A HISTOLOGIC DEMONSTRATION OF

BACTERIA

IN DENTAL GRANULOMAS

by

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INTRODUCTION

Should a non-vital tooth with radiographic evidence of apical pathology be subjected to dental treatment? The answer to this question is not agreed upon by all dentists. If there are no obvious symptoms, a goodly number of dentists will dismiss their patients with a word of caution to return if any trouble develops. Others insist upon immediate treatment of any tooth exhibiting these findings. The proper treatment depends, of course, upon the possibility of acute flare-ups in areas of previous chronic inflammation. As Kronfeld (5) states, "By far the largest number of acute dento-alveolar abscesses develop as an acute flare-up of a chronic periapical inflammation of long standing."

Another aspect of treatment is related to the possibility of focal infection. One hundred and fifty-four years after the cause and effect relationship between tooth removal and amelioration of systemic disease was first proposed (32), the problem of focal infection is still not solved to everyone's satisfaction. The dental granuloma, which most investigators readily agree represents a bodily defense mechanism, could be implicated in focal infection if it is shown that it may be septic in nature. For many years, research along this line has been directed at clarifying this point, but considerable criticism has been leveled at the procedures used and the conclusions drawn from them by the various investigators.

If granulomas can be shown to be infected in a significant number of cases, the proper treatment should be evident. This could be particularly decisive for the minority of dentists who do not believe (or are unaware of the fact) that chronic periapical inflammatory reactions can give rise to acute flare-ups (5). That this occurs is demonstrated by the fact that many acute periapical abscesses reveal radiolucencies at the time that they are noted. The bone destruction in these cases is a slow process and has occurred before the acute reaction due to the long-standing presence of a chronic inflammatory reaction, such as a dental granuloma. Also, the fact that the bulk of these acute flare-ups is due to the action of bacteria is shown by the fact that antibiotics, in almost all instances, cause the acute reactions to rapidly subside before therapy is instituted. These unpredictable, acute flare-ups result in pain, swelling, fever and other possible complications, such as osteomyelitis, cellulitis, bacteremia and maxillary sinusitis. Cavernous sinus thrombosis, brain abscess, and leptomeningitis and death have also been reported as arising from acute flare-ups of pre-existing dental granulomas (26), (34).

In 1809, Benjamin Rush (32) published a report that a woman suffering from chronic rheumatism was cured by the removal of a tooth. This was probably the first suggestion of a possible relationship between dental and systemic disease. Approximately one hundred years later, William Hunter, in an address at McGill University in Montreal, criticized the dental profession for producing oral sepsis by the improper placing of restorations in bacteriologically contaminated mouths. He listed a score of diseases which he thought were related to oral infection. Both

medical and dental practitioners seized upon this theory to cure their patients' diseases. Countless thousands of teeth were removed during the next quarter of a century. Gradually, however, the validity of the theory came under question in the face of relatively few permanent cures. A direct result of this dispute has been an increase in the number of publications on the subject.

In the last twelve years, over 400 articles on the subject of focal infection have appeared in the medical and dental literature, with a yearly high of 53 papers published in 1951. Even with this recent voluminous literature, it is surprising how few papers represent rigorous scientific investigation. Most of them consist of the authors' own views on the subject -- some more passionately written than others.

The theory of focal infection hinges upon the presence in the body of a circumscribed area of tissue which is infected with exogenous pathogenic microorganisms and which is usually located near a mucosal or cutaneous surface. This constitues a focus of infection. Focal infection, as such, refers to the metastasis or spread from the focus of infection by organisms (or their toxins or antigenic substances) to distant sites with resulting damage to affected tissues. There are, therefore, three generally-accepted mechanisms in the production of focal infection. The first actually occurs by the metastasis of organisms from an infected focus to distant areas by either lymphogenous or hematogenous spread. The second postulates the carrying of toxins or toxic products by way of the blood or lymph to other areas of the body where they may directly injure the tissues. The third is concerned with the possibility of distant or local antigen-antibody reactions on the part of specific

antibody-containing tissues which have been exposed previously to the bacterial antigens. A more recent variation implies that auto-immune reactions may be set off by the action of microorganisms on local tissue, thus producing auto-antigens, to which the body will respond by manufacturing auto-antibodies.

The four systemic diseases which most investigators believe can be related to focal infection are: rheumatic heart disease, subacute bacterial endocarditis, rheumatoid arthritis, and glomerulonephritis. Others may also be implicated, such as polyarteritis nodosa, disseminated lupus erythematosus, anaphylactoid purpura, iritis and various skin diseases. Several of these diseases have been directly or indirectly connected with a focus of infection consisting of group A hemolytic streptococci. In a recent investigation (25), material was obtained from 55 root canals, prior to root canal treatment, and cultured in tryptocase soy broth. Thirty-five percent of them revealed the presence of beta-hemolytic streptococci. Since bacteria in dental granulomas, in most instances, come from bacteria within an infected root canal, a dental granuloma could conceivably be a precursor to one of the above-mentioned diseases.

The serious nature of the majority of these diseases requires one to take a long, hard look at this problem. The three generally-accepted areas in the mouth which could serve as foci of infection are: (a) infected periapical lesions, such as the dental granuloma, (b) teeth with infected pulps (which eventually cause the production of a dental granuloma), and (c) periodontal disease with special reference

to periodontal pockets. In this thesis we shall be concerned only with the problem of the dental granuloma.

The essence of the problem is to prove whether bacteria or their products are actively present in granulomas. Therefore, the present study was instituted for the purpose of identifying the presence of bacteria, utilizing the histopathologic technique and the Brown and Brenn modification of the Gram stain (7). In addition, an attempt was made to learn as much as possible about the inflammatory reaction found in granulomas and possibly to correlate these findings with the presence of bacteria.

REVIEW OF THE LITERATURE

The typical dental granuloma consists of a fibrous capsule surrounding a more delicate fibrous tissue in which varying amounts of chronic and acute inflammatory cells are noted. The presence of neutrophiles is possibly due to a "burst" of bacteria, and other products, from the pulp canal which triggers an acute episode superimposed upon the previously quiescent, chronic, inflammatory situation. Considerable amounts of small blood vessels and young fibroblasts are present, as are tissue phagocytes, some of which have ingested lipids and are then known as "foam cells." Occasionally, cholesterol clefts are noted. Within this tissue, islands of epithelial cells are often present, which represent proliferating rests of Malassez (34).

Due to the presence of considerable numbers of small blood vessels, granulomas present an ideal focus of infection because of the ease with which either bacteria or their products can be introduced into the general circulation. On the other hand, these blood vessels also transport many of the body's defense materials and cells to the area of insult.

The problem of demonstrating the bacteriological status of a dental granuloma has been attacked in two basic ways: (a) by cultivation on artificial media of material obtained from dental granulomas, and (b) by microscopic examination of stained tissue sections from granulomas. The cultural techniques have been used most often in the past. Tissues for examination have been obtained in one of five ways:

(a) use of extracted teeth (6), (24), (18), (13), (27), (11), (37), (8), (22), (1), (31), (14), (15); (b) external approach using the surgical flap operation (17), (3), (27); (c) external approach using a drill and cannula (12); (d) use of cadavers (9), (28); and (e) approach through the canal and pulp chamber (23), (35).

The results of various investigators using cultural methods are as follows:

A. Extraction with no controls. Broderick (6) reported recovery of Streptococcus viridans in every examination he conducted. Kauffer (24) reported 67 positive cultures, and Goldberg (18), Cotton (13), Lucas (27), Cook (11), and Thoma (37) obtained similar results. Many types of organisms were cultured from material obtained in this manner. The most common were streptococci, diphtheroids, fusiform bacilli, cocco-bacilli and Hemophilus influenzae. Streptococci predominated in most studies. Bulleid (8) reported that the 25 granulomas which he studied bacteriologically contained streptococci in every instance.

B. Extracted teeth with controls. These consisted of extracting some normal, vital teeth as a sterility control, along with non-vital teeth which showed periapical radiolucent areas. Haden (22) cultured 400 vital, radiographically negative teeth and obtained 55 percent positive results. He attributed his high proportion of positive results to the use of glucose brain broth. Austin and Cook (1) examined 100 vital teeth and obtained positive results in only 4 instances. Growth was obtained in all but one of the 14 radiographically negative teeth with "normal" pulps which Rhoads and Dick (31) examined. This

correlated with a report by Cramer and Reith (14). From 42 teeth whose periapical tissues were roentgenologically normal, Ellingham (15) obtained bacterial growth in 57 percent of the cases.

The results of all these investigators are clouded with doubt after the work in 1936 by Fish and MacLean (16), and later by Tunnicliff and Hammond (38) and Gunter and Appleton (21). Fish and MacLean cauterized the surrounding periodontal and gingival areas of 2 teeth before extraction, and 2 others were extracted in the usual manner. After removal, they examined the pulp chambers histologically, using Gram's stain and discovered no organisms present in the blood vessels or perivascular lymphatics within the pulp, or within the pulp cells in those teeth around which cauterization had been done. But the teeth removed without prior cauterization had organisms in pulpal blood vessels and peri-vascular lymphatics but not within the pulp cells. They assumed that these bacteria were forced into the lymphatics and blood vessels at the time of extraction.

Tunnicliff and Hammond, and Gunter and Appleton showed by experiment that vital teeth are normally sterile, but that the act of extraction pumps bacteria into the vessels around the tooth. Grossman (19), in his book, "Root Canal Therapy" in 1955, stated: "It is a disconcerting but true statement to make that practically every investigation dealing with the pulpless tooth and made prior to 1936 is invalid in the light of recent studies." He also reasserted his stand in 1960, when he said: "These experiments of Fish and MacLean, of Tunnicliff and Hammond, and of Gunter and Appleton proved beyond a doubt that (1) bacteriologic examination of the root surfaces of

extracted teeth has no scientific standing unless the gingival tissue had been cauterized prior to extraction; (2) microorganisms can be pumped not only along the root surfaces during the act of extracting a tooth from its socket, but also can be forced into the pulp tissue itself because of the alternate positive and negative pressure used in rocking the tooth back and forth; and (3) pulps of vital, normal teeth are ordinarily sterile." (20)

C. External flap approach. This consists of sterilization of the oral mucosa (method not always stated) and subsequent removal of the buccal or labial plate of bone. By this method, Garvin (17) recovered bacteria from periapically x-ray negative teeth and no growth from roentgenologically positive teeth. From these results he concluded that the positive bacteria from x-ray negative teeth were due to contamination, and negative cultures from x-ray positive teeth were due to the fact that these granulomas were sterile. This investigation was based upon only 4 teeth, however. On the other hand, Berwick (3) cultured 71 periapical radiolucent areas obtained by this flap procedure and found 62 positive cultures. In 16 cases examined by Lucas (27), all grew out positive cultures.

D. Dental trocar method. This consists of the introduction of a trocar through the buccal or labial plate into the suspected area and withdrawal of tissue through the trocar. Coriell (12), who originally devised this method, stated: "In a great number of cases when I have attempted to obtain growth from x-ray positive teeth, the results have been negative, and in a greater number of teeth where radiographic evidence has been negative I have had luxuriant

growths."

E. Human necropsy studies. Burn (10) published an article in which he showed that samples of various organs obtained from human necropsies showed no significant growth of bacteria during the first one to twenty-four hours after death, providing the bodies had been placed at 10° C. shortly post-mortem. Burket (9), utilizing cadavers, cultured 419 granulomas obtained from areas showing periapical radiolucencies, and obtained 206, or 49 percent, positive cultures. He then cultured 283 periapical areas which showed no roentgenological change and were assumed to be normal, and from these obtained 30.6 percent positive cultures. Miller (28), using this same approach, studied 19 periapical areas and found Streptococcus viridans present in all.

F. In vivo approach, through the pulp canal. More recently, Hedman (23) used a cannula and stylet by inserting these through a previously sterilized pulp chamber, and obtained cultures from radiolucent areas. He obtained 56, or 68 percent, positive cultures and 26, or 31.5 percent, negative cultures from 82 cases. Similarly, Shindell (35), studied 63 cases, from which he obtained 3 positive cultures and 60 negative cultures.

The second general method of investigation -- the histologic method -- first appeared in print in 1916 when Thoma (36) reported the presence of actinomyces in two dental granulomas. The presence of bacteria in granulomas was also noted by Turner and Drew (39) in 1918. They determined to study the occurrence of bacteria in (a) living dental pulp; (b) pulp cavities and dentinal tubules of

dead teeth; (c) cementum; (d) periodontal membrane; (e) gum; (f) bone; and (g) granulomas. Although their article did not so state, it appears that they conducted their work on extracted teeth. Their procedure was to fix the tissues in 10 percent formol-saline or Muller's fluid, wash and cut them with a freezing microtome and infiltrate them with cherry gum. The tissues were mounted on thin slides and covered with 1 percent gelatine. The slides were then exposed to formol vapor for 1 minute and placed in 10 percent formol. They were then washed and stained by a modification of the Gram's stain, using Nile blue sulfate. The results, as far as the granulomas were concerned, were stated by the authors as, "sections of granulomata from the apices of teeth have invariably shown the presence of organisms." The number of granulomas examined was not divulged, but three photomicrographs accompanied the articles, one showing rod-shaped organisms in a lymphatic space and the other two showing cocci, which the authors interpreted as staphylococci, and diphtheroid organisms.

From the photomicrographs, the possibility of contamination cannot be eliminated. Similarly, any statement that granulomata invariably contained organisms might be challenged. In none of the photomicrographs was the presence of bacteria noted within phagocytic cells.

In 1929, Rhodenburg and Franken (30) published their observations on 165 dental granulomas. One hundred of these were examined histologically and 65 by bacterial culture methods. Although the authors do not so state, it appears that this material was obtained from

extracted teeth. They do not mention their staining procedures, or the culture media used in the study. No organisms were noted microscopically, but 11 of the 100 granulomas showed an acute inflammatory reaction, so the authors assumed that organisms were present. Photomicrographs accompanying the article demonstrate only the presence of an inflammatory reaction. Although all of their cultures were positive for microorganisms, there was no demonstration of them histologically. There were no controls and there was no effort to avoid contamination.

Investigations published by Bulleid (8) in 1928 and 1931 deal with his attempt to demonstrate the presence or absence of bacteria in dental granulomas, radicular cysts, chronic apical osteitis other than the granulomas, and acute alveolar abscesses. He used a combination of 2 approaches to obtain tissue for examination -- the "flap" operation and extraction. All, or nearly all, of those teeth showing granulomas appeared to have been obtained by extraction. Bulleid isolated the supporting tissue with cotton rolls, dried it with sterile wool rolls, flooded the tissues with absolute alcohol and dried with hot air. Tincture of iodine was applied to the teeth and supporting tissue, after which cultures were taken from the gum around the tooth. The tooth was extracted and washed in sterile saline. A little ether was then poured over the granuloma and set afire. The outside of the granuloma was cultured, incised with sterile scissors and material was obtained for the final cultures from the inside by the use of a sterile platinum wire.

He subjected 80 granulomas to both cultural and histological

examination. The only portion of his technique upon which he commented was the stain. The tissues were stained with either the Gram-Weigert or the Murray-Drew method. His paper contains 8 photomicrographs showing the presence of bacteria. These show bacilli, cocci and thread forms of microorganisms. From the article, we cannot determine from which portion of the granuloma the pictures were taken. Therefore, we do not know whether these represent contaminants or the preoperative presence of bacteria. Since the use of extracted teeth for demonstrating bacteria has been severely criticized, as noted earlier in this paper, the possibility of these representing contamination is a real one.

In 1934, Boyle (4) published the results of his histologic research on granulomas. His specimens were obtained from extracted teeth. He examined 200 granulomas and found only one which demonstrated intra-cellular bacteria. These were present in foam cells in the central portion of the granuloma. However, he did state that bacteria (number not mentioned) were occasionally found in areas of necrosis in the center of other granulomas. He also noted in his study that bacteria could be demonstrated regularly on the outer portion of the connective tissue capsule of the granuloma. No cellular reaction was noted to these organisms and they were, therefore, regarded as contaminants.

The technique he used in his examination was to embed tissue in paraffin and section to a thickness of 2 to 4 microns. Walbach's modification of Giemsa's stain, Gram's stain and eosin methylene-blue stain were used. The organisms were Gram positive, not acid

fast, colored deep purple by Giemsa's stain and measured 2 microns in length and 0.6 micron in breadth.

Since 1934, although much has been written about the dental granuloma, research in this field has consisted mainly of the bacterial culture approach. Because of the difficulty of bacteriological control and since the advent of better staining methods for revealing bacteria in tissue, it seems logical to once again use the histological approach. This method eliminates the possibility of contamination. If one can show bacteria within phagocytic cells or in the deeper portions of the granuloma where the probability of contamination is almost nil, one can state with some certainty that granulomas are septic in nature. The research underlying this thesis has been directed toward this end.

MATERIALS AND METHODS

Over the past ten years, all dental granulomas submitted to the Department of Oral Pathology at the University of Oregon have routinely been histologically sectioned. Selected sections from each granuloma have been stained by hematoxylin and eosin[1] and the Brown and Brenn tissue Gram stain.[2] The observation of bacteria deeply within many of these granulomas led to the use of this tissue bacteriological stain as the critical tool for this thesis. Basically, it was hoped to determine what percentage of the available dental granulomas contained visible bacteria which could not be accepted as contaminants and what relationship these findings bore to other observations, such as the types and relative amounts of inflammatory cells.

The sections examined were cut from 105 dental granulomas obtained from those coming through the department since 1953. These had been removed by various operators, the only common portion of handling being the immediate placement of the excised tissue in neutral 10 percent formalin.

Each granuloma was serially sectioned at an average thickness of about 7 microns. The number of tissue sections on each glass

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[1] Hereafter referred to as H & E.

[2] Hereafter referred to as B & B.

slide varied from 3 to 8, depending upon the size of the tissue being sectioned. The total number of sections per granuloma varied from 30 to 330, the mean being 92 and the mode 150. The slides were treated in the following sequence:

Slide # 1 was stained with H & E (average 20 sections per granuloma).

Slides # 2 and # 3 were stained with the B & B Gram stain for tissues (average 40 sections per granuloma).

Slide # 4 was stained with Gomori's Methenamine Silver Nitrate[1] Technique for fungi in tissue (20 sections per granuloma).

Slides # 5, # 6 and # 7 were mounted but left unstained to give ready availability to tissue if additional stains were desired.

THE B & B STAIN

Preliminary investigation. Since the ability of cells to retain the Gram stain is not a property applicable to all living matter in general, but is confined almost entirely to the yeasts and bacteria (2), (33), and since the B & B modification demonstrates blue to blue-black bacteria vividly against a yellow background, it was chosen as the major tool. (Appendix A) In a pilot study of 23 granulomas, it soon became apparent that often only a few intra-cellular bacteria would be seen in the whole granuloma, and that a 45x objective was the lowest power at which they could be defined as bacterial forms. Therefore, it was necessary to make a complete search of each B & B section under this magnification.

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[1] Hereafter referred to as GMS.

Present modification of B & B stain. Many variations in staining characteristics, particularly of the tissues, were noted. A problem inherent in the AFIP[1] modification of the B & B original technique is the fact that only 4 or 5 slides could be processed at a time. Because of the hundreds of B & B stained sections necessary in this investigation, an attempt at a 20 slide-staining schedule was made. During this experimentation, a means was also found to obtain consistent staining of the Gram-positive bacteria with only a very faint counterstain visible which permitted one to determine whether the bacteria were intra-cellular or extra-cellular. This depended on the finding that Gram-positive bacteria, in the granulomas and in the control sections (to be described) withstood decolorization of periods of time ranging from 1/2 to 4 minutes. As a matter of fact, the upper limit was never determined. In the meantime, while all the tissue cells (with the rare exception of the distinctive and large "Russell body") had lost the crystal-violet Gram's iodine stain, the Gram-positive bacteria alone retained it. Appendix A compares this modification of the Brown and Brenn technique with the original and with that of the AFIP.

Searching for bacteria. Initially, a meticulously complete search and bacterial count was planned for all of the tissue on each B & B section. It soon became apparent that, for the return, this was much too time-consuming for the 105 granulomas (each containing

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1 Armed Forces Institute of Pathology

an average of 40 B & B sections) since it took about 20 minutes to thoroughly and systematically search each section. This would have meant an average of 14 hours spent in searching each granuloma.

Therefore, four B & B stained sections from each granuloma, each section being taken from a successively deeper quartile than the preceeding one, were thoroughly searched. All the bacteria in these sections were localized with an ink dot always placed in the same relationship to the bacteria, which were identified by the following criteria:

Criteria for identifying bacteria.

1. The object must stain blue or blue-black as noted in our positive control slide.

2. The object must exhibit a typical spherical or rod shape.

3. The object must be of a size consistent with bacteria as observed in the control.

4. The object must be either definitely intra-cellular in location or present within the deeper portions of the granuloma.

Groups of bacteria.

1. Positive-for-bacteria[1] -- were those objects which fulfilled all four of the above-noted criteria. No further search of the remaining B & B stained sections was made after bacteria were positively identified in one or more of the initial four sections.

2. Questionable-for-bacteria[2] -- were those objects which met

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[1] Hereafter referred to as positive group.

[2] Hereafter referred to as questionable group.

one, two or three of the criteria, but not all four. Only the usual four slides were examined here.

3. Negative-for-bacteria[1] -- were those cases in which no objects were present, or which, if present, they did not satisfy any of the four criteria. (Sections showing bacteria, but located only on the periphery were included in this group.) All available B & B stained sections were thoroughly searched in each of these cases.

Locations of bacteria. When bacteria were present, they were classified relative to their focal or diffuse distribution as well as to their intra-cellular or extra-cellular location.

Quantitation of bacteria in sections. The appearance of bacteria as clumps or masses in tissues makes counting both difficult and somewhat meaningless. Since these aggregates can usually be discerned by means of a 10x objective in contrast to individually-dispersed bacterial cells, it can be assumed that they are present in greater numbers in the former case. This condition allows a rough division into two groups.

The ink-dotted areas on the B & B sections of the positive group were all re-examined under the 10x objective magnification, and those in which bacteria could be seen at this magnification were placed in the "10x" group[2]. The others which could be seen

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1 Hereafter referred to as negative group.

2 Hereafter referred to as the 100x magnification group.

only with the 45x objective were placed in the "45x" group.[1]

<u>Control for bacteria in sections</u>. In order to clarify the interpretation of bacteria in tissue, two control experiments were conducted:

A. Streptococcus faecalis RATCC 8043 was grown, suspended in sterile physiologic saline solution and 0.5 ml. injected into the gastrocnemius muscle of each of 2 rats. Injection of 0.5 ml. sterile physiologic saline solution into the gastrocnemius muscle of the opposite leg served as a control. In 24 hours, one rat was sacrificed and the muscles on both sides were dissected out, using a sterile technique. One half of each muscle tissue was placed in 10 percent formalin for histopathological diagnosis; the other portion was again cut in half and one piece placed in brain-heart infusion broth, the other in fluid thioglycollate medium.

The other rat was sacrificed after 48 hours and the same surgical, histological and cultural procedures were employed.

B. The second experiment consisted of the intramuscular and subcutaenous injection of a week-old culture of Staphylococcus aureus, Phage type # 47, into an adult rabbit. A 24-hour culture was first tried, but no tissue reaction was evoked. By the end of one week, sufficient toxic products were present in the broth to produce a good tissue reaction. Forty-eight hours after injection, the animal was sacrificed and the tissues were removed. One half was used for culture and phage typing, the remainder was serially

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[1] Hereafter referred to as the 450x magnification group.

sectioned and stained with H & E and B & B, using the method as previously outlined for dental granulomas.

THE H & E STAIN

Relative numbers of the various inflammatory cells in the areas containing bacteria. The H & E sections adjacent to the bacteria-containing sections, as shown by the B & B stains, were examined in their corresponding areas, using a net reticule[1] and a 25x objective -- the lowest at which the various inflammatory cells could be identified with certainty. Of the 25 squares into which the net reticule was divided, only those at the four corners and center were used in counting the numbers of neutrophiles, eosinophiles, lymphocytes, plasma cells and histiocytes. In order to substantiate the accuracy of this procedure, a sample of five H & E stained granulomas was selected. An area which corresponded to that containing bacteria in the adjacent B & B stained slide was chosen and a cell count was done. In each of the five granulomas this particular field was counted using the five squares as noted above. The procedure was repeated using all the 25 squares. At the 5 percent level, no significant difference between the two methods could be demonstrated using the sign test statistic.

Relative numbers of various inflammatory cells in the negative and questionable groups. In the cases where bacteria were absent, the area with the largest number of neutrophiles was selected.

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1 American Optical # 1421, 20 mm. diam., 5 mm. sq., ruled into 1.0 mm. squares.

The reticule was placed over this area and the cells were counted. If the presence of bacteria was questionable, an area was selected where these questionable objects were in evidence and the cells were counted in the same way as stated for the presence of definite bacteria.

CLINICAL INFORMATION

Where available, other information was recorded for each case:

1. Age, sex and race of patient.
2. Presence or absence of pain -- through patient history.
3. Presence of a fistulous tract (this was determined by means of the patient's history).

All of this information was collected in an attempt to show a possible relationship between the presence or absence of bacteria in the dental granuloma and any of this clinical information.

FINDINGS

BACTERIA

Positive group. Of the 105 granulomas investigated, 53 were found to contain bacteria which could be definitely identified as such. In 25 of these, forms which were recognized as bacteria could be seen at a magnification of 100x. The remaining 28 contained bacteria which could be seen only at a magnification of 450x.

Questionable group. Forty-four of the granulomas were considered questionable because the structures seen in them did not meet the four criteria as previously described.

Negative group. Eight of the granulomas revealed no forms that could be considered to be bacteria.

Findings from this portion of the project are recorded in Table 1.

Location. The bacteria observed in a granuloma and not considered contaminants were found in focal accumulations both intra-cellularly and extra-cellularly. Less often they were distributed diffusely, but here again they were intra-cellular and extra-cellular in location. When located within cells, these cells were either neutrophiles or histiocytes. These observations are shown in Table 2.

At the beginning of the investigation, an attempt was made to identify several types of microorganisms; namely, streptococci, staphylococci, bacilli, thread forms and fungi. It soon became

Table 1. Bacterial Status of the Granulomas

STATUS	NUMBER
Positive at 100x magnification	25
Positive only at 450x magnification	28
Total Positive Cases	53
Questionable	44
Negative	8
Total Granulomas	105

Table 2. Location of Bacteria in the 53 Positive Granulomas

BACTERIAL STATUS OF GRANULOMAS	NUMBER	FOCAL INTRACELLULAR	FOCAL INTRA. & EXTRACELLULAR	DIFFUSE EXTRACELLULAR	DIFFUSE INTRA & EXTRACELLULAR	COMBINA-TION
Positive at 100x magnification	25	2	16	0	6	1
Positive at 450x magnification	28	4	20	3	1	0
Total positive	53	6	36	3	7	1

evident, however, that coccal forms of bacteria occurred in tissue only singly or in diplococcal arrangements. Therefore, streptococci and staphylococci could not be identified as such. In no instance did bacilli occur alone, but always in combination with coccal forms (see Table 3). No spirochetal or "thread" forms were seen in deep cellular portions of any of the granulomas. Examination for fungi was discontinued after unsuccessfully searching GMS stained sections from the preliminary group of 23 granulomas.

Controls. Organisms isolated from the first control animal, sacrificed 24 hours after injection, grew on sheep blood plates as small, white, slightly elevated colonies in 17 hours. These were typical of streptococcal colonies. No contaminants were present. The broth was streaked on a glass slide and Gram stained. Long chains of streptococci were noted. Growth in brain-heart infusion agar with 6.5 percent NaCl was accepted as evidence this organism was Streptococcus faecalis.

The formalinized tissue was serially sectioned, mounted and the slides were stained in sequential series as follows:

One slide with H & E stain.

Two slides with B & B stain.

Three slides were mounted but left unstained.

The H & E stain showed the presence of neutrophiles, lymphocytes, plasma cells and histiocytes collected over the surface of the muscle bundles. In places, they extended into and were associated with destroyed muscle bundles and appeared to be progressing down interfascicular fascial planes.

Table 3. Types of Bacteria Present in Positive Granulomas

BACTERIAL STATUS OF GRANULOMAS	NUMBER	NUMBER SHOWING COCCI ONLY	NUMBER SHOWING BACILLI & COCCI
Positive at 100x Magnification	25	9	16
Positive only at 450x Magnification	28	8	20

The B & B stained slides (which were stained by the present modification previously described and outlined in Appendix A) showed the presence of cocci. These were mainly present as single organisms, but some occurred as diplococci. The organisms staining blue to blue-black in color, were practically all the same shape and size, and were noted within neutrophiles and histiocytes, as well as outside inflammatory cells. The microscopic appearance of the bacteria was similar to some of those seen in the dental granulomas.

In 48 hours, the other rat was sacrificed. The results were the same as those obtained from the first one.

In the animal inoculated with Staphylococcus aureus Phage type 47, the organisms cultured from the infected tissue and appearing culturally as Staphylococcus aureus were phage typed once again and found to be type 47. The H & E sections revealed considerable acute inflammatory reaction, and the B & B sections demonstrated gram-positive cocci, similar to those seen in the dental granulomas in this study. These organisms met the four criteria as previously noted for identification of bacteria in tissues. No inflammatory reactions or bacteria were seen in the tissues removed from the sites of the sterile injections.

INFLAMMATORY REACTION

For the 53 positive cases, the total number of each cell type was determined by the cell count previously described. The mean number of each cell type was then determined and expressed as a percentage. Thus, it was noted that the ratio of lymphocytes to neutrophiles was about 4 to 1 for the total of all 53 granulomas.

The actual numbers, however, varied considerably; for example, the neutrophile count among granulomas varied from 0 to 71 per field, and lymphocytes from 4 to 81 per field. The number of granulomas which exhibited the presence of the various cell types was also recorded. Lymphocytes were present in every field of every granuloma. Next in frequency came histiocytes, plasma cells and, lastly, neutrophiles. These and similar observations for positive granulomas in which organisms could be seen at 100x magnification compared with those in which organisms could be seen only at 450x magnification and those granulomas in which bacteria were absent are recorded in Table 4. Although the number of cases is insufficient for statistical evaluation, it is of interest that, in the group positive at 100x magnification, a mean of 10.32 neutrophiles was seen in the areas of bacterial presence; whereas; in the negative group, only 4.13 neutrophiles was the mean maximum number found.

CLINICAL HISTORY RELATIVE TO HISTOLOGIC FINDINGS

Pain. In an attempt to relate clinical symptoms to the presence or absence of bacteria, histories were obtained from as many patients as possible, particularly in reference to the presence or absence of pain in or about the affected tooth. The results are shown in Table 5. Patients having granulomas containing greater quantities of bacteria showed the highest percentage of pain.

Fistulous tract. An attempt was made to relate the presence of a fistulous tract with the quantitative presence of bacteria. Only 12 histories could be obtained for patients in each of the

Table 4. Inflammatory Cell Composition of 105 Granulomas

BACTERIAL STATUS OF GRANULOMAS	CELL TYPES			
	NEUTROPHILES	LYMPHOCYTES	PLASMA CELLS	HISTIOCYTES
1. Total positive cases (53)*				
a. Mean number of each cell type present	8.33	29.56	6.58	5.50
b. % of each cell type present	17	59	13	11
c. Number of the 53 granulomas having a given cell type	44	53	49	52
d. Variation in actual cell count among granulomas	0-71	4-81	0-74	0-22
e. Total cells counted in all 53 granulomas	442	1567	349	292
2. Positive cases at 100x* magnification (25)				
a. Mean number of each cell type present	10.32	25.44	7.92	6
b. % of each cell type present	21	51	16	12
c. Number of the 25 granulomas having a given cell type	20	25	23	25
d. Variation in actual cell count among granulomas	0-71	4-63	0-74	1-22
e. Total cells counted in all 25 granulomas	258	636	198	150

Continued

* Counts of inflammatory cells were made in the areas where bacteria were present.

Table 4. Continued

BACTERIAL STATUS OF GRANULOMAS	CELL TYPES			
	NEUTROPHILES	LYMPHOCYTES	PLASMA CELLS	HISTIOCYTES
3. Positive cases seen only at 450x magnification (28) *				
a. Mean number of each cell type present	6.57	33.25	5.39	5.07
b. % of each cell type present	13	66	11	10
c. Number of the 28 granulomas having a given cell type	24	28	26	27
d. Variation in actual cell count among granulomas	0-21	6-81	0-34	0-21
e. Total cells counted in all 28 granulomas	184	931	151	142
4. Negative cases (8) **				
a. Mean number of each cell type present	4.13	37	7.38	5
b. % of each cell type present	8	69	14	9
c. Number of the 8 granulomas having a given cell type	7	8	8	8
d. Variation in actual cell count among granulomas	0-19	11-72	1-36	3-9
e. Total cells counted in all 8 granulomas	33	296	59	40

* Counts of inflammatory cells were made in the areas where bacteria were present.

** Counts of inflammatory cells were made in the areas of greatest concentration of these cells.

Table 5. Bacterial Status of Granulomas and the Presence of Pain

BACTERIAL STATUS OF GRANULOMAS	TOTAL NUMBER OF CASES WITH HISTORY	NUMBER OF CASES WITH PAIN
Negative (8)	7	2
Positive at 100x magnification (25)	12	8
Positive only at 450x magnification (28)	23	6
Total Positive (53)	35	14

100x magnification group and the 450x magnification group. The results of this portion of the investigation are recorded in Table 6.

Table 6. Bacterial Status of Granulomas Versus the Presence of a Fistulous Tract

BACTERIAL STATUS OF GRANULOMA	TOTAL NUMBER OF HISTORIES AVAILABLE	NUMBER GIVING HISTORY OF PRESENCE OF FISTULOUS TRACT
Positive at 100x magnification	12	2
Positive only at 450x magnification	12	6

DISCUSSION AND SUMMARY

In this investigation, an attempt was made to attack an age-old problem by a little-used approach. For many years, and by a variety of investigators, the cultural method has been used to evaluate the bacteriological status of dental granulomas. Its drawbacks are many, as emphasized by the variety of conflicting results published by its proponents. The difficulties of the cultural methods are: (1) the culture medium must be suitable for each of the organisms to grow or false negative results might be obtained; (2) the temperature must be precisely controlled; (3) optimum growth time must be definitely determined; and (4) optimum oxygen tension must be adjusted to meet the needs of each of the organisms.

The possibility, or even probability, of contamination when removing material for culture from a granuloma is extremely high. Many surgical approaches have been devised to eliminate or minimize this problem, but all have their questionable aspects. The oral cavity is known to contain many microorganisms of various types. Formulating a surgical method adequate to draw a piece of tissue through this environment without contamination requires considerable ingenuity and skill.

On the other hand, too vigorous sterilization of root canals could conceivably kill or attenuate bacteria in a dental granuloma so that subsequent cultures would be negative.

The other most obvious aspect, but the one that draws least comment, is the action of the body defenses upon bacteria. A micro-organism within viable tissue is subjected not only to the action of phagocytic cells, but also to the presence of a variety of humoral antibacterial substances. It is possible that most bacteria in such an environment are either killed or attenuated to such a point that they would be unable to grow on artificial culture media. The film, "Dynamics of Phagocytosis: The Interaction Between Group A Strepto-cocci and Human Neutrophiles in Vitro" (29), strikingly shows growth inhibition of bacteria which have undergone phagocytosis for a brief period of time and are then freed from the phagocytic cell. Perhaps this happens occasionally within granulomas, and accounts for some of the negative results when attempts are made to culture this tissue.

One method used by 2 investigators previously cited in this paper (23), (35) employed a cannula and stylet placed in root canals to recover material from a periapical granuloma.

Since the results vary considerably in the two reports, one has to attempt an explanation. The technique varied between the two investi-gators in that Hedman used 7 stylets for each granuloma, whereas Shindell used only one. Shindell attempted to revolve this single stylet 360° to obtain a good tissue sample for culturing. Since a granuloma is a rather resilient, firm tissue, it is possible that this stylet did not move 360°, but rather stayed in the area where it was first introduced. This would help explain his high percentage of negative cultures.

The amount of material recovered by this means hardly seems adequate for proper bacteriological evaluation. Indeed, the results of the present investigation reveal the fact that bacteria in granulomas are, in many instances, very scarce, and diligent microscopic search is necessary to find them. It is, therefore, difficult to imagine the odds against obtaining culturable bacteria in sufficient numbers by using a stylet in one small area of the total granuloma.

Hedman recorded his bacteriological procedures quite specifically, whereas Shindell recorded his procedures in very general terms. One is not told how long his media were incubated or whether the growth of slow-growing organisms was encouraged.

It is a well-known bacteriological principle that, in order to obtain a true growth of bacteria which have been previously subjected to bactericidal or bacteriostatic substances, such as presumably occurs in dental granulomas or in prior root canal treatment, one must use large enough quantities of broth to dilute the sterilizing substance enough for bacteria to grow. If a specific blocking agent to the antibacterial substance is available (such as penicillinase) this should also be included in the cultural media. In neither study was it stated whether either one of these methods was employed in the investigation.

The use of cadavers for such an investigation seems questionable also. The high percentage of recovery of bacteria, reported in Burket's work, from otherwise normal-appearing teeth certainly casts doubt upon the validity of this method. If this represents a true finding, one would have to assume that approximately 1/3 of all

radiographically normal-appearing apices have bacteria present in their vicinities. This assumption would be an exception to the generally-accepted concept that all viable sub-surface tissues are sterile. Since all normal body defense mechanisms cease at death, or shortly after, it seems logical to assume that this finding may represent a postmortem bacterial penetration, possibly from the surface periodontium through the periodontal membrane to the tooth apices.

Probably the best surgical approach to the problem is by means of the "surgical flap" operation, provided sterilization and operative procedures are adequately controlled. Proper care of the specimen to avoid contamination, and correct selection of handling culture media are necessary to obtain acceptable results. If any one of these is neglected or subjected to less than ideal standards of meticulous technique, the validity of the results would be doubtful.

The histopathological method has been used very sparingly in previous years. Many of the past observations and results leave much to be desired. The obvious advantages of this procedure over the cultural methods are:

1. Bacterial contamination is of no consequence, so surgical procedures are not critical. Contamination can be ruled out by observing the location of offending organisms.

2. It is unnecessary to have an "ideal" culture medium.

3. It is unnecessary to have proper atmospheric conditions, and time and temperature for incubation for bacteria in cultural media.

4. There is no need for concern if the bacteria have been attenuated by either transient passage through a phagocyte or intimate

contact with one or more of the many humoral substances present in the body.

5. Adequate amounts of material can be obtained in nearly every instance.

The obvious shortcoming of this method is the danger of calling something a bacterium which is not. To eliminate or minimize this hazard, rigid criteria for identification have to be formulated. Considerable investigation of the bacterial specificity of the tissue Gram stain must be undertaken. These two requisites, together with many hours of microscopic identification of known bacteria, from deliberately inoculated control animals, present in tissue, are necessary to minimize inherent error. The use of above-mentioned procedures allows considerable confidence to be placed in this method of investigation and its findings.

Fifty-three, or 50.4 percent, of the 105 granulomas which were used in this study, showed unquestionable presence of bacteria. Bacteria in these graulomas met all the four criteria for identification as previously outlined. No attempt was made to identify Gram-negative bacteria because previous investigations of the bacterial flora of non-vital teeth, from which bacteria in dental granulomas presumably emanate, reveal the presence of considerably more Gram-positive than Gram-negative organisms. Admittedly, this omission would prevent the identification of additional bacteria which may be present in the granuloma. Also, as noted by Brown and Brenn (7) in their original article, dead and disintegrated organisms stain Gram-negative regardless of their original Gram specificity. This observation also

would presuppose that not all organisms present were identified in this study.

Forty-four of the 105 granulomas showed the presence of questionable bacteria; that is, those which did not meet all four criteria previously mentioned. Perhaps some of these represented dead or disintegrating Gram-positive organisms. Additional research on this aspect of the stainability of bacteria within tissues would materially aid in a study of this kind. Since these results are based upon the examination of only 4 slides per granuloma, perhaps definite bacteria could have been demonstrated if additional slides had been searched.

No objects could be observed in 8 of the 105 granulomas which met any of the 4 criteria for identification. Although all the B & B stained slides on these cases were thoroughly searched, without success, this represents an observation of only 2/7 of the tissue available, since only 2 of every 7 slides were stained with B & B. Possibly, if <u>all</u> the tissue were examined for this phase of the investigation, definite, or at least questionable, bacteria might have been located. On the other hand, since it is assumed that bacteria enter the dental granuloma in "bursts" from the root canal, these 8 may represent the quiescent stage between bursts, during which previously disseminated bacteria have been engulfed and destroyed by the available phagocytes.

Of the 53 granulomas classified as positive, 25 revealed the presence of bacteria which could be visualized in 100x magnification and identified as bacteria at higher magnification. These cases demonstrated the presence of a greater number of bacteria in focal

collections as did also the other 28 cases in which bacteria could be seen only at 450x or greater magnification.

A combination of cocci and bacilli occurred more often in the total positive group than did cocci alone. The 450x magnification group seemed to show a greater number of bacillus and coccus combinations than the 100x magnification group, but this was not statistically significant. The 100x magnification group of granulomas revealed more bacteria present in diffuse, intra-cellular and extra-cellular location than the 450x magnification group. This is explainable if one considers that this group represents a situation in which the bacteria are multiplying more rapidly than the tissue defenses can eliminate them. One would then expect to see bacteria not only within phagocytes but rapidly multiplying diffusely in the adjacent tissue. The 450x magnification group can, conversely, be considered that situation in which the inflammatory cells are keeping bacterial multiplication at a minimum by their rapid and effective phagocytosis and destruction, possibly by humoral substances as well. In this case, one would not expect to see a lot of bacteria diffusely spread through the tissue, but in focal collections and within phagocytes, which was so noted in this study.

When comparing the total positive cases with the negative cases, relative to inflammatory cellular composition, there appears to be a mean of twice as many neutrophiles present in the positive group as in the negative group. The variation of cells in each granuloma is considerable and the number of negative cases is small, so little value can be placed upon this observation. Although not significant

statistically, it is of interest to note that the 100x magnification group contained 21 percent neutrophiles (of all inflammatory cells) in the area of bacteria, as compared with 13 percent in the 450x magnification group, and 8 percent in the negative group. If, in a larger study, this were shown to be a valid observation, it might be explained by the fact that more bacteria are present in the 100x magnification group and more neutrophiles would presumably accumulate to combat them. The lymphocyte, plasma cell and histiocyte count varied little between the groups, so no definite comment can be made in their regard. However, in all probability, the lymphocytes and plasma cells are equally active in all groups in antibody activity. The histiocytes probably "clean up" the debris caused by antigen-antibody reactions, dead neutrophiles and bacteria, as well as phagocytising some of the bacteria directly.

The relationship between the bacterial status of the granuloma and presence or absence of pain is very interesting. There is no statistically significant difference between the groups which were positive for bacteria and the group negative for bacteria in this respect, but there is a significant difference between the 100x magnification and the 450x magnification groups. Pain was more common in the 100x magnification group. This difference could be explained on the assumption that the presence of greater quantitites of bacteria produce a more severe inflammatory reaction which, in turn, causes increased pain.

The bacterial status of the granulomas relative to the presence or absence of a fistulous tract was not significantly different.

Again, a slightly larger sample may have shown statistical significance. It appears that some granulomas, which contain few organisms and few neutrophiles, are chronic in nature because a fistula allows the escape of bacteria and necrotic debris into the oral cavity. In this situation, there is need for very few neutrophiles in the granuloma itself during the draining periods.

The histopathological technique for studying the bacterial status of dental granulomas is not new, but, since the advent of new and better tissue staining methods, and for other reasons noted earlier in this paper, it was selected for this investigation. Refinements of this technique (which will undoubtedly yield more definite information) can be used in future research. Such a technique would be the use of fluorescent antibodies and fluorescence microscopy. By these means, it would be unnecessary to set up criteria for the identification of bacteria, since fluorescence would identify them. The problem of natural tissue fluorescence would have to be considered and resolved in this type of study, but much valid information could thus be obtained.

Tissue staining procedures vary so greatly from day to day when only a few slides at a time are stained, that good microscopic interpretation is made difficult. Freshly prepared solutions and rigidly timed staining procedures (by a clock) are necessary to eliminate this problem. The very best possible staining is necessary to help minimize erroneous conclusions when attempting to identify organisms under the microscope. A little less than ideal staining is worthless.

Additional problems encountered in this work included the presence in several slides of foreign material in and on the tissue sections. Some of these appeared to be crystalline in nature and were birefringent, as noted by polarized light. This did not interfere with interpretation because they were never noted to take a blue or blue-black stain, and they revealed typical crystaloid morphology, such as sharp edges. Other foreign substances were occasionally noted, which were not crystalline in nature. However, they did not meet the criteria for bacteria, and, in most cases, were readily identified as foreign material.

Several of the dental granulomas were noted surrounding the apices of extracted teeth. In many of these cases, it was possible to identify colonies of bacteria within the root canal and in the granuloma near the root apices where they were being ingested by phagocytes. In some instances, they could also be traced up carious dentinal tubules to the surface of the tooth. These observations added considerable strength to the argument that, not only are many dental granulomas septic, but that the bacteria probably enter them via an infected root canal.

In all probability, many of the granulomas placed in the questionable group contained bacteria, either in stages of disintegration with loss of normal morphology and staining characteristics, or present in some of the other sections which were not examined in this investigation.

It is hoped that the results of this investigation have demonstrated that a good many dental granulomas do indeed contain bacteria,

and, because of the possible consequence of their presence in the body, adequate procedures should be instituted to eliminate them.

CONCLUSIONS

Over one-half of the 105 dental granulomas examined in this investigation revealed the presence of bacteria. These bacteria were similar and, in many cases, identical to known bacteria injected into control animals. It is very possible that an even greater percentage of the granulomas contained bacteria, but the staining procedure used in this study was unable to demonstrate more.

Some granulomas contain more bacteria than others. When more were present, their location in the tissues was diffusely intra-cellular and extra-cellular for the most part. This is opposed to the case when few bacteria were present. In these instances, the majority were noted in focal, intra-cellular and extra-cellular location.

Because of the few negative granulomas, statistical significance cannot be attached to many possible correlations. However, greater numbers of neutrophiles were present in those cases containing bacteria than in those which did not. More neutrophiles were also noted in the granulomas which had large numbers of bacteria present, as opposed to those that had few. The presence of lymphocytes, plasma cells and histiocytes was approximately the same for those groups of granulomas having definite, questionable and no bacteria present. Those patients who had granulomas which contained large numbers of bacteria showed a statistically higher incidence of pain than those whose granulomas contained few bacteria.

It is, also, of interest to note that the presence of a fistulous tract was more common in granulomas having fewer bacteria than those with many.

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APPENDIX A

BROWN AND BRENN STAIN FOR BACTERIA IN TISSUE

ORIGINAL	AFIP MODIFICATION	PRESENT MODIFICATION
	Slides are individually stained on a staining rack.	Slides are stained in lots of 20 placed in an immersible nickel silver staining rack. 350 cu. cm. of solution was placed in clear glass staining dishes for each of the following procedures.
1. (a) Stain is freshly filtered alum-hematoxylin (Harris) for 2-5 min. (b) Wash in acid alcohol (3% HCl in 95% ROH) until light pink. (c) Wash in ammonia H_2O (1cc. of aqua ammonia in 100 cc H_2O until blue.	1. Pour on approximately 1.0 cc. (or 20 drops of 1% crystal violet solution), and add 5 drops of 5% sodium bicarbonate solution for 1 minute. Agitate gently.	1. Place tray containing slides in 280 cc. of 1% crystal violet to which 70 cc. of 5% sodium bicarbonate has been added just prior to use. Leave in solution 1 minute. Agitate gently.
2. (a) Wash in water. (b) Mix 5 drops of 5% aqueous solution of $NaHCO_3$ with about 0.75 cc. of 1% gentian violet (stain slide with this for 2 minutes). (c) Wash quickly with water.	2. Wash in water.	2. Wash in water until excess dye is removed.
3. Cover slide with Lugol's iodine for 1 minute.	3. Flood slides with Gram's iodine solution for 1 minute.	3. Place in Gram's iodine solution for 1 minute.
4. Wash in H_2O and blot.	4. Rinse in water and blot with filter paper to complete dryness.	4. Wash in water until all excess iodine solution is removed.

5. Decolorize with 1 part ether plus 3 parts acetone, dropping it onto the slide until no more color runs off, and blot.	5. Decolorize with mixture of equal parts of ether and acetone dropped on slides until no more blue color runs off.	5. Place in equal parts of ether and acetone for 2 minutes with constant agitation.
6. Stain for 5 min. with rosanilin hydrochloride (0.005 gm/100 cc.) or basic fuchsin (0.1 cc saturated alcohol solution per 100 cc. H_2O).	6. Stain with working 0.1% basic fuchsin solution for 1 minute.	6. Place in 0.1% basic fuchsin solution for 30 seconds.
7. Wash in H_2O. Blot but don't allow section to dry.	7. Wash in water; blot gently, but not completely dry.	7. Wash in water for 1 minute.
8. Pass through acetone.	8. Dip in acetone to start the reaction.	8. Place in acetone for 15 seconds.
9. Decolorize and differentiate by dropping over the section a solution of 0.1 gm. of picric acid in 100cc. of acetone until section becomes a yellowish-pink.	9. Differentiate immediately with 0.1% picric acid-acetone solution until sections are yellowish-pink.	9. Place in 0.1% picric acid-acetone solution for 15 seconds.
10. Pass successively through acetone and equal parts of acetone and xylol.	10. Rinse quickly in acetone and in mixture of equal parts of acetone and xylene.	10. Place in acetone for 15 seconds. Place in mixture of equal parts of acetone and xylene for 5 minutes.
11. Clear in xylol.	11. Clear in several changes of xylene and mount in Permount.	11. Clear in one or more solutions of xylene for 5 minutes each and mount.

APPENDIX B

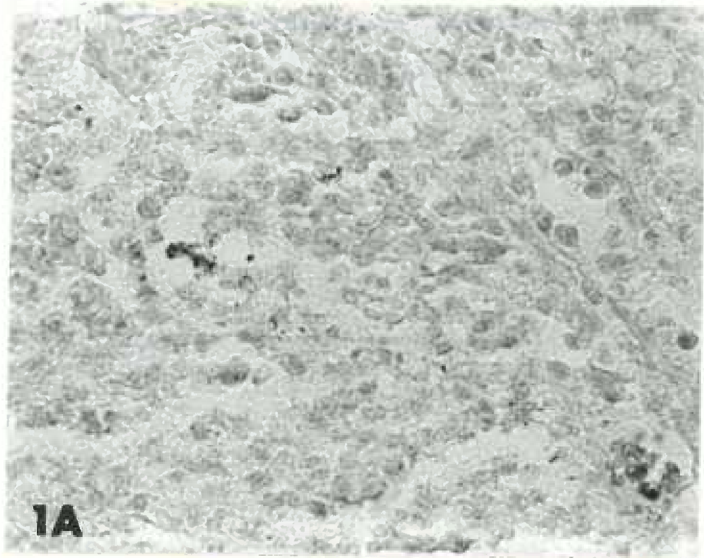
PLATE I

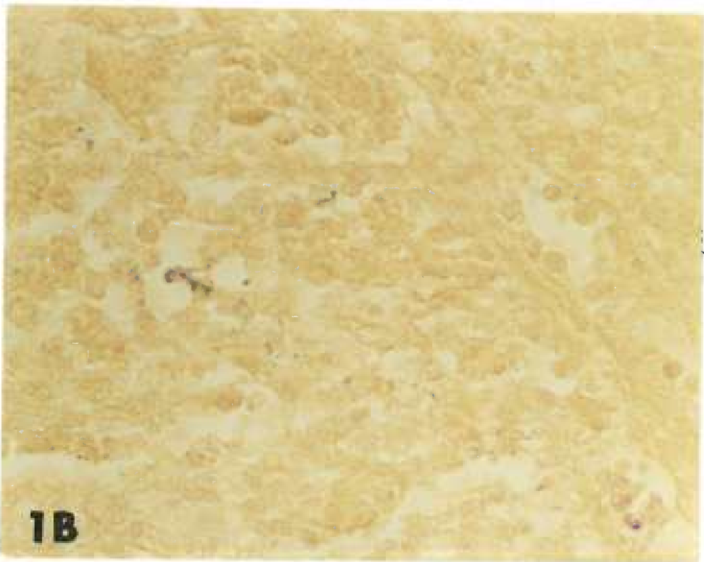
Bacteria and Associated Inflammation in Granulomas

1A The cocci and bacilli seen are deeply situated within a dental granuloma. They are diffusely distributed and are both intra- and extra-cellular in location. (Black and white print of B & B stained section 1B 500x.)

1B Color photograph of a Brown & Brenn stained section demonstrating the typical blue-black coloration of Gram positive bacteria. (500x)

1C The cellular infiltrate in the area of the bacteria seen in 1B consists of neutrophiles, lymphocytes, plasma cells and histiocytes. (Black and white print of an H & E section adjacent to 1B 250x)


1A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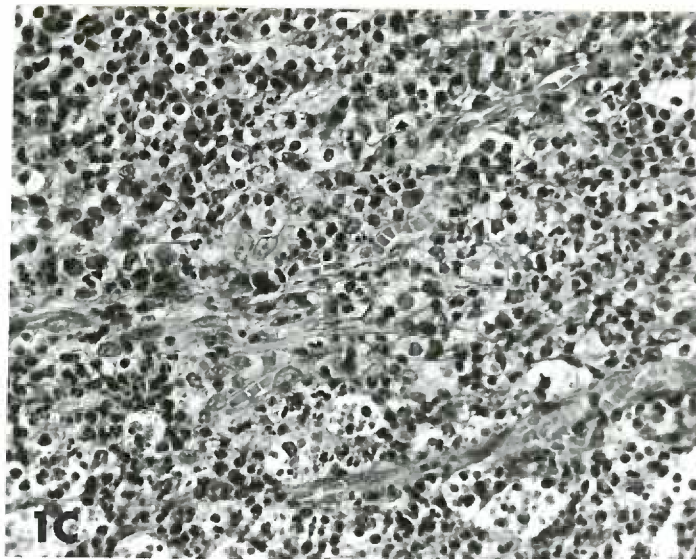

1C

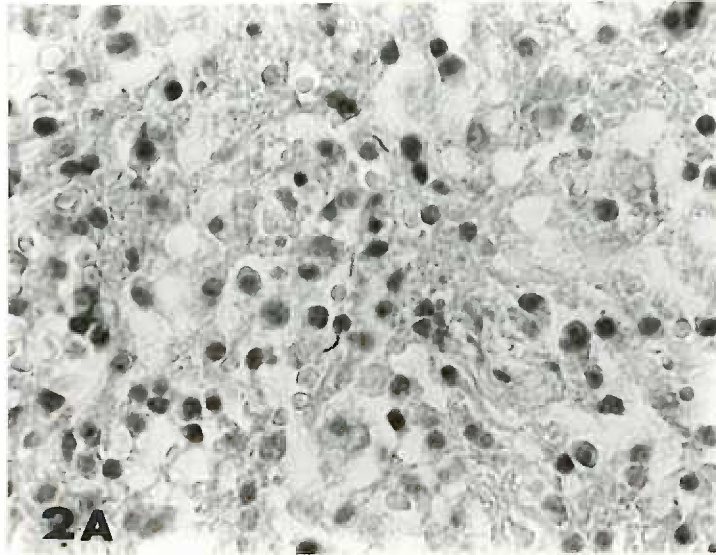
PLATE II

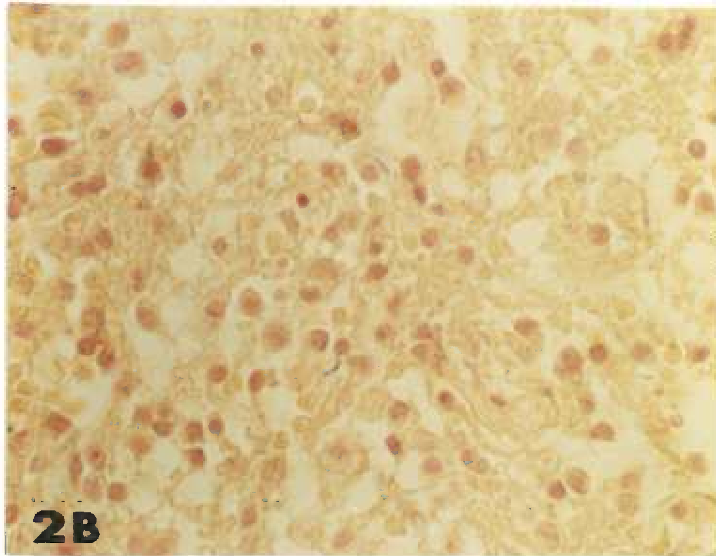
Bacteria and Associated Inflammation in Granulomas

2A Two curved, rod-shaped organisms present in the central portion of a granuloma. Both organisms appear to be extra-cellular in location. In the same general area, but not shown in the photograph, were several cocci. (Black and white print of B & B stained section 2B. 500x)

2B Color photograph of a Brown & Brenn stained section demonstrating the typical blue-black coloration of Gram positive bacteria (500x).

2C The cellular infiltrate in the area of the bacteria in 2B consisting of lymphocytes, plasma cells and histiocytes is shown in this black and white print of an H & E stained section adjacent to 2B (250x)


2A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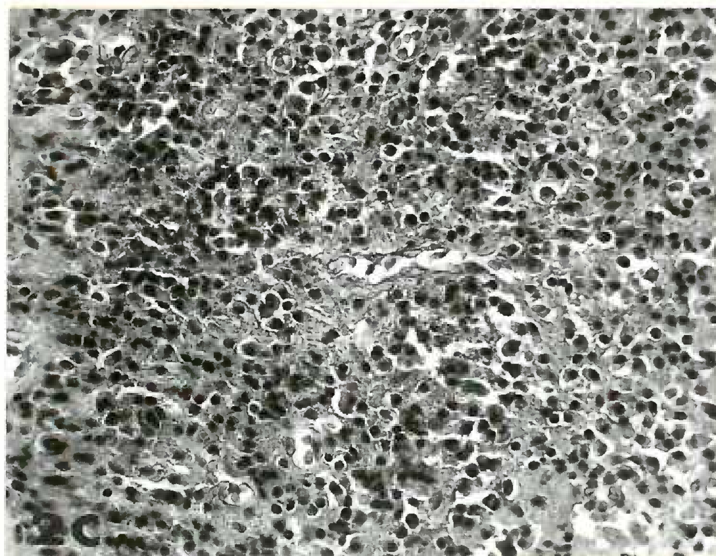

2C

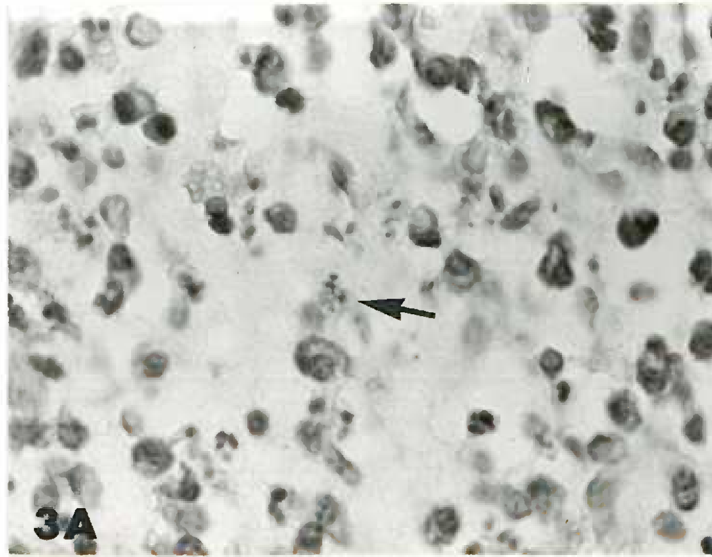
PLATE III

Bacteria and Associated Inflammation in Granulomas

3A Cocci present singly and in focal accumulation. The small focus shows up well (arrow) in this photograph. (Black and white print of a B & B stained section 3B. 500x)

3B Color photograph of a Brown & Brenn stained section depicting the single organism present within a phagocytic cell (arrow). The organisms shown in Figure 3A may also be seen, but the plane of focus in 3B is at the level of the single organism. (500x)

3C Neutrophiles and lymphocytes with lesser numbers of plasma cells and histiocytes are shown in this black and white print of an H & E stained section adjacent to 3C. (250x)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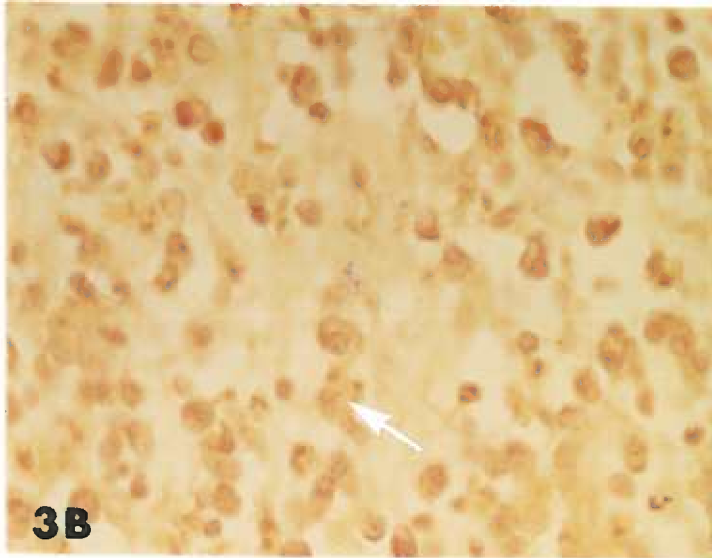

3B

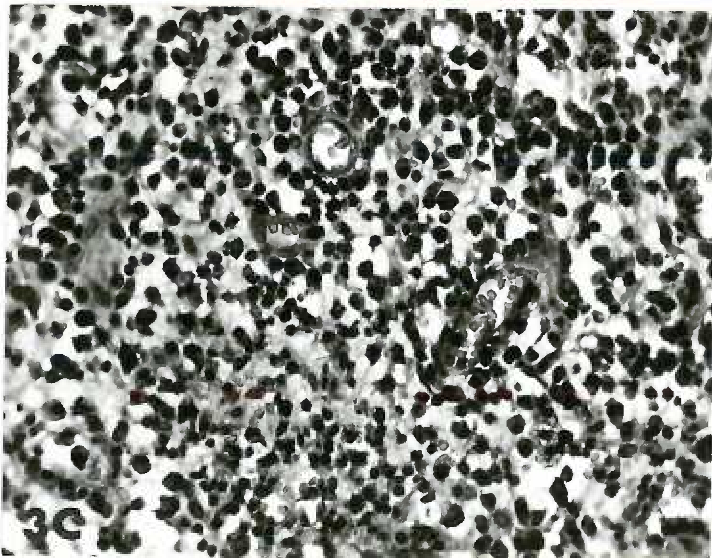

3C

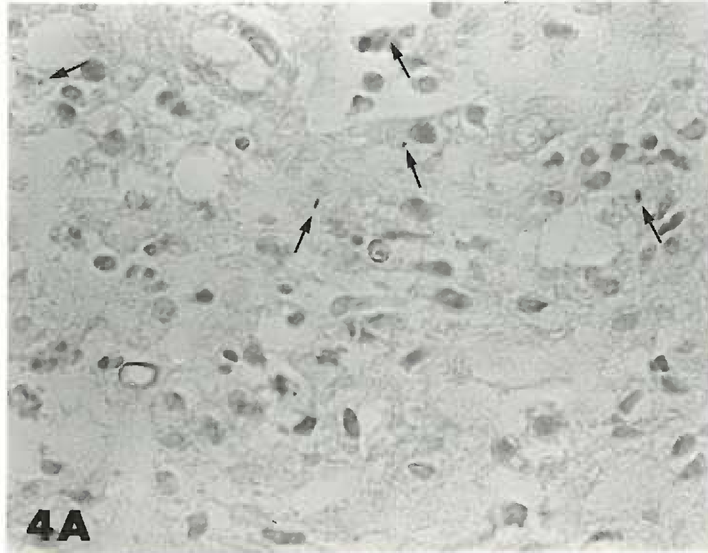
PLATE IV

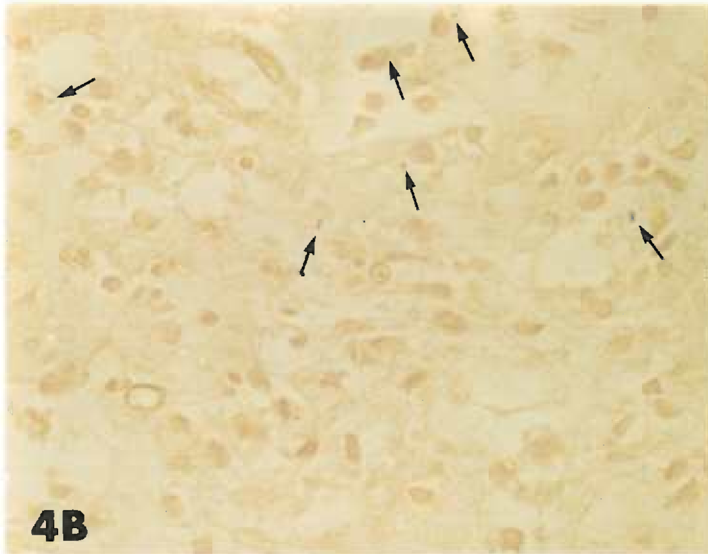
Bacteria and Associated Inflammation in Granulomas

4A The bacilli and cocci in diffuse intra-cellular and extra-cellular locations are sharply defined in this black and white print of B & B stained section 4B. Other bacteria present, not shown by arrows, are not in the same focal plane as those shown by the arrows. (500x)

4B Note the typical Gram-positive staining of the same organisms as shown by arrows in Figure 4A. Color photograph of B & B stained section. (500x)

4C The majority of the cells shown in this black and white print of an H & E stained section adjacent to 4B are lymphocytes and histiocytes, with lesser numbers of neutrophiles and plasma cells. (250x)


4A


4B

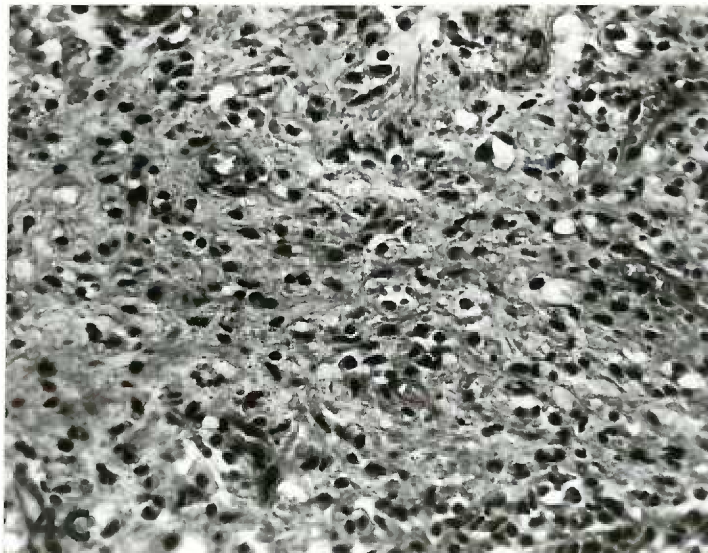

4C

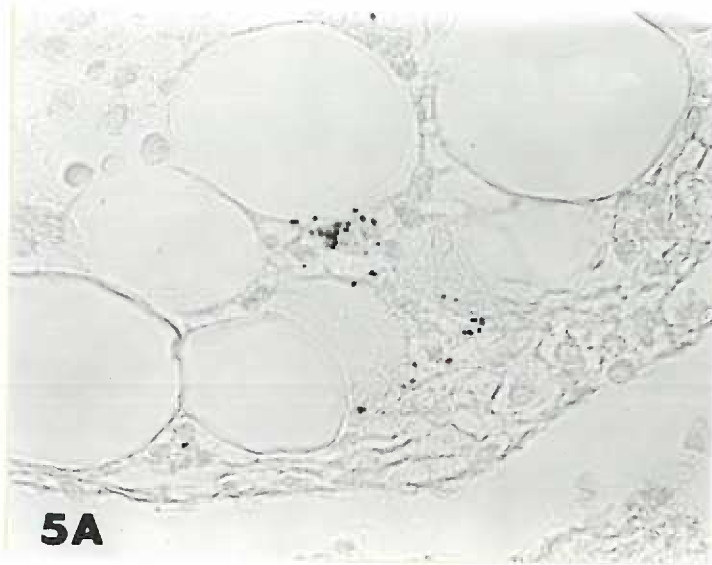
PLATE V

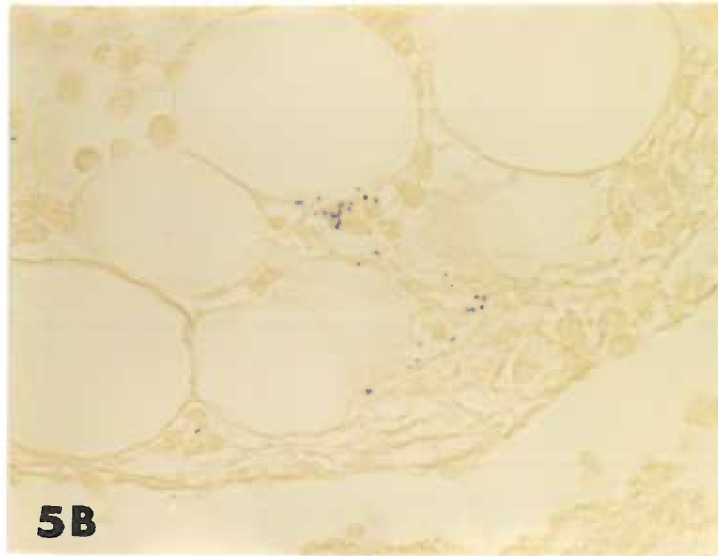
Bacteria and Associated Inflammation in Control Tissues

5A Streptococcus faecalis in the subcutaenous tissues of an inoculated control animal (rat). Notice that in this tissue the organisms do not occur in chains. This same organism, after it was recovered and grown in broth, demonstrated typical streptococcal chain arrangement. (Black and white print of B & B stained section 5B. 500x)

5B Typical Gram-positive and a few Gram-negative cocci can be noted both intra and extra-cellularly in this color print of a B & B stained section. (500x)

5C Lobules of fat are present, between which are noted lymphocytes and neutrophiles with an occasional plasma cell and histiocyte. Black and white print of an H & E stained section adjacent to 5B. (250x)


5A


5B

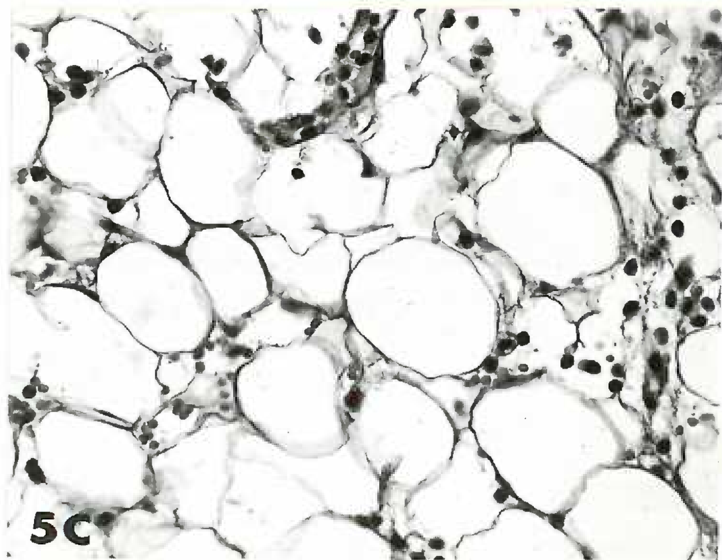
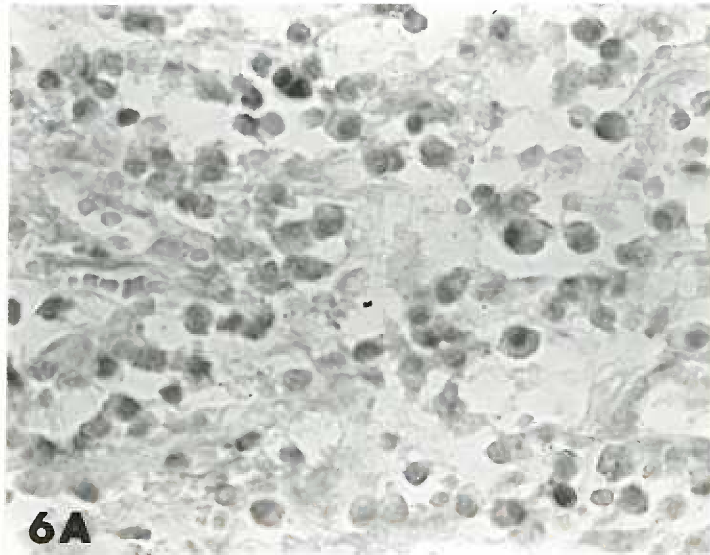

5C

PLATE VI

"Questionable" Bacteria

6A Black and white print of a B & B stained section showing the presence of a bacterium-like object which may represent a diplococcus or a bacillus. The size and shape of this object makes its identity as a bacterium doubtful, hence it was placed in the questionable-for-bacteria group. (500x)

6B Colored photomicrograph of the same area. Note that the object does take the blue-black stain of Gram-positive bacteria. Nevertheless, it was not considered as positive, since all the criteria for such a designation were not fulfilled. (500x)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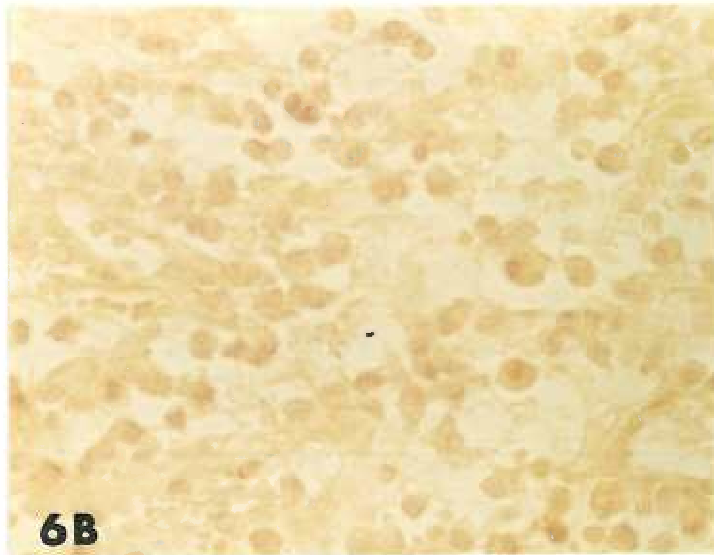

6B

PLATE VII

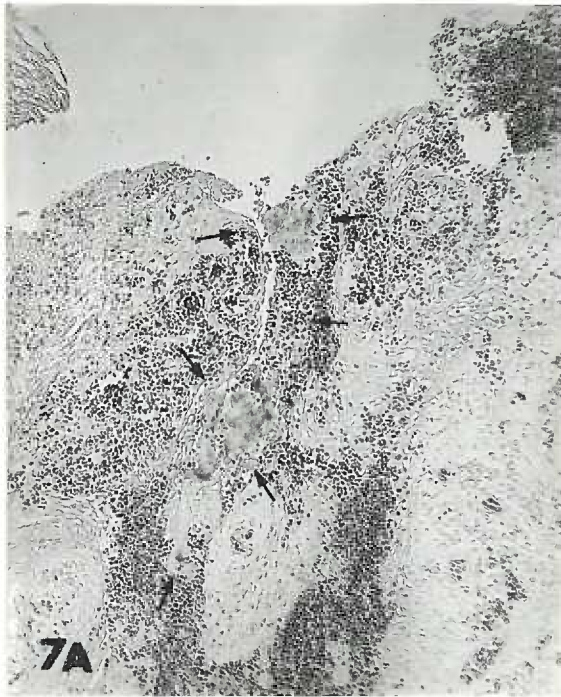
Bacteria in Granulomas

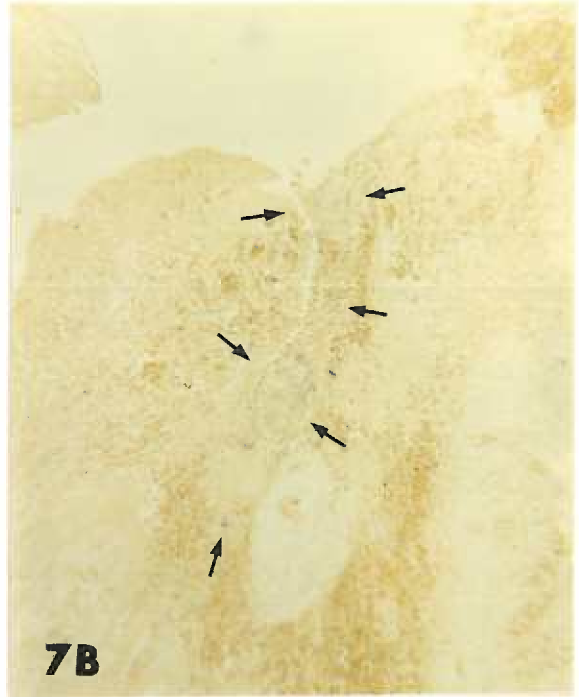
7A Areas outlined with arrows demonstrate colonies of bacteria which -- in this black and white print of the B & B stained section in 7B -- appear to be amorphous necrotic areas. (100x)

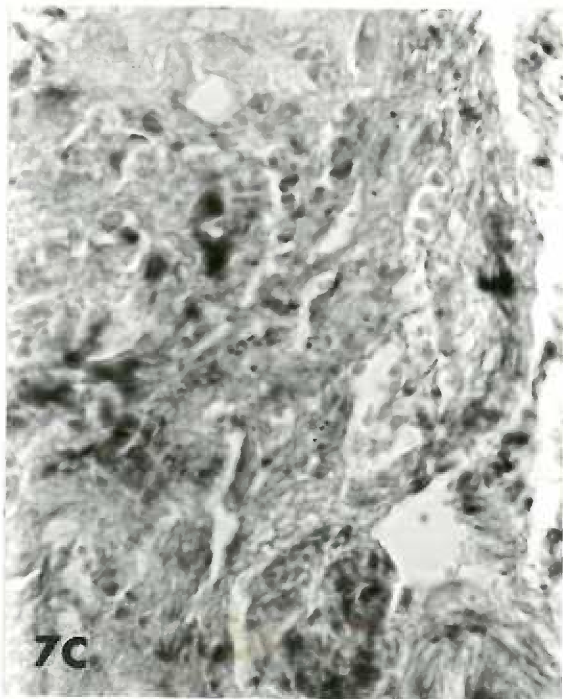
7B Color print of a B & B stained section of a dental granuloma at 100x magnification. Here the blue-black staining unique to Gram-positive bacteria can be seen. Higher magnifications permitted their identification as bacilli and cocci. (100x)

7C Black and white print of a B & B stained section showing the presence of bacteria in diffuse intra-cellular and extra-cellular locations. An occasional bacillus may be seen but the majority are cocci. (500x).

7D Colored photomicrograph of the same area shown in 7C. The contrast in staining between organisms and tissue is well demonstrated (500x)


7A


7B


7C

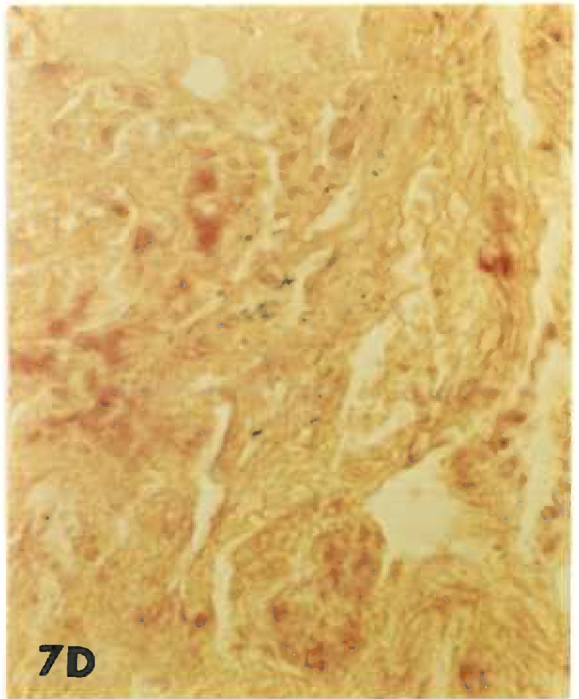
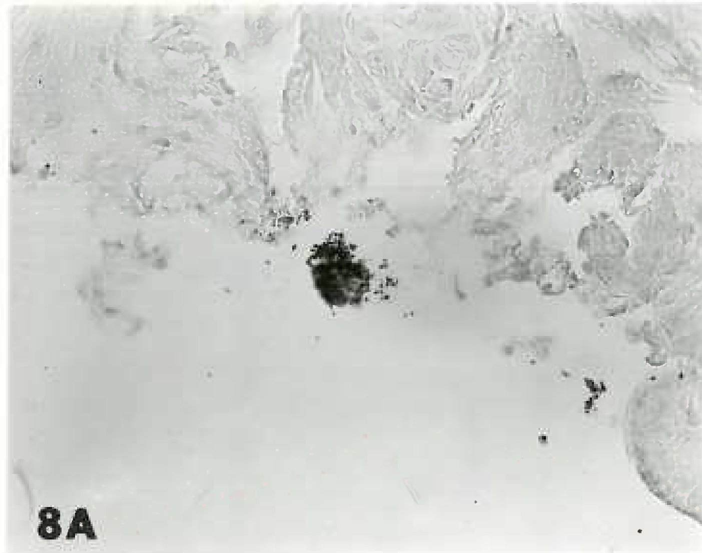

7D

PLATE VIII

Bacterial Contamination of a Granuloma

8A Black and white print of stained section 8B showing an example of contamination. The organisms are present in one large clump with others spread over the outer edge of the tissue section. (250x)

8B Colored photomicrograph of the same area shown in 8A. (The brown line is an artifact present in the colored negative. 250x)


8A

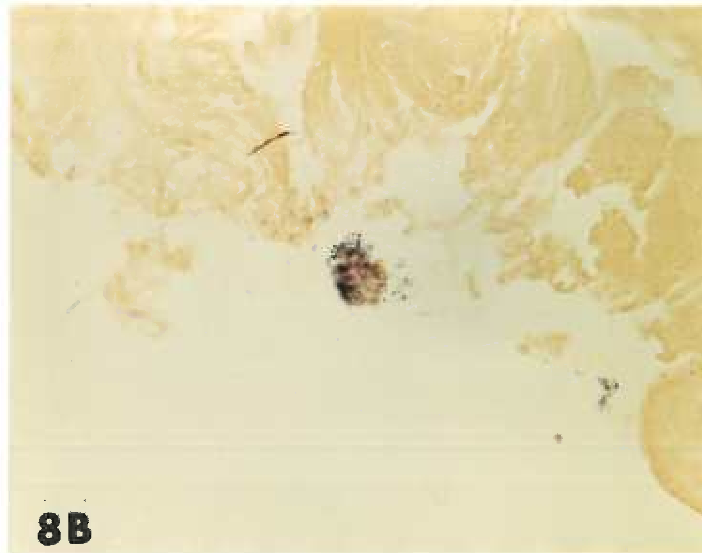

8B

PLATE IX

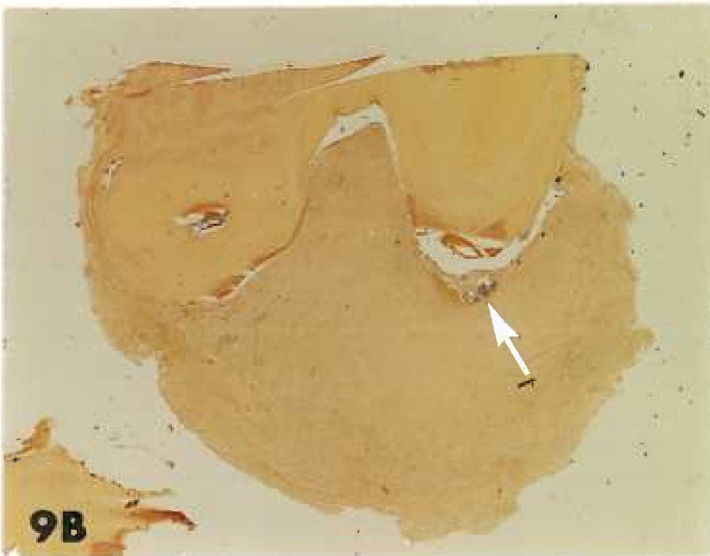
Periapically Infected Dental Granuloma

9A Black and white print of a B & B stained section (9B) showing the presence of a granuloma in toto surrounding the apex of a multirooted tooth. (8x)

9B Colored photomicrograph of the same area shown in 9A. The arrow points to a large bacterial colony. A higher magnification of this area is shown in 9C. Note the location of the organisms within the dental granuloma and near the apex of the root. (8x)

9C Higher magnification of the area shown by arrow in 9B. The organisms consist of cocci, bacilli and thread forms, which are proliferating in a small area of necrotic debris. The surrounding inflammatory reaction revealed the presence of neutrophiles, lymphocytes, plasma cells and histiocytes. (250x)

9A


9B

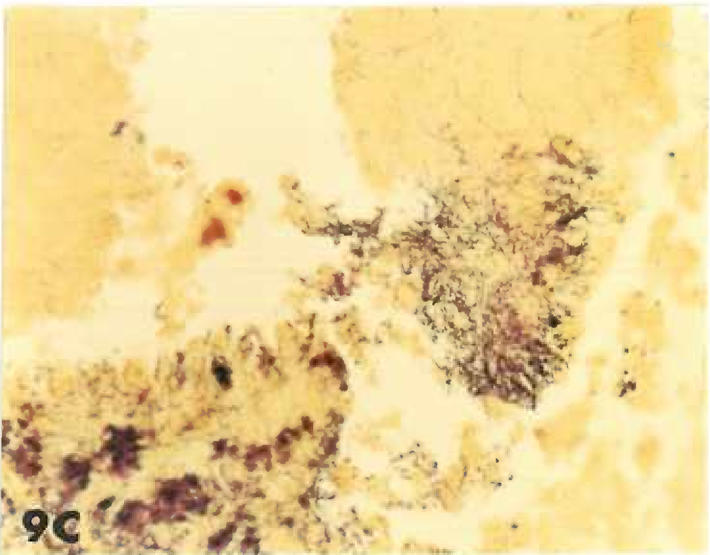
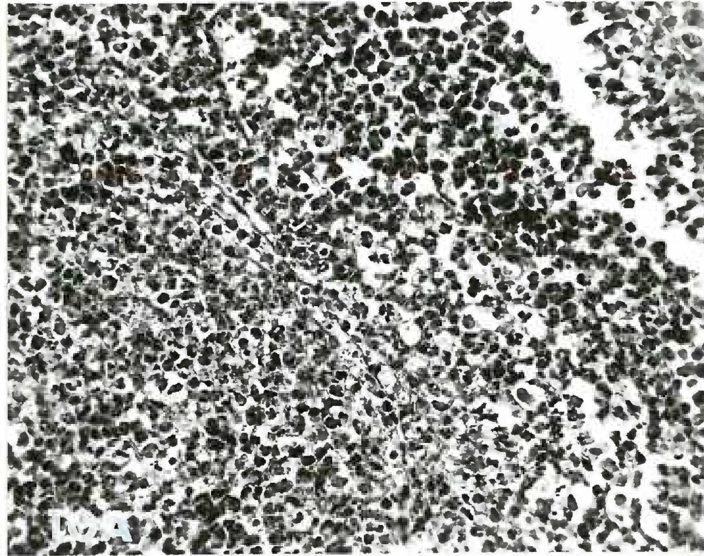

9C

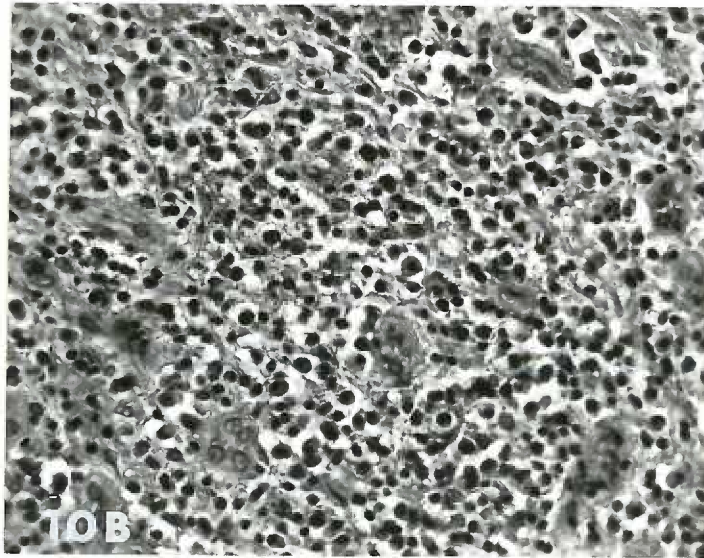
PLATE X

Types of Inflammation

10A ACUTE -- H & E stained tissue section showing a focus of inflammatory reaction in a dental granuloma. Most of the infiltrate consists of neutrophiles, many of which are undergoing disintegration. A few lymphocytes and plasma cells can also be seen. (250x)

10B CHRONIC -- H & E stained tissue section showing a chronic inflammatory reaction in a dental granuloma in which several giant cells are in evidence. (Giant cells were noted in only 8 of the 105 granulomas investigated.) The remainder of the cellular infiltrate consists of lymphocytes, plasma cells and histiocytes. (250x)


10A


10B