholm and others identified lymph vasculature through use of FVIII-RA and a two-layer conjugated immunoperoxidase technique. They reported a weaker stain with lymphatic vessels than with blood vessels (49).

Schinglemann and others recommended PAL-E as an accurate marker of blood vasculature endothelial cells (50). In frozen section, PAL-E stained the endothelium of capillaries, small and medium-sized veins, and venules. There was little or no reaction with small, medium, or large arteries or with large veins or lymphatics.

Russell-Jones and colleagues used the monoclonal antibodies EN-4 and PAL-E to differentiate between lymph and blood vasculature in Kaposi's sarcoma (51). Citing previous studies (50, 52), the authors used the staining characteristics of the antibodies to

identify the vessels in question. EN-4-stained lymphatic capillaries, small- and medium-sized arteries, arterioles, blood capillaries, venules, and small- and medium-sized veins. PAL-E did not react with lymphatic capillaries, gave weak or negative results with small- or medium-sized arteries and arterioles, and moderately stained blood capillaries, venules, and small- and medium-sized veins.

Nadimi and others were successful in using EN-4 to stain lymph vessels selectively. They also reported that PAL-E stained only blood vasculature. Additionally, the staining properties of LN-3, FVIII-RA, the basement membrane laminin, and fibronectin were determined (53-54).

Barsky and others (55) conducted a study with monoclonal antibody to Type IV collagen. They determined that the antibody did not react with lymphatic capillaries while exhibiting strongly positive reactions with blood vessel capillaries. Type IV collagen is present in the basement membrane of blood vessels. Lymphatics, known to lack a basement membrane, consequently do not stain when treated with the antibody.

Through incorporation of these varied markers in this research, lymphatic vessel presence and its disposition in the dental pulp tissue will be verified.

MATERIALS AND METHODS

Human dental pulp tissue was removed from 37 freshly extracted human teeth. Reasons for extraction of the teeth included orthodontic considerations, deep caries, painful pulpitis, apical abscess, and impaction. Upon recovery, the teeth were placed in either fresh neutral buffered ten-percent Formalin or phosphate-buffered saline, depending on the type of tissue processing and sectioning to follow.

The pulp tissue was removed without trauma, using an endodontic broach after conventional endodontic access. The pulp tissue of those specimens initially preserved in Formalin was fixed in fresh neutral buffered ten-percent Formalin for 24 hours before being routinely processed and embedded in paraffin. Initial classification of the specimen was accomplished after staining 4um sections with hematoxylin and eosin.

The pulps of those samples placed in phosphate-buffered saline were removed as soon as possible following extraction. In most cases, this was within one hour. The tissue was immediately embedded and frozen at -60° Centigrade. Again, 4um sections were prepared and examined with hematoxylin and eosin staining for preliminary categorization.

Paralleling the classification system of Seltzer (2), pulp tissue samples, in various states of health and disease, were identified.

In frozen section, samples were stained with either the monoclonal antibodies EN-4, PAL-E, or FVIII-RA using an indirect immunoperoxidase method. Specifically, the alkaline phosphatase anti-alkaline phosphatase technique was employed (59-60). This method is dependent on labeling with an antigen-antibody complex made up of alkaline phosphatase and monoclonal antibody against alkaline phosphatase (Figure 1). A link antibody, against antibody of the same animal species, is used to react with both the

primary antibody and the antibody of the enzyme complex. This reaction ensures the labeling of the antigen site (60). A simultaneous coupling method using alpha naphthyl-AS-phosphate and a hexazonium compound ensures histochemical visualization of the localized phosphatase (59).

Frozen sections of human pulp tissue, along with control tissues of human tonsil and mouse spleen, were cut at 4um. The sections were collected on poly-L-lysine-coated slides and immediately fixed for 30 seconds in absolute methanol at room temperature. The samples were air-dried for one hour and then stored in airtight plastic containers with Drierite dessicant at -60° Centigrade.

Prior to use, the slides were brought back to room temperature while still in the airtight containers. This avoided condensation of water vapor on the specimen. The slides were postfixed in absolute acetone at -20° Centigrade for 30 minutes and then dried briefly at 60° Centigrade.

Immunohistochemical staining was assisted mechanically through the use of the Jung Histostainer Ig instrument (Australian Biomedical Co., Mount Waverly, Australia).

The monoclonal antibody PAL-E (Accurate Chemical and Scientific, Westbury, New York) has its source in mouse spleen cells. The mouse was initially immunized with human melanoma tissue. The mouse spleen cells were fused with the myeloma cells. The cells which proceeded to produce antibody were analyzed on thin sections of human tissue (Figure 2). The PAL-E clone was identified by its positive reaction to the endothelial cells of blood vessels.

The PAL-E is of IgG2a subclass. Positive staining of human, goat, rabbit, and pig blood vessels is visualized in frozen section only. Arterial and lymphatic endothelium is not stained (50).

The EN-4 monoclonal antibody (Accurate Chemical and Scientific Co., Westbury, New York) is cultured in mouse spleen tissue. A CBA/Balbc mouse is immunized with

cultured human umbilical vein endothelial cells. Mouse spleen cells are fused with murine myeloma cells. The antibody producing cells are selected for their reaction with cultured umbilical vein endothelial cells using ELISA. The extracted clones produce high-titer antibodies (61).

All human blood and lymphatic vessels are stained with EN-4 showing equal reactivity of both large and small vessels. There have been favorable results with frozen sections, but previous studies have yielded the best data using periodate, lysine, paraformaldehyde-fixed sections (52). There is no other background staining of structures in the skin, heart, kidney, tonsils, or spleen with EN-4. This antibody is of IgM subclass.

FVIII-RA (DAKO A/S, Glostrup, Denmark) is a mouse monoclonal antibody of IgG1, kappa isotype. Staining of both cryostat and Formalin-fixed, paraffin-embedded tissue has been accomplished. The antibody reacts with von Willebrand factor in endothelial cells. A granular pattern of reactivity is the norm (62).

The PAL-E was prepared at a dilution of 1:20 in a tris-buffered saline/one-percent bovine serum albumin solution (TBS/BSA). The antibody was applied mechanically to each slide with a 30 minute incubation period. Three washes in TBS of three minutes each followed.

TBS is composed of 6g of tris (hydroxymethyl) aminomethane base in 900ml deionized water. To obtain a pH 7.6, 1N hydrochloric acid is added. Finally, deionized water to make one liter of solution is added.

Goat antimouse immunoglobulin link antibody (DAKO Co., Glostrup, Denmark) was prepared in 1:25 dilution in TBS/BSA and was applied for 30 minutes. Again the slides were washed in TBS as before.

Finally, the mouse alkaline phosphatase anti-alkaline phosphatase, APAAP, (DAKO Co., Glostrup, Denmark) at a 1:50 concentration in TBS/BSA was administered

via the instrument and incubated 30 minutes. The slides were washed in three changes of TBS for three minutes each to complete the mechanical cycle.

Manually, the slides were then rinsed in three changes of deionized water and covered with substrate solution for 45 minutes. The substrate solution (Histomark Red Reporter System, Kirkegaard-Perry Laboratories, Gaithersburg, Maryland) was prepared just prior to use per the manufacturer's instructions. It is an alpha-naphthyl-AS-phosphate hexazonium compound. After substrate incubation, the specimens were rinsed several times in deionized water. Counterstaining was accomplished with Harris hematoxylin for two minutes followed by brief differentiation in acid alcohol, bluing in ammonia water, and final washing in tap water. The slides were mounted from xylene in Accumount after air drying.

The EN-4-treated slides underwent an identical regimen with the exception of trials at two different dilutions of primary antibody (1:20 and 1:5). Similarly, those frozen sections stained with FVIII-RA were prepared as before, using a 1:25 dilution.

Formalin-fixed, paraffin-embedded sections were stained via the avidin-biotin-peroxidase technique (Figure 3). As developed by Hsu (63), this method uses a secondary antibody conjugated with biotin to detect the primary antibody. Peroxidase conjugated streptavidin is localized to the secondary antibody by the reaction of streptavidin with biotin. The streptavidin is a protein with four identical biotin binding subunits. It is made from the bacteria Streptomyces avidinii (64).

Prior to treatment with the primary antibody, each section is treated with a blocking agent of normal goat serum (Figure 4). This blocks potential nonspecific immunoglobulin binding sites and reduces background staining. The blocking serum is derived from the same species as the second layer antibody (64).

The lectin Ulex europaeus 1, UEA-1, was used with Formalin-fixed paraffin-embedded sections. The UEA-1 (ICN Biomedicals, Costa Mesa, California) is supplied in

a biotinylated form, thus eliminating the need for the secondary antibody previously mentioned. UEA-1 is a glycoprotein of molecular weight 63,000 daltons. It binds to many glycoproteins and glycolipids containing an alpha-linked fucose residue. It selectively binds group O red blood cells and has been used to determine secretor status. It has recently been established as a marker for human endothelial cells (46).

For both UEA-1 and FVIII-RA staining with Formalin-fixed, paraffin-embedded staining, sections were cut at 4um and mounted on slides previously treated with poly-L-lysine. The slides were air-dried at room temperature. Human tonsil served as a control.

All of the sections were preheated for 20 minutes at 60° Centigrade and then immediately transferred to three washes of five minutes each in xylene. Two rinses in absolute alcohol of two minutes duration followed.

Endogenous peroxidase and hemoprotein were inactivated by soaking the slides in a solution of .075 percent hydrochloric acid in absolute alcohol (.1ml concentrated acid/50 ml alcohol) for 15 minutes.

The sections were brought through graded alcohols (100-95-70) to water, with two minutes in each solution.

Trypsinization is required for proteolytic digestion of Formalin-fixed, paraffin-embedded tissues being stained with UEA-1 and FVIII-RA. The processing procedures negatively affect the antigenicity of the samples. Incubation in the protease, trypsin and restore the antigenicity (65). The slides were placed in a solution of 50mg pancreatic trypsin and 50mg calcium chloride dihydrate in 50ml of tris buffer (TBS) for five minutes.

In order to inhibit the enzymatic reaction, the sections were washed for five minutes in cold water. The slides were then washed in TBS for five minutes.

At this point, the staining procedure proceeded mechanically. The FVIII-RA slides were treated with normal goat serum blocking agent (Kirkegaard-Perry Laboratories, Gaithersburg, Maryland) for 30 minutes. The UEA-1-treated slides received a TBS rinse.

The slides were machine dried with a burst of air prior to the application of the UEA-1 or FVIII-RA. The FVIII-RA was prepared at a dilution of 1:20 in TBS/BSA containing one-percent normal goat serum; whereas, the UEA-1 was diluted 1:500 in ten-percent bovine albumin (dilution of 30-percent pentex bovine albumin, ICN Biomedicals, Costa Mesa, California, in TBS/BSA). The incubation period was 30 minutes. Following antibody application, the slides were washed in TBS three times for three minutes each.

Biotinylated goat antimouse immunoglobulin (Kirkegaard-Perry Laboratories, Gaithersburg, Maryland) was applied to the FVIII-RA treated slides as the UEA-1 slides remained in the buffer. The goat antimouse immunoglobulin was left in contact with the sections for 30 minutes.

After an additional three washes in TBS, all specimens were reacted with streptavidin-peroxidase reagent (Kirkegaard-Perry Laboratories, Gaithersburg, Maryland) for 30 minutes. Another three rinses in TBS followed as the automated portion of the staining concluded. The slides were rinsed in deionized water prior to treatment with peroxidase substrate.

Brown staining of positively reacting antigen was accomplished with diaminobenzidine substrate (DAB). For this preparation, DAB tablets (Sigma Laboratory, St. Louis, Missouri) are added to 50ml of room temperature buffer solution. The tablets are given time to dissolve, and the solution is filtered. Immediately prior to use, 90uL of three-percent hydrogen peroxide (per 50ml solution) is added.

The buffer solution is 3.85g of ammonium acetate in 900ml deionized water. Ten-percent citric acid is added to obtain pH 5.5. More water, as necessary, to make one liter of solution is added.

The specimens were placed in the DAB solution and left in a dark chamber for three minutes.

The slides were washed in water and counterstained with hematoxylin for four minutes. Differentiation in acid alcohol, bluing in ammonia water, and washing followed. Dehydration in a series of alcohols (80-95-95-100-100) preceded clearing in xylene and mounting in Accumount.

Another series of Formalin-fixed, paraffin-embedded specimens were double-stained with either UEA-1 and Type IV collagen or FVIII-RA and Type IV collagen. Samples stained solely with either FVIII-RA or Type IV collagen were included as controls. Type IV collagen is a mouse monoclonal antibody of subclass IgG1, kappa. The immunogen is prepared as antibody to purified pepsin fragments of human Type IV collagen from human kidneys (66). The antibody is targeted against Type IV collagen which is present in the basement membrane. The prepared antibody reacts with the basement membrane of many tissues and organs, including kidney, skin, striated and smooth muscle, spleen, lymph node, lung, placenta, and tendon. The discontinuous basement membrane of spleen and lymph node sinusoids exhibits fragmented staining. Other blood vessels show a continuous pattern. The basement membrane of corneal epithelium does not stain. No other structures are stained (66).

Type IV collagen works best with Formalin-fixed, paraffin-embedded tissue. Enzymatic predigestion with either pepsin or trypsin is required.

Initial processing proceeded as before with all specimens, mounted on silanized slides, undergoing preheating followed by xylene and alcohol rinses. Samples being stained with Type IV collagen and UEA-1 were treated with pepsin (DAKO Co., Glostrup, Denmark) for ten minutes to restore antigencity lost in fixing and embedding. Next, the slides were washed in cold running water for ten minutes. The Type IV collagen processing was done mechanically.

The slides were treated with normal goat serum blocking agent for 30 minutes. The antibody was diluted to a 1:50 concentration in ten-percent TBS/BSA containing one-

percent normal goat serum. After three washes in TBS, the antibody was applied for 30 minutes. Three TBS washes of three minutes duration followed. As before, biotinylated goat antimouse immunoglobulin was applied to the samples and left in contact 30 minutes. After three additional TBS washes, a 1:50 solution of mouse alkaline phosphatase anti-alkaline phosphatase was added to the samples for 30 minutes. The mechanical component of the process concluded with three more washes in TBS.

The red chromogen from the Histomark Red Reporter System was chosen to color the Type IV collagen reaction. The slides were left in contact with the solution for 45 minutes. Next, samples were treated with 1M citric acid for 15 minutes to inactivate excess alkaline phosphatase. After brief washing in TBS, a ten-percent solution of BSA/TBS was applied to the sections in preparation for the UEA-1 treatment.

The slides were placed in a moist covered tray, and UEA-1 at a 1:500 dilution in ten-percent BSA/TBS was reacted with them for 30 minutes at room temperature. The usual TBS washes were followed with the addition of streptavidin alkaline phosphatase in TBS/BSA (DAKO Co., Glostrup, Denmark) for 30 minutes. After TBS washing, BCIP/NBT substrate chromogen in .015 sodium nitrite buffer was applied for ten minutes to activate color change. The samples were washed briefly in deionized water and placed in .1M acetate buffer (pH 4.6) for five minutes. Counterstaining for nuclei was accomplished with a five minute treatment in one-percent methyl green (in .1M acetate buffer, pH 4.6). Final rinsing in deionized water followed by a series of rinses in isopropyl alcohol (95-100-100-100) preceded xylene treatment and mounting.

Those samples being treated with Type IV collagen and FVIII-RA and the controls underwent a similar regimen with a few alterations.

Primarily, initial enzyme digestion was accomplished with trypsinization for five minutes. The Type IV collagen staining and chromogen application was as explained. Following the citric acid rinse, the slides were prepared for the FVIII-RA application. The

FVIII-RA processing was done by hand using covered, moistened slide trays. Normal goat serum was applied for 20 minutes, followed by rinsing and the addition of the antibody at a 1:20 dilution in one-percent TBS/BSA. Antibody incubation was for 30 minutes at room temperature. After TBS rinses, goat antimouse link antibody was applied for 30 minutes. Rinsing preceded the addition of a 1:50 concentration in TBS/BSA of mouse alkaline phosphatase anti-alkaline phosphatase for a 30 minute time period. Again, the slides were treated with BCIP/NBT substrate chromogen for ten minutes after having been washed in TBS. The procedure concluded with counterstaining, dehydration, and mounting as before.

Finally, a series of slides were stained with both UEA-1 and periodic acid Schiff reagent (PAS). The procedure for the UEA-1 staining was as previously described, with the addition of the PAS stain prior to counterstaining with hematoxylin.

The PAS stain is visible on the basement membrane of endothelial cells. It also reacts with connective tissue and nonspecific glycoproteins (55).

After completion of the UEA-1 staining, the slides were reacted with one-percent periodic acid for ten minutes, washed in water, reacted with Schiff reagent for 30 minutes, and rinsed in three changes of .5-percent sodium metabisulfite for two minutes each. The samples were washed for five minutes in running water prior to hematoxylin staining.

The processed slides were examined for the presence of blood and lymphatic vessels in accordance with the initial histological classifications of the hematoxylin and eosin stained specimens.

RESULTS

A total of 34 human dental pulp tissue specimens were examined under light microscope following H-E and immunohistochemical staining. Twenty-six of the samples were Formalin-fixed, paraffin-embedded, and stained differentially with H-E. Of these, 11 were classified as being chronically inflamed due to the presence of plasma cells, lymphocytes, macrophages, eosinophils, and polymorphonuclear leukocytes. Increased fibrosis was also often evident. In six of these cases, calcifications were present. Figures 5, 6, 7, and 8 exhibit the classical histological picture of chronic pulpitis. In Figure 8, vessels resembling lymphatics are located near the interface of the chronically inflamed and healthy areas of the pulp tissue sample. No definitive designation as lymphatic vasculature was assigned after H-E staining, alone. Figure 9 shows a case of chronic pulpitis with a resultant degeneration of the tissue. Inflammatory cells have accumulated at a central area with accompanying breakdown of the peripheral pulp tissue.

One of the 26 Formalin-fixed, paraffin-embedded samples showed signs of both acute and chronic inflammation and was placed under the chronic calcification scheme. There were no other specimens exhibiting the classic signs of acute pulpitis. That is, a tissue showing the presence of polymorphonuclear leukocytes and edema without an accumulation of plasma cells and leukocytes.

Another seven of these 26 specimens were diagnosed as normal pulp tissue (Figure 12). These samples were found to have a physiologically sound distribution of fibroblasts, blood vessels, and nerves. Vessels were found in close proximity to nerve tissue. The zones of the pulp tissue were identified with odontoblastic, cell-free, and cell-rich areas recognized. This picture is seen in Figure 12.

Finally, eight of 26 tissues preserved with Formalin and embedded in paraffin were given a designation of calcific. This classification was applied to normal pulp tissue exhibiting signs of calcific change. In particular, discrete calcifications were evidenced. In contrast, chronically inflamed pulp tissue with calcification was placed under the chronic inflammation appellation.

Eight of the original 34 specimens were initially preserved in frozen section. Of these, five were found to be normal pulp tissue, two were examples of pulp tissue calcification, and one exhibited chronic pulpitis.

Tissue sections were selectively stained with FVIII-RA, Type IV collagen, EN-4, PAL-E, PAS, and UEA-1 lectin. Staining characteristics of arterioles, capillaries, venules, and lymphatic vessels were noted.

The basic structural characteristics of each type of vessel served as guidelines in identifying the vascular components. Arterioles are found to be approximately 50um in diameter with several layers of surrounding smooth muscle. Capillaries are 8 to 10um in diameter with a single layer of lining epithelium. They possess a basement membrane with no circumferential smooth muscle. There are often reticular and collagenous fibers located around the vessel. Venules are usually the same diameter as arterioles while lacking the surrounding smooth muscle of the afferent vessels. Consequently, the lumen of a venule appears larger than that of an arteriole. Lastly, the lymphatics are epithelially lined vessels without basement membranes. They have irregularly shaped lumens with diameters similar to the capillaries (18). Figure 13 illustrates each blood vessel after H-E staining of Formalin-fixed, paraffin-embedded chronically inflamed human pulp tissue.

These basic morphological criteria were combined with the data obtained from the immunohistochemical staining to establish an immunoprofile for lymphatic vasculature in human dental pulp tissue.

Characteristics of each of the immunohistochemical markers as determined by other investigators (44-58) were used. The monoclonal antibody FVIII-RA is known to react positively with arterioles, venules, and capillaries. It does not stain lymphatic vessels. On the other hand, UEA-I lectin stains blood vessels very strongly with a weak reaction to lymph vessels. In frozen section, EN-4 reacts with all vasculature; whereas PAL-E stains only blood vessels with a negative lymphatic reaction. Type IV collagen is specific for basement membrane; thus its characteristic staining of blood vessels accompanied by its failure to stain lymphatics was informative. The PAS stain is also reactive with basement membrane but is less specific than Type IV collagen.

Lymphatics were identified as capillary-sized vessels lined with a single layer of epithelium. They were weakly reactive to UEA-1 staining and nonreactive to FVIII-RA staining. Lacking a basement membrane, they show negative results with Type IV collagen staining and with PAS staining.

Capillaries differed in their staining characteristics. The vessels were strongly stained with UEA-1. FVIII-RA and Type IV collagen stained slightly less intensely. PAS did not stain capillaries.

These distinctly different responses to the immunohistochemical reactions simplified differentiation of the vessels.

Nineteen of the 26 Formalin-fixed, paraffin-embedded specimens were stained with UEA-1 lectin. Generally, UEA-1 strongly stained arterioles, capillaries, and venules. Lymphatic vessels were stained weakly. Figure 14 shows the positive reaction of UEA-1 with blood vasculature. Figure 15 shows a case of chronic pulpitis with blood vessels staining positive for UEA-1. Nonstaining vessels are believed to be lymphatics.

Additional sections of the same 19 samples were treated with FVIII-RA. The FVIII-RA produced weaker staining of the blood vasculature. Lymphatic vessels reacted very weakly or not at all. Figure 16 shows a partially staining blood vessel in association with a

negatively reactive lymphatic in conjunction with chronic pulpitis. Figure 17 demonstrates a similar situation. Figure 18 also shows negatively staining lymphatics using a methylene green counterstain. Only 16 of the 19 stained tissues yielded interpretable data as three tissues were lost during the procedure.

A trial of two of the 19 specimens diagnosed as exhibiting chronic pulpitis were stained with both Type IV collagen and FVIII-RA. FVIII-RA strongly stained all the blood vessels, with no lymphatic reaction. The Type IV collagen reacted similarly. Figure 19 demonstrates a nonreactive lymphatic vessel in chronically inflamed pulp tissue as seen following this staining regimen.

A different sampling of seven of the original 26 Formalin-fixed, paraffin-embedded specimens were stained with a combined application of PAS and UEA-1. The UEA-1 exhibited staining characteristics similar to those obtained in the single marker technique with intensely staining blood vessels and weakly staining lymphatics. The PAS strongly stained only arterioles with most of its activity being viewed as background coloration. One sample of these seven was lost in the procedure.

Finally, five specimens from the set of seven stained with the combined UEA-1 and PAs were stained with both UEA-1 and Type IV collagen. Again, the UEA-1 strongly stained blood vessels with a slight, weak reaction with lymph vasculature. The Type IV collagen was not visible on the slides. Of the five original samples, two were lost from the slide during the staining, thus failing to yield any results.

A set of tonsillar control tissues was Formalin-fixed and embedded in paraffin. Arterioles, capillaries, and venules all reacted strongly with UEA-1. Lymphatics were weakly stained. Figure 23 exemplifies the control reaction with UEA-1. FVIII-RA exhibited less of a staining reaction with the blood vasculature and failed to stain the lymphatic vessels in the tonsil. PAS stained only arterioles with capillaries, venules, and lymphatics not reacting. There was considerable background staining, as seen in Figure 24.

The Type IV collagen showed positive reactivity with all blood vessels and did not stain lymphatic vasculature. Figure 25 shows this reaction. A section of Formalin-fixed, paraffin-embedded placental tissue was also stained with Type IV collagen, as seen in Figure 26.

A total of eight samples was preserved as fresh frozen tissues. Sections from six of the specimens were stained, on separate slides, with FVIII-RA and PAL-E. The FVIII-RA reacted weakly with arterioles, capillaries, and venules. No lymphatics were identified. Figure 27 demonstrates the staining characteristics. The frozen sections were more difficult to interpret, with considerable tissue destruction occurring during processing.

The samples stained with PAL-E were equally difficulty to analyze. The staining of arterioles, capillaries, and veins was very weak. No lymphatics were seen. As visualized in Figure 28, the stains are muted.

Attempts to achieve noteworthy results were futile when staining with EN-4. Cryostat sections of two of the initial eight frozen sections were treated with the monoclonal antibody, with negative results. No vascular structures were stained. Two trials at differing dilutions (1:20 and 1:5) were attempted in order to achieve stronger staining. They proved unsuccessful.

Placenta, spleen, and tonsil were used as fresh frozen tissue controls. Frozen sections of tonsil were stained with FVIII-RA, and the results were similar to those garnered with Formalin-fixed, paraffin-embedded tissue. Arterioles, capillaries, and venules stained moderately positive; whereas, lymphatic vessels were not detected. Placenta and spleen in cryostat section were stained with PAL-E and EN-4. Similar to the reactions obtained with the pulp tissue, there was very weak coloration of blood vasculature and no detection of lymph vessels when PAL-E was used. Figures 29 and 30 show the spleen control tissue for FVIII-RA and EN-4, respectively. Figure 31 shows the PAL-E reaction in placental tissue.

The immunohistochemical staining results were tabulated in regard to the H-E diagnosis of the pulp tissue specimen (Table 2). The staining characteristics of the control tissues are condense in Table 1. Evaluation of the stained tissues revealed a distinctive pattern. All cases in which lymph vessels were identified showed the histological profile to be one of chronic pulpitis. Lymphatic vasculature was not found in normal pulp tissue.

DISCUSSION

The major thrust of this investigation concerned the attempt to identify lymphatic vasculature in dental pulp tissue. Previous studies relied on light and electron microscopic analysis (4-16) of pulp tissue samples. Staining procedures were nonspecific and often dependent upon intrapulpal injection techniques.

Through the use of the immunohistochemical markers, FVIII-RA, UEA-1, and Type IV collagen, lymphatic vessels were identified in chronically inflamed human pulp tissue. FVIII-RA stained arterioles, venules, and capillaries. Lymphatic vessels did not stain with this monoclonal antibody. The UEA-1 lectin reacted with arterioles, venules, and capillaries to a stronger extent than did the FVIII-RA. Lymphatic vessels stained weakly. Type IV collagen reacted with the basement membrane of arterioles, venules, and capillaries. Lymphatics, lacking a basement membrane, did not stain.

No other studies have reached the conclusion offered here. Among those who have isolated lymphatics, most investigators cite an incidence in all states of pulpal health (4-16). For instance, Bernick (9) stated that lymph vessels were present in the normal and inflamed pulp tissue samples he examined. He purported the theory that collapsed lymphatics are present in normal pulp tissue and become distended during inflammation. He used PAS-hematoxylin or iron-hematoxylin stains to visualize the constricted lymph vessels.

Baratieri and others identified lymphatics in normal and inflamed pulp tissue with conventional staining techniques. This investigator, much like Bernick, sees lymphatics as being more dilated and more numerous in cases of inflammation.

In this study, through use of very sensitive immunohistochemical techniques, no lymphatics were isolated in healthy, normal human pulp tissue. As is prevalent in other

body tissues, it is possible that pulpal lymphatic vasculature proliferates only when there is a need to clear the area of the byproducts of inflammation. Limitations of this study preclude any attempts to develop actual theory. Sample sizes were too small to establish significant numerical data in regard to the prevalence of lymph vessels.

There were numerous difficulties encountered in obtaining pulp tissue samples, thus lessening the scope of the investigation. The removal of dental pulp specimens from the extracted teeth proved challenging, with many teeth lacking usable tissue. Immediately, this skewed the study, as necrotic pulps were virtually impossible to remove intact. Often if a necrotic sample was obtained, the specimen was so small and preservation so difficult that no attributable data could be obtained. It proved to be that the samples which were best preserved and stained were the larger, usually healthier, tissues.

Additionally, no examples of acutely inflamed pulp tissue were garnered. It would be beneficial to analyze this stage of pathology. All of the samples examined were from teeth extracted due to impaction, orthodontic considerations, painful pulpitis, apical abscess, or deep caries. Thus the preponderance of chronically inflamed or normal pulp tissue is explained. It appears that histologically diagnosed acute pulpitis is a less bothersome clinical entity, rarely leading to extraction or endodontic therapy. Most cases of patients presenting with acute pulpal pain are acute exacerbations of a chronic situation. It would seem likely that lymph vessels exist in purely acute inflammatory situations, but to a lesser extent.

Finally, there were some other procedural difficulties warranting mention. Once seemingly good sections were obtained, problems developed in assuring that the sample remained fixed to the slide for the entire staining procedure. Two different types of coated slides were used in attempts to firmly secure specimens. Nonetheless, there did not seem to be a difference between the poly-L-lysine-coated and silanized slides. A good number of samples were lost in staining, again lessening the scope of the study. There seemed to be

no correlation between size of specimen and likelihood of its loss during processing. Some of the larger, well-defined specimens after H-E staining were lost while undergoing the immunohistochemical staining battery. Additionally, another area of difficulty involved the preservation of the fresh frozen sections. As is to be expected with this method, the specimens were often damaged and slides were hard to interpret.

Further studies employing a wider variety of pulp tissues would be enlightening. The benefits of using human pulp tissue precluded the use of animal models in which more control of sample histology is possible. For instance, it may have been possible to induce histologically identifiable acute pulpitis in an animal. At any rate, verification of lymphatic vessels in human pulp was an important task.

With lymphatic vessels identified, articles by Heyeraas and Kvinnsland and by Bishop create further interest (66-67). Bishop proposes that teeth have differing amounts of lymphatic vasculature. His study identifying lymph vessels in feline dental pulp showed all teeth to contain lymphatics with different distributions and quantities of vessels noted (16). In a later work, Bishop (66) theorizes that the severity of patient pain may correlate with the complexity of the lymphatic network within the tooth. Accordingly, those teeth with well-developed lymphatic vasculature are less likely to become painful following noxious stimuli; whereas even minor irritations may cause discomfort in teeth with a scarcity of lymphatic vessels. From the results garnered here, it is believed that lymphatic vasculature develops in response to infection or inflammation. The complexity of the lymph system does not appear to be a matter of chance. Theoretically, as a lesion becomes chronic, lymphatic vasculature develops, concurrently, thus lessening the likelihood of pressure-induced pain.

Heyeraas and Kvinnsland (67) discuss pulpal blood flow in inflammatory situations. As pressure increases within the closed confines of the pulp chamber, vessels may become constricted and blood flow will be compromised. The authors feel that

lymphatic drainage helps to reduce intrapulpal pressure by preventing vascular collapse. Blood flow is maintained and the inflammation is cleared. These authors believe that pulpal lymphatic vessels are significantly involved in the healing process.

The monoclonal antibodies and lectin used in this study reacted as reported in previous studies (44-58). Their usefulness in morphological analysis of pulp tissue is indisputable. Expanded versions of this investigation may provide more definitive answers as to the occurrence of pulp tissue lymphatic vasculature.

The identification of lymph vessels in the pulp tissue creates questions as to the scope of its regenerative capabilities. It is possible that pulp tissue may be more resilient than currently believed. With the recognition of these vessels solely in chronically inflamed tissues, it appears that dental pulp tissue mounts a strong defense before succumbing to harmful stimuli.

CONCLUSION

In review, 34 samples of human dental pulp tissue were examined for the existence of lymphatic vasculature using immunohistochemical markers. After preliminary H-E staining to determine states of health or disease, the tissues were stained with a battery of monoclonal antibodies and one lectin. Those monoclonal antibodies, EN-4 and PAL-E, used in frozen section did not yield noteworthy discoveries. Staining with FVIII-RA and Type IV collagen was successful using Formalin-fixed, paraffin-embedded sections. These monoclonal antibodies reacted strongly with blood vasculature but did not stain lymphatics. In some instances, FVIII-RA produced a less intense stain than did Type IV collagen. The UEA-1 lectin intensely stained arterioles, venules, and capillaries. Lymphatic vessels achieved a very weak stain.

After staining normal, normal and calcific, and chronically inflamed human pulp tissue, the findings indicated an existence of lymphatic vessels solely in the inflamed tissues. No lymph vasculature was identified in healthy pulp tissue.

TABLE 1.

IMMUNOLOGIC PROFILE OF CONTROL TISSUES

Tissue	EN-4	PAL-E	FVIII-RA	Type IV Collagen	UEA-1	PAS
Tonsil						
arterioles	ND	NA	2	3	3	2
capillaries	ND	NA	2	2	3	0 - 1
venules	ND	NA	2	1	3	0 - 1
lymphatics	ND	NA	0 - 1	0	1	0
Placenta						
arterioles	0 - 1	0 - 1	NA	NA	NA	NA
capillaries	0 - 1	0 - 1	NA	NA	NA	NA
venules	0 - 1	0 - 1	NA	NA	NA	NA
lymphatics	ND	ND	NA	NA	NA	NA
Spleen						
arterioles	0 - 1	0 - 1	2 - 3	NA	NA	NA
capillaries	0 - 1	0 - 1	2	NA	NA	NA
venules	0 - 1	0 - 1	2	NA	NA	NA
lymphatics	ND	ND	ND	NA	NA	NA

ND - no vessel detected NA - no staining attempted

Rating scale 0 - 3 O - vessel not stained 3 - vessel intensely stained

TABLE 2.

IMMUNOLOGIC PROFILE OF HUMAN DENTAL PULP TISSUE

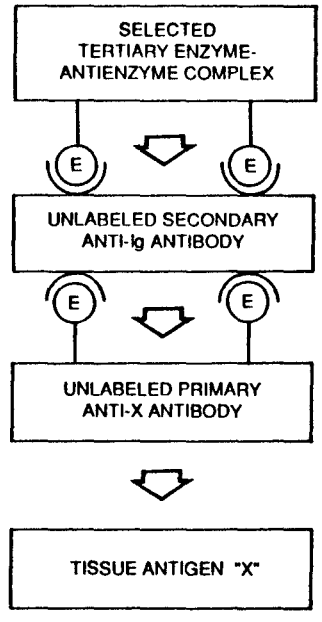
Tissue	EN-4	PAL-E	FVIII-RA	Type IV Collagen	UEA-1	PAS
Normal pulp						
arterioles	ND	0 - 1	2 - 3	3	3	3
capillaries	ND	0 - 1	2 - 3	3	3	1
venules	ND	0 - 1	2 - 3	3	3	0 - 1
lymphatics	ND	ND	ND	ND	ND	ND
Calcific pulp						
arterioles	ND	0 - 1	2	NA	3	2
capillaries	ND	0 - 1	2	NA	3	1
venules	ND	0 - 1	2	NA	3	0 - 1
lymphatics	ND	ND	ND	NA	ND	ND
Chronic pulpitis						
arterioles	ND	0 - 1	2 - 3	3	2 - 3	O
capillaries	ND	0 - 1	2 - 3	3	2 - 3	O
venules	ND	0 - 1	2 - 3	3	2 - 3	O
lymphatics	ND	ND	O	O	0 - 1	O

ND - no vessel detected NA - no staining attempted

Rating scale 0 - 3 O - vessel not stained 3 - vessel intensely stained

FIGURE 1

SCHEMATIC REPRESENTATION OF ALKALINE PHOSPHATASE ANTI-ALKALINE PHOSPHATASE STAINING TECHNIQUE

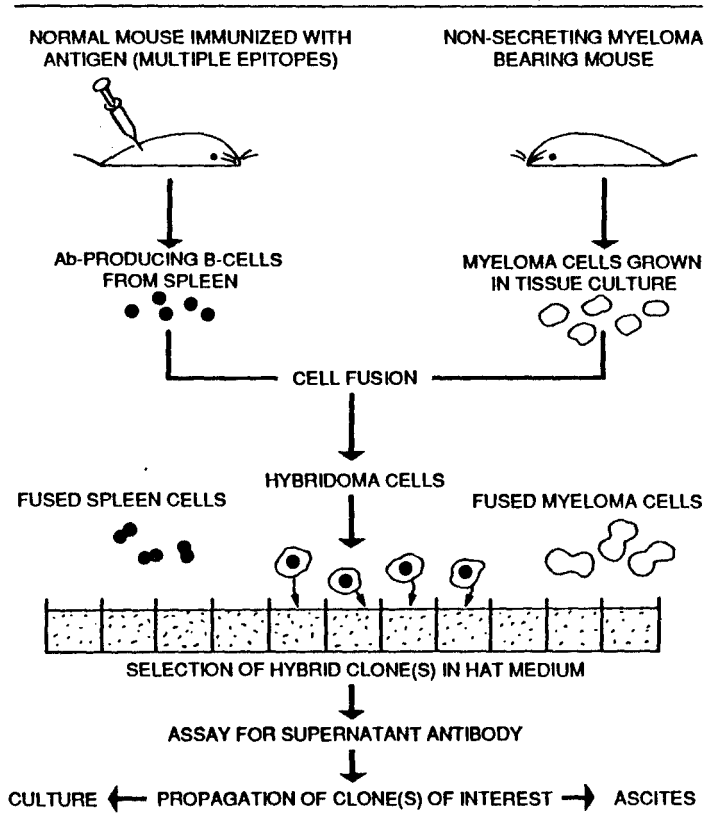


Unlabeled anti-X antibody is the chosen tissue specific monoclonal antibody with epitopes (E) for the unlabeled secondary antibody (link antibody). Accordingly, the alkaline phosphatase anti-alkaline phosphatase, termed the tertiary enzyme-antienzyme complex, has epitopes specific for the link antibody.

(from Immunohistopathology: A practical approach to diagnosis, ASCP, Chicago, 1990).

FIGURE 2

TECHNIQUE EMPLOYED IN GENERATION OF MONOCLONAL ANTIBODIES

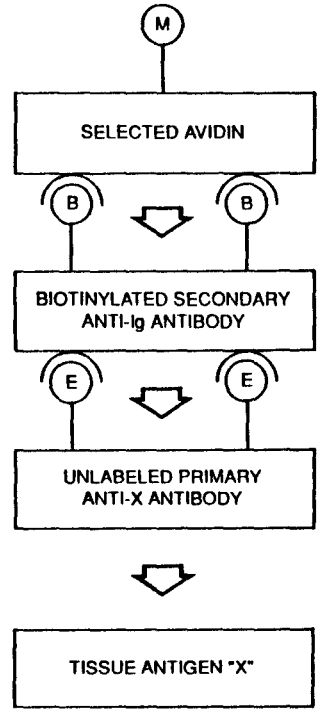


HAT (hypoxanthine, aminopterin, and thymidine) medium promotes growth of hybrid cells only. The fused spleen and myeloma cells do not survive. The collected hybridoma cells are analyzed for particular antibody protection

(from Immunohistopathology: A practical approach to diagnosis, ASCP, Chicago, 1990).

FIGURE 3

SCHEMATIC REPRESENTATION OF AVIDIN-BIOTIN-PEROXIDASE STAINING TECHNIQUE

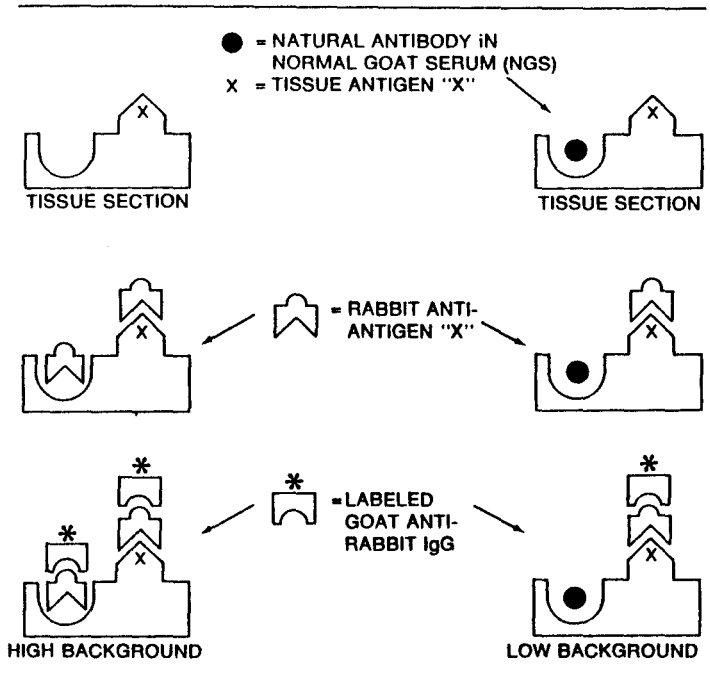


Unlabeled anti-X antibody is the chosen tissue specific monoclonal antibody with epitopes (E) for the biotinylated secondary antibody. The streptavidin, with a peroxidase marker (M), binds to the biotinylated antibody.

(from Immunohistopathology: A practical approach to diagnosis, ASCP, Chicago, 1990).

FIGURE 4

USE OF BLOCKING AGENT TO REDUCE BACKGROUND STAINING



Normal goat serum is used to block possible cross-reactive sites.

(from Immunohistopathology: A practical approach to diagnosis, ASCP, Chicago, 1990).

FIGURE 5

H-E STAIN OF CHRONIC PULPITIS WITH EOSINOPHILS (E), LYMPHOCYTES (L), PLASMA CELLS (P) IDENTIFIED.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 250X.

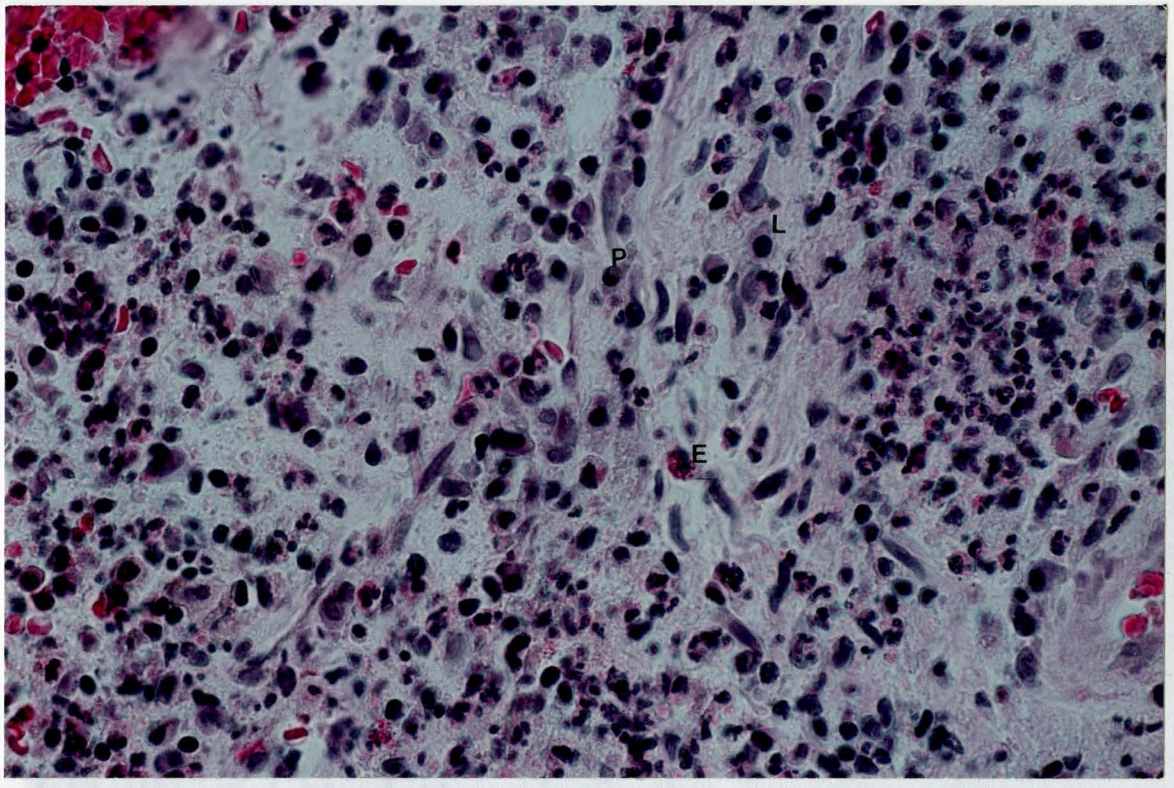


FIGURE 6

H-E STAIN OF CHRONIC PULPITIS WITH LYMPHOCYTES (L), PLASMA CELLS (P), POLYMORPHONUCLEAR LEUKOCYTES (N), FIBRIN DEPOSITS (F), AND ENGORGED BLOOD VESSELS (V).

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 250X.

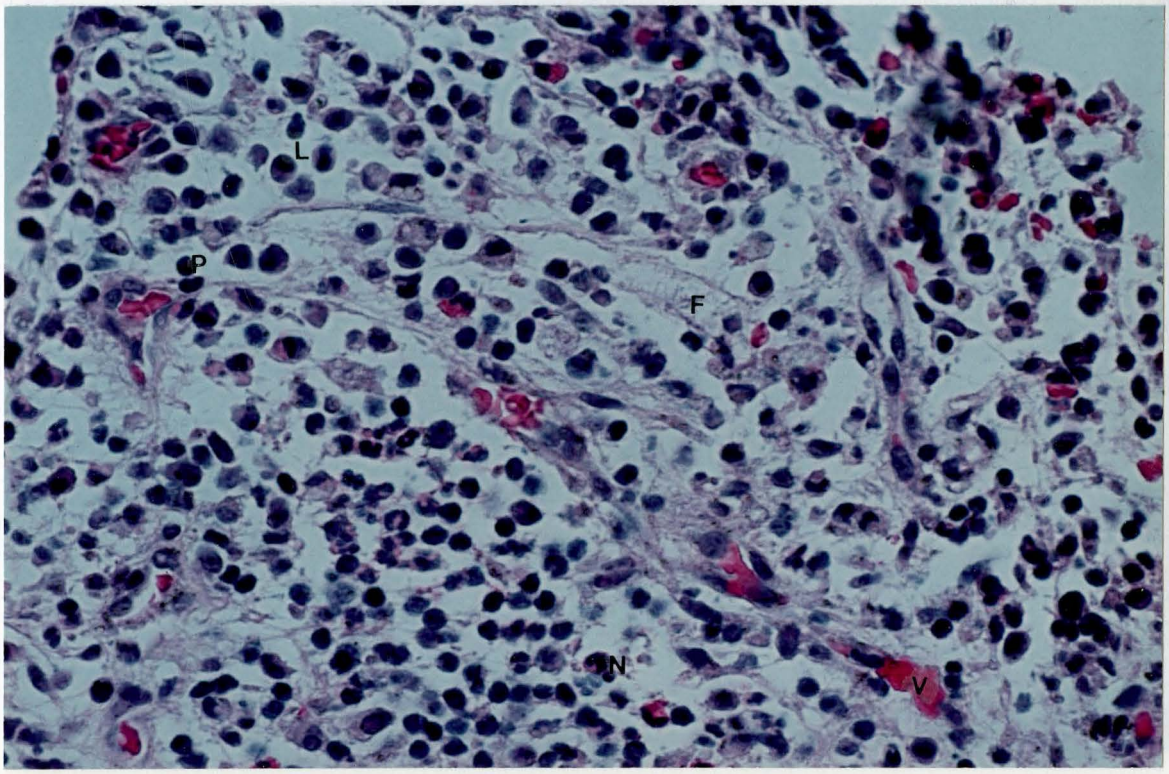


FIGURE 7

H-E STAIN SHOWING ZONES OF HEALTHY (H) AND CHRONICALLY INFLAMED (I) TISSUE. AREAS OF ABSCESS FORMATION (A), CALCIFICATION (C), ENGORGED BLOOD VESSELS (V), AND NERVE TISSUE (N) ARE SEEN.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 125X.

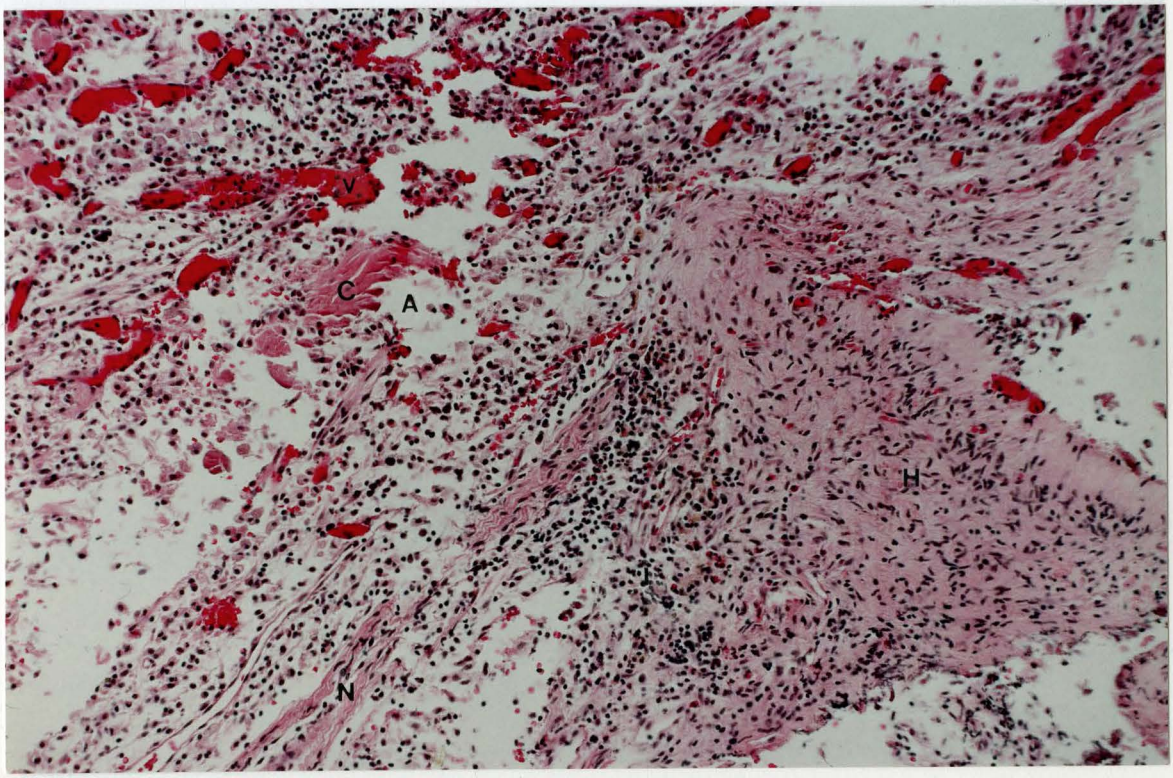


FIGURE 8

H-E STAINED SECTION WITH SIGNS OF CHRONICALLY INFLAMED AND HEALTHY PULP TISSUE. NERVE TISSUE (N), ENGORGED BLOOD VESSELS (V), AND VASCULATURE RESEMBLING LYMPHATICS (L) ARE SEEN.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 125X.

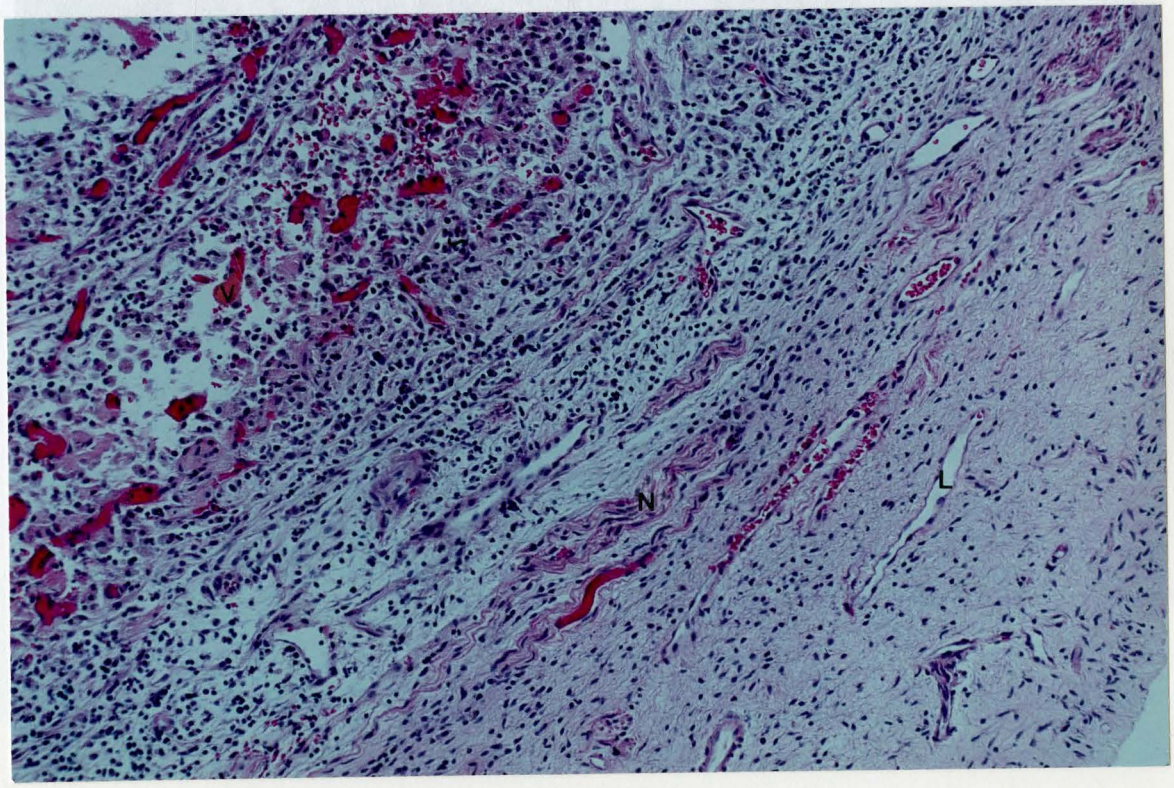


FIGURE 9

H-E STAIN OF DEGENERATING CHRONIC PULPITIS. ACCUMULATION OF INFLAMMATORY CELLS (I) AND BREAKDOWN OF TISSUE (B) ARE NOTED.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 125X.

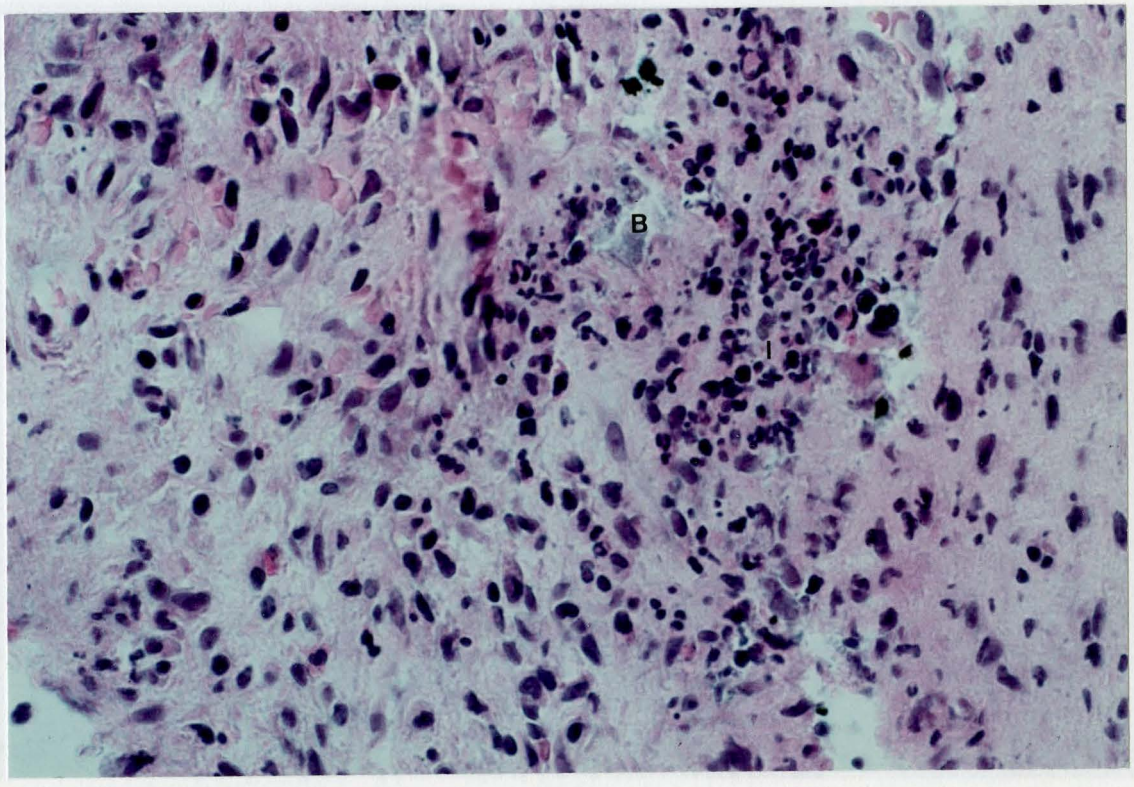


FIGURE 10

H-E STAIN OF CHRONICALLY INFLAMED PULP TISSUE EXHIBITING AREAS OF FIBROSIS AND CALCIFICATION.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 40X.

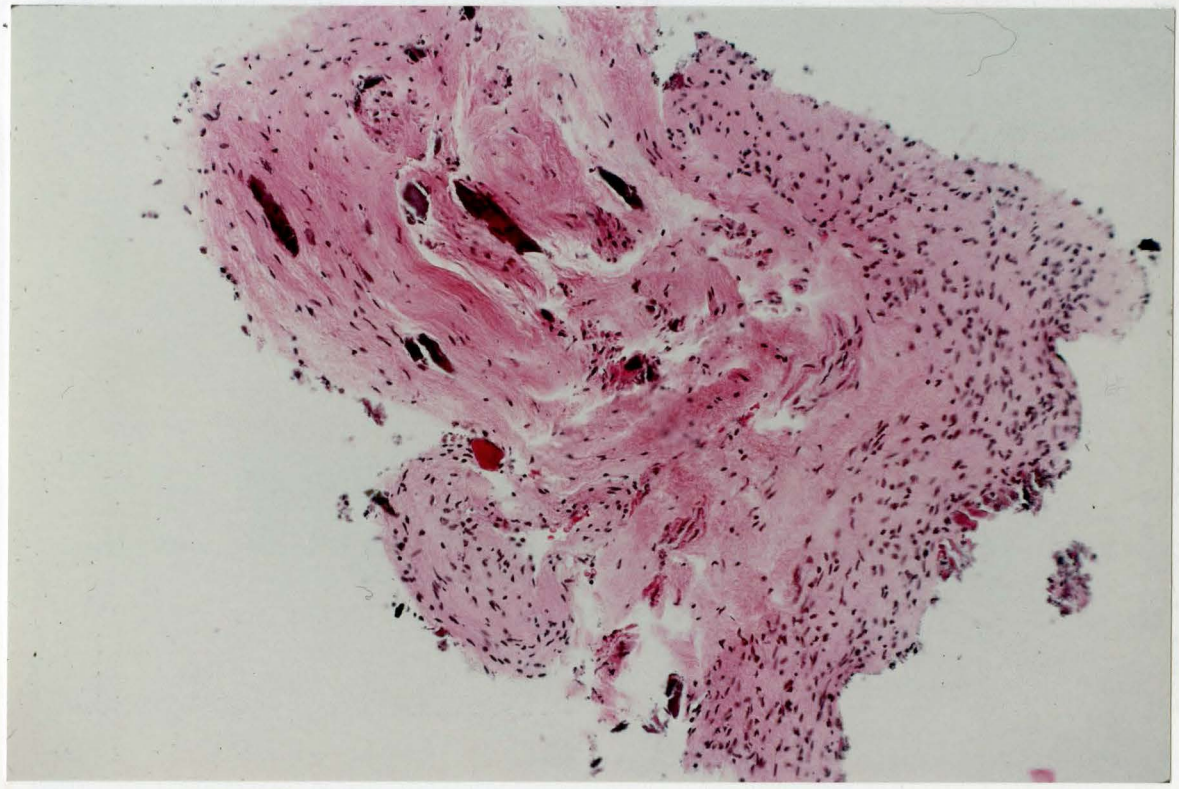


FIGURE 11

H-E STAIN OF NORMAL PULP TISSUE.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 125X.

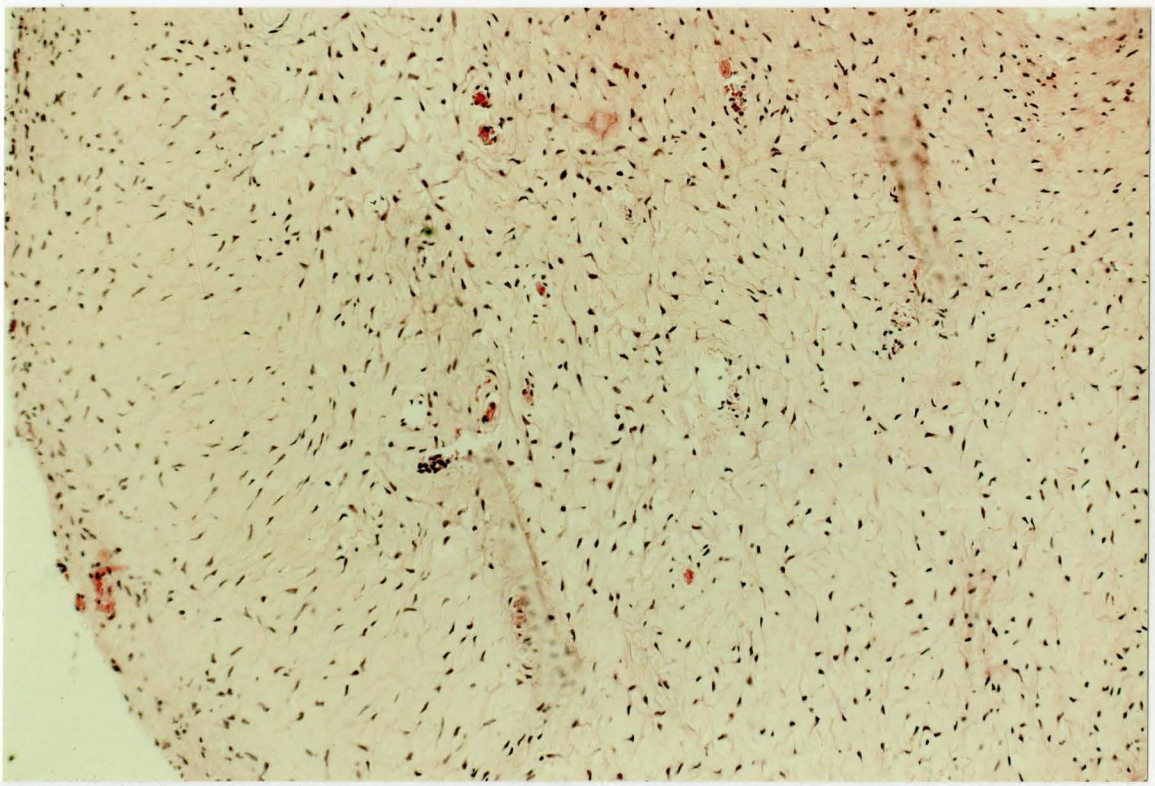


FIGURE 12

H-E STAIN SHOWING THE ZONES EXHIBITED IN NORMAL PULP TISSUE. ODONTOBLASTS (O), CELL-FREE ZONE (F), CELL-RICH ZONE (R). AREAS OF VACULONS DEGENERATION ARE ALSO SEEN (D).

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 40X.

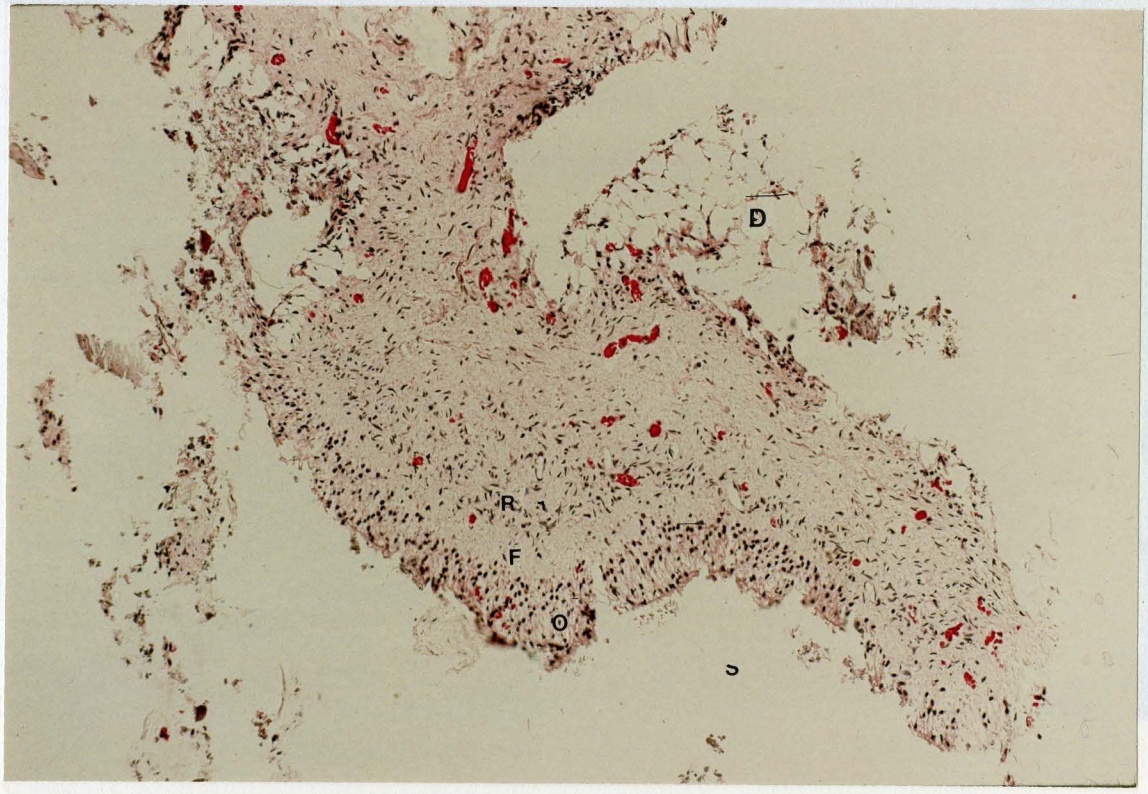


FIGURE 13

CHRONICALLY INFLAMED PULP TISSUE EXHIBITING TERMINAL ARTERIOLE (T), VENULE(V) AND CAPILLARY (C).

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 125X.

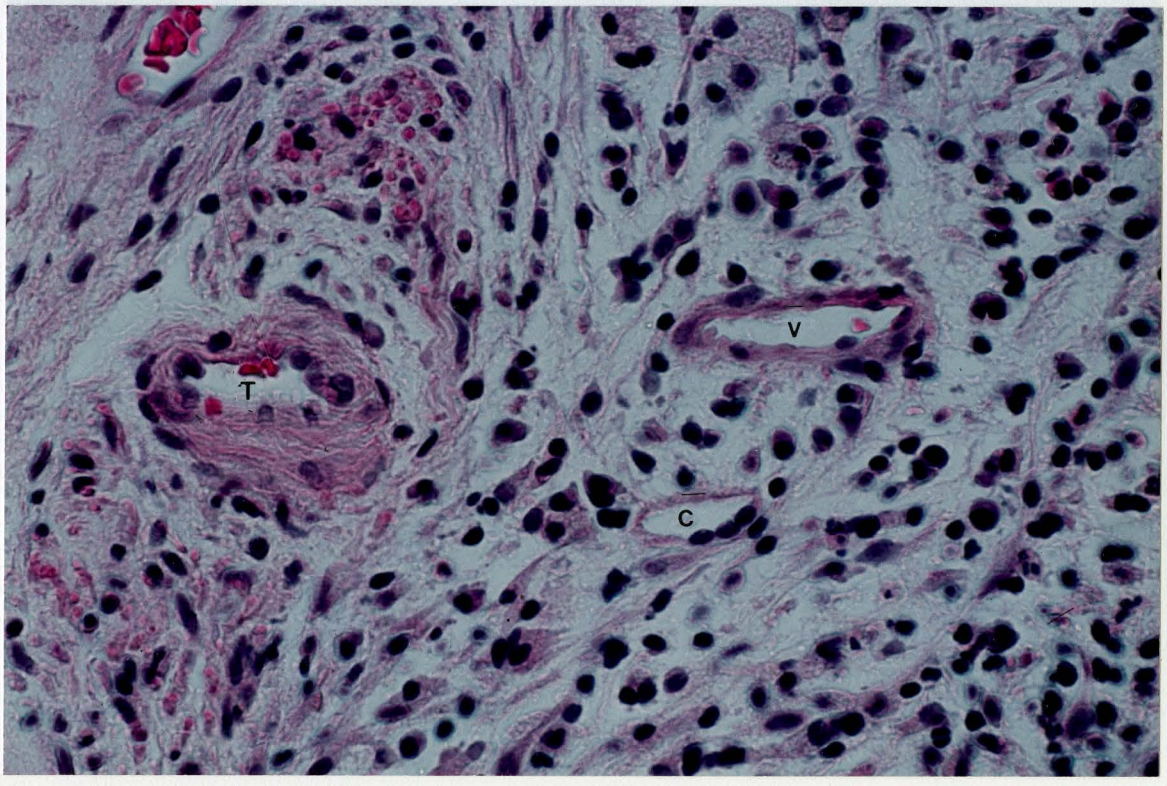


FIGURE 14

PULP TISSUE BLOOD VESSELS (V) STAINING POSITIVELY FOR UEA-1.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 250X.

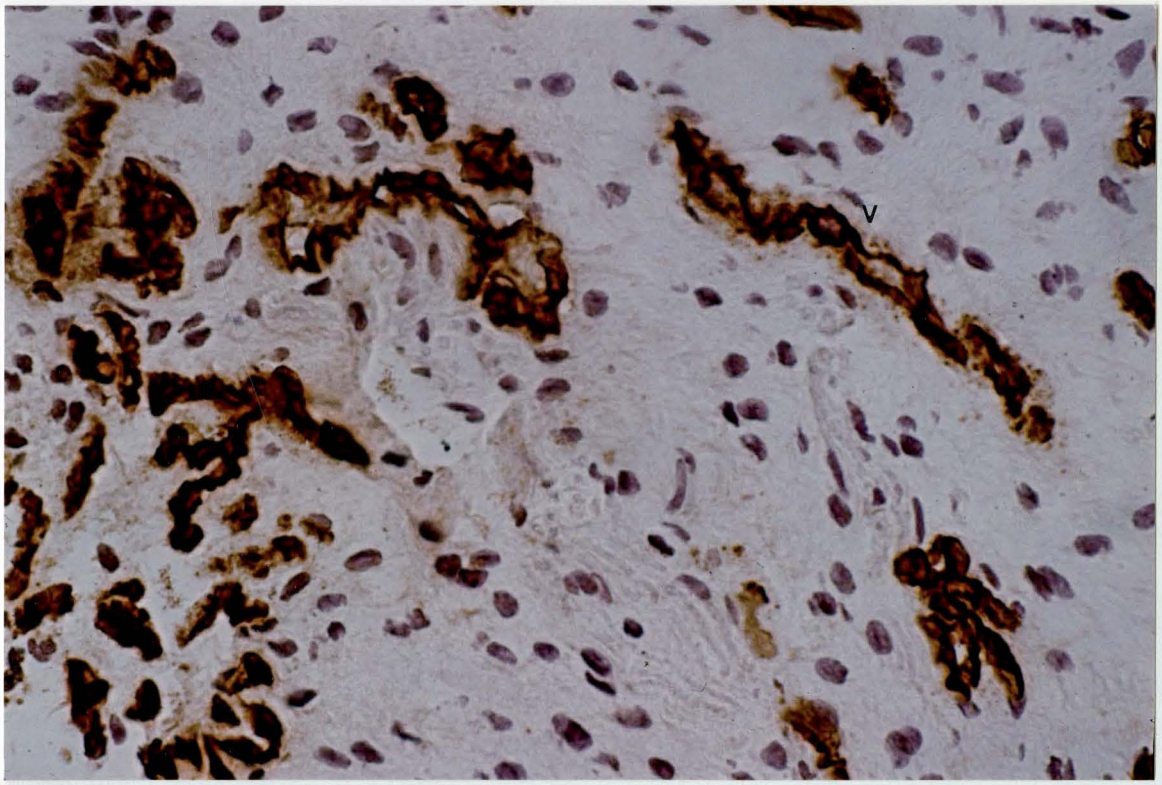


FIGURE 15

UEA-1 STAIN OF CHRONIC PULPITIS SHOWING REACTIVE BLOOD VESSELS (V) AND NONSTAINING LYMPHATICS (L).

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 125X.

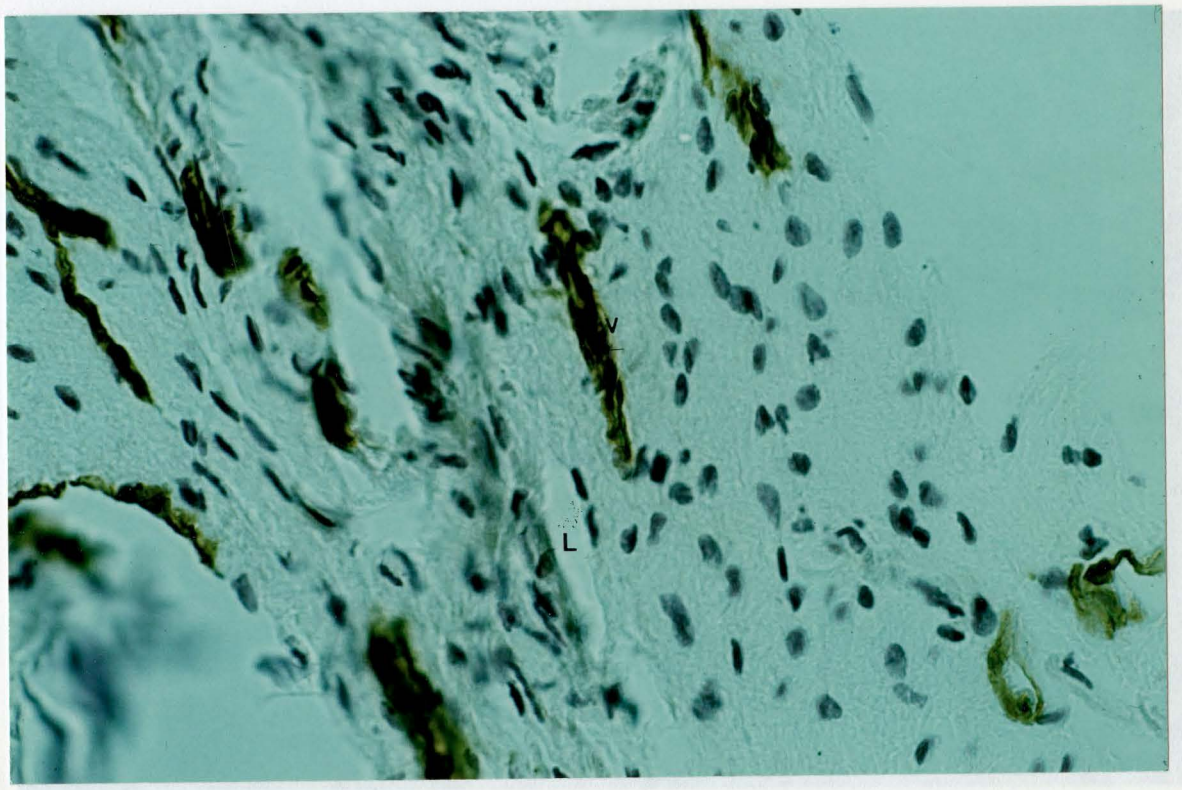


FIGURE 16

CHRONIC PULPITIS EXHIBITING NONREACTIVE LYMPHATIC (L) VESSEL WITH FVIII-RA. BLOOD VESSEL (V) SHOWS POSITIVE REACTION.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 250X.

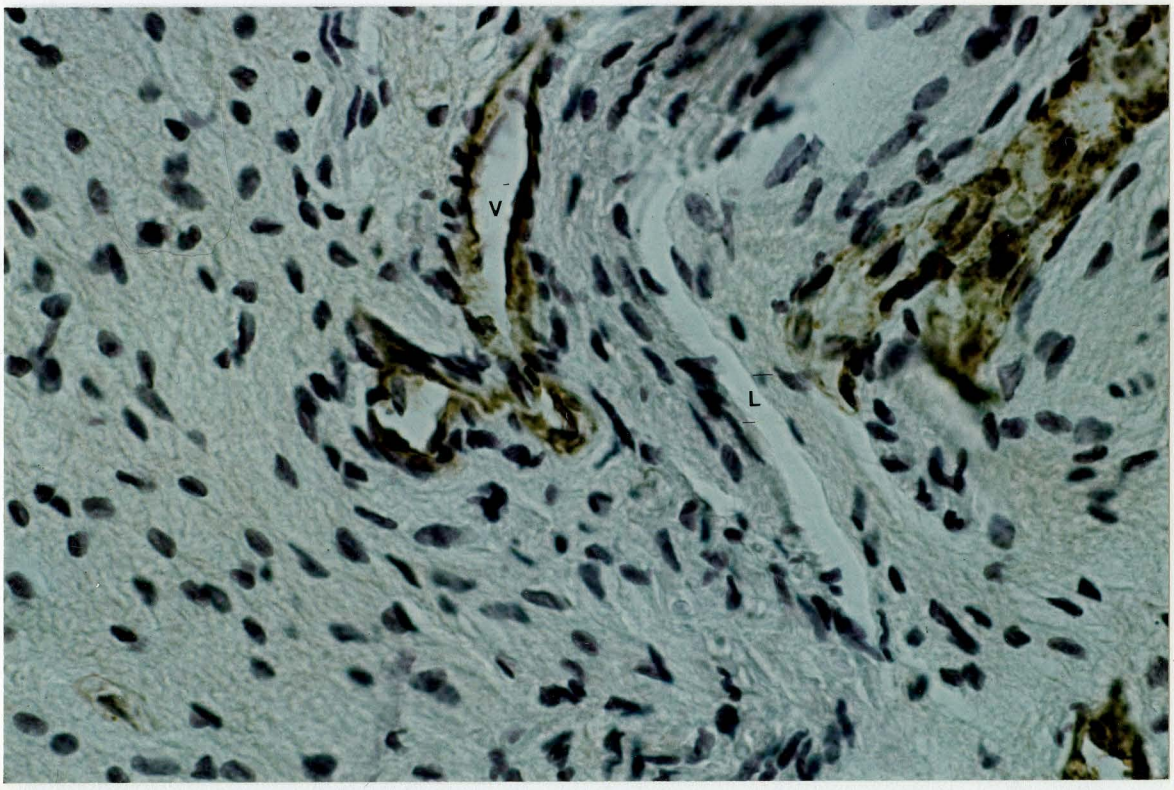


FIGURE 17

SECTION OF CHRONICALLY INFLAMED PULP TISSUE STAINED WITH FVIII-RA. BLOOD VESSELS REACT POSITIVELY WITH NONREACTIVE LYMPH (L) VESSELS.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 250X.

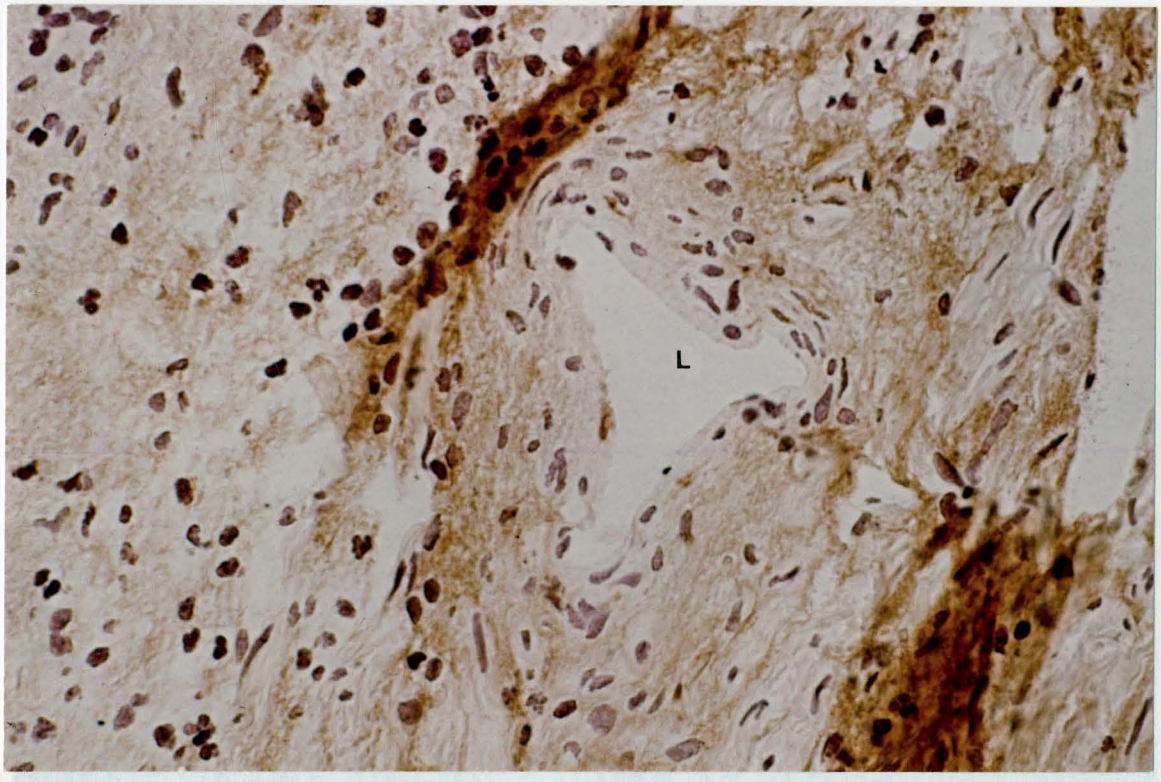


FIGURE 18

CHRONICALLY INFLAMED PULP TISSUE STAINED WITH FVIII-RA. BLOOD VESSELS (V) ARE POSITIVE. NONSTAINING VASCULATURE IS SUGGESTIVE OF LYMPHATICS (L). METHYLENE GREEN COUNTERSTAIN.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 125X.

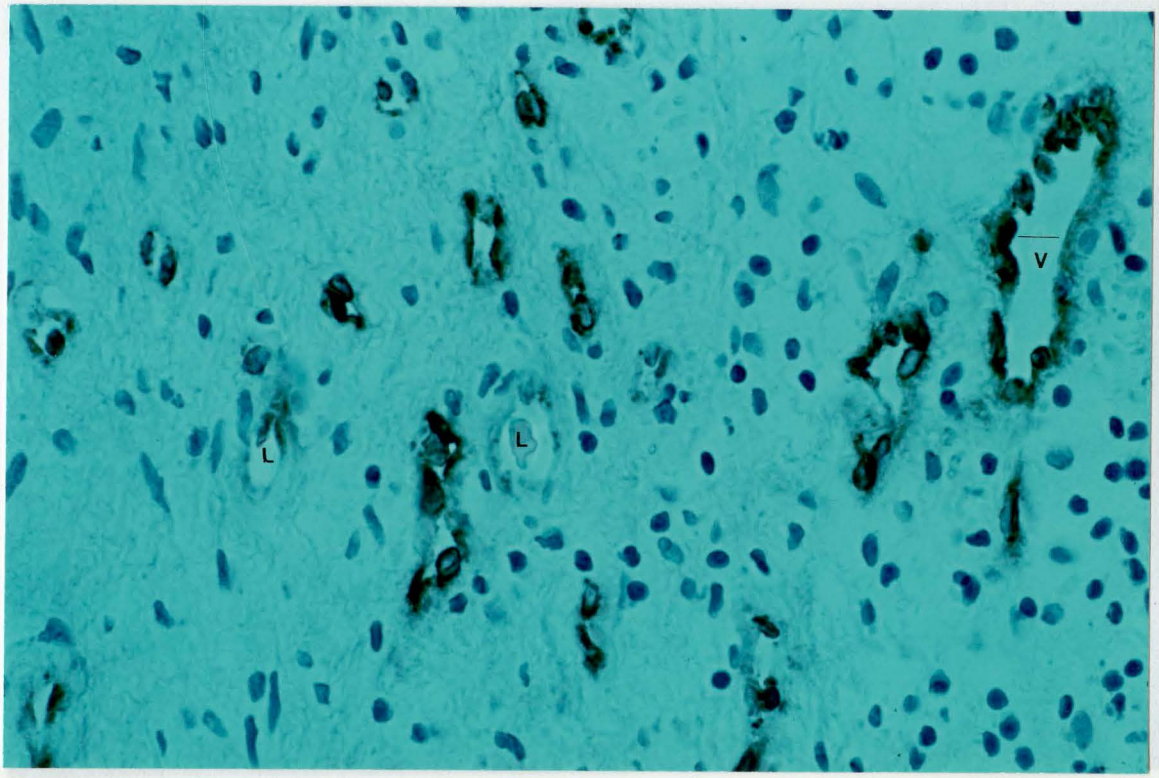


FIGURE 19

COMBINATION TYPE IV COLLAGEN AND FVIII-RA STAIN SHOWING NONREACTIVE LYMPHATIC VESSEL (L) IN CHRONICALLY INFLAMED TISSUE. POSITIVELY STAINING BLOOD VESSELS (V). CELL NUCLEI ARE STAINED GREEN.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 250X.

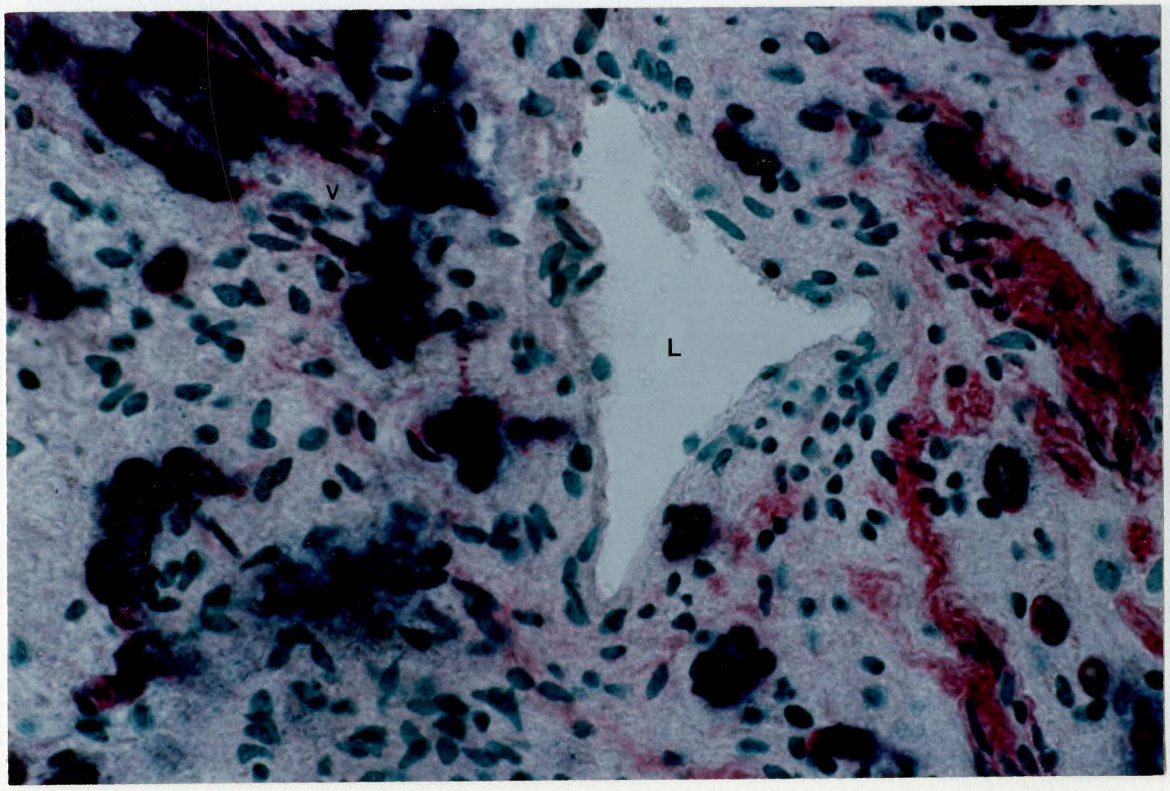


FIGURE 20

TERMINAL ARTERIOLE (T) REACTING POSITIVELY WITH UEA-1 AND PAS STAINING. DEGENERATING TISSUE (D) DOES NOT STAIN AND MAY POSSIBLY BE LYMPHATIC. NERVE TISSUE (N).

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 250X.

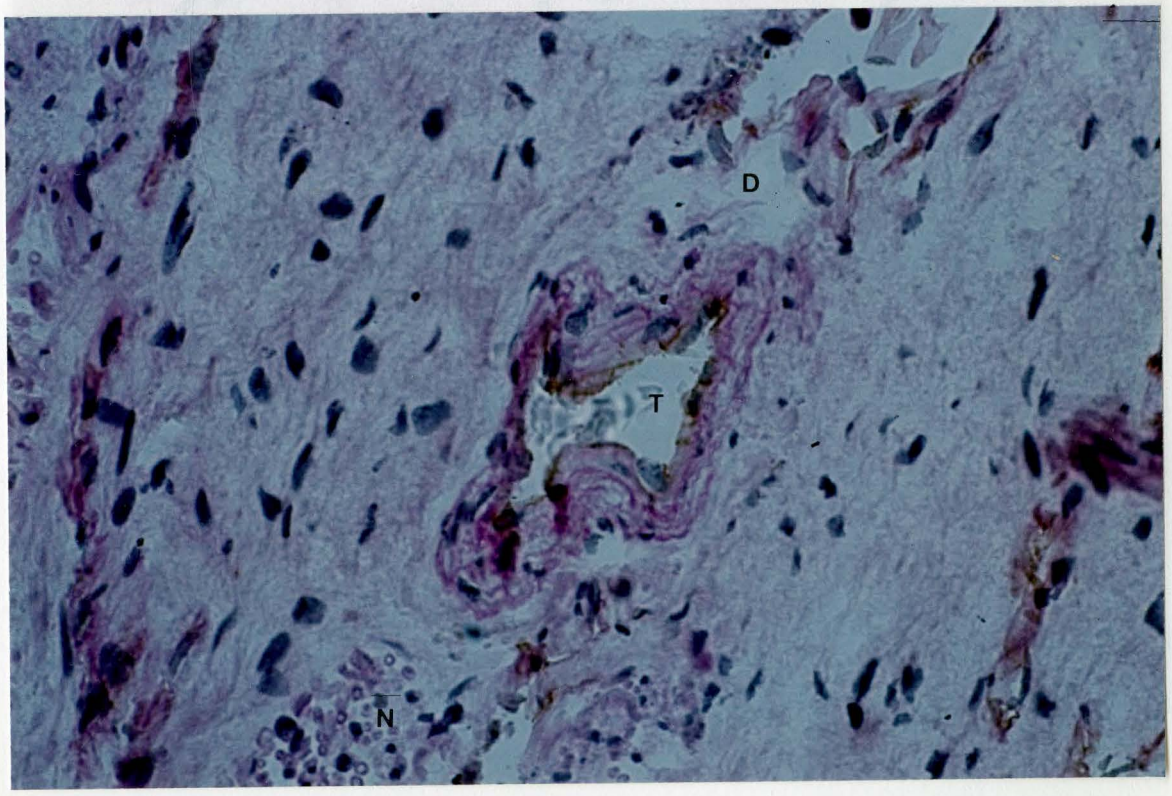


FIGURE 21

DISCRETE PULP TISSUE CALCIFICATION (C) EXHIBITED WITH COMBINATION PAS AND UEA-1 STAIN. POSITIVELY STAINING BLOOD VESSEL (V) IS ADJACENT.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 250X.

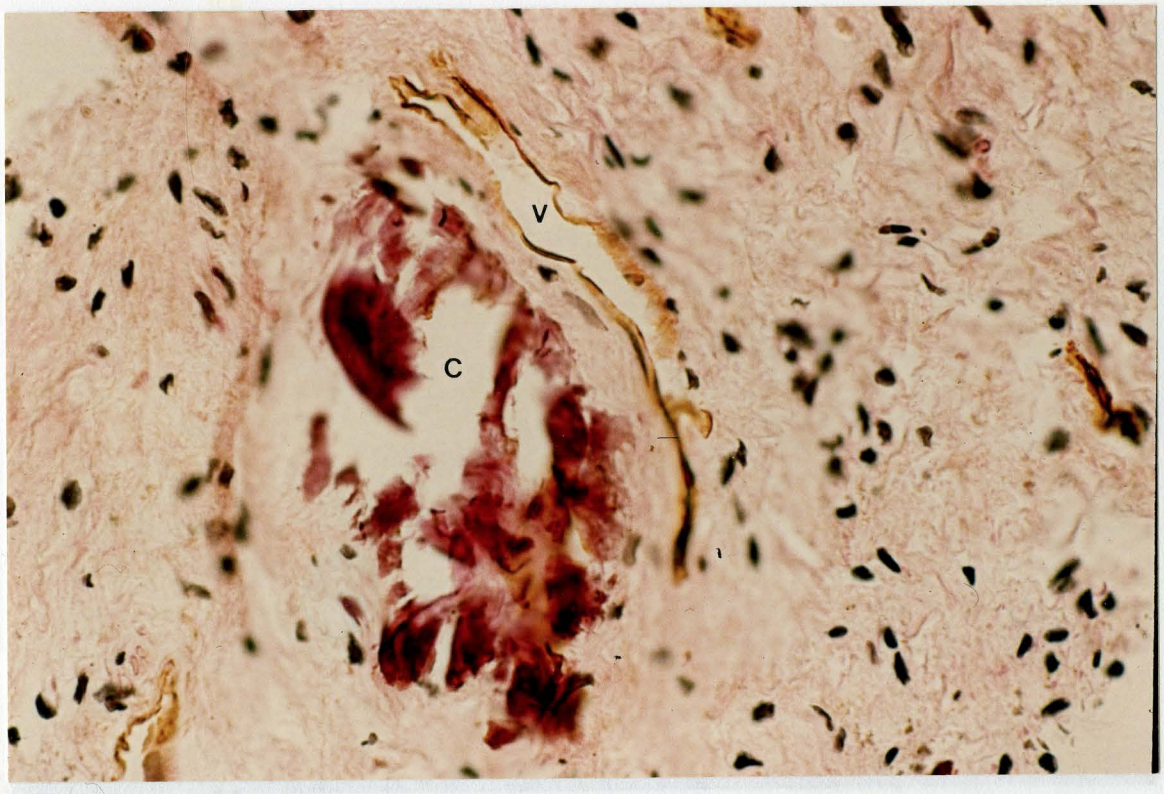


FIGURE 22

UEA-1 AND PAS STAIN OF NORMAL PULP TISSUE. NONSTAINING AREA AT BOTTOM LEFT IS A VACUOLE.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 125X.

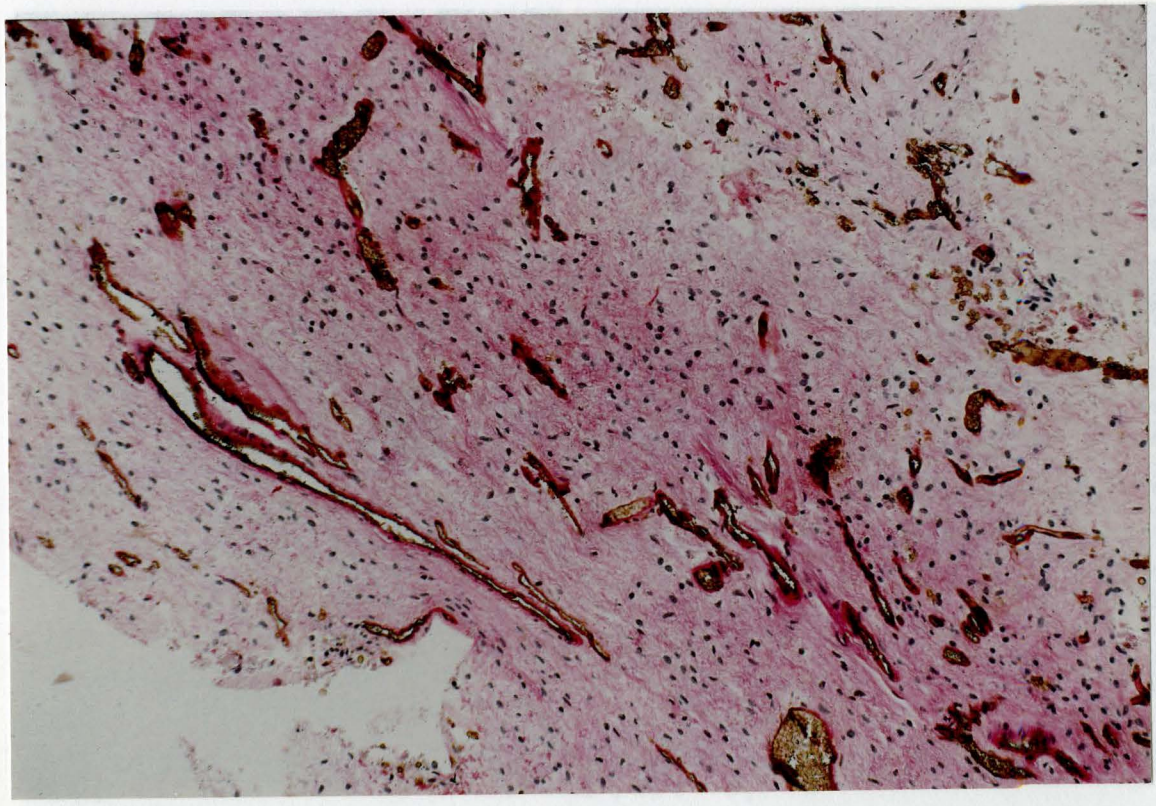


FIGURE 23

UEA-1 STAINING OF TONSIL TISSUE. LYMPHATICS (L) STAIN WEAKLY WITH ALL SIZES OF BLOOD VESSELS (V) REACTING POSITIVELY.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 250X.

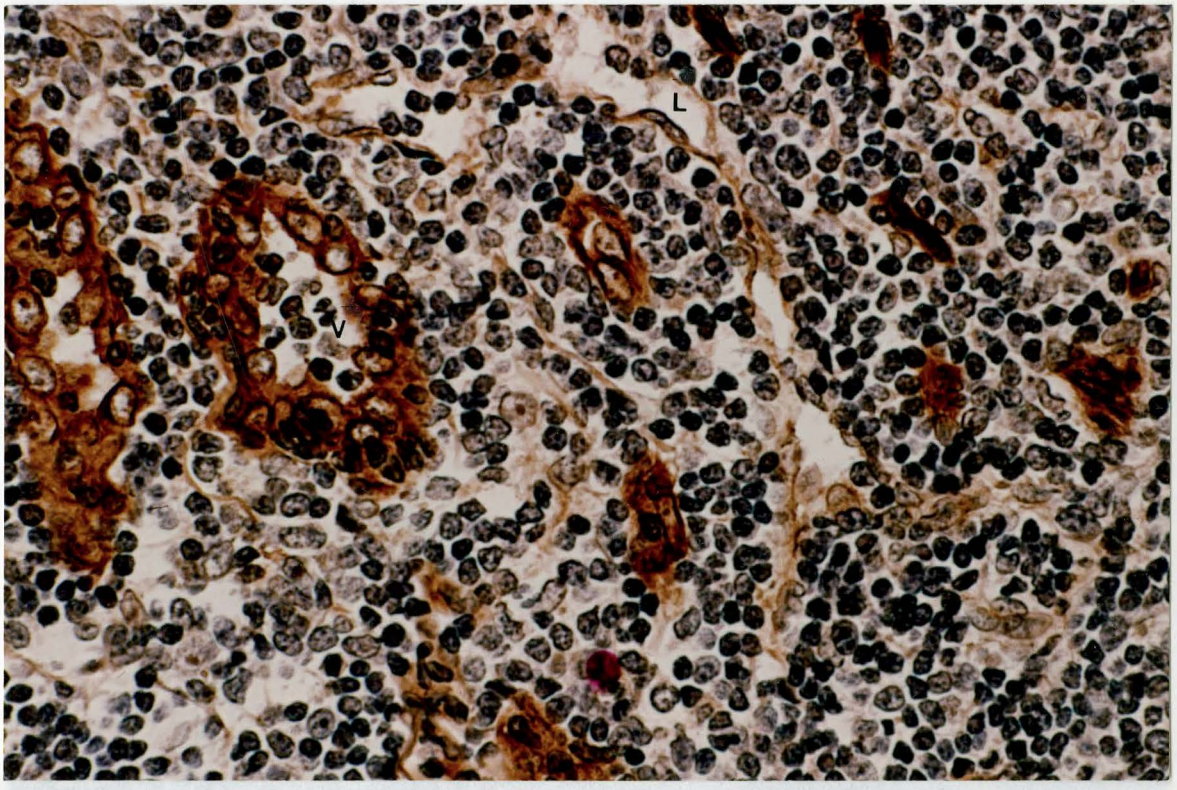


FIGURE 24

UEA-1 AND PAS STAIN OF TONSIL TISSUE. TERMINAL ARTERIOLES (T) REACT STRONGLY WITH BOTH STAINS. THE PAS STAIN LOSES INTENSITY AS VESSEL CALIBER DECREASES. LYMPHATICS (L) ARE STAINED WEAKLY WITH UEA-1. PAS DOES NOT REACT WITH LYMPH VESSELS.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 125X.

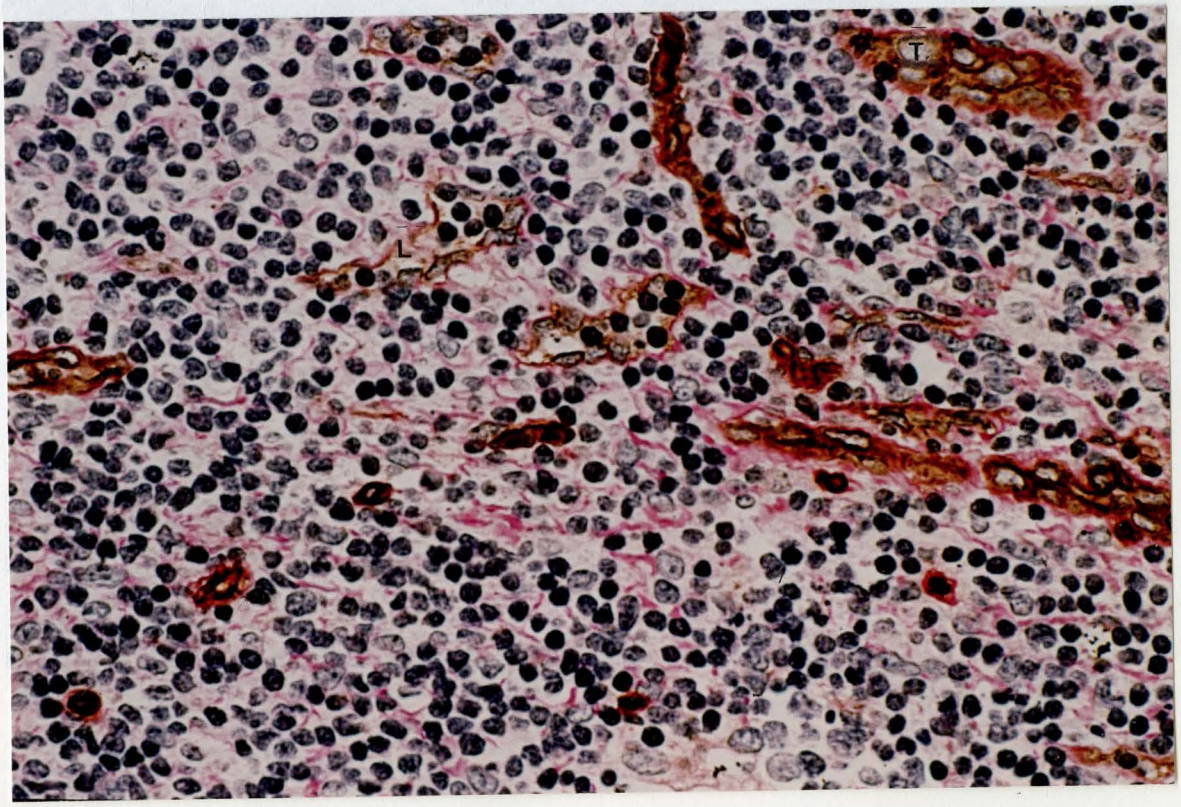


FIGURE 25

TYPE IV COLLAGEN STAIN OF TONSIL TISSUE. BLOOD VESSELS (V) SHOW POSITIVE REACTIVITY. LYMPHATICS (L) APPEAR NONREACTIVE.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 125X.

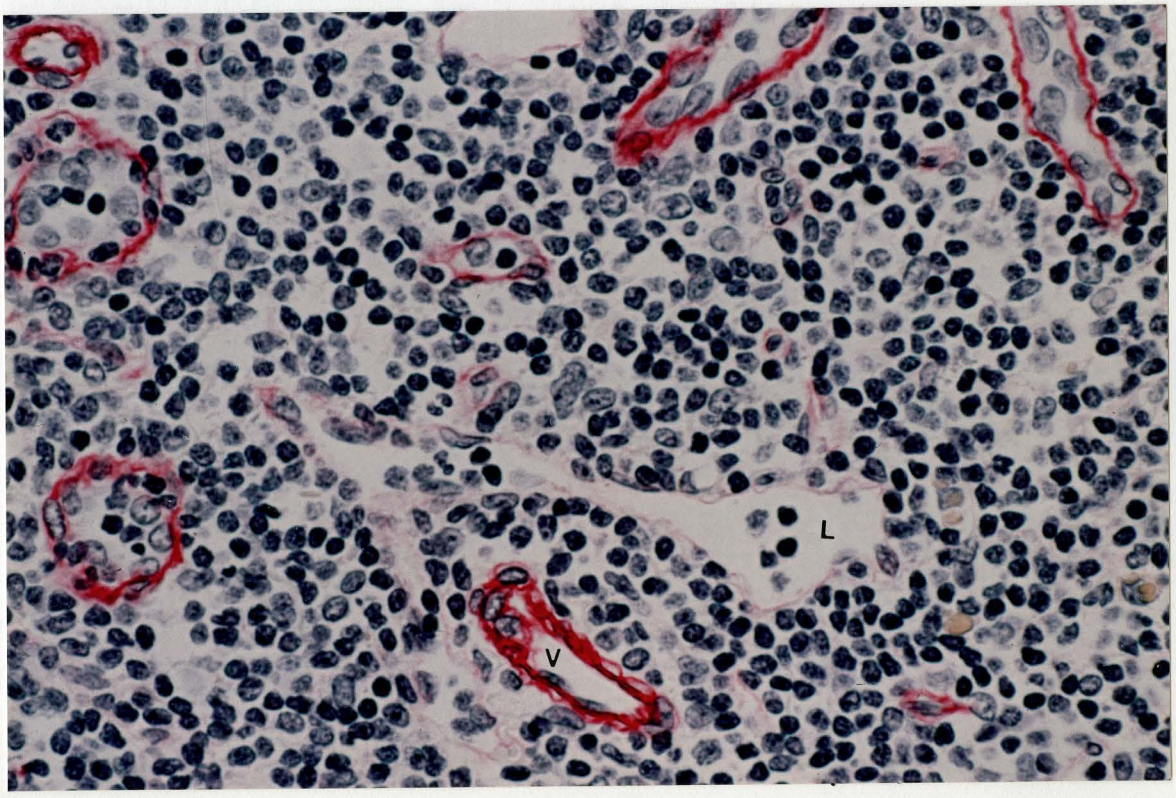


FIGURE 26

PLACENTAL TISSUE STAINED WITH TYPE IV COLLAGEN. LYMPH CHANNELS (L) DO NOT STAIN. BLOOD VESSELS (V) ARE REACTIVE.

FORMALIN-FIXED, PARAFFIN-EMBEDDED, 125X.

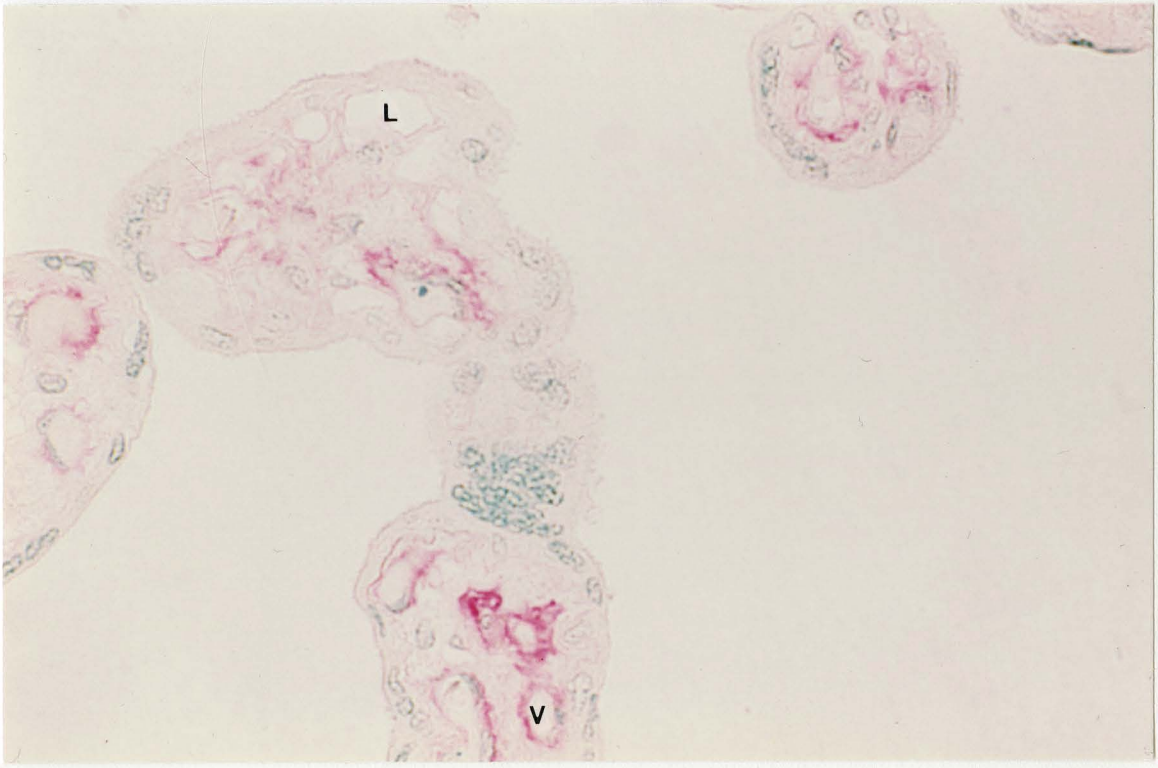


FIGURE 27

NORMAL PULP TISSUE STAINED WITH FVIII-RA. BLOOD VESSELS (V) ARE STAINED.

FRESH FROZEN TISSUE, 125X.

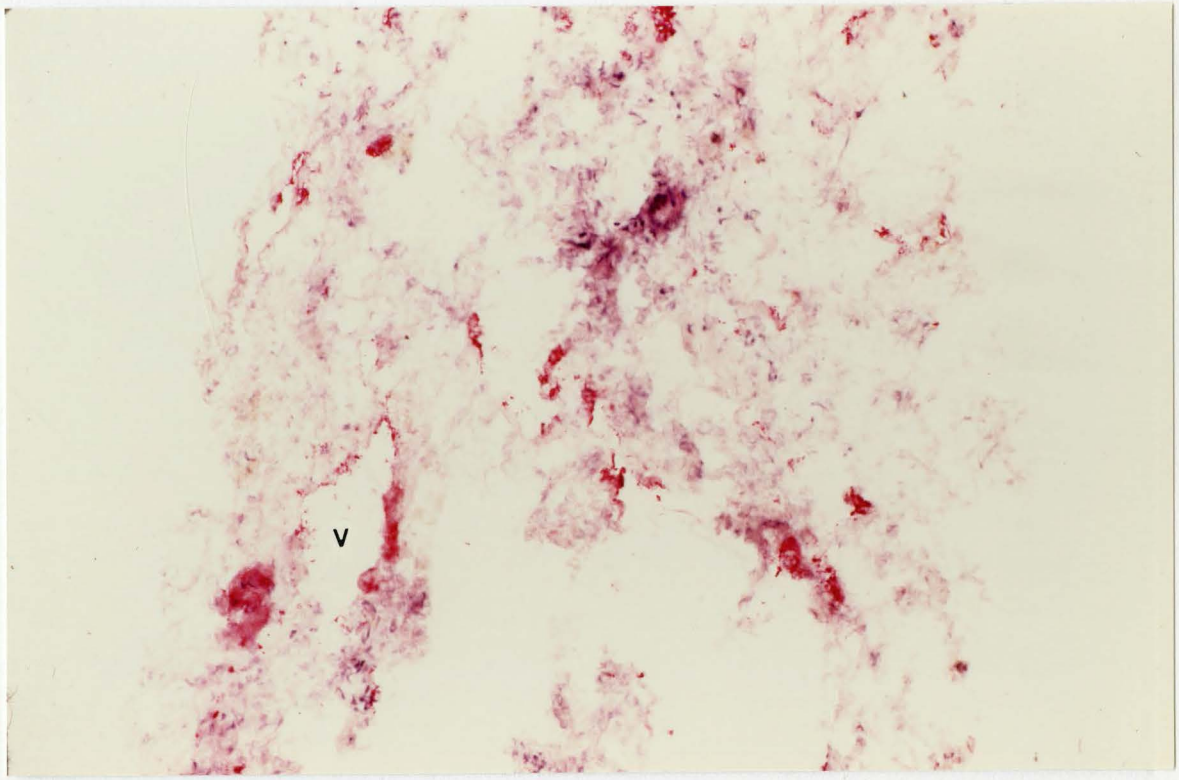


FIGURE 28

PAL-E STAIN OF NORMAL PULP TISSUE WITH POSITIVELY REACTIVE BLOOD VESSELS (V).

FRESH FROZEN TISSUE, 125X.

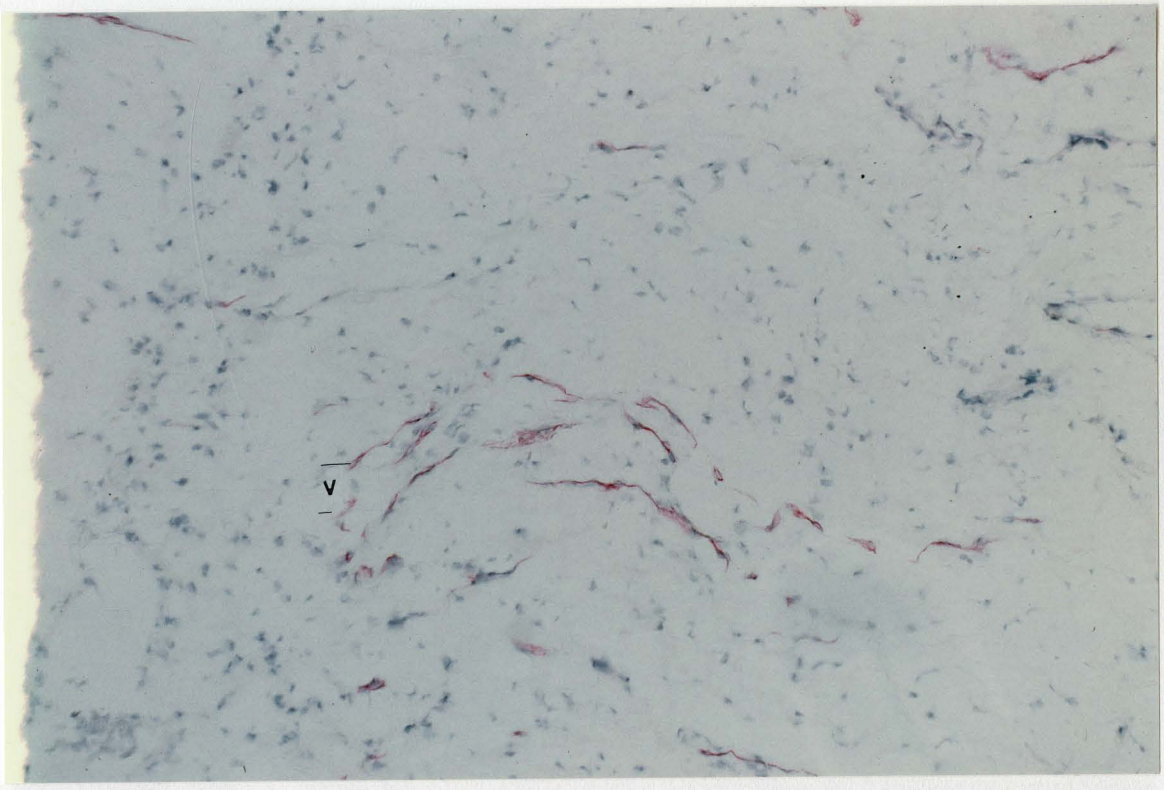


FIGURE 29

FVIII-RA STAIN OF SPLEEN TISSUE WITH TERMINAL ARTERIOLE (T) POSITIVELY REACTIVE.

FRESH FROZEN TISSUE, 125X.

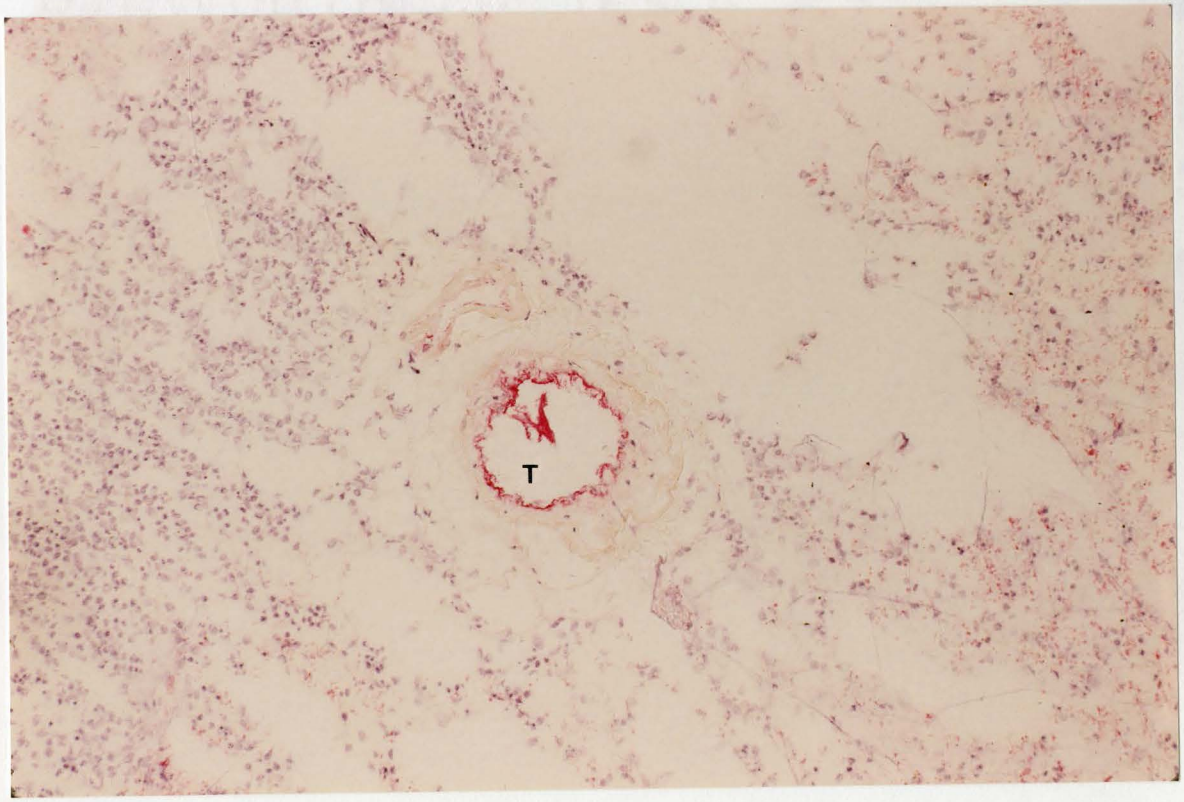


FIGURE 30

SPLEEN TISSUE STAINED WITH EN-4. BLOOD VESSELS (V) REACT POSITIVELY.

FRESH FROZEN TISSUE, 125X.

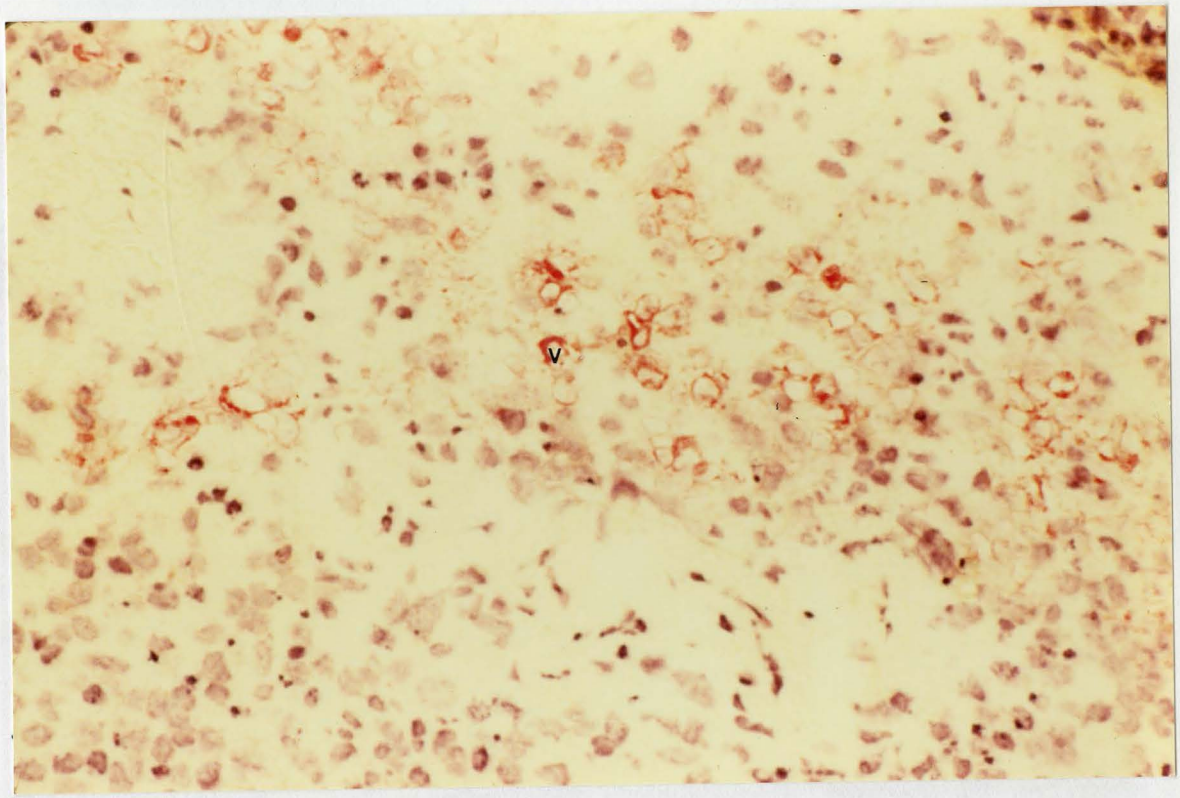
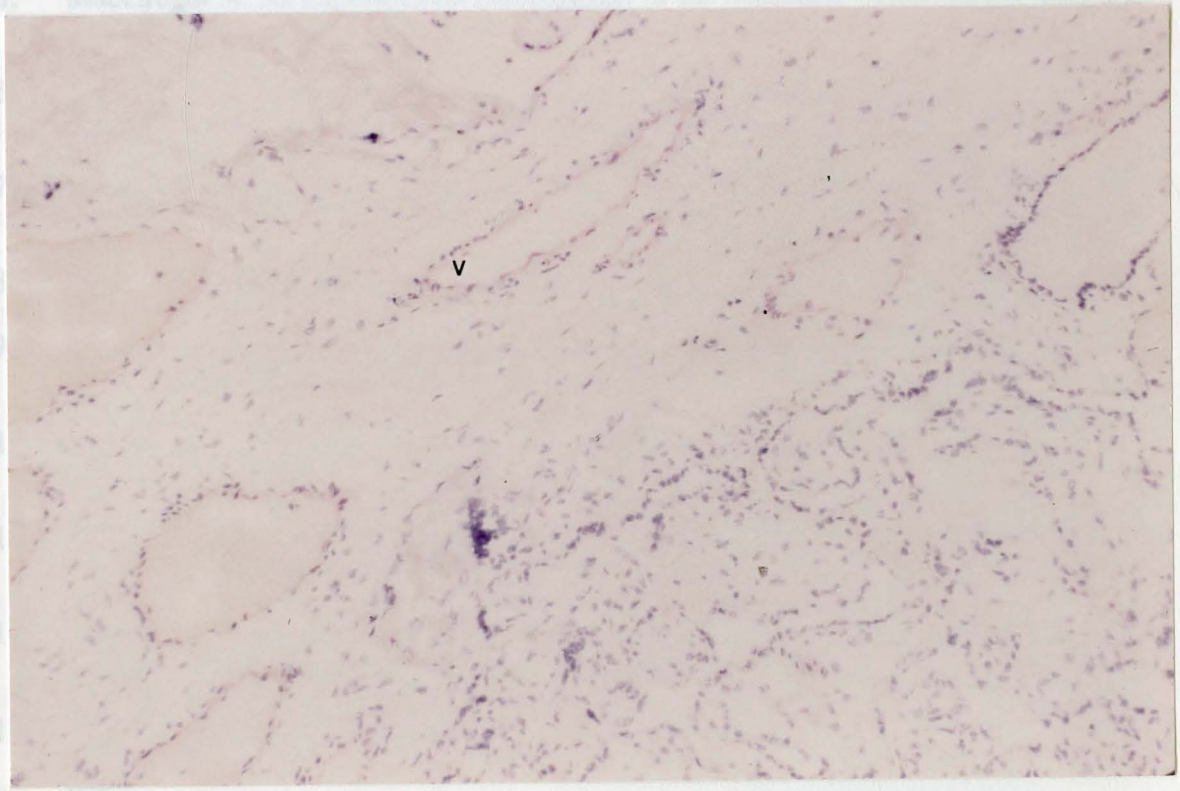


FIGURE 31

PAL-E STAIN OF PLACENTAL TISSUE SHOWING POSITIVE REACTION WITH BLOOD VESSELS (V).

FRESH FROZEN TISSUE, 125X.



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The Masters Thesis submitted by Dr. Beth Shenden has been read and approved by the following committee:

Dr. Hasan Nadimi, D.M.D., M.S., Director
Assistant Professor, Pathology
Loyola University College of Dentistry

Dr. Franklin Weine, D.D.S., M.S.D., Professor and Director
Post Graduate Endodontics
Loyola University College of Dentistry

Dr. Joseph Gowgiel, D.D.S., PhD.
Associate Professor and Chairman Dental Anatomy
Loyola University College of Dentistry

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

The Masters Thesis is, therefore, accepted in partial fulfillment of the requirements for the degree of Master of Science

6.25.93
Date

Hasan Nadimi
Director's Signature