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Determination of Antibody Levels in Periapical Lesions Against Sixteen Oral Microorganisms by the ELISA Technique

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Abstract

DETERMINATION OF ANTIBODY LEVELS IN PERIAPICAL LESIONS AGAINST SIXTEEN ORAL MICROORGANISMS BY THE ELISA TECHNIQUE

by

Sammee Lee Jones

The root canal system, which includes the periapical region of the tooth, may become infected by several types of microorganisms which are normally associated with these tissues. A bacteria-specific antibody response may occur at the local level, where various classes of immunoglobulins have been found and reported in the literature. The purpose of this study was to quantitatively measure locally produced immunoglobulin levels in periapical lesion abscess materials and to determine the specific reactivity of these antibodies to several microorganisms which are normal flora, as well as being previously associated with the development and pathogenesis of endodontic infections. Periapical lesions were developed into tissue culture explants. Antibodies produced within these tissues were released into the cell culture fluid and measured, utilizing the ELISA technique. In addition, an ELISA Amplifier System was incorporated into the assay, greatly increasing the sensitivity of detection of antibody. The data indicate that localized immunoglobulins of the IgG, IgA, and IgM classes were released from these tissues, and that specific reactivity against sixteen oral microorganisms was observed, indicating that these infections are probably multibacterial in nature and not caused exclusively by a single organism.

LOMA LINDA UNIVERSITY

Graduate School

DETERMINATION OF ANTIBODY LEVELS IN PERIAPICAL LESIONS AGAINST SIXTEEN ORAL MICROORGANISMS BY THE ELISA TECHNIQUE

by

Sammee Lee Jones

A Thesis in Partial Fulfillment of
the Requirements for the Degree Master
of Science in Microbiology

June 1988

Each person whose signature appears below certifies that this thesis in his opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.

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INTRODUCTION

Endodontics is that branch of dentistry which involves the root canal system of a tooth. Each tooth has at its center the pulp, living tissue which provides the blood and nerve supply to support it. The periapical region is around its tip, between the root and its place of insertion into the jawbone. Any of these areas may become infected and result in pulpitis, periodontitis, or gingivitis (an early stage of periodontitis), conditions which are all characterized by acute pain and inflammation.

A periapical abscess, or "lesion", is an area of dense inflammatory response which results in destruction of the connective tissue and bone supporting the tooth. In the United States, more teeth are lost as a result of periodontal infections than due to dental caries (Russell, 1968). A less common, but more important, consideration is that those bacteria which are involved in the formation of a lesion may also cause a generalized bacteremia. This may lead to potentially life-threatening consequences in rheumatic heart disease patients and those who develop bacterial endocarditis (Joklik, Willett, and Amos, 1984). The bacteria generally believed to be etiologically involved in the development of periodontitis are indigenous oral microorganisms, previously implicated in the carious process, which gain access to the periapical region through one or more of the following ways:

1. via the root canal system (Cochrane, 1969; Morse, Lasater, and White, 1975; Torabinejad and Bakland,

1978).

2. from closely associated infected tissues, as in the case of pulpitis (Keudall, Conte, Fujimoto, Ernest, and Berry, 1976).
3. from dental plaque, an accumulation of oral bacteria, mucus, and food particles shown to be the source of bacteria in gingivitis (Berglund, 1971).
4. via exposure of the periodontal tissue to other oral bacteria as a result of physical damage to the tooth or surrounding area.

The identification of these microorganisms has been investigated, with an estimated 200 or more species of oral bacteria known to inhabit these areas (Holt, Ebersole, Felton, Brunsvold, and Kornman, 1988). Isolation studies from the periapical region as well as from lesions have shown that the majority of these organisms are anaerobic. Of this group, gram negative, rod-shaped bacteria are the most common and include:

- several species of the genus Bacteroides, such as B. intermedius (Slots, Bragd, Wikstrom, and Dahlen, 1985), B. endodontalis, B. gingivalis, and B. oralis (Williams, McCann, and Schoenknecht, 1983).
- Fusobacterium nucleatum, which has been found to be a part of the normal flora of the root canal and periapical region (Melville and Birch, 1967), as well as a frequent isolate from lesions (Williams et. al., 1983).

- Actinobacillus actinomycetemcomitans; closely related to adult periodontitis and the accepted etiological agent of Localized Juvenile Periodontitis (Slots et. al., 1985; Slots, 1986).

Gram positive anaerobic rods have also been isolated, though not as frequently. These include Actinomyces israelii and Actinomyces odontolyticus (Oguntebi, Slee, Tanzer, and Langeland, 1982).

Gram positive anaerobic cocci include Peptostreptococcus micros (Oguntebi et. al., 1982) and three species from the genus Streptococcus (viridans group): S. intermedius, S. intermedius-MG, and S. sanguis, all of which are also normal flora of the root canal and periapical region (Melville et. al., 1967).

The gram negative diplococcus Veillonella sp. is part of the normal flora and has also been implicated in pulpitis (Falkler, Martin, Tolba, Siegel, and Mackler, 1987); however, their isolation from an actual abscess has not yet been reported.

Aerobic organisms, which are easier to isolate because of their less stringent O_2 requirements, were once thought to be the most dominant of the oral microorganisms. A role in periodontal disease etiology has been described for the gram positive cocci Streptococcus mutans (Melville et. al., 1967), another bacterium from the viridans-group streptococci, and Staphylococcus epidermidis (Williams et. al., 1983), a common skin bacterium and isolate. Lactobacillus casei, a gram positive rod, is a normal flora organism (Melville et. al., 1967) and has been cited in the induction of periodontitis (Falkler et. al.,

1987).

In addition to reports of isolation from oral lesions, many studies have been done to determine the mechanisms by which these bacteria induce their immunopathogenic effects on the host. Organisms which invade and colonize the periapical tissues stimulate a variety of immune response reactions, with the consequences of these being a breakdown of soft tissue and bone which permits further penetration by these organisms. This may propagate a cycle which can continue until the organisms have been removed or the tooth is lost (Torabinejad et. al., 1978; Slots, 1986). Periodontitis is associated with a dense infiltration of immunoreactive cells (Taubman, Ebersole, and Smith, 1981), the predominant types being T- and B-lymphocytes and B cell-derived plasma cells, whose primary function is the production of antibodies (Liljenberg and Lindhe, 1980). Also found are phagocytic cells (e.g. polymorphonuclear cells and macrophages), mast cells, and other leukocytes whose exact role has not been fully elucidated (Genco and Slots, 1984). The microorganisms and these components may interact in several different immune pathways:

1. Antigen-antibody complexes
   - binding of antibody to bacteria (i.e. antigen) allows immediate neutralization of antigen and may block further attachment and colonization (Genco et. al., 1984).
   - complexes involving immunoglobulins of the IgG or IgM classes may bind complement, activating the classic pathway whereby phagocytic cells are attracted and

release lysozymes which break down tissue (Golub and Spitznagel, 1965).

2. Aggregated IgG
   - may act like an immune complex to trigger the complement sequence (Platt, Crosby, and Dalbow, 1970).
3. Lymphoproliferation of T cells (hypersensitivity)
   - the presence of bacteria may stimulate proliferation of previously sensitized T cells, which release lymphokines which can destroy tissue (Platt et. al., 1970; Torabinejad et. al., 1978).
4. Lymphoproliferation of B cells
   - bacteria may stimulate a non-specific proliferation of B cells which are also capable of producing and releasing lymphokines (Mackler, Altman, Wahl, Rosenstreich, Oppenheim, and Mergenhagen, 1974; Genco et. al., 1984).
5. Bacterial endotoxins (lipopolysaccharide)
   - shown with Bacteroides gingivalis to be mitogenic for lymphoproliferation (Genco et. al., 1984).
   - may trigger the alternate complement pathway, its end result also involving tissue damage (Snyderman, Gewurz, and Mergenhagen, 1968).
   - these toxins have been shown with B. melaninogenicus to promote bone resorption by osteoclasts (Hausman, Raisz, and Miller, 1970).

Identification of the immunogenic bacteria is necessary to further

understand these mechanisms. It is understood that bacteria-specific antibodies play a significant role in the immunopathology of periodontitis (Taubman et. al., 1981). Although diagnosis of this disease is made based on clinical criteria, studies are now underway to determine the predictive and/or prognostic value of antibody assays (Taubman et. al., 1981). Their presence in the serum of patients suffering from this condition has been studied based on the concept that serum levels of specific antibodies should be indicative of both the overall immune status of a patient and any amplified response(s) occuring as a result of oral microorganisms involved in an acute or chronic phase of the disease. This relationship has been demonstrated by several researchers for organisms such as:

Actinobacillus actinomycetemcomitans, especially in the case of juvenile periodontitis where increased levels of IgG are detected in approximately 90% of these patients (Genco et. al., 1984).

Actinomyces israelii and Fusobacterium nucleatum, where significantly increased levels of IgG, IgA, and IgM have been found (Tolo and Brandtzaeg, 1981).

Bacteroides gingivalis, which promotes high levels of IgG in adult periodontitis, although levels are low or normal in juvenile periodontitis, where this organism is rarely isolated (Slots, 1986).

B. intermedius is also associated with significantly high serum IgG levels (Slots, 1986). These antibodies have also been detected in saliva, particularly secretory IgA (Taubman et. al., 1981).

Another important consideration in fully understanding the nature

of the humoral response is where these specific antibodies are actually produced by plasma cells. Whereas the presence of these cells in periapical lesions has been verified by researchers, on-site synthesis by localized plasma cells is supported by preliminary evidence only. Naidorf (1975) has demonstrated, using granulomatous periapical tissue, localized antibodies which he suggests are derived from the lesions themselves, as opposed to serum exudate associated with the tissue. In a study by Falkler and co-workers (1987), cell cultures of extracted pulp tissues were found to contain antibodies specifically reactive to endodontically-implicated microorganisms including: Streptococcus mutans, Lactobacillus casei, Actinomyces israelii, Peptostreptococcus micros, and Veillonella parvula. They also provide some evidence which suggests local synthesis of these antibodies. In another tissue culture study involving the gingiva, Berglund (1971) used a protein synthesis-inhibitor to demonstrate the de novo synthesis of antibody to Fusobacterium nucleatum.

This study was undertaken in order to determine the levels of locally-produced antibodies found in periapical tissues with specific reactivity to a panel of 16 oral microorganisms believed to be the etiological agents of endodontic periapical abscesses.

MATERIALS AND METHODS:

Periapical Lesions:

The tissues used in this study were obtained from patients who were treated for chronic periapical lesions by therapeutic removal of abscess material. A total of thirty-two lesions were collected. Fifteen of these were provided by Loma Linda University School of Dentistry (Department of Endodontics) and the Faculty Dental Offices. An additional seventeen lesions were provided by Mahmoud Torabinejad, D.M.D., M.S.D. (Upland, CA) from private endodontic practice. Lesions were transferred directly from each patient to RPMI-1640 cell culture medium supplemented with 10% fetal calf serum and 0.05 mg of gentamycin per ml. As soon as possible after their extraction, the lesions were placed into a sterile petri dish and aseptically sectioned into pieces approximately 2 mm in diameter. Each piece was placed into one well of a 24-well cell culture plate (Costar, Cambridge, MA) and covered with 0.5 ml of fresh RPMI medium. The plates were then covered and placed into a CO_2-incubator at 37°C for approximately seven days. After incubation, the cell culture supernatents from each lesion were collected, pooled, and stored at -20°C, after being aliquoted into replicate samples. Twenty of these samples were subsequently used in the actual test.

Microorganisms:

A panel of sixteen oral microorganisms were selected for the determination of specific antibody reactivity, based on their previous association with the development of endodontal diseases. The following

organisms were chosen: (*American Type Culture Collection)

1.	Actinobacillus actinomycetemcomitans	ATCC*29522
2.	Actinomyces israelii	ATCC 12102
3.	Actinomyces odontolyticus	ATCC 17929
4.	Bacteroides endodontalis	ATCC 35406
5.	Bacteroides gingivalis	ATCC 25611
6.	Bacteroides intermedius	ATCC 33277
7.	Bacteroides oralis	ATCC 33269
8.	Fusobacterium nucleatum	Clinical Isolate
9.	Lactobacillus casei	ATCC 4646
10.	Peptostreptococcus micros	ATCC 33270
11.	Staphylococcus epidermidis	ATCC 14990
12.	Streptococcus intermedius	Clinical Isolate
13.	Streptococcus intermedius-MG	Clinical Isolate
14.	Streptococcus mutans	Remel Laboratories LRA-280281
15.	Streptococcus sanguis	Clinical Isolate
16.	Veillonella parvula	ATCC 10790

All species of the genus Bacteroides, as well as A. israelii, F. nucleatum, P. micros, S. intermedius, S. intermedius-MG, and S. sanguis were subcultured onto Brucella blood agar plates (Anaerobe Systems, Santa Clara, CA) and placed into a GASPAK anaerobic jar (BBL Microbiology Systems) along with a GASPAK H_2-CO_2 generator (BBL, cat.no. 71040). The plates were incubated at 37°C for 7-10 days, until moderate growth could be observed on the plates. Stocks of these

bacteria were maintained in meat broth culture (Difco Laboratories, Detroit, MI) by subculturing 0.1 ml of cell suspension into fresh broth every 10 days.

A. actinomycetemcomitans, A. odontolyticus, and V. parvula were grown in tightly-capped tubes containing Thioglycolate broth supplemented with vitamin K and hemin (Anaerobe Systems, cat.no. AS-801), an anaerobic medium, and incubated at 37°C for approximately 3-5 days, until a turbid suspension appeared.

L. casei, S. epidermidis, and S. mutans were inoculated into tubes of Todd-Hewitt broth (Difco) and incubated at 37°C for 3-5 days. After the plated bacteria had been grown as described, they were removed from the agar with sterile, cotton-tipped applicator sticks and stirred into 30 ml of 0.5% formalin in Tris buffered saline (pH 7.5). Broth suspensions of bacteria were also added to 30 ml of formal saline, and all the bacteria thus collected were stored at 4°C for 12-18 hours for effective killing. Next, the microorganisms were centrifuged (International Equipment Co., CENTRA-7R) at 4°C for 20 minutes at 20,000 x g to form a pellet. The supernatents were discarded. Each pellet was resuspended in 20 ml of carbonate coating buffer, 0.1M $NaCO_3$ + 0.20% NaN_3 (pH 9.6), and centrifuged again to wash the bacteria. This process was repeated two more times, so that the bacteria were washed a total of three times. After the final wash, each pellet was resuspended in 10 ml of carbonate buffer and a sample 1:10 dilution of each was made and its optical density at 580 nm was measured (Bausch and Lomb: Spectronic-2000). The dilution factor for each species was

adjusted to obtain an O.D. reading of approximately 0.238, equalling roughly 10^8 cells per ml. This concentration of bacteria has been shown to provide optimum bacterial coating on the wells and also to promote optimum removal of antibody reactivity from human serum (Ebersole et. al., 1980).

Enzyme-linked Immunosorbent Assay (ELISA):

For this study, we have utilized the ELISA procedure which has proven to be very useful for both quantitative and qualitative antibody determinations (Ebersole et. al., 1980; Taubman et. al., 1981; Falkler et. al., 1987). The ELISA has several advantages over other techniques, as follows:

- the ELISA is simple to perform and is well-suited for automation.
- it provides sensitivity comparable to radioimmunoassay, yet no radiation hazards are involved (Taubman et. al., 1981).
- it allows testing of small amounts of biological fluids and secretions (Taubman et. al., 1981).
- it allows differentiation of immunoglobulin classes.
- reagents are relatively inexpensive and may be stored for several months.
- results are digitally measured and, therefore, objective.

The concept of this assay is based on the fact that an antigen (e.g. microorganisms) will bind to a solid medium such as a tube or the wells of a microtiter plate. An antibody molecule may be conjugated to

reactive enzymes without adverse effects on the activities of either the antibody or enzyme. Therefore, an antigen may be coated onto the wells of a microtiter plate, and a test fluid can then be added. Any specific antibody present will attach to the antigen. After the excess (unreacted) antibody has been washed away, an enzyme-conjugated antiglobulin may be added, which will bind to the antigen-specific antibody complex fixed in the wells. Again, excess antiglobulin is washed away. Upon addition of an appropriate substrate for the enzyme, a color change will occur which may be measured by a spectrophotometer. The amount of color produced will be proportional to the concentration of specific antibody present in the test liquid.

Procedure:

In the first step of the ELISA, 200 ul of each bacterial suspension (previously diluted to an O.D. of 0.238 at 580 nm) in the carbonate coating buffer (pH 9.6) were added to the wells of a 96-well polystyrene microtiter plate (Dynatech Laboratories, Immulon-2, cat.no.011-010-3450). The plates were covered and incubated at 37°C for 90 min., after which they were incubated at 4°C for 12-18 hours. The bacterial suspensions were aspirated from the wells, which were then washed three times with 0.1M Tris buffered saline (pH 7.5) containing 0.05% (vol/vol) Tween-20 (Sigma Chemical Co., cat.no. P-1379). The wells were filled with 1% (wt/vol) bovine serum albumin (Sigma, cat.no. A-4503) in TBS-Tween and kept at 25°C for 90 minutes so that all antigen-free sites on the wells were tied up. This solution was discarded from the wells, which were washed again three times in

TBS (pH 7.5).

One hundred ul of various dilutions of the tissue culture fluid (TCF) samples were added, in duplicate, to the wells. TCF dilutions of 1:5, 1:7, 1:10, or 1:15 were made in TBS, based on the total volume of supernatent collected from each lesion, in order to obtain the minimum dilution which would still provide sufficient fluid for the assay. The plates were re-covered and placed into a CO_2-incubator at 37°C for 12-18 hours. After this incubation, the TCF was removed. The wells were again washed three times with TBS, followed by the addition of appropriate dilutions of the enzyme conjugates. Dilutions were made based on previous titrations for optimal conjugate concentrations performed by William Payne, Determination of Antibody Levels in Endodontic Patients Against Eleven Microorganisms Using IFA and ELISA Techniques. Thesis for Master of Science Degree, 1987:

Assay for specific IgG:

- Alkaline phosphatase-conjugated, affinity-purified goat anti-human IgG (Cooper Biomedical-Cappel, cat.no. 8601-0121) was diluted 1:500 in TBS and 200 ul per well were added.

Assay for specific IgA:

- Alkaline phosphatase-conjugated, affinity-purified goat anti-human IgA (cat.no. 8601-0021) was diluted 1:1000 in TBS and added, at 200 ul per well.

Assay for specific IgM:

- Alkaline phosphatase-conjugated, affinity-purified goat

anti-human IgM (cat.no. 8601-0201) was diluted 1:5000 in TBS and added to the wells as for IgG and IgA.

After the addition of conjugate, the covered plates were again placed into the CO_2-incubator at 37°C for 90 minutes. The conjugate reagents were then removed and the plates were washed four times in the final wash buffer, 0.05M Tris-HCl + 0.15M NaCl (pH 7.5). The substrate for alkaline phosphatase was provided in an ELISA Amplification System (Bethesda Research Laboratories, cat.no. 9689SA. For details concerning the enzyme system, refer to product insert.). This kit consists of two pre-formed reagents: the "substrate" and the "amplifier" solution. In the first step, 50 ul per well of the substrate is added to all the wells and allowed to incubate for 15 minutes at 25°C. During the second step, the substrate remains in the wells, and 50 ul per well of the amplifier mixture is then added. The plates are incubated at 25°C for an additional 15 minutes, after which the reaction is quenched by adding 0.3M H_2SO_4, 50 ul per well. The absorbance values of the wells are then read at 490 nm by an ELISA reader (BIO-TEK Instruments, model EL-308).

Throughout the assay, two types of control systems were used. In the first, a mixture of sterile bacterial growth media was substituted in place of the bacterial suspensions used to pre-coat the wells. This served to remove from the test values any non-specific binding of either the TCF antibody or the enzyme conjugates to the solid phase. As a second control, TBS was added in place of the TCF samples in order to compensate for any non-specific binding of conjugate to the

bacterial coat.

Selection of Test Samples:

Of the thirty-two TCF samples collected, as previously described, twenty of these were actually tested in our ELISA. Their selection was based on a pre-screening ELISA procedure designed to detect the total amount of immunoglobulin in the fluid (this assay was performed by Christine A. Molinaro, M.S., Loma Linda University). Briefly, microtiter plates (Dynatech) were coated, 50 ul per well, with an appropriate dilution of goat anti-human immunoglobulin antiserum in TBS (pH 7.5) and incubated overnight at 37°C. Nonspecific binding sites were blocked with 1% ovalbumin in TBS, at 37°C for 30 minutes. Wells were washed four times with running tap water and allowed to air dry. Twenty-five ul per well of nondiluted TCF was added, in triplicate, followed by a 60 minute incubation at room temperature. The wells were washed again as described. Next, horseradish peroxidase-conjugated goat anti-human immunoglobulin antibody, diluted 1:3000 with 1% ovalbumin in TBS + 0.05% Tween-20, was added to the wells for 60 minutes at 25°C. This reagent was then discarded, and the wells were washed one time with 150 ul of TBS-Tween per well, followed by six additional washes under running water. The substrate added, at 100 ul per well, was O-phenylenediamine (Abbot Laboratories), 10 mg per 25 ml of phosphate-citrate buffer (pH 5.0) + 10 ul of 30% H_2O_2. Plates were kept at 25°C, in the dark, until the reaction was quenched with 25 ul of 4N H_2SO_4 per well. Absorbance was read at 492 nm (Biorad spectrophotometer). Those test samples that contained more than 1 ug

of immunoglobulin per ml were subsequently used in our ELISA procedure.

Determination of a Standard Curve:

In order to produce a standard curve for IgG, IgA, and IgM from which the test O.D. values could be converted to ugs per ml, it was first necessary to perform the ELISA procedure using standard concentrations of these antibodies.

For IgG:

The wells were coated with 200 ul per well of goat anti-human IgG antibody (Cooper Biomedical, cat.no. 0601-0121) diluted to 15 ug per ml in carbonate coating buffer. Plates were incubated for 90 minutes at 37°C, followed by overnight refrigeration (4°C). The wells were washed three times with TBS-Tween, and non-antibody coated sites were blocked with 200 ul per well of 1% bovine serum albumin in TBS-Tween. After 90 minutes at 25°C, the wells were washed again three times, with TBS. Serial two-fold dilutions of human IgG (cat.no. 6001-0080), ranging from 25 ug per ml down to 0.003 ug per ml in TBS were made. 200 ul per well of each dilution were added, in triplicate, this time for an overnight incubation at 37°C. After the incubation, the wells were again washed three times, and 200 ul per well of alkaline phophatase-conjugated goat anti-human IgG, diluted 1:500 in TBS, were added to all wells. The next incubation of 90 minutes at 37°C was followed by four washes in the final wash buffer, before the addition of the substrate-amplifier system reagents as previously described. Absorbance values at 490 nm were used to plot a standard curve which correlated O.D. values to IgG concentration. Blanking wells contained TBS in place of

IgG.

For IgA:

The same procedure was done as for IgG, with the exception that the wells were coated with goat anti-human IgA (cat.no. 0601-3141), the serial dilutions of human IgA (cat.no. 6001-0020) ranged from 25 ug per ml down to 0.10 ug per ml, and the alkaline phosphatase-conjugated goat anti-human IgA was diluted at 1:1000. Blanking wells were the same as for IgG.

For IgM:

The wells were coated with goat anti-human IgM (cat.no. 0101-0201); human IgM (cat.no. 6001-1590) was serially diluted from 25 ug per ml down to 0.10 ug per ml; and alkaline phosphatase-conjugated goat anti-human IgM was diluted 1:5000. Blanking wells were as for IgG and IgA.

RESULTS

The data show that immunoglobulins of the IgG, IgA, and IgM classes were present in the periapical tissue samples at levels detectable by the ELISA technique. IgG was found in all 20 of the lesions tested. Also, for every test sample, IgG antibody reacted with at least 14 out of the 16 microorganisms utilized (Table 1a., 1b.). Those bacteria for which IgG was found in the highest levels include:

A. israelii

S. intermedius

P. micros

F. nucleatum

S. mutans

B. endodontalis

A. israelii, S. intermedius, P. micros, and F. nucleatum were the four bacteria with the uppermost reactivity levels. Figure 1 illustrates the average IgG levels which were found for each test bacterium.

IgA was also found to be present in all samples tested. In this case, each lesion was shown to contain antibodies of this class with specific reactivity to at least 13 out of the 16 microorganisms (Table 2a., 2b.). The bacteria which caused the highest IgA levels to develop include:

A. israelii

S. intermedius

P. micros

S. sanguis

S. mutans

B. gingivalis

Again, A. israelii, S. intermedius, and P. micros were among the four most reactive species. S. sanguis was the fourth member of this group for IgA. Figure 2 illustrates the IgA levels which were found.

Tables 1a. through 3b.: Levels of Specific Antibodies Against Sixteen Oral Microorganisms by ELISA.

A. act. = Actinobacillus actinomycetemcomitans
A. isr. = Actinomyces israelii
A. odo. = Actinomyces odontolyticus
B. end. = Bacteroides endodontalis
B. gin. = Bacteroides gingivalis
B. int. = Bacteroides intermedius
B. ora. = Bacteroides oralis
F. nuc. = Fusobacterium nucleatum
L. cas. = Lactobacillus casei
P. mic. = Peptostreptococcus micros
S. epi. = Staphylococcus epidermidis
S. int. = Streptococcus intermedius
S. intM = Streptococcus intermedius-MG
S. mut. = Streptococcus mutans
S. san. = Streptococcus sanguis
V. par. = Veillonella parvula

TABLE 1a. IgG Antibody Levels Against Sixteen Oral Microorganisms by ELISA*

TCF#	A.act.	A.isr.	A.odo.	B.end.	B.gin.	B.int.	B.ora.	F.nuc.
7	0.119	0.780	0.138	0.248	0.265	0.120	0.117	0.900
8	0.144	0.217	<0.00	0.298	0.270	0.313	0.249	0.230
11	0.135	0.398	0.112	0.144	0.790	0.130	0.130	0.288
13	0.180	0.925	0.124	0.955	0.275	0.745	0.800	0.840
14	1.970	1.800	0.350	1.870	0.462	0.560	0.314	≥2.00
16	1.350	2.450	0.623	0.441	0.720	0.900	1.163	≥3.00
24	1.000	1.700	0.267	1.160	0.525	0.825	0.285	≥2.00
25	0.400	0.515	0.127	0.615	0.132	0.575	0.535	0.840
28	0.240	0.915	0.433	0.715	0.154	0.530	0.368	≥1.00
29	0.178	0.630	0.176	0.268	≥1.40	0.174	0.193	0.469
30	0.288	0.645	0.228	0.515	0.410	ND	1.280	0.955
31	0.260	1.850	0.450	0.340	0.400	ND	0.222	≥2.00
32	0.288	0.585	0.295	0.320	1.030	ND	0.209	0.930
33	0.350	1.470	0.284	1.600	0.388	ND	1.950	≥2.00
34	0.232	1.470	0.282	0.415	0.368	ND	0.280	≥2.00
35	0.167	0.750	0.168	0.750	0.225	ND	≥1.00	0.945
36	ND	0.840	0.365	0.438	0.985	ND	0.800	0.925
37	ND	0.750	0.161	0.350	≥1.00	ND	0.117	0.825
39	ND	0.640	0.130	0.655	0.233	ND	0.335	0.565
40	ND	0.720	0.243	0.780	0.328	ND	0.160	0.490

ND= Not Done

* The wells were coated with the bacteria, incubated with tissue culture fluid, and revealed with an enzyme-linked anti-IgG antibody. The mean absorbance of duplicate samples were tabulated in micrograms per ml.

TABLE 1b. IgG Antibody Levels Against Sixteen Oral Microorganisms by ELISA*

TCF#	L.cas.	P.mic.	S.epi.	S.int.	S.intM	S.mut.	S.san.	V.par.
7	0.114	0.157	0.165	0.570	0.143	0.171	0.368	0.122
8	0.140	0.154	0.145	0.148	0.151	0.151	0.158	0.142
11	0.108	0.205	0.115	ND	0.187	0.210	0.180	0.118
13	0.128	0.975	0.185	0.605	0.438	0.253	0.483	0.290
14	0.275	1.220	0.965	1.940	0.810	0.930	1.300	0.465
16	1.163	1.478	1.800	1.995	1.313	2.205	≥2.22	0.758
24	≥2.00	0.490	≥2.00	≥2.00	1.340	≥2.00	≥2.00	1.500
25	0.120	0.915	0.245	0.970	0.185	0.245	0.440	0.174
28	0.143	≥1.00	0.535	0.785	0.273	0.515	0.780	0.263
29	0.231	0.179	0.227	0.340	0.195	0.294	0.263	0.184
30	0.205	≥2.00	0.273	1.130	0.267	0.365	0.400	0.244
31	0.207	1.640	0.600	0.760	0.645	0.373	0.400	0.378
32	0.248	1.680	0.252	1.890	0.330	0.800	1.030	0.635
33	0.383	0.805	0.490	0.490	0.390	0.450	0.645	0.425
34	0.228	0.424	0.455	0.330	0.419	0.560	0.385	0.415
35	0.233	0.345	0.162	0.288	0.280	0.275	0.500	0.233
36	0.165	0.850	0.435	0.575	0.255	0.150	0.665	0.400
37	0.117	0.535	0.215	0.223	0.143	0.323	0.295	0.163
39	0.116	0.625	0.170	0.665	0.135	0.248	0.230	0.111
40	0.135	0.920	0.348	0.950	0.268	0.278	0.393	0.235

ND= Not Done

* The wells were coated with the bacteria, incubated with tissue culture fluid, and revealed with an enzyme-linked anti IgG antibody. The mean absorbance of duplicate samples were tabulated in micrograms per ml.

Figure 1.: Average Levels of IgG Antibody Detected in Periapical Lesions Against Sixteen Oral Microorganisms by ELISA.

A.a = Actinobacillus actinomycetemcomitans
A.i = Actinomyces israelii
A.o = Actinomyces odontolyticus
B.e = Bacteroides endodontalis
B.g = Bacteroides gingivalis
B.i = Bacteroides intermedius
B.o = Bacteroides oralis
F.n = Fusobacterium nucleatum
L.c = Lactobacillus casei
P.m = Peptostreptococcus micros
S.e = Staphylococcus epidermidis
S.i = Streptococcus intermedius
S.M = Streptococcus intermedius-MG
S.m = Streptococcus mutans
S.s = Streptococcus sanguis
V.p = Veillonella parvula

AVERAGE IgG ANTIBODY LEVELS FROM PERIAPICAL LESIONS AGAINST 16 ORAL MICROORGANISMS BY ELISA

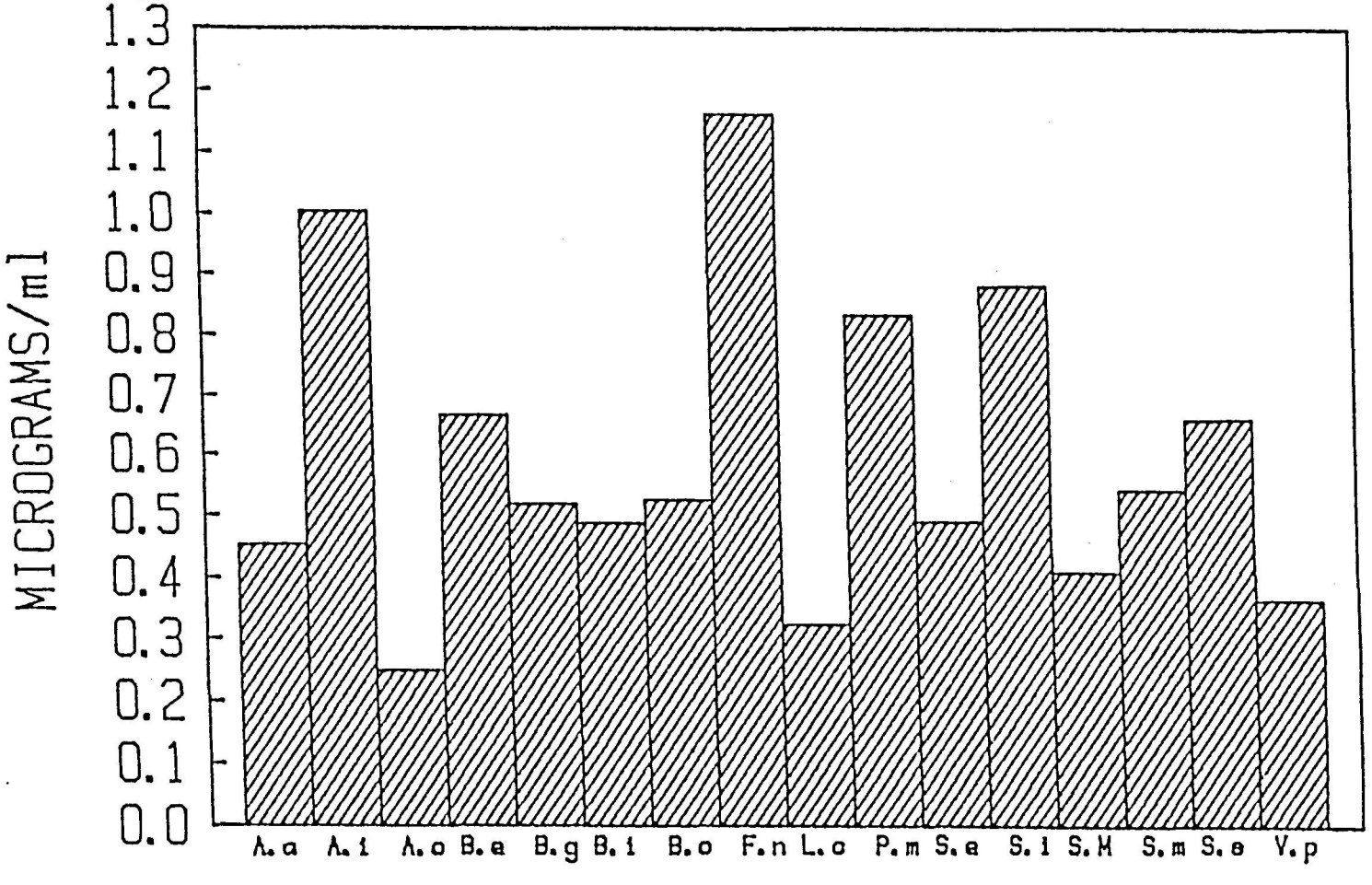


TABLE 2a. IgA Antibody Levels Against Sixteen Oral Microorganisms by ELISA*

TCF#	A.act.	A.isr.	A.odo.	B.end.	B.gin.	B.int.	B.ora.	F.nuc.
7	0.253	0.315	0.253	0.255	0.265	0.255	0.250	0.300
8	0.000	0.354	0.357	0.354	0.354	0.392	0.347	0.350
11	0.248	0.333	0.253	<0.00	0.540	0.250	0.258	0.255
13	0.260	0.478	0.263	0.410	0.283	0.328	0.283	0.295
14	1.250	0.930	0.560	0.535	0.535	0.545	0.500	0.615
16	0.953	1.560	1.160	0.878	1.088	0.840	0.900	1.050
24	0.555	0.950	0.540	0.740	0.630	0.560	0.530	0.635
25	0.260	0.278	0.250	0.250	0.258	0.250	0.260	0.258
28	0.268	0.440	0.270	0.283	0.265	0.278	0.263	0.340
29	0.357	0.368	0.357	0.749	<0.00	0.357	0.364	0.357
30	0.505	0.680	0.530	0.535	0.530	ND	0.580	0.585
31	0.510	0.585	0.525	0.510	0.520	ND	0.500	0.580
32	0.520	0.850	0.550	0.530	1.530	ND	0.550	0.615
33	0.500	0.660	0.540	0.600	0.585	ND	0.635	0.680
34	0.505	0.585	0.530	0.520	0.550	ND	0.525	0.600
35	0.260	0.315	0.260	0.325	0.280	ND	0.340	0.358
36	ND	0.335	0.378	0.283	0.413	ND	0.280	0.400
37	ND	0.285	0.263	0.313	0.850	ND	0.265	0.265
39	ND	0.315	0.303	0.295	0.283	ND	0.275	0.258
40	ND	0.333	0.270	0.465	0.310	ND	0.253	0.315

ND= Not Done

* The wells were coated with the bacteria, incubated with tissue culture fluid, and revealed with an enzyme-linked anti-IgA antibody. The mean absorbance of duplicate samples were tabulated in micrograms per ml.

TABLE 2b. IgA Antibody Levels Against Sixteen Oral Microorganisms by ELISA*

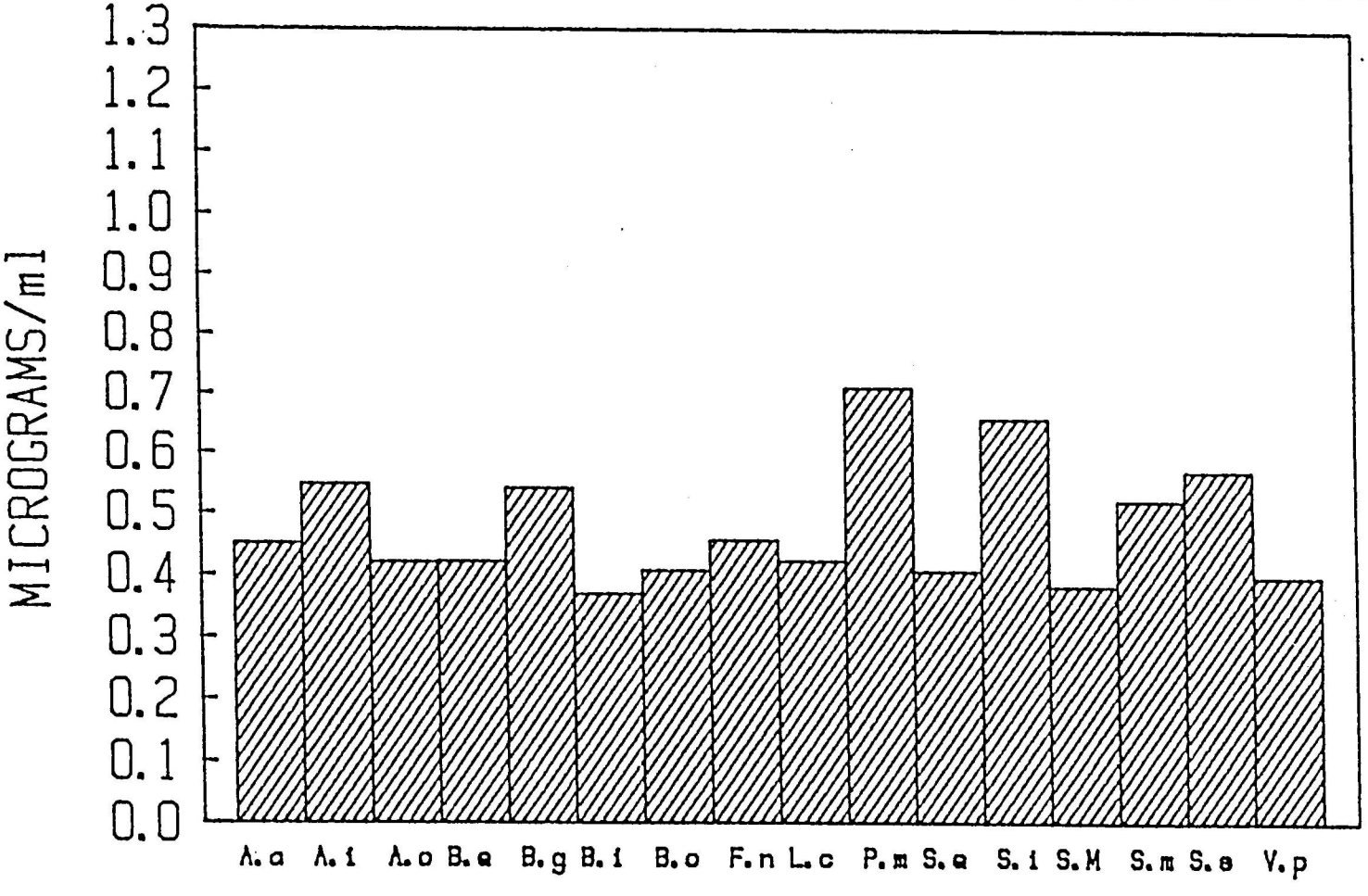
TCF#	L.cas.	P.mic.	S.epi.	S.int.	S.intM	S.mut.	S.san.	V.par.
7	0.250	0.263	0.258	0.278	0.255	<0.00	0.275	0.258
8	≤0.35	0.375	0.347	0.357	0.350	0.368	0.347	<0.00
11	0.250	0.265	0.250	ND	0.250	0.253	0.280	<0.00
13	0.258	0.565	0.270	0.333	0.275	0.263	0.285	0.265
14	0.525	0.670	0.620	0.780	0.600	0.540	0.675	0.520
16	0.953	2.370	0.998	≥2.93	0.945	1.545	2.550	0.915
24	0.990	0.780	0.600	0.885	0.605	1.020	0.815	0.560
25	0.250	0.335	0.255	0.315	0.258	0.268	0.273	0.258
28	0.273	0.825	0.288	0.413	0.288	0.363	0.315	0.268
29	0.357	0.378	0.368	0.378	0.357	0.364	0.382	0.378
30	0.505	1.900	0.570	0.580	0.520	0.670	0.565	0.520
31	0.500	0.760	0.525	0.710	0.520	0.550	0.590	0.525
32	0.585	1.400	0.610	1.620	0.570	1.600	1.120	1.030
33	0.535	0.620	0.550	0.565	0.510	0.585	0.625	0.550
34	0.545	0.570	0.585	0.565	<0.00	0.550	0.595	0.545
35	0.265	0.308	0.248	0.290	0.273	0.265	0.290	0.268
36	0.265	0.380	0.270	0.465	0.265	0.270	0.365	0.268
37	0.258	0.290	<0.00	0.258	0.260	0.260	0.260	0.270
39	0.280	0.400	0.260	0.288	0.260	0.260	0.323	0.260
40	0.275	0.790	0.275	0.465	0.283	0.403	0.420	0.273

ND= Not Done

* The wells were coated with the bacteria, incubated with tissue culture fluid, and revealed with an enzyme-linked anti-IgA antibody. The mean absorbance of duplicate wells were tabulated in micrograms per ml.

Figure 2.: Average Levels of IgA Antibody Detected in Periapical Lesions Against Sixteen Oral Microorganisms by ELISA.

AVERAGE IgA ANTIBODY LEVELS FROM PERIAPICAL LESIONS AGAINST 16 ORAL MICROORGANISMS BY ELISA



For IgM, every lesion produced levels which were demonstratable against at least four organisms from the test group. The overall average for the 20 samples tested was a specific reaction toward 12 of the 16 microorganisms (Table 3a., 3b.). The most reactive bacteria for IgM include:

A. israelii

S. intermedius

S. sanguis

F. nucleatum

B. oralis

A. actinomycetemcomitans

F. nucleatum produced the highest reactivity levels for IgM; whereas S. intermedius produced the second highest levels. Figure 3 illustrates these IgM levels.

For 12 of the 16 microorganisms, levels of IgG against a particular species were found to be greater than the corresponding levels of IgA. Also, for 15 of the 16 species, the levels of IgA toward a single organism were greater than those of IgM for that same organism (see Figures 4 through 7).

TABLE 3a. IgM Antibody Levels Against Sixteen Oral Microorganisms by ELISA*

TCF#	A.act.	A.isr.	A.odo.	B.end.	B.gin.	B.int.	B.ora.	F.nuc.
7	0.270	0.290	<0.00	0.265	<0.00	0.685	0.335	0.265
8	<0.00	0.354	0.385	<0.00	<0.00	<0.00	0.378	<0.00
11	<0.00	<0.00	<0.00	<0.00	<0.00	0.260	0.255	0.255
13	≤0.25	0.290	0.265	<0.00	0.258	<0.00	0.270	<0.00
14	0.510	0.540	0.505	0.505	0.300	<0.00	<0.00	<0.00
16	0.810	<0.00	<0.00	<0.00	0.780	0.765	0.795	<0.00
24	0.540	0.505	0.505	0.505	0.520	≤0.50	0.530	0.540
25	0.295	0.370	0.315	0.275	0.258	0.270	0.305	0.350
28	0.293	<0.00	0.253	0.265	0.248	0.253	0.295	<0.00
29	0.406	<0.00	0.371	0.427	0.368	<0.00	0.427	<0.00
30	0.610	1.080	0.670	0.610	0.515	ND	0.770	2.150
31	0.540	0.570	0.570	0.560	0.520	ND	0.530	0.620
32	≤0.50	0.570	0.570	0.560	0.530	ND	0.510	0.570
33	0.630	0.780	0.700	0.640	0.660	ND	0.710	0.690
34	0.550	0.600	0.540	0.500	0.530	ND	0.560	1.250
35	0.280	0.345	0.285	0.280	0.263	ND	0.335	0.280
36	ND	0.345	0.330	0.275	0.253	ND	0.285	0.280
37	ND	0.380	0.290	0.265	0.255	ND	0.265	0.275
39	ND	0.395	0.325	0.350	0.263	ND	0.525	0.290
40	ND	0.620	0.330	0.640	0.300	ND	0.280	0.600

ND= Not Done

* The wells were coated with the bacteria, incubated with tissue culture fluid, and revealed with an enzyme-linked anti-IgM antibody. The mean absorbance of duplicate samples were tabulated in micrograms per ml.

TABLE 3b. IgM Antibody Levels Against Sixteen Oral Microorganisms by ELISA*

TCF#	L.cas.	P.mic.	S.epi.	S.int.	S.intM	S.mut.	S.san.	V.par.
7	0.255	0.270	0.280	0.295	0.280	<0.00	0.290	0.265
8	<0.00	0.399	<0.00	0.357	<0.00	<0.00	<0.00	0.357
11	<0.00	<0.00	<0.00	ND	0.255	<0.00	<0.00	<0.00
13	0.275	0.265	<0.00	0.265	≤0.25	<0.00	0.260	0.275
14	0.520	0.530	0.530	0.530	0.510	<0.00	0.520	<0.00
16	0.855	0.810	0.825	0.885	0.840	0.855	0.975	0.825
24	0.610	0.540	0.550	0.630	0.510	0.580	0.600	0.540
25	0.285	0.380	0.310	0.320	0.310	0.280	0.310	0.300
28	≤0.25	<0.00	<0.00	0.275	<0.00	<0.00	<0.00	0.310
29	0.462	<0.00	0.357	0.371	<0.00	<0.00	<0.00	<0.00
30	0.590	0.660	0.610	0.670	0.690	0.640	0.700	0.560
31	0.530	<0.00	0.530	0.580	0.570	0.570	0.590	0.560
32	0.530	<0.00	0.520	0.590	<0.00	0.560	0.640	0.590
33	0.560	0.590	0.530	0.670	0.600	0.610	0.920	0.690
34	<0.00	<0.00	0.510	0.510	0.510	0.530	0.660	0.530
35	0.300	0.310	<0.00	0.320	0.305	0.310	0.365	0.285
36	0.275	≤0.25	<0.00	0.270	0.300	<0.00	0.345	0.290
37	0.285	0.253	≤0.25	<0.00	0.290	0.255	0.335	0.280
39	0.300	0.280	<0.00	<0.00	0.310	0.270	0.285	0.310
40	0.255	0.335	0.295	0.415	0.365	0.300	0.450	0.295

ND= Not Done

* The wells were coated with the bacteria, incubated with tissue culture fluid, and revealed with an enzyme-linked anti-IgM antibody. The mean absorbance of duplicate samples were tabulated in micrograms per ml.

Figure 3.: Average Levels of IgM Antibody Detected in Periapical Lesions Against Sixteen Oral Microorganisms by ELISA.

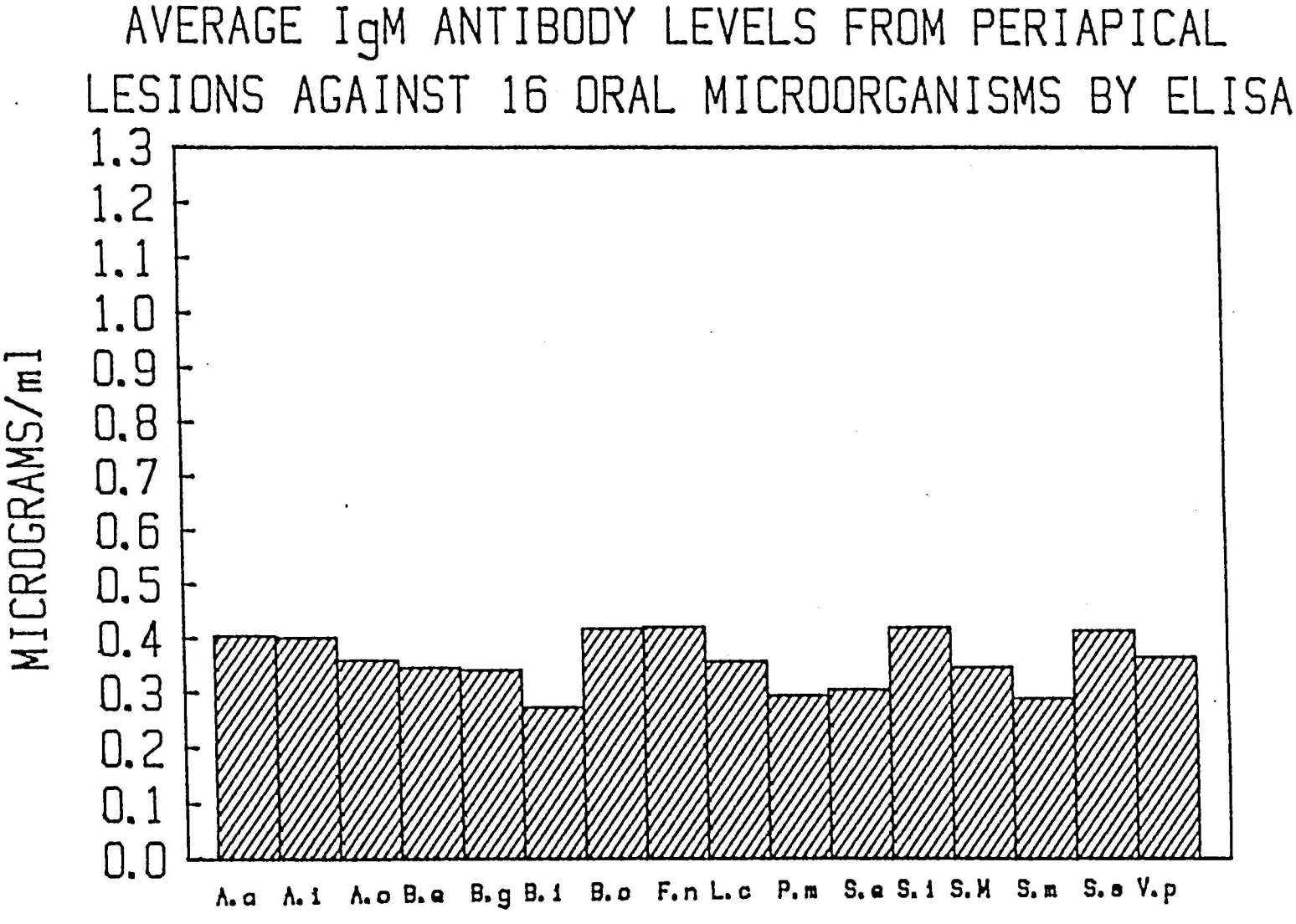

AVERAGE IgM ANTIBODY LEVELS FROM PERIAPICAL LESIONS AGAINST 16 ORAL MICROORGANISMS BY ELISA
MICROGRAMS/ml
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A.a A.i A.o B.a B.g B.i B.o F.n L.c P.m S.a S.i S.M S.m S.s V.p

Figure 4.: Comparison of IgG, IgA, and IgM Antibody Levels Detected in Periapical Lesions Against Sixteen Oral Microorganisms by ELISA for:

A.act = Actinobacillus actinomycetemcomitans

A.isr = Actinomyces israeli

A.odo = Actinomyces odontolyticus

B.end = Bacteroides endodontalis

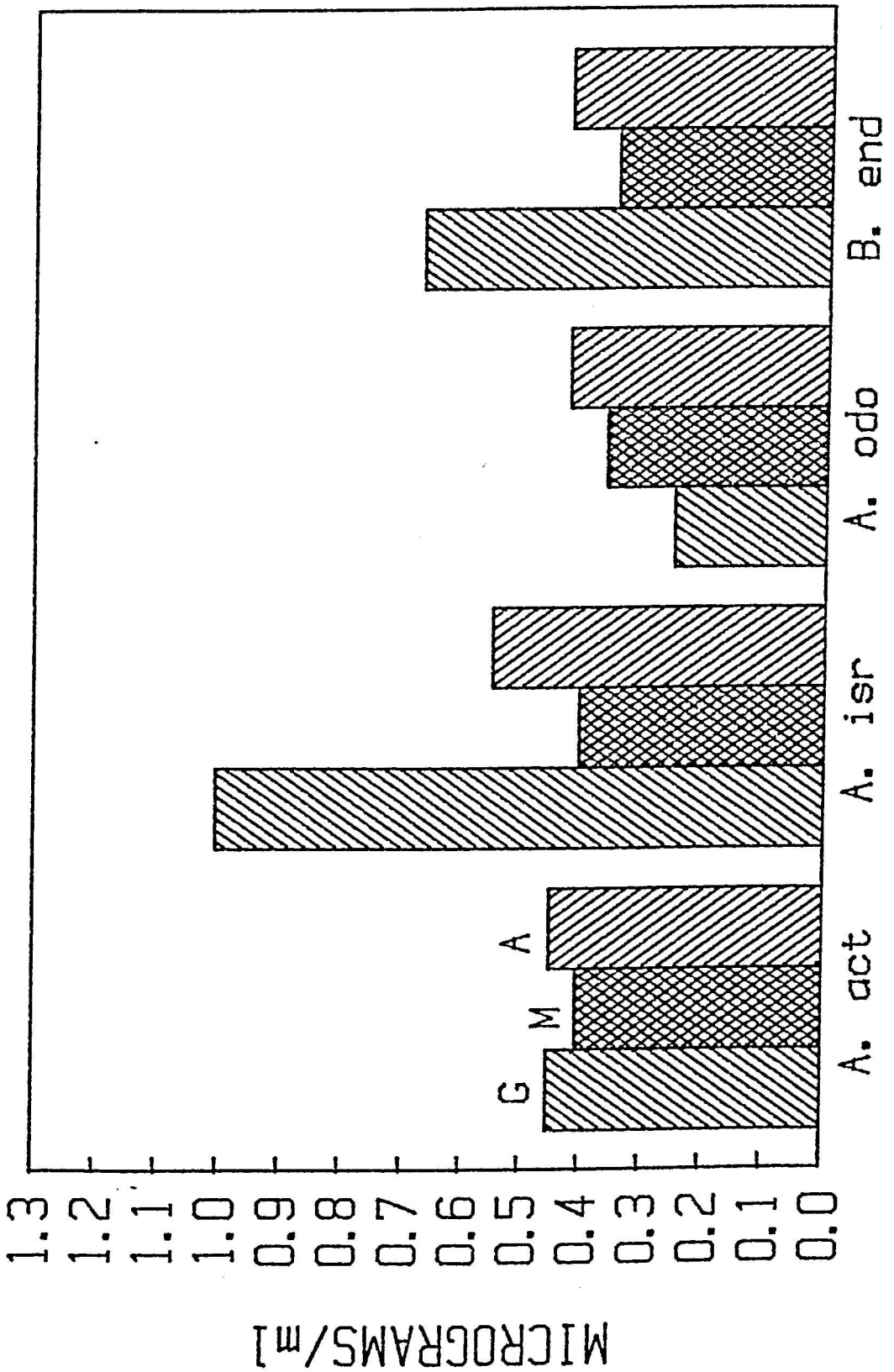

MICROGRAMS/ml
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
G
M
A
A. act
A. isr
A. odo
B. end

Figure 5.: Comparison of IgG, IgA, and IgM Antibody Levels Detected in Periapical Lesions Against Sixteen Oral Microorganisms by ELISA for:

B.gin = Bacteroides gingivalis

B.int = Bacteroides intermedius

B.ora = Bacteroides oralis

F.nuc = Fusobacterium nucleatum

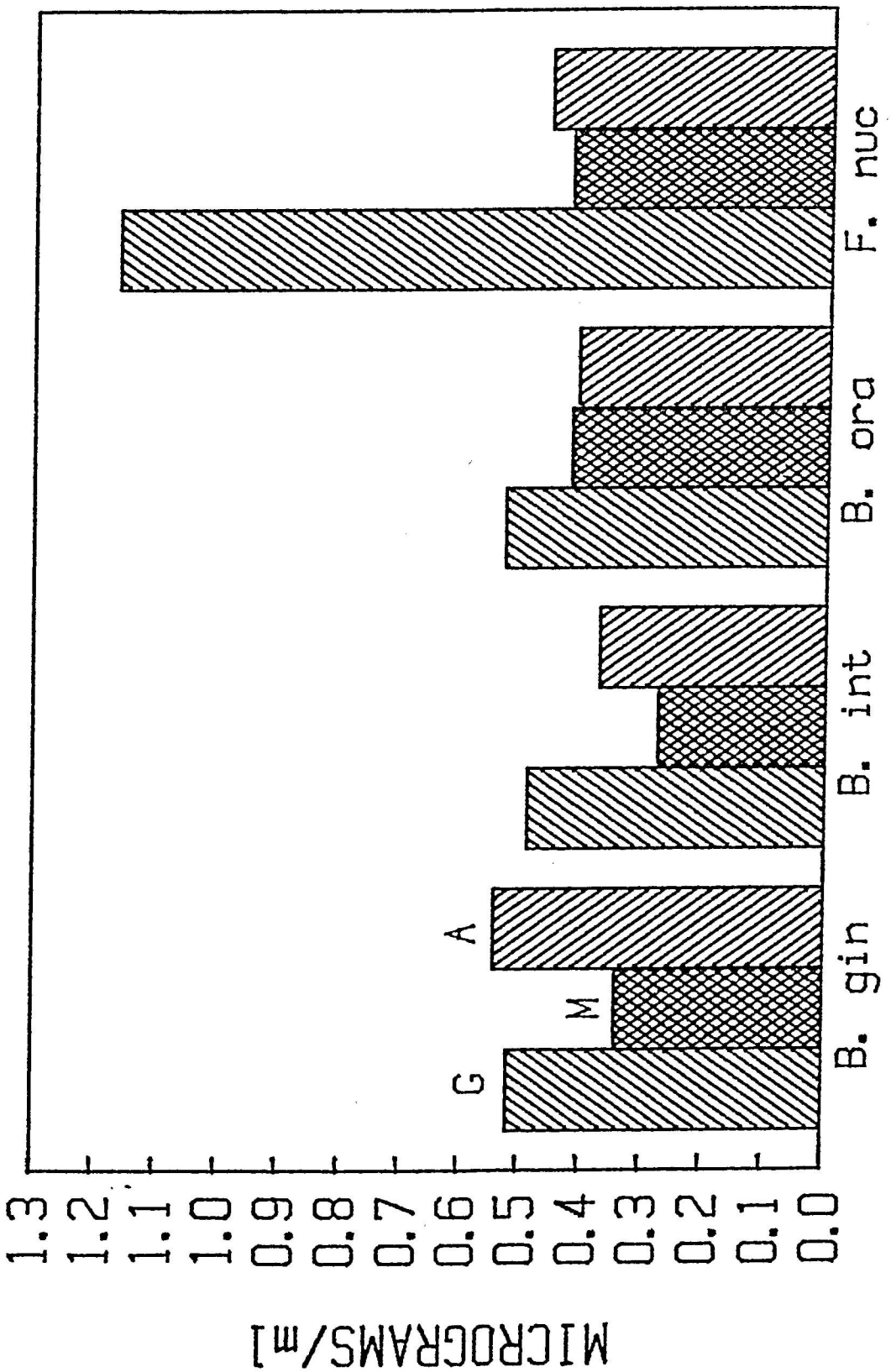

MICROGRAMS/ml
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
G
M
A
B. gin
B. int
B. ora
F. nuc

Figure 6.: Comparison of IgG, IgA, and IgM Antibody Levels Detected in Periapical Lesions Against Sixteen Oral Microorganisms by ELISA for:

L.cas = Lactobacillus casei

P.mic = Peptostreptococcus micros

S.epi = Staphylococcus epidermidis

S.int = Streptococcus intermedius

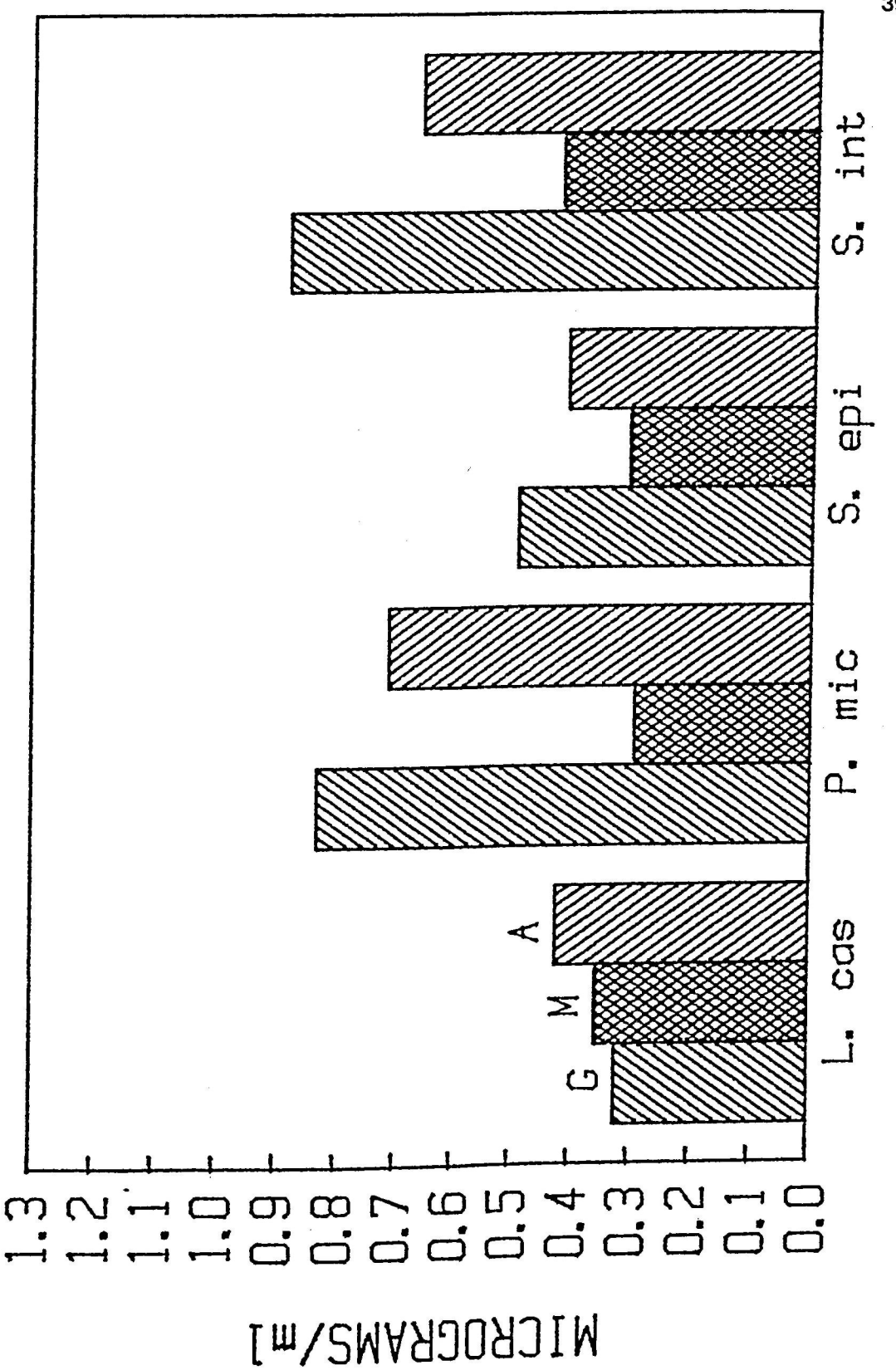

MICROGRAMS/ml
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
G
M
A
L. cas
P. mic
S. epi
S. int

Figure 7.: Comparison of IgG, IgA, and IgM Antibody Levels Detected in Periapical Lesions Against Sixteen Oral Microorganisms by ELISA for:

S.intM = Streptococcus intermedius-MG

S.mut = Streptococcus mutans

S.san = Streptococcus sanguis

V.par = Veillonella parvula

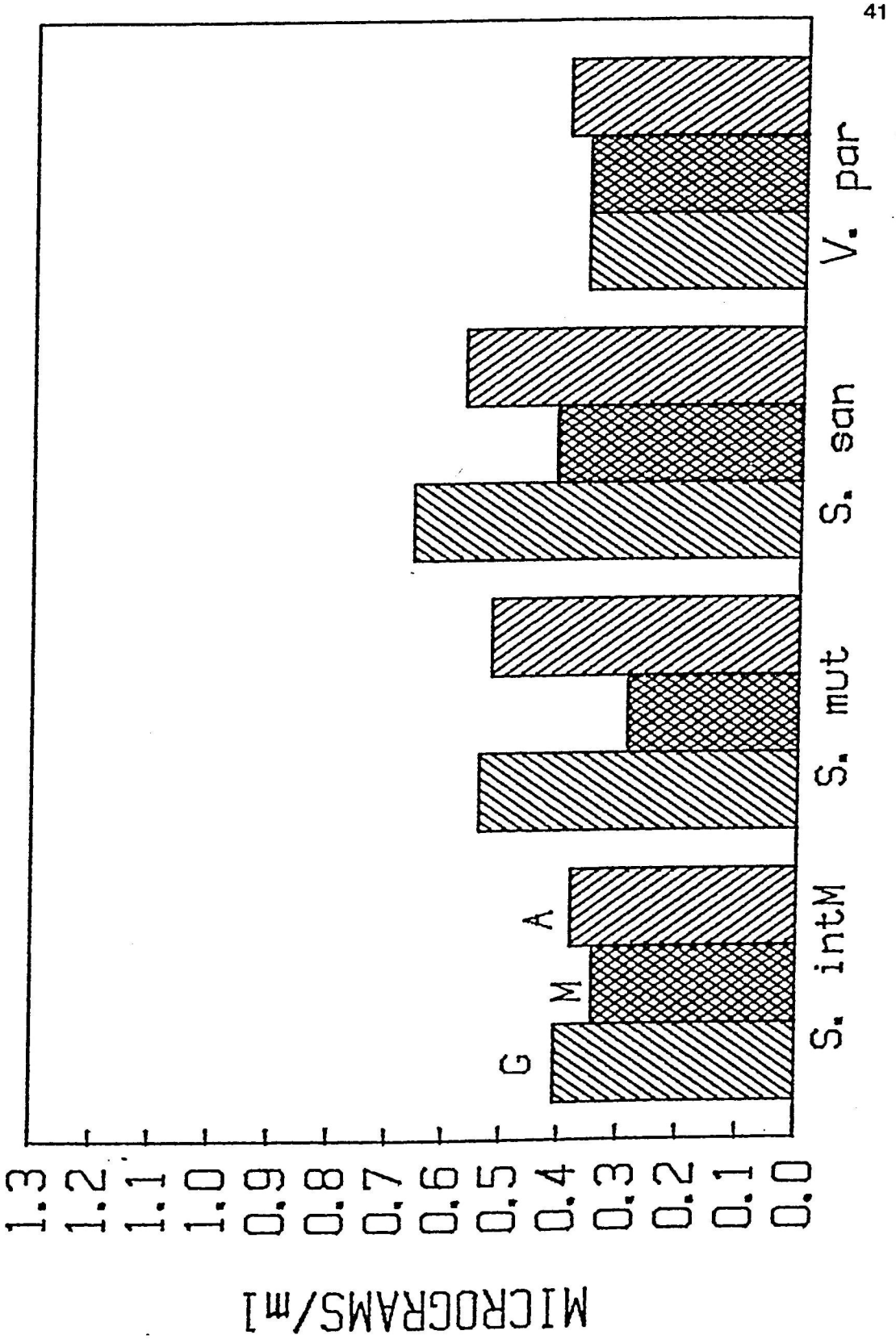

MICROGRAMS/ml
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
G
M
A
S. intM
S. mut
S. san
V. par

Figure 8.: Standard Curve for IgG Antibody Used to Correlate Values of Optical Density to Micrograms per Milliliter.

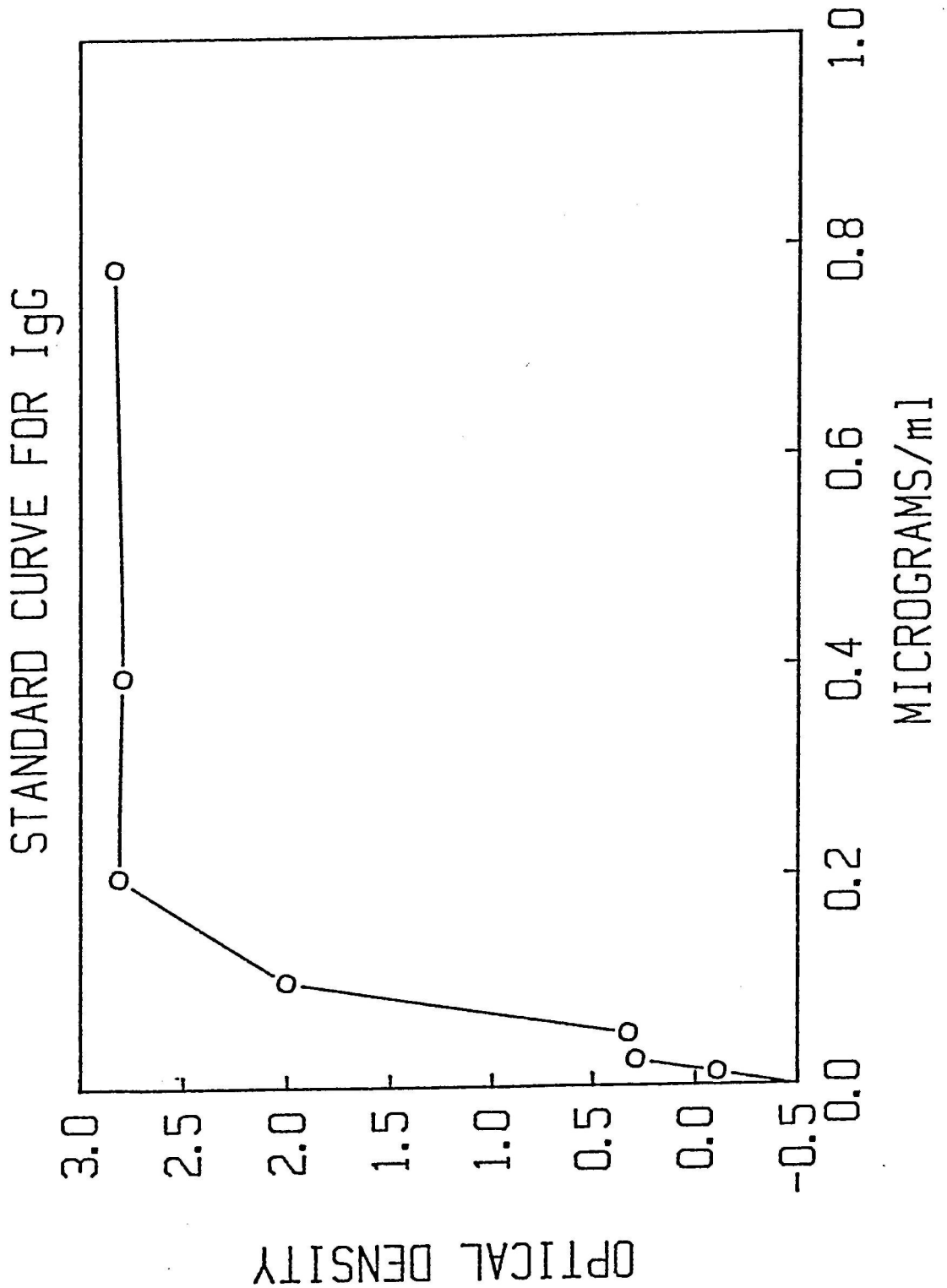

STANDARD CURVE FOR IgG
OPTICAL DENSITY
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0.0
0.2
0.4
0.6
0.8
1.0
MICROGRAMS/ml

Figure 9.: Standard Curve for IgA Antibody Used to Correlate Values of Optical Density to Micrograms per Milliliter.

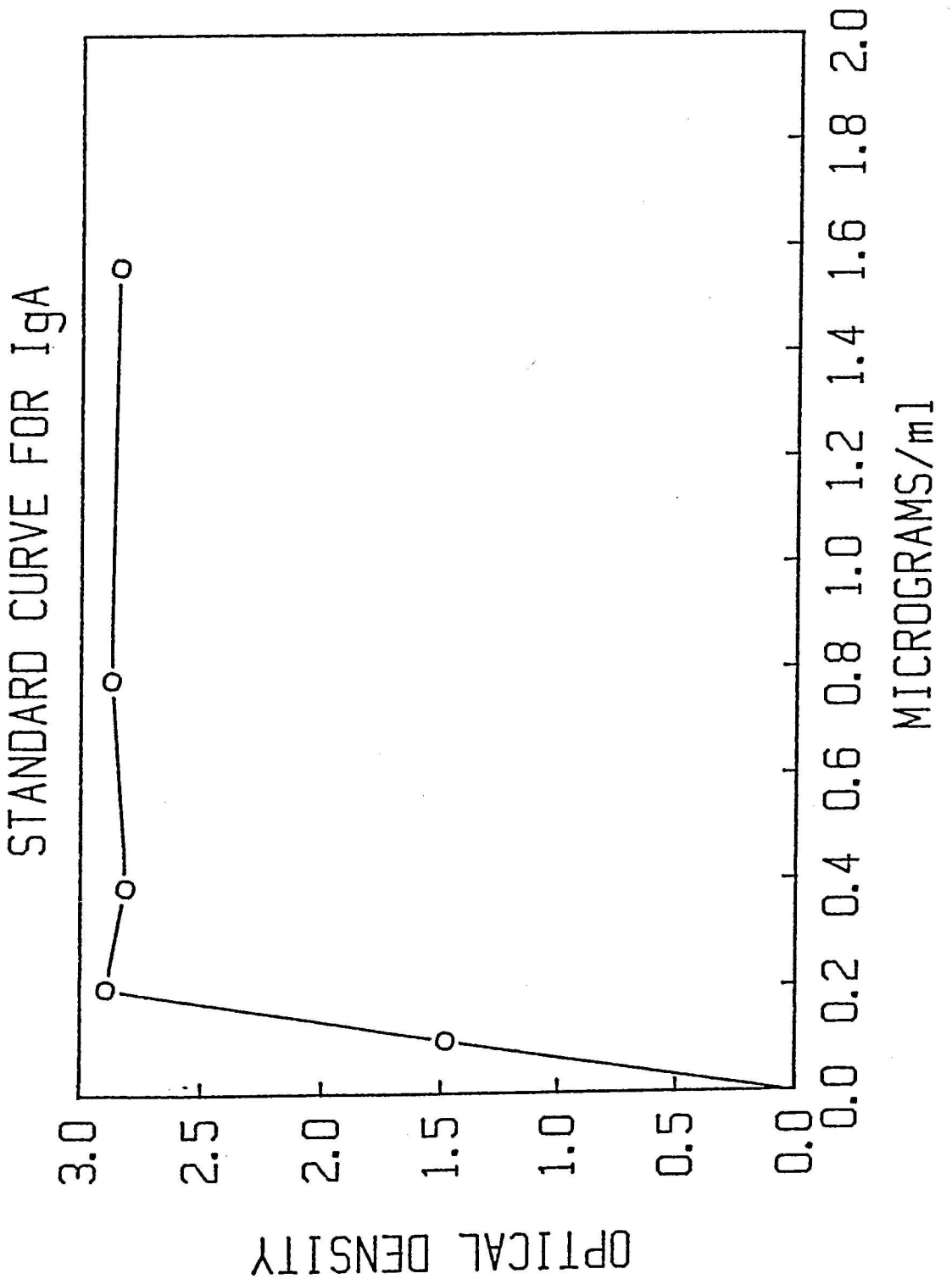
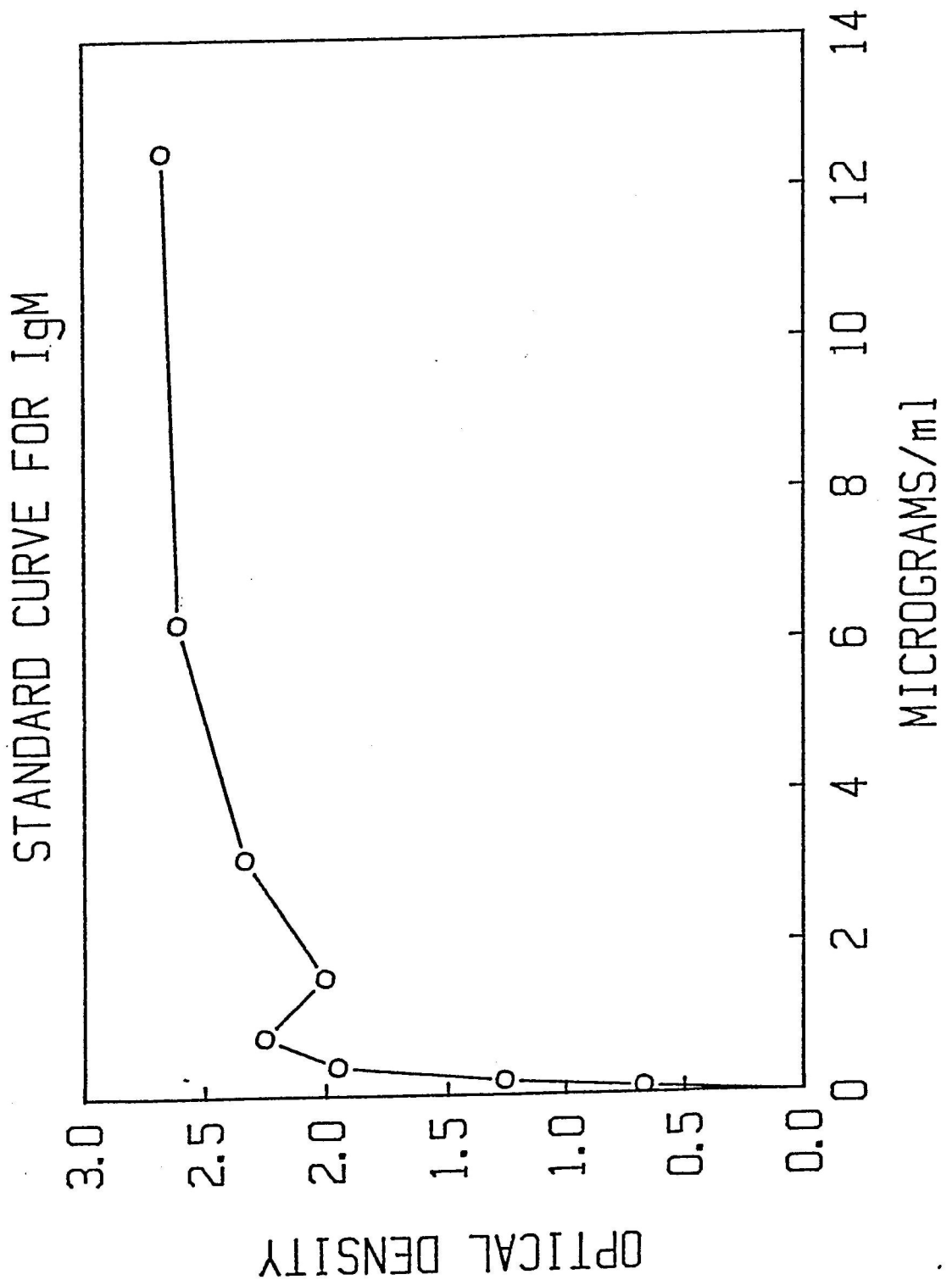

STANDARD CURVE FOR IgA
OPTICAL DENSITY
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
MICROGRAMS/ml

Figure 10.: Standard Curve for IgM Antibody Used to Correlate Values of Optical Density to Micrograms per Milliliter.


STANDARD CURVE FOR IgM
OPTICAL DENSITY
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
MICROGRAMS/ml

DISCUSSION

The development of periodontal infection has been associated with several different species of microorganisms which may also be normal inhabitants of the root canal and oral cavity (Melville et. al., 1967). The identities of several of those bacteria which are most commonly isolated from such infected sites have been determined by several researchers (Oguntebi et. al., 1982; Williams et. al., 1983). It has also been shown that the pathogenesis of endodontic lesions involves both the specific bacteria as well as the immune mechanisms of the host (Torabinejad et. al., 1978; Genco et. al., 1984). Their interactions in patients suffering from periodontal lesions has been described and has brought attention to the role of immunoglobulins in the pathogenesis of the disease (Taubman et. al., 1981; Genco et. al., 1984). Certain classes of these antibodies have been found in serum (Taubman et. al., 1981), gingival tissues (Berglund, 1971), pulp tissues (Falkler et. al., 1987), and periapical granulomas (Naidorf, 1975). In many of these studies, these antibodies have been shown to demonstrate specific reactivity with those same microorganisms which are most often associated with and isolated from the infected areas (Falkler et. al., 1987). The presence of such antibodies in periapical lesions has not yet been reported.

It was the purpose of this study to obtain from periapical lesions, via tissue culture explants, localized antibodies and to

determine their specificity against a panel of microorganisms chosen because of their previous implication in the development of periodontal abscesses.

Our results have shown that indeed, three distinct classes of immunoglobulins are present in the lesions and that they could be demonstrated by a modified ELISA technique which incorporated an Amplifier system. This finding is significant, in that this is the first evidence for the presence of locally-produced antibodies in periapical abscesses. IgG class antibodies were found to be present in the highest levels, followed by IgA, and, finally, IgM levels were found in the lowest concentrations. Because of the nature of the test conditions used to obtain these antibodies, we suggest that these antibodies have been locally produced while in tissue culture explants and subsequently released into the medium. In support of this is the fact that a high degree of variability of response could be seen between individual patients from whom the samples were extracted. We have demonstrated for the first time that these locally-produced antibodies also show specific reactivity toward those bacteria implicated in literature reports in the development of these lesions. The reactivity of all three classes of these antibodies revealed a striking similarity for several of the microorganisms tested, in that the highest reactivity levels for both IgG and IgA were directed toward four common bacteria:

A. israelii

S. intermedius

P. micros

S. mutans

IgM levels were also highest for A. israelii, S. sanguis, and S. mutans. It may then be inferred that these bacteria may be the most immunogenic in the development and pathogenesis of periapical abscesses in the patients tested in this study.

F. nucleatum showed the highest degree of reactivity with both IgG and IgM class antibodies, indicating the possibility that this organism might be involved in the etiology of the lesions as well.

Another interesting observation is that each class of Ig demonstrated a high reactivity to a different species of Bacteroides, so that it may be said that this genus does seem to also have an etiological role in this type of infection.

It may be seen from these results that the etiology of periodontal lesions appeared to involve not just one, but several different microorganisms. Also, a combination of several normal flora species may very well induce and propagate the pathogenesis of a lesion. From this we can conclude that the development of a periodontal lesion is multibacterial in nature. This may be a significant aspect in the diagnosis and/or treatment of patients suffering from periodontitis or endodontitis.

In summary, we have shown for the first time that periapical lesion tissues do contain immunoglobulins of the IgG, IgA, and IgM classes and that these antibodies demonstrate specific reactivity toward a common group of bacterial species, indicating that these

bacteria are highly immunogenic, and may possibly be etilogical agents of periodontitis in humans.

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