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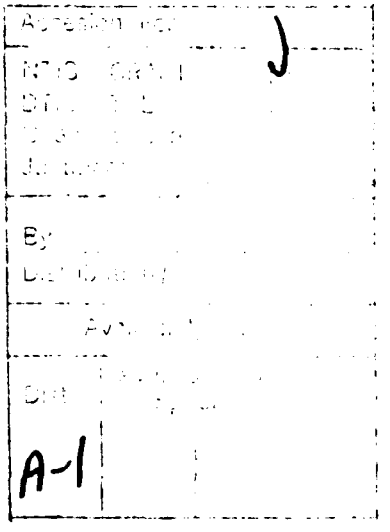
SERUM ANTIBODY RESPONSES TO ORAL MICROORGANISMS IN NONHUMAN PRIMATES

A
THESIS

Presented to the Faculty of
The University of Texas
Graduate School of Biomedical Sciences
at San Antonio
in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

By
Anthony Gary Giardino, D.D.S.



San Antonio, Texas

May 1991

SERUM ANTIBODY RESPONSES IN HONHUMAN PRIMATES TO ORAL MICROORGANISMS

Anthony Gary Giardino

APPROVED:

Supervising Professor

May 17, 1991

Date

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DEDICATION

This thesis is dedicated to my beloved wife Jane for her support and understanding during the past three years of my life. Although this residency has been very demanding of my time and energy I have been continually sustained by Jane's efforts and I will always be grateful to her. I have borrowed from her constant strength to get me through the tougher times. To my children Michael, William and Thomas; I hope that the perseverance and dedication embodied in this thesis may someday encourage your pursuit of worthwhile ambitions.

SERUM ANTIBODY RESPONSES TO ORAL MICROORGANISMS IN NONHUMAN PRIMATES

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Porphyromonas gingivalis and *Prevotella intermedia* have been associated with periodontitis in humans and nonhuman primates (NhP). This investigation characterized serum antibody levels, isotypes and subclass distribution and specificity to *P. gingivalis*, *P. intermedia* and *Bacteroides fragilis* prior to, and after immunization with these bacteria and during ligature-induced periodontitis. Serum from 20 adult, female cynomolgus monkeys was obtained at baseline, after 3 intramuscular injections with 10^9 bacteria emulsified in incomplete Freund's adjuvant (IFA) or with IFA, and during 30 wks. of ligature placement. IgG, IgM and IgA isotypes and IgG1-4 subclass antibody were assessed in an ELISA. Baseline levels of IgG/M/A antibody were ~4-20 fold higher to *P. intermedia* (90/20/20 EU) than to *P. gingivalis* (26/5/1 EU) in the NhP. Immunization increased IgG/M/A antibody by 16-260 fold to *P. gingivalis* (389/102/263 EU) and 5-70 fold to *P. intermedia* (948/534/1411 EU). IgM/A responses subsided by 8-13 wks. post-immunization, while IgG was maintained through 25-30 wks. Negligible cross-reactivity was detected except for a 3-fold increase in

IgM antibody to *P. intermedia* in the *P. gingivalis* or *B. fragilis* immunized groups. No detectable changes were noted in IgG/M/A antibody to *P. gingivalis* or *P. intermedia* in any group following ligation. Nearly 75% of natural IgG antibody was comprised of IgG1 to *P. gingivalis* and *P. intermedia*, while IgG3 (62%) and IgG2 (33%) predominated to *B. fragilis*. The IgG response to *P. gingivalis* and *P. intermedia* after immunization was comprised primarily of IgG1 (86-98%), IgG2=IgG4 (~4-10%) and minimal IgG3. Anti-*B. fragilis* responses were IgG1 (49%), IgG2=IgG3 (18-21%) and IgG4 (12%). These results demonstrate the ability to induce a highly specific antibody response in NhP following immunization with oral microorganisms. While all isotypes were elicited, only the IgG antibody was maintained through the ligation interval. The natural and induced IgG response to the oral bacteria was primarily IgG1. Due to the extensive capsule that has been described on these microorganisms, this type of IgG response may be ineffective in protecting from disease due to its inability to opsonize these microorganisms.

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I. INTRODUCTION

A. Human Periodontal Diseases

1. Clinical Characteristics

The progression of untreated periodontal disease may lead to eventual tooth loss. Gingival bleeding, suppuration, tooth mobility, and changes of the gingival margin location are clinical signs of periodontitis. Alveolar bone loss is evident radiographically. Histological examination of the gingival tissue shows the presence of severe acute and chronic inflammatory reactions.

The results of studies concerning the prevalence and severity of periodontitis have shown a great deal of variation. Marshall-Day *et al.* (1955) showed the prevalence of periodontitis to reach nearly 100% by age 35. More recent studies (Douglass *et al.* 1983 and Brown *et al.* 1989) have found a much lower prevalence, but no epidemiological studies have distinguished the different forms of periodontitis.

The pattern of chronic adult periodontal disease progression does not appear to be continuous. Destructive disease activity probably occurs by site-specific periods of exacerbation followed by longer periods of remission. Studies of untreated adult periodontitis patients by Lindhe *et al.* (1983) and Haffajee *et al.* (1983) have shown that progression occurs in only a small proportion of individuals, and almost not at all in others. Furthermore, progression at any given site occurs only infrequently. Other studies have looked at groups of patients with particularly severe periodontal disease (Lavine *et al.* 1979, Smith *et al.* 1980). Unlike localized juvenile periodontitis, no

specific pattern of bone loss has been established in this severe form of periodontitis. Rapidly progressive periodontitis may be localized to single teeth, but commonly, more than half of the dentition is severely affected, with attachment loss reaching 90% of affected teeth. Acute florid inflammation of the gingival tissues is seen. Attachment loss occurs rapidly, usually documented to have occurred within a period of months. The disease is seen most commonly in the 20- to 35- year age group, but may occur at any age beyond puberty (Page and Schroeder, 1982). The prevalence of rapidly progressive periodontitis is lower than that for chronic adult periodontal disease; however, the morbidity in terms of tooth-loss appears to be much higher for the former.

2. Microbiological Characteristics

The presence of microorganisms in the gingival crevice is considered to be the etiologic agent responsible for initiating periodontal disease. Although several hundred bacterial species have been found to inhabit the oral cavity, the list of putative periodontal pathogens is relatively short (Moore *et al.* 1982).

The studies by Loe *et al.* (1965) and Theilade *et al.* (1966) demonstrated a clear association between the accumulation of bacterial plaque and the subsequent development of gingivitis. This led to the postulation of the "non-specific" theory of periodontitis (Loesche, 1976). Plaque associated with healthy gingiva consists of primarily Gram-positive bacteria. Approximately 85% of the plaque is made up of *Streptococcus* and *Actinomyces* species (Slots, 1979). Gingivitis has been characterized as a shift from a *Streptococcus* dominated plaque to a plaque dominated by *Actinomycetes*. Listgarten and Hellden (1978) found an association between gingivitis and increased proportions of motile bacteria and spirochetes.

a. Black-pigmenting species

The pathogenic potential of members of black-pigmenting oral bacteria is well established. Mixtures of indigenous oral bacteria containing *Bacteroides* species were shown to cause severe transmissible subcutaneous infections by MacDonald *et al.* (1963). They mediate bone resorption by the production of endotoxins (Hausmann *et al.* 1970). These organisms can impair polymorphonuclear leukocytes *in vitro* which can inhibit phagocytosis and killing of other bacteria. *Bacteroides* species can also produce proteolytic enzymes such as collagenase and trypsin-like enzymes (Gibbons, 1974).

Zambon *et al.* (1981) found bacterial dental plaque to be the most common intraoral site for *Bacteroides* colonization, followed by the tonsillar area. Five periodontally-healthy children under age 6 were examined and no cultivable black-pigmented *Bacteroides* were detected. *Bacteroides* species were recovered from 9 out of 10 adult patients with moderate to severe alveolar bone breakdown. Although they were detected in 3 of the 5 healthy adult controls, when seen in these subjects, *Bacteroides* comprised 2% or less of the cultivable microflora in all sample sites. The adult periodontitis group exhibited cultivable *Bacteroides* in 47% of the subgingival plaque samples constituting as much as 59% of the isolates. Based on the results of the study, the authors suggested that these organisms may contribute to the pathogenesis of certain forms of periodontal disease.

Close association of *Porphyromonas gingivalis* with advancing periodontitis lesions having rapidly progressing alveolar bone destruction has been established by several studies (Slots 1977, Spiegel *et al.* 1979, Tanner *et*

al. 1979). *P. gingivalis* was not found at healthy gingival sites; however, at sites with severe inflammation this microorganism constituted a major part of the subgingival microbiota. Additionally, their relative numbers were significantly higher than in samples from "normal" sulci suggesting that *P. gingivalis* has a role in the etiology of severe periodontitis in adults. In a retrospective study, Bragd *et al.* (1987) found the combination of *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* at the same subgingival site was associated with progressive periodontitis as evaluated by radiographic bony changes. Van Winklehoff *et al.* (1988) reviewed the role of black-pigmenting bacteria in oral infections and concluded that *P. gingivalis* is the most pathogenic species of these microorganisms based on the virulence factors it possesses. *P. gingivalis* and *P. intermedia* were found in elevated levels at active periodontal sites as assessed by probing attachment loss over a two month period (Dzink *et al.* 1988). Thus, the common finding of elevated levels of *P. gingivalis* and *P. intermedia* at sites of periodontal breakdown implicates these microorganisms as having a major role in destructive periodontal disease. *P. intermedia* genotypes have been associated with a wide range of periodontal pathoses. They have been associated with chronic gingivitis with little or no alveolar bone loss in several studies (Slots *et al.* 1978, Spiegel *et al.* 1979, White & Mayrand 1981). *P. intermedia* appears to be involved in the development of experimental gingivitis (Loesche & Syed, 1978) as well as pregnancy gingivitis (Kornman & Loesche, 1980). Slots and Zambon (1982) studied 4 acute necrotizing ulcerative gingivitis patients and found *P. intermedia* to be a significant constituent of the microflora. This microorganism has also been a major isolate recovered from advanced periodontitis sites (Tanner *et al.* 1979, Zambon *et al.* 1981).

B. Nonhuman Primate Model of Periodontal Disease

Although specific bacteria are associated with periodontal disease, a positive cause and effect relationship between these microorganisms and the initiation of disease has not been determined. It is very difficult to obtain useful data concerning the progression of gingivitis to periodontitis in humans. Longitudinal investigations on human subjects are limited due to the variability of clinical measurements and lack of precision of these measurements. Also, by the time a "burst of activity" has been detected, that particular site may have a different microbial environment. The systemic immunological response also may change after a period of disease activity.

Cross-sectional studies in humans appear to be unable to define a cause and effect relationship between specific pathogens and periodontal disease (Slots, 1979). These studies cannot determine which microorganisms initiate pathological changes versus those microorganisms which come secondary to the disease. Additionally, identification of critical virulence antigens for the pathogens may be impossible to discern from human studies. Likewise, the differentiation between protective and destructive components of the host response in the pathogenesis of periodontal disease cannot be addressed in humans and the frequent radiographic monitoring necessary to closely monitor alveolar bone changes cannot be done in humans. Ethical treatment considerations also restrict the use of humans in longitudinal periodontitis studies.

To enable research to progress in elucidating these processes, several studies have used nonhuman primates to study periodontitis. Heijl *et al.* (1976) demonstrated the conversion of chronic gingivitis to periodontitis in squirrel monkeys. Kennedy and Polson (1973) studied induced marginal periodontitis in the squirrel monkey. Staple *et al.* (1977) studied diphenylhydantoin-induced gingival enlargement in the nonhuman primate *Macaca arctoides*.

Experimentally-induced gingivitis in *Macaca speciosa* was studied by Krygier *et al.* (1973) who found a general similarity of clinical, microbiological and histological findings to human gingivitis. Slots and Hausmann (1979) studied ligature-induced periodontitis in *Macaca arctoides* in which they used a radioactive iodine labelling technique to measure alveolar bone changes. These authors also showed that significant increases of *Bacteroides melaninogenicus* subsp. *asaccharolyticus* (*P. gingivalis*) were correlated with the ligature-related alveolar bone loss.

Kornman *et al.* (1981) clearly established the nonhuman primate (Nhp) as an excellent animal model system for longitudinal studies on the progression of adult periodontal disease. In this model naturally occuring chronic gingivitis can be converted to progressing periodontitis.

1. Clinical Characteristics

The naturally occuring chronic gingivitis in the cynomolgus monkey is consistent clinically with human gingivitis. Visual signs of inflammation are present and the gingiva bleed on gentle probing. Radiographically the bone crest approaches the cementoenamel junction. Progression to periodontitis can be initiated by ligature placement, and by 8-12 weeks this disease is consistent clinically, histologically, and radiographically with adult periodontitis in humans. Kornman *et al.* (1981) divided this transition into four clinical stages which correlated with changes in the microflora. Stage I was the naturally occurring gingivitis, Stage II (3 weeks post-ligation) pseudopocketing and 100% bleeding on probing, Stage III (4-7 weeks post-ligation) severe inflammation, increased pocket depths, radiographic bone loss. Stage IV (8-17 weeks post-ligation) was characterized by increased pocket depths, but no increase of radiographic bone loss. Initially, ligature-induced periodontitis in the Nhp is an acute

lesion which probably parallels the exacerbated phase of human periodontal disease (Slots & Hausmann, 1979).

Morphological studies on ligature-induced periodontal disease in the cynomolgus monkey were reported by Nabandian *et al.* (1985) and Brecx *et al.* (1985, 1986). Histological specimens of naturally occurring gingivitis and ligature-induced gingivitis are identical, resembling the established lesion in humans. The inflammatory infiltrate is dominated by plasma cells, while leukocytes are present throughout the inflamed tissue and neutrophils are seen at the plaque and soft tissue interface. Although signs of perivascular inflammation are seen in the transseptal fiber region, the alveolar crest remains intact.

As the disease progresses, changes similar to descriptions of human periodontitis continue to develop. Subgingival plaque is apparent and bacteria are seen around and within the ligature. The connective tissue infiltration of leukocytes becomes more dense with a high proportion of plasma cells. Numerous polymorphonuclear neutrophils are seen in a zone associated with the ligature. The 4-7 week specimens show destrucion of the collagenous attachment to cementum and apical migration of the epithelial attachment. This advanced lesion also shows signs of osteoclastic bone resorption. These morphological changes are similar to descriptions of human periodontitis (Page & Schroeder, 1982) and support the use of the cynomolgus monkey as a model to study human periodontitis.

2. Microbiological Characteristics

Early attempts to characterize the oral microflora of nonhuman primates demonstrated that the types of bacteria present in plaque and saliva samples are similar

to those found in humans. Bowen (1963) and Cock & Bowen (1967) used culturing, dark-field microscopy, and Gram staining to study mixed saliva and plaque samples of *Macaca fascicularis*. Most of the animals harbored a microbiota including *Bacterionema matruchotii, Veillonella, Neisseria,* black-pigmenting *Bacteroides, Fusobacterium, Lactobacillus*, spirochetes, and vibrios. Krygier *et al.* (1973) studied the sulcular microbiota of *Macaca speciosa* in health and during a 7-day period of no oral hygiene measures. After debridement and 2 weeks of toothbrushing the sulcular flora was dominated by Gram-positive cocci and rods. By 7 days after cessation of toothbrushing, Gram-negative cocci and rods became dominant, filamentous bacteria and spirochetes increased dramatically, and *Actinomyces, Veillonella,* and *Bacteroides* were present.

Kornman *et al.* (1981) found the subgingival microbiota of *Macaca fascicularis* to parallel that of humans with gingivitis and adult periodontitis. Gingivitis was characterized by a predominance of Gram-positive cocci and rods. One to three weeks following ligature placement, a significant increase in motile and surface translocating Gram-negative rods was detected. By 4-7 weeks post-ligation, a Gram-negative anaerobic flora predominated with *P. gingivalis* as the dominant cultivable organism.

The Nhp model should allow the determination of the pathogenic potential of specific microorganisms in the complex ecosystem of the periodontal pocket. In this model the local microbiota can be manipulated to select for specific microorganisms by the use of systemic antibiotics (Holt *et al.* 1988). When rifampin-resistant mutants of *P. gingivalis* were placed subgingivally, rapid and significant bone loss occurred. This "burst" of bone loss demonstrated the ability of *P. gingivalis* to induce progression of periodontitis.

C. *Immune Responses in Periodontal Disease*

The cell-mediated and humoral host immune responses to oral microorganisms and their relationship to periodontal disease have been examined in many studies. In general, an elevated immune response to specific microorganisms has been associated with increasing severity of periodontal disease.

1. Human Studies - Immune Reactions

The shift from gingival and periodontal health to a state of disease has been closely associated with both cell-mediated and humoral immune responses. The initial immune response to bacterial plaque antigens appears to be a result of cell-mediated mechanisms. The presence of large proportions of lymphocytes in gingival tissues associated with human periodontitis led early researchers to attempt to define the role of these cells. Ivanyi and Lehner (1970) compared *in vitro* lymphocyte blastogenesis of gingivitis and periodontitis patients to normal healthy controls. The peripheral blood mononuclear cells of the gingivitis and periodontitis patients underwent a blastogenic response and produced lymphokines. This study and others (Ivanyi & Lehner 1971, Horton *et al.* 1972) supported the idea of an important role for T-lymphocytes and delayed hypersensitivity in the pathogenesis of gingivitis and periodontitis (Page, 1982). Substances from bacterial plaque have since been shown to activate both T- and B-lymphocytes and both T- and B-lymphocytes produce lymphokines (Mackler *et al.* 1974). B-lymphocytes and plasma cells predominate in the gingiva in human periodontitis (Page and Schroeder, 1976). Since T-lymphocytes and macrophages would predominate in a delayed hypersensitivity reaction, the immune response in periodontitis apparently involves other major mechanisms.

The role of T-cells and cell-mediated hypersensitivity was more clearly defined by Wilde *et al.* (1977). T-cells are normally present in small numbers in healthy gingival tissue and they are most numerous in the early lesion of gingivitis. At this stage, tissue damage is slight and reversible. As gingivitis progresses, the lesion becomes dominated by B-lymphocytes and plasma cells lessening the apparent role of cell-mediated hypersensitivity.

In another attempt to define the role of T-lymphocytes in periodontal disease Stashenko *et al.* (1983) studied the *in vitro* blastogenic response of T-cells to sonicates of oral microorganisms. They grouped the patients based on the level of their response and positive correlation was seen between low responders and signs of increased gingival inflammation. Scores for gingival redness and bleeding on probing were higher for this group, but no differences were noted for attachment level, pocket depth or bone loss. Stashenko *et al.* (1985) also compared the ratio of helper (T4) to suppressor (T8) T-cells in normal subjects and periodontitis patients. T lymphocyte blastogenic responses were also examined. T4/T8 ratios were the same for periodontitis patients and healthy subjects, however the subgroup of low-responder patients had decreased T4/T8 ratios. These patients had increased signs of gingival inflammation, redness and bleeding on probing, but did not differ with respect to attachment levels or pocket depths. These studies did not demonstrate a significant difference of T-cell proliferative response between healthy controls and periodontitis patients.

When unstimulated lymphocyte blastogenesis was studied some significant differences appeared (Seymour *et al.* 1986). When cultured in media alone, lymphocytes from periodontitis patients consistently had lower blastogenic responses than lymphocytes from healthy controls. The blastogenesis is thought to be due to

autologous mixed lymphocyte reactions (AMLR) and lower AMLR reactions may represent a defect in the immune response capabilities.

In contrast to these more generalized descriptions of host immune capacity, recent evidence has indicated that different forms of periodontal disease have a distinctive microbiota colonizing the disease sites (Savitt & Socransky, 1984, Loesche *et al.* 1985 and Slots, 1986). As an extension of these investigations, the systemic antibody responses to some of the proposed periodontopathogens have been examined. Bacterial-specific elevated serum antibody responses have implicated several bacteria as being associated with the pathogenesis of periodontal disease in humans.

Early studies by Genco *et al.* (1980) in localized juvenile periodontitis (LJP) patients demonstrated the presence of precipitating serum antibodies to sonicates of *Actinobacillus actinomycetemcomitans*. Serum from the normal subjects in the study did not have antibodies reactive for this microorganism. Ebersole *et al.* (1980) confirmed these results using an enzyme-linked immunosorbent assay (ELISA) they developed for measuring antibodies to surface antigens of *A. actinomycetemcomitans*. Subsequent serum antibody studies (Taubman *et al.* 1982) on a variety of subjects including patients with LJP, generalized juvenile periodontitis (GJP), adult periodontitis, and acute nectrotizing ulcerative gingivitis (ANUG) demonstrated antibody reactivity to a wide selection of putative periodontopathogens. In a study of the pooled sera from a group of periodontitis patients Tolo *et al.* (1981) used an ELISA to examine serum IgG, IgM and IgA antibody to several microorganisms commonly associated with diseased sites and determined that there was elevated antibody to all of the microorganisms.

Ebersole *et al.* (1982) reported a significantly elevated serum IgG antibody response to *A. actinomycetemcomitans* in 90% of LJP patients compared to only 10% of healthy controls. These results suggested that this microorganism is an important pathogen in patients with LJP. Zambon (1985) reviewed the pathogenic potential of *A. actinomycetemcomitans* providing further support for the pathogenic potential of this microorganism in periodontitis. Although *A. actinomycetemcomitans* is harbored in the dental plaque of 36% of the normal population, this colonization was always at low levels.

There have been numerous serum antibody studies which have delineated the characteristics of host antibody responses to oral black-pigmenting microorganisms. An early study by Mouton *et al.* (1981) demonstrated a positive correlation between age and IgG, IgM and IgA antibody to *P. gingivalis* in both normal controls and periodontitis patients. The results suggested that the elevated antibody response seen in the older subjects without disease was primarily a protective response, since most normal subjects are not colonized by *P. gingivalis*. Taubman *et al.* (1982) confirmed these early findings showing a significantly increased IgG antibody level to *P. gingivalis* in adult and generalized juvenile periodontitis patients compared to LJP, ANUG and normal subjects.

Patters and Kornman (1982) expanded these serum antibody findings using an ELISA involving whole bacteria. Significantly elevated IgG/M/A antibody levels to *P. intermedia* were found in both the periodontitis and normal subjects. However, elevated IgG and IgA antibody levels to *P. gingivalis* were only seen in the periodontitis group and elevated antibody levels were more related to disease status than to age. The findings of this study were extended by Ebersole *et al.* (1986) who characterized serum antibody responses to a battery of nine oral microorganisms in

LJP, advanced destructive periodontitis (ADP), adult periodontitis (AP) and normal subjects. Elevated IgG antibody to *P. gingivalis* was found in the ADP and AP groups, while elevated IgM to *P. gingivalis* was seen in all diseased groups versus normal subjects. No significant differences in IgA levels were seen between the groups and only negligible IgE antibody was detected to *P. gingivalis*. Additional antibody specificity studies as part of this same investigation revealed that antibody in the patients' sera reacted with unique antigens on *P. gingivalis* and *P. intermedia*. The authors suggested that the elevated antibody responses in the periodontitis patients reflect a colonization of patients with these bacteria indicating their pathogenic potential. Additional studies involving serum antibody responses have corroborated these findings (Tew *et al.* 1985, Farida *et al.* 1986 and Aukhil *et al.* 1988).

2. Rodent Studies - Immune Manipulation

The ability to manipulate the immune system of mice and rats allows several different approaches to study host responses and infections. Studies involving immune manipulation of rats in response to Gram-positive microorganisms have shown that induced serum and salivary antibody may have provided some protection from bone loss resulting from infection with *S. mutans* and *A. naeslundii* (Crawford *et al.* 1978). Another study in rats used the T-lymphocyte inhibiting agent Cyclosporine A to affect immune responses and disease (Guggenheim *et al.* 1981). The results indicated very little effect on the severity of periodontal disease when the rats were infected with *A. viscosus*, despite the dramatic decrease detected in serum agglutinins to *A. viscosus* in the Cyclosporine A-treated rats. The authors indicated that multiple pathogenic mechanisms are responsible for periodontal disease in addition to T-cell mediated hypersensitivity.

Johnson *et al.* (1978) and Behling *et al.* (1981) induced periodontitis in germ-free rats monoinfected with *E. corrodens*. An increased systemic antibody response to the bacteria was detected during disease progression. Subsequently, immunization with whole bacterial cell sonicates elicited elevated antibody levels and resulted in less bone loss. No antibody was detected to bacterial lipopolysaccharide and the authors proposed that the pathogenic mechanism of periodontal disease may include delayed-type hypersensitivity and endotoxic effects. Several studies (Taubman *et al.* 1983, 1984 and 1988) have suggested a "mixed-type hypersensitivity" reaction as a contributor to the progression of periodontal disease. After immunizing germ-free rats with formalinized *A. actinomycetemcomitans*, they were infected with *A. actinomycetemcomitans*. A significant elevation in serum IgG to this organism was seen for both the immunized group and the sham-immunized controls. Increased antibody level appeared to exacerbate the disease process even though fewer bacteria were detected in the immunized group. Yoshie *et al.* (1985, 1987) demonstrated the protective and detrimental potentials of the cell-mediated immune response. The transfer of *A. actinomycetemcomitans*-sensitized T lymphocytes into athymic (nude) rats allowed the production of elevated levels of immunoglobulins and exacerbated the disease after *A. actinomycetemcomitans* infection. The nude rats which received normal T cells produced normal levels of antibody with protection from increased bone loss.

Several studies using the rodent model and *P. gingivalis* infections have been reported. Wyss and Guggenheim (1984) implanted *P. gingivalis* and *A. viscosus* in rats and although they were able to implant *P. gingivalis* alone, *P. gingivalis* implanted most successfully when combined with *A. viscosus*. Rats colonized with *P. gingivalis* formed serum antibodies to the microorganism; however, levels of disease were similar for the *P. gingivalis*-infected group and the dual-infected group. In a study designed to

test the effect of active immunization with *P. gingivalis* or *P. intermedia*, Chen and coworkers (1987) immunized BALB/c mice which induced specific serum antibody responses. Only homologus immunization protected the mice from an invasive lesion and systemic infection after subcutaneous challenge with viable microorganisms.

Undoubtedly, the rodent model will continue to be used to study the effects of immune manipulation on periodontal disease. However, there are several drawbacks to the use of mice and rats as models for periodontitis. The small dimensions of the alveolar housing of the teeth and soft tissues make it difficult to draw conclusions which would seem easily applicable to human disease. Rapid attrition of the rodent dentition and continuous tooth eruption probably alter the progression of periodontal disease in a manner which has no parallel in the human dentition. Periodontal disease in rats also may have a different initiating mechanism than man. The rat appears to be extremely resistant to naturally-occuring periodontal disease (Page and Schroeder, 1982). When fed a sucrose-rich diet an overgrowth of Gram-positive bacteria occurs and signs of periodontitis become manifest. Furthermore, when the disease progresses there is a conspicuous absence of a chronic immunopathologic infiltrate. For these reasons, studies concerning the initiation and pathogenesis of periodontal disease in the rat are limited in their ability to extrapolate to the human condition.

The nonhuman primate has been studied for use as an experimental model of periodontitis. The teeth and dentoalveolar structures of nonhuman primates are of similar size and morphology as the human dentition. Nonhuman primates have a primary and permanent dentition which includes molars, premolars, canines and incisors. As has been described above, the supragingival and subgingival microbiota are comprised of similar to that of man bacteria in both periodontal health and disease.

3. Nonhuman Primates - Immune Response Effects on Disease

Manti *et al.* (1984) studied peripheral blood lymphocytes for changes associated with the progression of gingivitis to ligature-induced periodontitis in the cynomolgus monkey. The proportion of helper T cells decreased while B cells increased. The proportion of suppressor T cells remained the same; however, their total numbers increased. The proportion of *P. gingivalis* cultured from subgingival sites increased from 5.3-16.6% during disease progression. When the animals were treated with the immunomodulating agent thymopoietin the helper/suppressor T cell ratio increased and *P. gingivalis* levels decreased. The authors suggested that lymphocyte subsets have the potential to affect subgingival levels of periodontopathogens.

Studies by Ebersole *et al.* (1987) of the systemic humoral immune response in *Macaca fascicularis* established the fact that antibody responses can be detected that accompany ligature-induced periodontitis. Using an ELISA to measure serum IgG, IgM, and IgA antibodies to several periodontopathogens they found that the responses are generally directed to *P. gingivalis* in both naturally-occurring infections and after implantation. Significant elevations in systemic antibody were also induced by immunization with formalinized *P. gingivalis*. When implantation of *P. gingivalis* resulted in progressing periodontal disease, a significant increase of systemic antibody was detected (Holt *et al.* 1988). Clark *et al.* (1988) reported on naturally-occurring IgG antibody to *P. gingivalis* in squirrel monkeys. Subgingival black-pigmented *Bacteroides* (BPB) were found in 50-70% of squirrel monkeys and increased levels were associated with increased serum IgG antibody specific for *P. gingivalis*. In an attempt to modulate colonization of BPB, McArthur *et al.* (1989) immunized a group of squirrel monkeys with a formalinized squirrel monkey isolate of *P. gingivalis*. A 100- to 500-fold increase of serum IgG antibody to this microorganism over baseline

was detected. During a 14 week ligation period, there was also a trend toward decreased BPB in the immunized monkeys. Similarly, Nisengard *et al.* (1989) showed that immunization with whole formalinized microorganisms induced elevated serum IgG antibody specific for *Bacteroides macacae*. This antibody production correlated with decreased levels of *B. macacae* compared to sham immunized cynomolgus monkeys.

These recent studies using the non-human primate model have shown that immunization with formalin-fixed whole bacteria appeared to have the ability to reduce colonization of the gingival crevice by black-pigmented *Bacteroides*. Also, it has been suggested that a specific elevated humoral immune response to periodontopathogens can be induced by parenteral immunization of the nonhuman primate. However, further characterization of the humoral immune response in the nonhuman primate is necessary to establish the nonhuman primate as a reliable animal model for the study of periodontitis. Additionally, efforts can then be made to discover the importance of the antibody response as a protective or destructive factor.

As detailed above, it is clear that the nonhuman primate has been defined as an excellent model for studying the clinical and microbiological progression of periodontitis. Although the clinical, microbiological, and histological events in the progression of ligature-induced periodontitis in the nonhuman primate are well understood, there is little information relevant to the immunological responses of the animals to the microbiota of progressing disease or the function of this immune response.

II. MATERIALS AND METHODS

A. *Monkeys and Serum Samples*

Twenty systemically healthy adult female cynomolgus monkeys with erupted third molars were used in these studies. They had no preexisting periodontitis, and their Plaque Index and Gingival Index scores were >1.0. Serum samples were obtained at baseline and every 5 weeks for 8 months post-ligation. The serum was collected from venipuncture blood samples that were allowed to clot for 2 hours at room temperature and centrifuged at 5,000 x g for 15 minutes. Standardized oral examination, radiographs, and subgingival plaque samples were also obtained at these times as part of a larger study that assessed the effects of immunization on the colonization and/or emergence of *P. gingivalis* and *P. intermedia.* The animals were maintained in accordance with the guidelines established at the University of Texas Health Science Center at San Antonio which is accredited by the American Association of Laboratory Animal Care.

B. *Microorganisms*

Anaerobic culturing of the microorganisms was done at 37°C in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. The microorganisms were grown on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) with 5% sheep blood and supplemented with 5 μg/ml of hemin and 1 μg/ml of menadione. The stock solutions were prepared by dissolving 100 mg of hemin in 2 ml of 1 M NaOH, and bringing to a total volume of 100 ml with deionized water and by dissolving 50 mg of menadione in 50 ml of 95% ethanol, brought to 100 ml with DH_2O, filter sterilized with a 0.22 μ filter, and stored at 4°C.

For antigen preparation, the organisms were grown in mycoplasma broth base (BBL) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml) under anaerobic conditions at 37^{o}C. Each liter of media was prepared by dissolving 21 gm of Mycoplasma Broth Base (BBL) in 1 liter of DH_2O to which was added 5 ml of the hemin stock solution. The media was autoclaved and cooled to 50^{o}C and then 2 ml of the filter-sterilized menadione solution was added. The media was placed in an anaerobic chamber overnight before the *P. gingivalis* innoculum was added. The cultures were grown to stationary phase (~48 hours) and culture purity was assessed by gram staining and streaking onto blood-agar plates to identify colony morphology. A total of 16 liters of bacterial suspension were grown. The microorganisms were prepared for immunization and antibody analysis according to the method of Ebersole *et al.* (1986). Bacteria were harvested by centrifugation (13,000 X g; 20 minutes) at 4^{o}C, washed 3 times in phosphate-buffered saline (0.02 M phosphate; PBS) containing 1 mM EDTA (PBSE), and killed with 0.5% buffered formal saline by incubation at room temperature for 16-18 hours. The organisms were again washed 3 times in PBSE, and resuspended in 100 ml of PBSE. Bacterial cells were dispersed by expressing the suspension through a 26 gauge needle and stored at 4^{o}C. Bacterial density was determined by counting the formalinized organisms in a Petroff-Hauser chamber.

C. *Immunization and Reference Serum Standards*

The Nhps were immunized with a series of 3 subcutaneous injections with 10^9 bacteria emulsified in incomplete Freund's adjuvant (IFA) or with IFA alone (sham-immunized group) during a 1 month interval.

To obtain reference serum standards, two cynomolgus monkeys (N 21 and N 45) were immunized with *P. gingivalis* strain 3079.03, *P. intermedia* strain 6235.2 and *B. fragilis* (ATCC 25258). The antigens were prepared by pelleting 3 ml of killed bacteria (10^8 cells/ml)

by centrifugation (10,000 x g; 10 min.). The pellet was resuspended in 1.5 ml PBS, added to 1.5 ml IFA, and the suspension emulsified using a vortex mixer. Each monkey received 1 ml of the immunogen injected subcutaneously in the thigh. Immunizations were repeated at 7 and 14 days. The serum standard for *Bacteroides fragilis* was obtained from a monkey with high levels of antibody to this microorganism. Sera were subsequently obtained by venipuncture and stored at 4°C until used in the ELISA assays.

D. ELISA Development for Immunoglobulin G, M, and A Isotypes

Formalin-killed microorganisms diluted in 0.1M $NaCO_3$ buffer at pH 9.6 containing 0.02% NaN_3 were attached to polystyrene microtiter plates (Linbro) at 37°C for 4 hours. The antigen-coated plates were stored at 4°C until used. The assay procedure is composed of 4 steps:

1) incubation of washed, antigen-coated plates with the experimental serum diluted in phosphate buffered saline (PBS) containing 0.05% Tween 20 (Fischer Scientific Co.) and 0.02% NaN_3 for 2 hours at room temperature on a rotator;

2) three 5 minute washes with 0.9% NaCl, pH 7.4 containing 0.05% Tween 20 followed by incubation with affinity purified goat anti-human IgG, IgM, or IgA antisera (Calbiochem IgG #401441, IgM #401901, and IgA #401131) for 2 hours at room temperature;

3) washing (as described above) followed by incubation with affinity purified rabbit anti-goat IgG conjugated to alkaline phosphatase (Sigma Chemical Co.) for 16-18 hours at room temperature; and

4) addition of p-nitrophenylphosphate (1 mg/ml in 0.05M $NaCO_3$, pH 9.8 with 1mM $MgCl_2$; Sigma 104 Phosphate Substrate) as the substrate. The reactions were terminated by the addition of 1N NaOH.

The extent of the reaction was determined spectrophotometrically at 410 nm. (Dynatech). A serum standard was included in each plate and the level of antibody in the experimental sera was determined by comparison to a linear regression analysis relating the optical density (OD) to the logarithm of the antibody activity in the standard. The experimental NhP sera were diluted so that the OD readings fell within the linear range of the standard curve.

Specificity of the anti-isotype antisera was assessed using an ELISA. For this determination purified polyclonal human IgG (Sigma), IgM (Sigma) and IgA (Calbiochem) were used to coat wells at 2 μg/well. The assay was developed with the isotype-specific antisera and rabbit anti-goat IgG enzyme conjugate as above. The results are shown in **Table 1** and demonstrate the specificity of the antisera.

E. ELISA Development for Immunoglobulin G Subclasses

The antigens used for determination of subclass antibodies were as described for the isotype identifications. The monoclonal antibodies used in the ELISA were directed to human IgG subclasses and comprised: anti-IgG1 (HP6001, Calbiochem); anti-IgG2 (HP6002, Calbiochem; HP6014, Zymed); anti-IgG3 (HP6047, Calbiochem) and anti-IgG4 (HP6025, Calbiochem). The system was developed using affinity purified goat anti-mouse IgG conjugated to biotin (Zymed) and alkaline phosphatase conjugated Streptavidin (Zymed). The relative levels of the IgG subclasses were estimated by adjusting the development system so as to detect the maximum level of each subclass (OD measurement) in the reference sera.

TABLE 1

Specificity of Anti-Isotype Antisera used in ELISA for Measurement of Nhp Antibodies

	% Reactivity Compared to Homologous Immunoglobulin		
Antisera Specificity	**IgG**	**IgM**	**IgA**
Anti-IgG	100	0.02	0.02
Anti-IgM	0,8	100	0.04
Anti-IgA	0.25	0.05	100

The results describe the mean of triplicate determinations for each combination of isotype specific antisera and immunoglobulin used as antigen to coat the plates. Each homologous reactivity was assigned a value of 100%. The antibody activity to the heterologous antigens was then compared to this control. The range of replicates from the mean values was <8% of the mean.

As such, the dilution of the sera and the absolute OD could be compared to estimate the relative activity of each subclass. As was described for assessment of specificity of the isotype antisera, the monoclonal antibodies were examined using an ELISA in which wells were coated with purified human myeloma IgG1 (Calbiochem), IgG2 (Calbiochem), IgG3 (Calbiochem) and IgG4 (Calbiochem). The results in **Table 2** demonstrate the specificity of the murine monoclonal antibodies.

The subclasses of antibody were expressed as a proportion of the total IgG antibody activity, based upon their individual ELISA Unit (EU) relationships.

For example:

IgG1 max. OD = 1.0 @ 1:25 diln. (std.); therefore,	50
IgG2 max. OD = 1.0 @ 1:50 diln. (std.); therefore,	100*
IgG3 max. OD = 0.5 @ 1:10 diln. (std.); therefore,	14
IgG4 max. OD = 0.75 @ 1:10 diln. (std.); therefore,	18
SUM in ELISA Units =	182

* Assigned relative EU of the serum standard

Total IgG = 147 determined by isotype ELISA.

Therefore,

IgG1 = 50(147)/182 = 40.4 EU
IgG2 = 100(147)/182 = 80.8 EU
IgG3 = 14(147)/182 = 11.3 EU
IgG4 = 18(147)/182 = 14.5 EU

F. Experimental Protocol

Baseline serum samples were obtained 2 weeks prior to immunization, during the immunization phase and throughout the duration of the experiment. **Figure 1** describes the protocol with sampling intervals in weeks. Six nonhuman primates (Nhp) were immunized with *P. intermedia*, 5 Nhp with *P. gingivalis*, 5 Nhp with *B. fragilis* and 4 Nhp with incomplete Freund's adjuvant as a control group. *B. fragilis* was included as a non-cross reacting species to emphasize the importance of the response specificity.

TABLE 2

Specificity of Anti-Subclass Monoclonals used in ELISA for Measurement of Nhp Antibodies

	% Reactivity Compared to Homologous Immunoglobulin			
Anti-Subclass Monoclonal Specificity	**IgG1**	**IgG2**	**IgG3**	**IgG4**
Anti-IgG1	100	0.01	0.9	0.02
Anti-IgG2	0.03	100	0.03	0.07
Anti-IgG3	1.2	0.02	100	0.2
Anti-IgG4	0.5	0.02	0.05	100

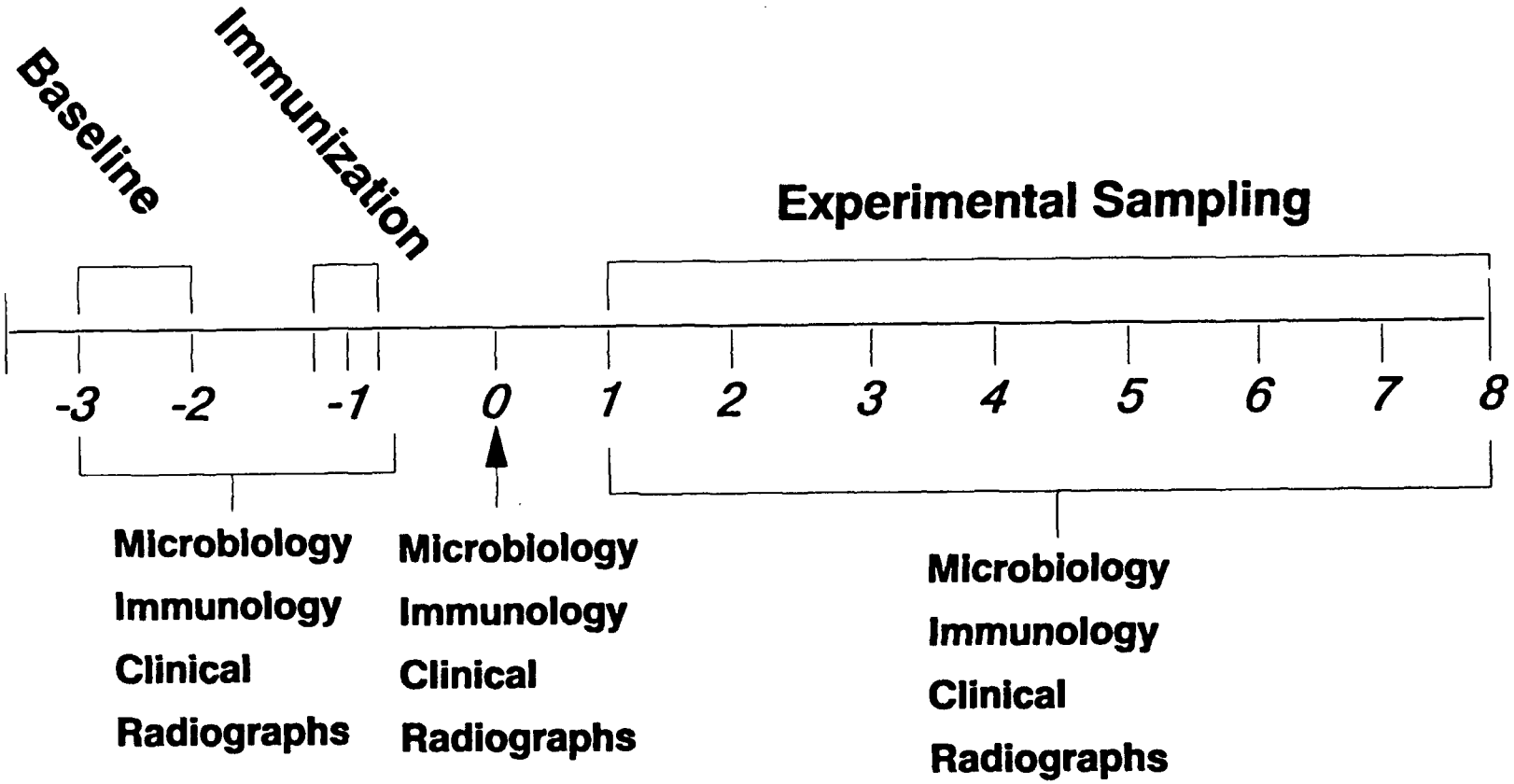
The results describe the mean of triplicate determinations for each combination of subclass specific antisera and myeloma used as antigen to coat the plates. Each homologous reactivity was assigned a value of 100%. The antibody activity to the heterologous antigens was then compared to this control. The range of replicates from the mean values was <11% of the mean.

The monkeys received ligatures 2 weeks after the last immunization. Silk sutures were ligated at the cementoenamel junction of all mandibular left second premolars and second molars to convert the pre-existing gingivitis to periodontitis. The teeth in the mandibular right quadrant served as non-ligated controls.

G. Specificity Studies

To determine the specificity of the antibody responses to the bacteria, adsorptions of sera with the formalinized microorganisms were performed. A 1:25 dilution of four nonhuman primate sera (1.0 ml) was incubated with 10^9 organisms in 1.0 ml of PBS and mixed for 1 hr at 37^{o} C. The organisms were removed by centrifugation, and the resulting 1:50 serum dilution was used to test for residual antibody activity. Antibody activity in the Nhp sera was expressed as ELISA units (EU) defined by a reference curve prepared by a linear regression analysis. Each Nhp serum was assigned a value of 100 EU prior to adsorption. Antibody activity in the sera after the adsorption procedures were each related to the homologous untreated activity.

Experimental Protocol



H. Statistical Analyses

Descriptive statistics were obtained for data from all groups using a microcomputer program, Statgraphics (STSC) on an IBM PC. Differences between the placebo and treatment groups were determined using a Mann-Whitney U nonparametric rank analysis. Correlation studies to examine relationships of antibody responses among the groups were performed using a Spearman rank correlation analysis.

III. RESULTS

A. Development of Conditions for Isotype Determinations

Experiments to determine the ELISA conditions for isotype measurement were performed using a serum standard derived from hyperimmunized nonhuman primates (Nhp) and a checkerboard assessment of the developing system. Serial dilutions of the serum were tested and concentrations for which an ideal standard curve was achieved were used as a comparision for the test sera. Final optimal concentrations are listed in **Table 3.**

The serum standard derived from hyperimmunized Nhps was included in each ELISA plate. The level of antibody in the experimental sera was determined by comparison to a linear regression analysis relating the optical density (OD) to the $\log_{10}$ of the ELISA Units (EU) in the standard. The experimental NhP sera were diluted so that the OD readings fell within the linear range of the standard curve. After the animals were immunized, sera were examined at 4-8 fold greater dilutions for the immunized group tested against the homologous antigen.

TABLE 3

ELISA Characteristics for Measurement of Nhp Isotype Antibodies

Sample	IgG	IgM	IgA
P. gingivalis Baseline	1:100	1:25	1:25
P gingivalis Immunized	1:800	1:100	1:100
Polyclonal Anti-Isotype	1:500	1:500	1:500
Reference Standard	1:400 100 EU	1:50 12.5 EU	1:40 10 EU
P. intermedia Baseline	1:200	1:100	1:100
P. intermedia Immunized	1:1600	1:800	1:800
Polyclonal Anti-Isotype	1:500	1:500	1:500
Reference Standard	1:800 200 EU	1:400 100 EU	1:400 100 EU
B. fragilis Baseline	1:100	1:400	1:100
B. fragilis Immunized	1:800	1:3200	1:1600
Polyclonal Anti-Isotype	1:500	1:500	1:500
Reference Standard	1:400 100 EU	1:1600 400 EU	1:200 50 EU

B. Development of Conditions for Subclass Determinations

Determination of concentrations for IgG subclasses using mouse monoclonal antibody was also done via serial dilutions of the reference standard. Mouse monoclonal antibodies prepared to human IgG subclasses also react with molecules in the serum of nonhuman primates. Studies by Bauman (1990) have shown the similarity in reactivities between the human and nonhuman primate serum components. Although exact identification of molecular homology was not determined, each of the monoclonal antibodies was exquisitely specific for the homologus human myeloma protein. The optimal system and reactivity of the reference sera are shown in **Table 4.**

C. Isotype Responses

1. Natural Antibody Levels

Baseline serum immunoglobulin levels to *Prevotella intermedia*, *Porphyromonas gingivalis* and *Bacteroides fragilis*: All of the nonhuman primates that were included in this study exhibited baseline antibody levels to *P. gingivalis*, *P. intermedia* and *B. fragilis*.

The baseline serum IgG antibody response to *P. intermedia* ranged from 20-184 ELISA units (EU). The baseline serum IgG response to *P. gingivalis* ranged from 2-95 EU and the values for *B. fragilis* were from 3-10 EU (**Figure 2**). A wide variability of responses to the different bacteria among the monkeys was also observed.

The baseline serum IgM antibody response to *P. intermedia* ranged from 5-70 EU. The baseline serum IgM response to *P. gingivalis* ranged from 2-16 EU and the

values for *B. fragilis* were from 75-1,253 EU (**Figure 3**). As was noted with IgG, a wide variability of IgM responses among the Nhps was seen.

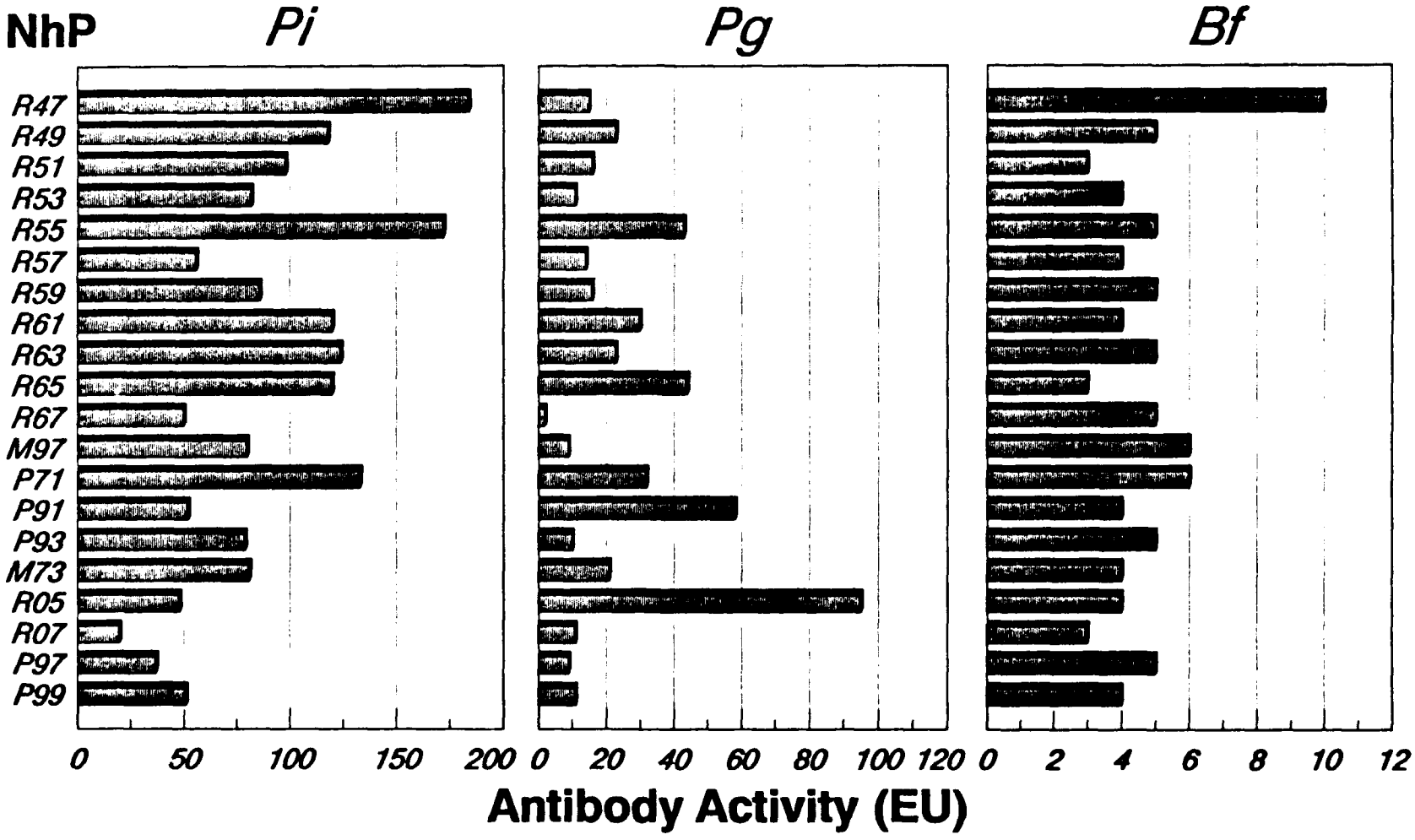
The baseline serum IgA antibody levels to *P. intermedia* ranged from 7-39 EU. The baseline serum IgA levels to *P. gingivalis* ranged from 1-6 EU and IgA values for *B. fragilis* were from 5-43 EU (**Figure 4**).

TABLE 4

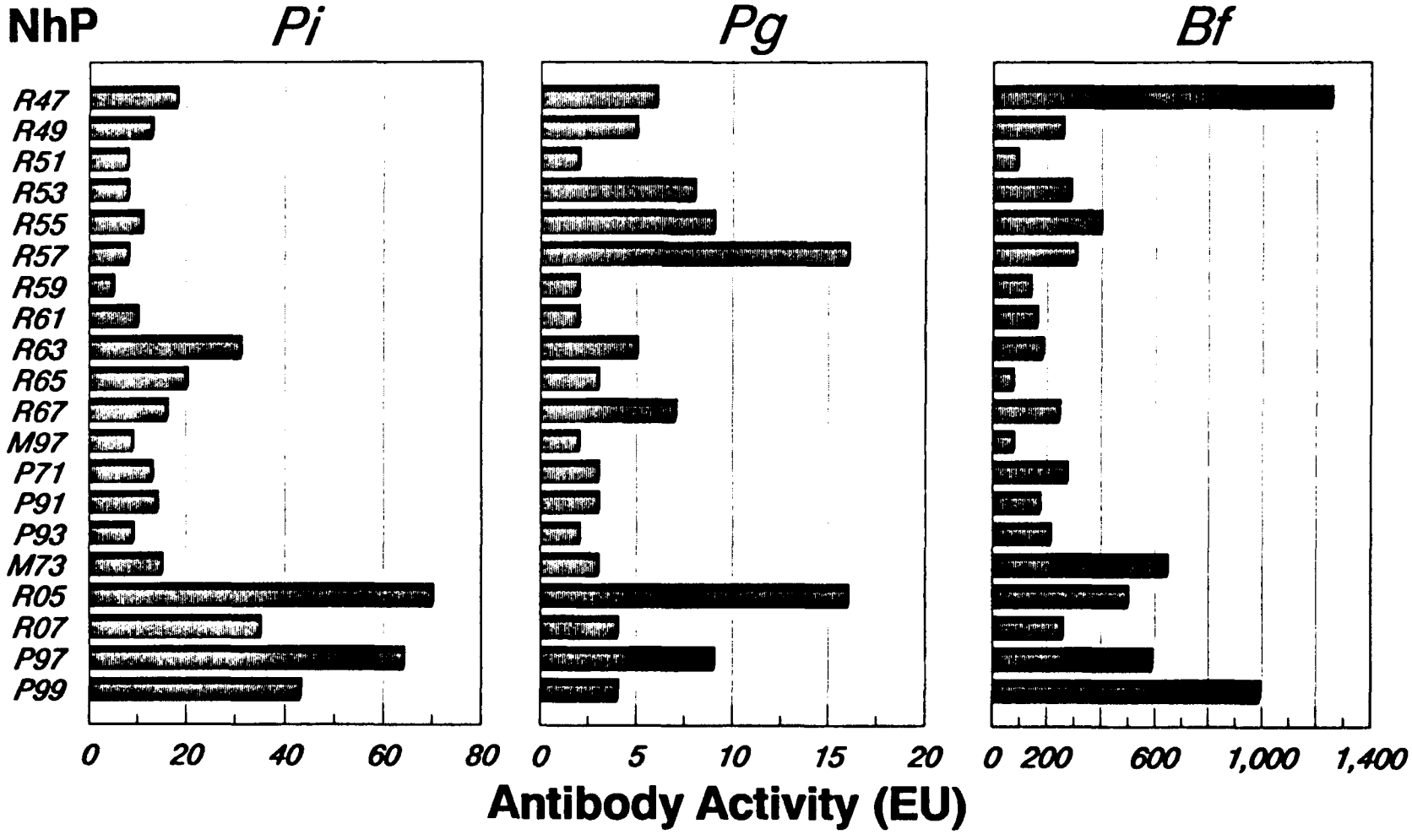
ELISA Characteristics for Measurement of Nhp Subclass Antibodies

Sample	IgG1	IgG2	IgG3	IgG4
P. gingivalis Baseline	1:50	1:5	1:10	1:50
P. gingivalis Immunized	1:400	1:10	1:20	1:100
Monoclonal Anti-Subclass	1:200	1:200	1:3200	1:800
Reference Standard	1:200 50 EU	1:5 1.25 EU	1:10 2.5 EU	1:50 12.5 EU
P. intermedia Baseline	1:50	1:50	1:50	1:50
P. intermedia Immunized	1:400	1:100	1:100	1:200
Monoclonal Anti-Subclass	1:1600	1:400	1:6400	1:3200
Reference Standard	1:200 50 EU	1:50 12.5 EU	1:50 12.5 EU	1:100 25 EU
B. fragilis Baseline	1:50	1:50	1:50	1:50
B. fragilis Immunized	1:100	1:50	1:50	1:50
Monoclonal Anti-Subclass	1:3200	1:1600	1:1600	1:400
Reference Standard	1:50 12.5 EU	1:25 6.25 EU	1:25 6.25 EU	1:25 6.25 EU

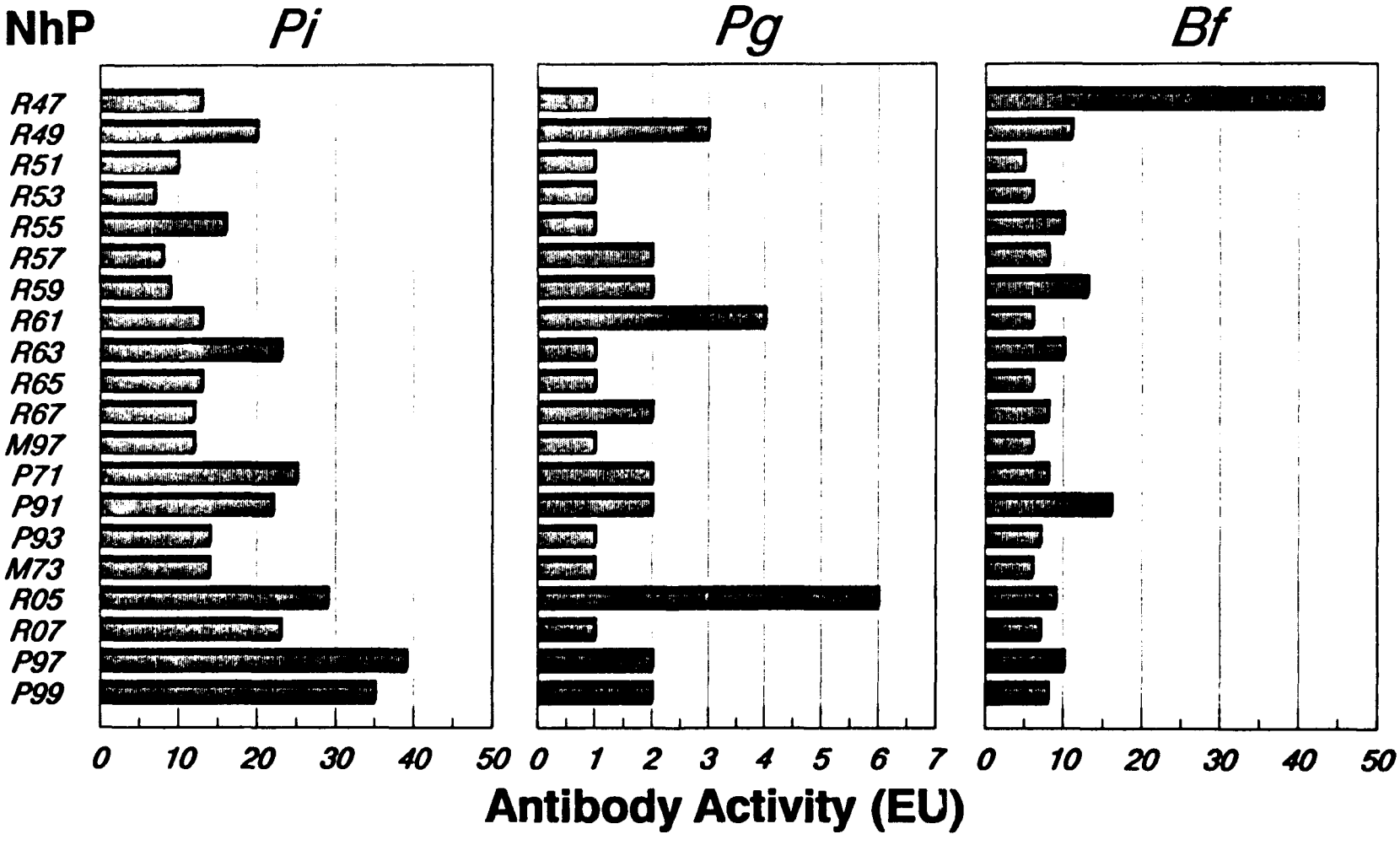
Baseline Serum IgG Antibody Responses in Nhp



Baseline Serum IgM Antibody Responses in NhP



Baseline Serum IgA Antibody Responses in NhP



As shown in **Table 5**, the predominant isotype to the oral microorganisms was IgG, with antibody levels to *P. intermedia* (89.6 $\pm$ 9.8 EU) being 4-fold greater than those to *P. gingivalis* (24.7 $\pm$ 4.9 EU). In contrast, the principal response to the non-oral gut microorganism, *B. fragilis*, was of the IgM isotype. In this case the levels were 30- 100-fold greater than either IgG or IgA antibody. In a group of human subjects similar differences of IgG responses were observed.

2. Serum Antibody Responses to Active Immunization

Levels of all antibody isotypes in all four groups of monkeys were determined after immunization and the results demonstrated significantly elevated responses. Antibody responses in monkeys not immunized to the bacteria showed no significant changes from baseline values. Interestingly, the level of serum IgA antibody was increased to a greater degree than the other isotypes with each of the microorganisms.

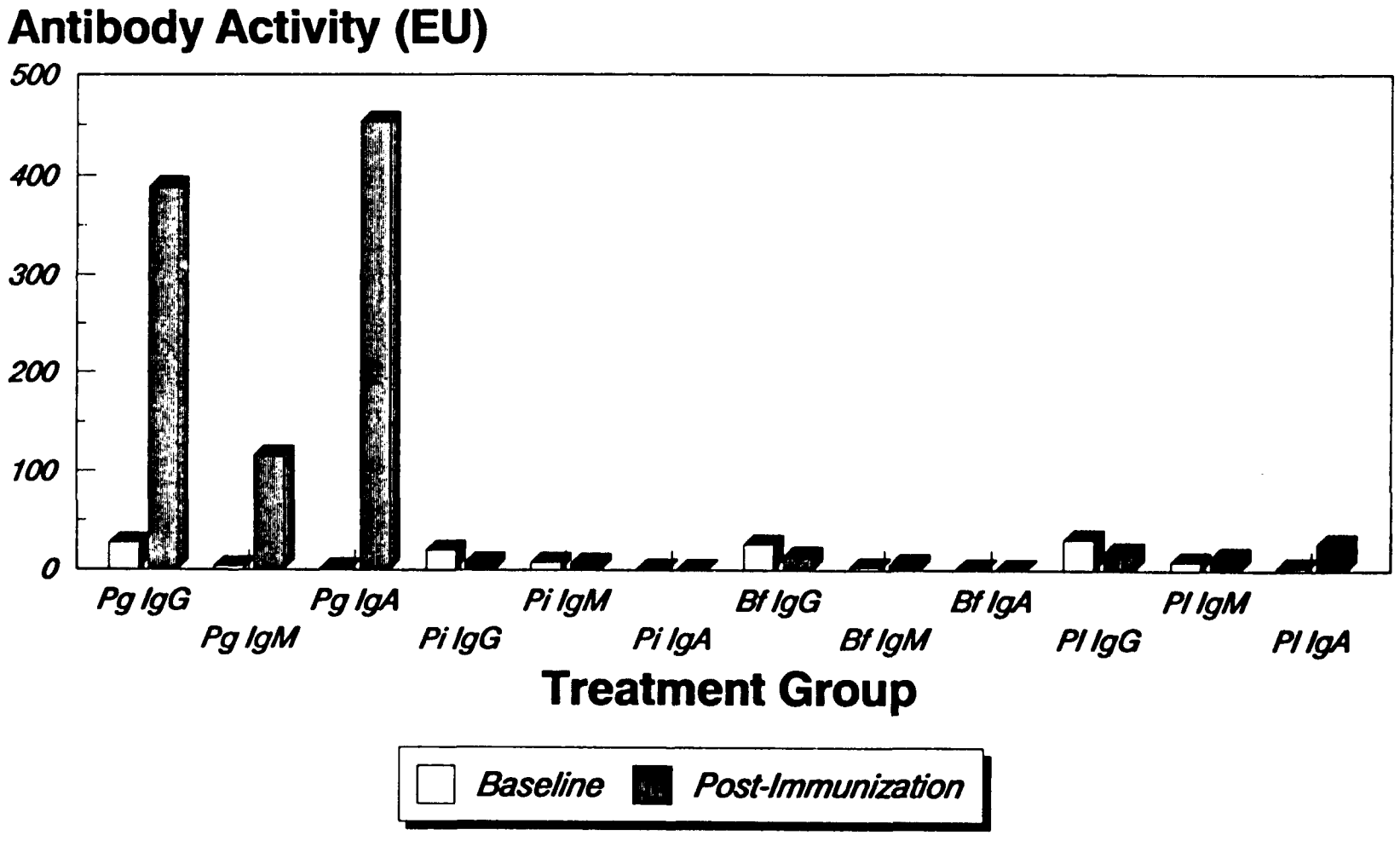
Immunization increased IgG, IgM, and IgA antibody by 16-260 fold to *P. gingivalis* (389/102/263 EU), 5-70 fold to *P. intermedia* (948/534/1411 EU) and 20-138 fold to *B. fragilis* (610/5048/690 EU) (**Figures 5-7**). There was a significant increase of homologous antibody levels to all three microorganisms tested. This demonstrates both the dramatic increase of antibody level post-immunization and also the specificity of the responses. The antibody response to *P. gingivalis* in the three groups not immunized with *P. gingivalis* showed no significant changes from their baseline reactivity to *P. gingivalis* demonstrating the specificity of the response. These same characteristics of a dramatic antibody response and a highly specific response were also noted for the monkeys immunized with *P. intermedia* and *B. fragilis*.

TABLE 5

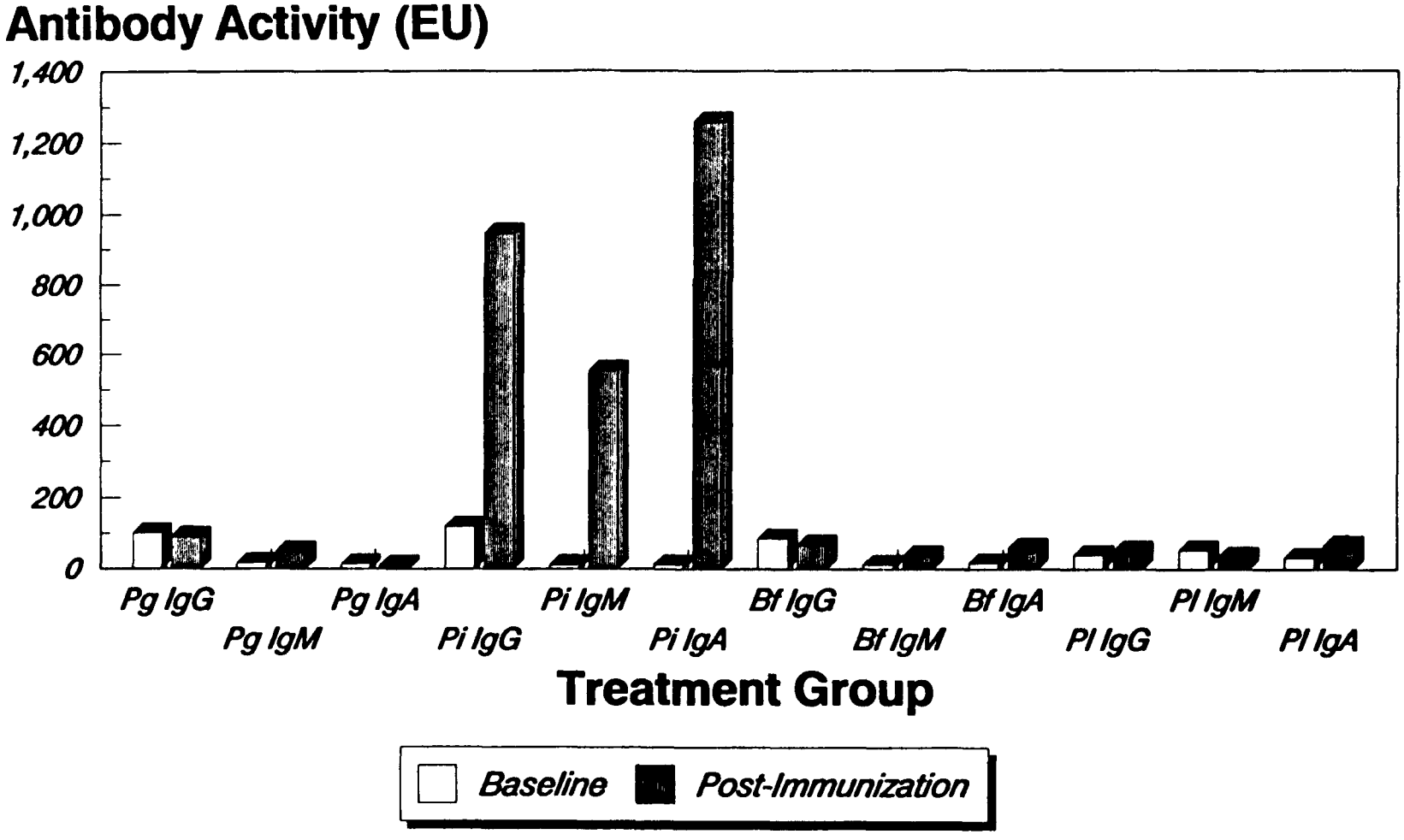
Descriptive Statistics of Serum Antibody Levels in *Porphyromonas/Prevotella/Bacteroides* Species

Species	Isotype	Sample Size	Mean	Median	SD	SEM	Min	Max
Nhp	Pi IgG	20	89.6	81.5	44.0	9.8	20	184
	Pi IgM	20	21.0	13.5	18.6	4.2	5	70
	Pi IgA	20	17.9	14.0	8.9	2.0	7	39
	Pg IgG	20	24.7	16.0	21.8	4.9	2	95
	Pg IgM	20	5.6	4.0	4.3	1.0	2	16
	Pg IgA	20	1.9	1.5	1.3	0.3	1	6
	Bf IgG	20	4.7	4.5	1.5	0.3	3	10
	Bf IgM	20	353.8	255.0	308.5	69.0	75	1,253
	Bf IgA	20	10.2	8.0	8.2	1.8	5	43
Human	Pi IgG	82	63.0	58.0	63.0	7.0	9	168
	Pg IgG	82	9.6	8.9	20.7	2.3	1	27
	Bf IgG	34	74.0	66.0	90.9	15.6	27	202

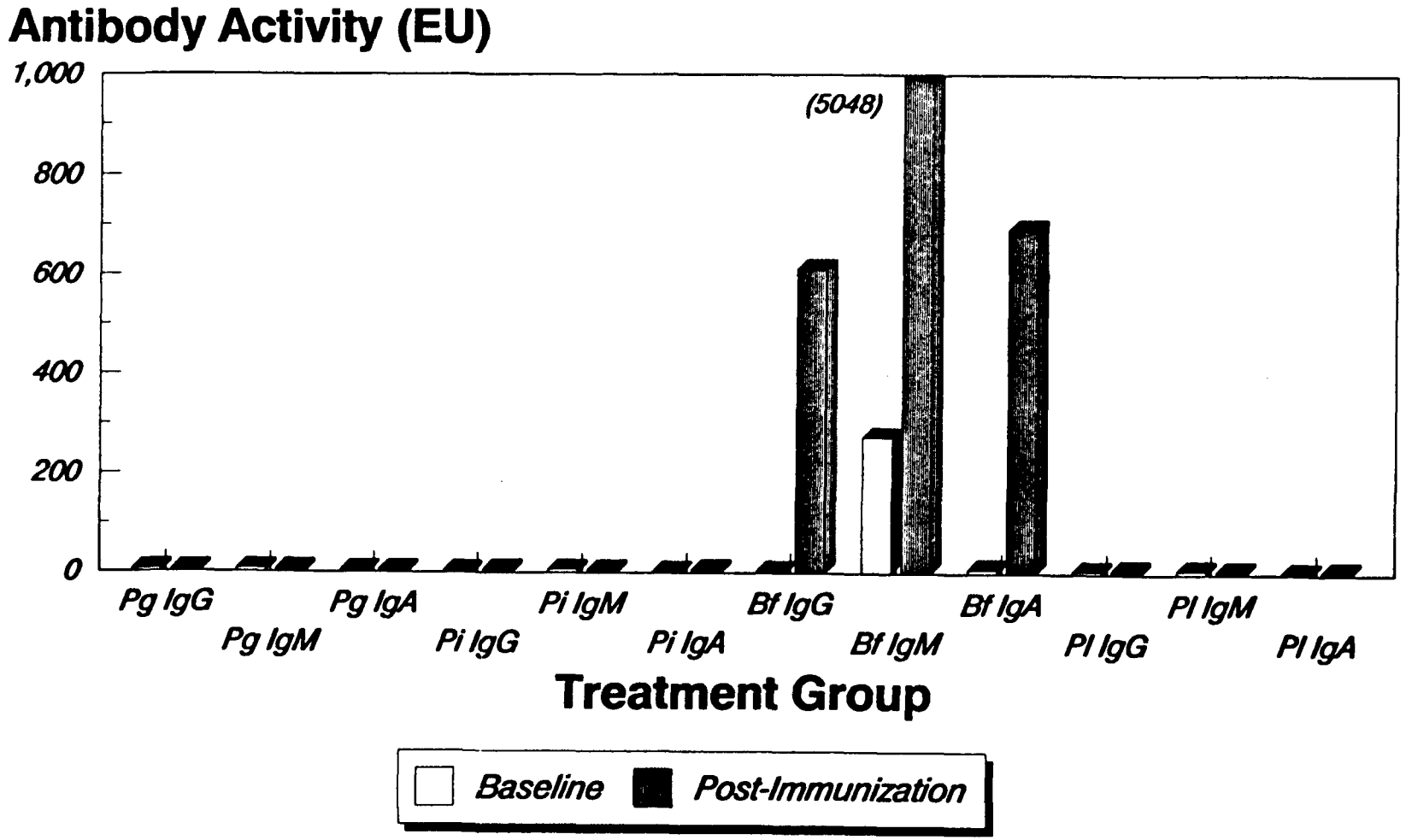
Serum Antibody Responses in NhP to Porphyromonas gingivalis



Serum Antibody Responses in NhP to Prevotella intermedia



Serum Antibody Responses in NhP to Bacteroides fragilis



D. *Dynamics of Isotype Response*

The kinetics of serum IgG responses to the bacterial antigens are seen in **Figures 8-10.** As noted previously, there was a substantial increase in the antibody response following active immunization. However, the dynamics of this antibody response post-immunization and post-ligation differed with respect to each of the bacteria tested. Antibody to *P. gingivalis* increased through week 16 (11 weeks post-ligation) of the experiment and remained elevated through week 22 of the study. The IgG antibody level to *P. intermedia* peaked at 2 weeks following the third immunization and decreased post-ligation to near baseline levels by week 16. Anti-*B. fragilis* IgG antibody increased to peak levels by 6 weeks post-ligation, at which time it decreased substantially but remained significantly above the baseline through 22 weeks of the experiment. The kinetics of serum IgM and IgA responses in all immunized groups were generally similar with peak responses attained approximately 4 weeks post-immunization and levels dropping during the ligation interval **(Figures 11-16).**

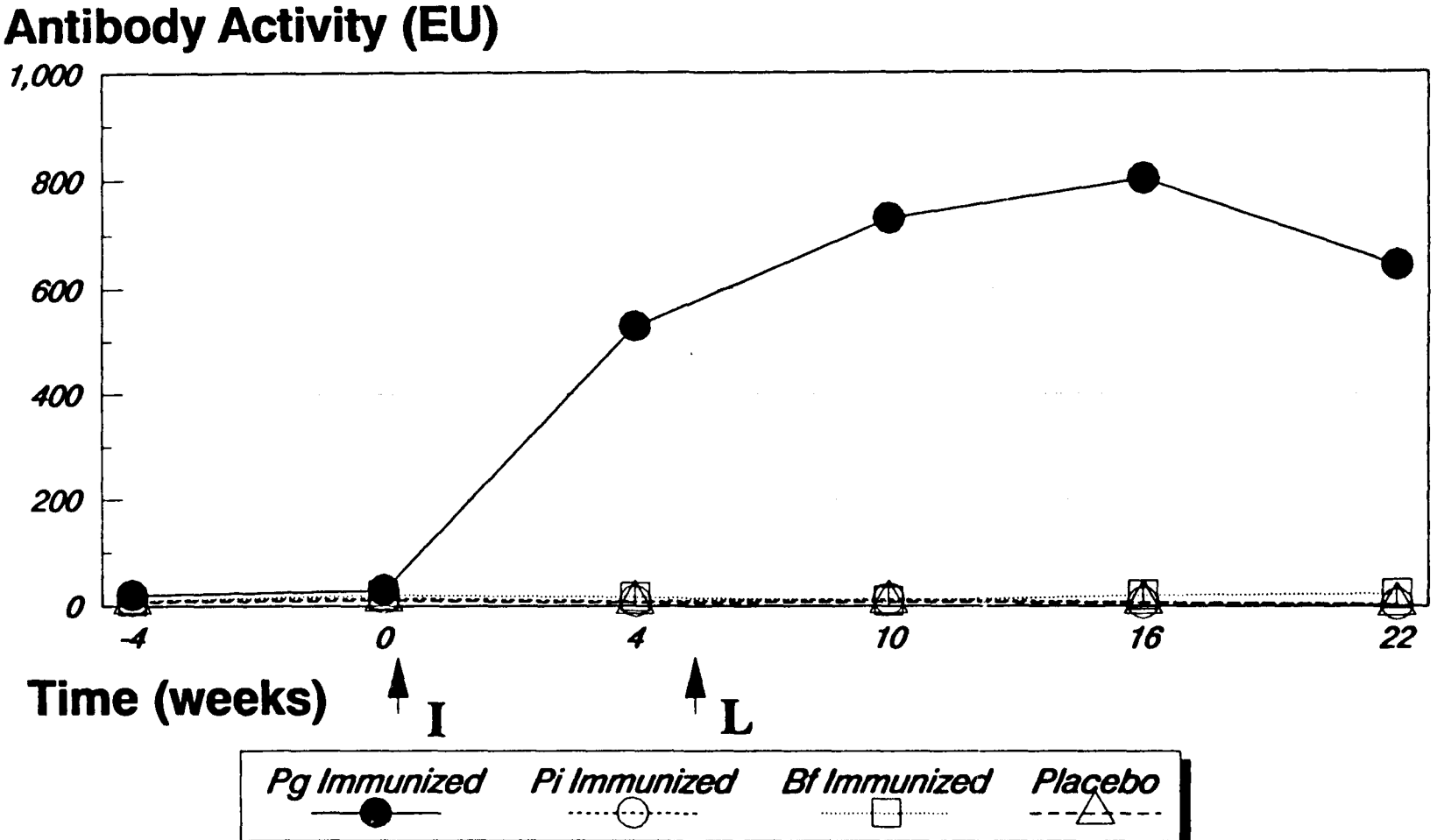
E. *Subclass Response Characteristics*

Nearly 75% of natural IgG antibody was comprised of IgG1 to *P.gingivalis* and *P. intermedia*, while IgG2 and IgG3 predominated to *B. fragilis* **(Figures 17-19).**

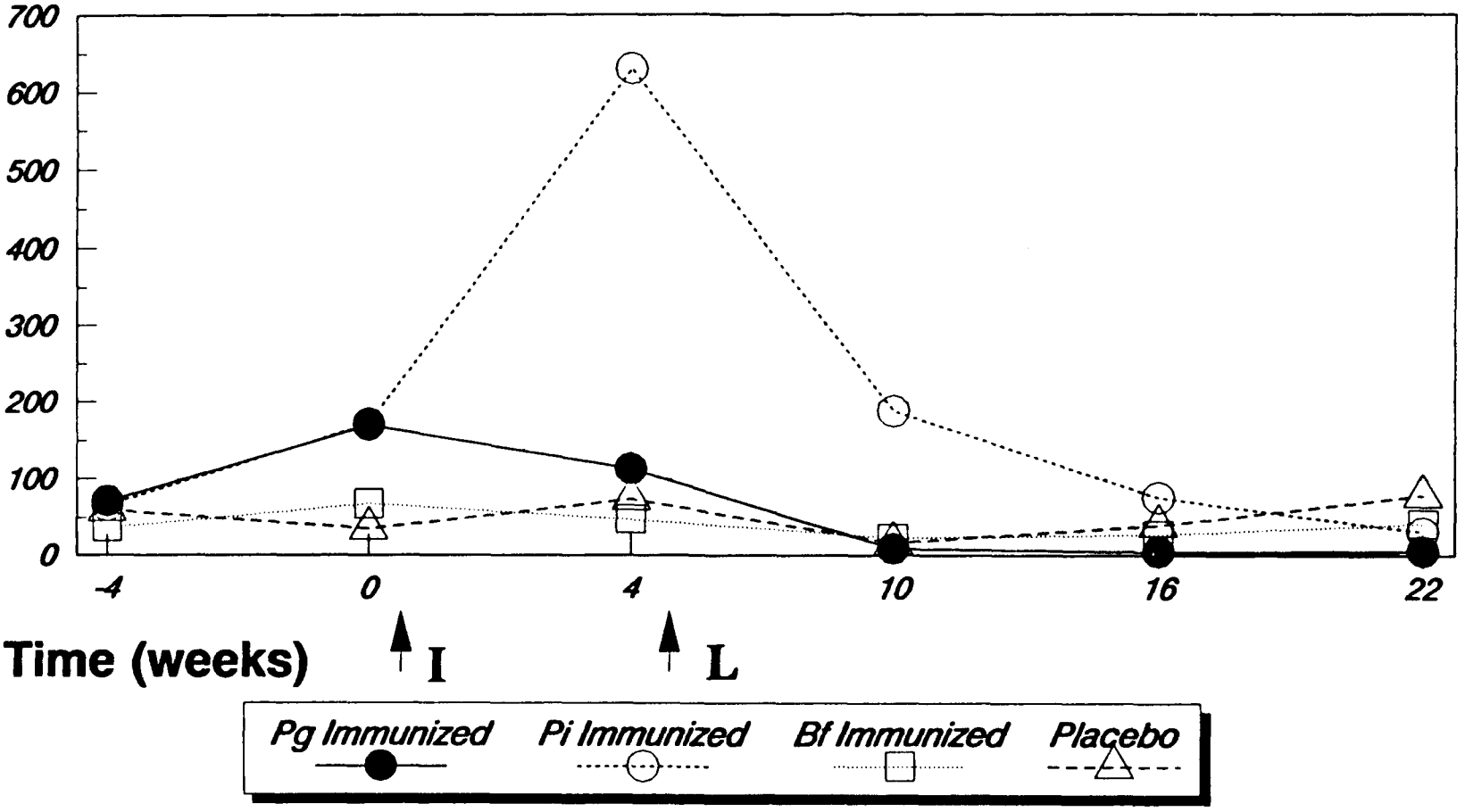
The IgG response to *P. gingivalis* after immunization was comprised primarily of IgG3 and IgG4, while the post-immunization IgG response to *P. intermedia* was largely IgG1. For *B. fragilis*, the post-immunization IgG response was almost entirely comprised of IgG1 **(Figures 17-19).**

Analysis of subclass proportions midway through the ligation phase of the experiment demonstrated a conversion back to a large proportion of the IgG response to *P. gingivalis*

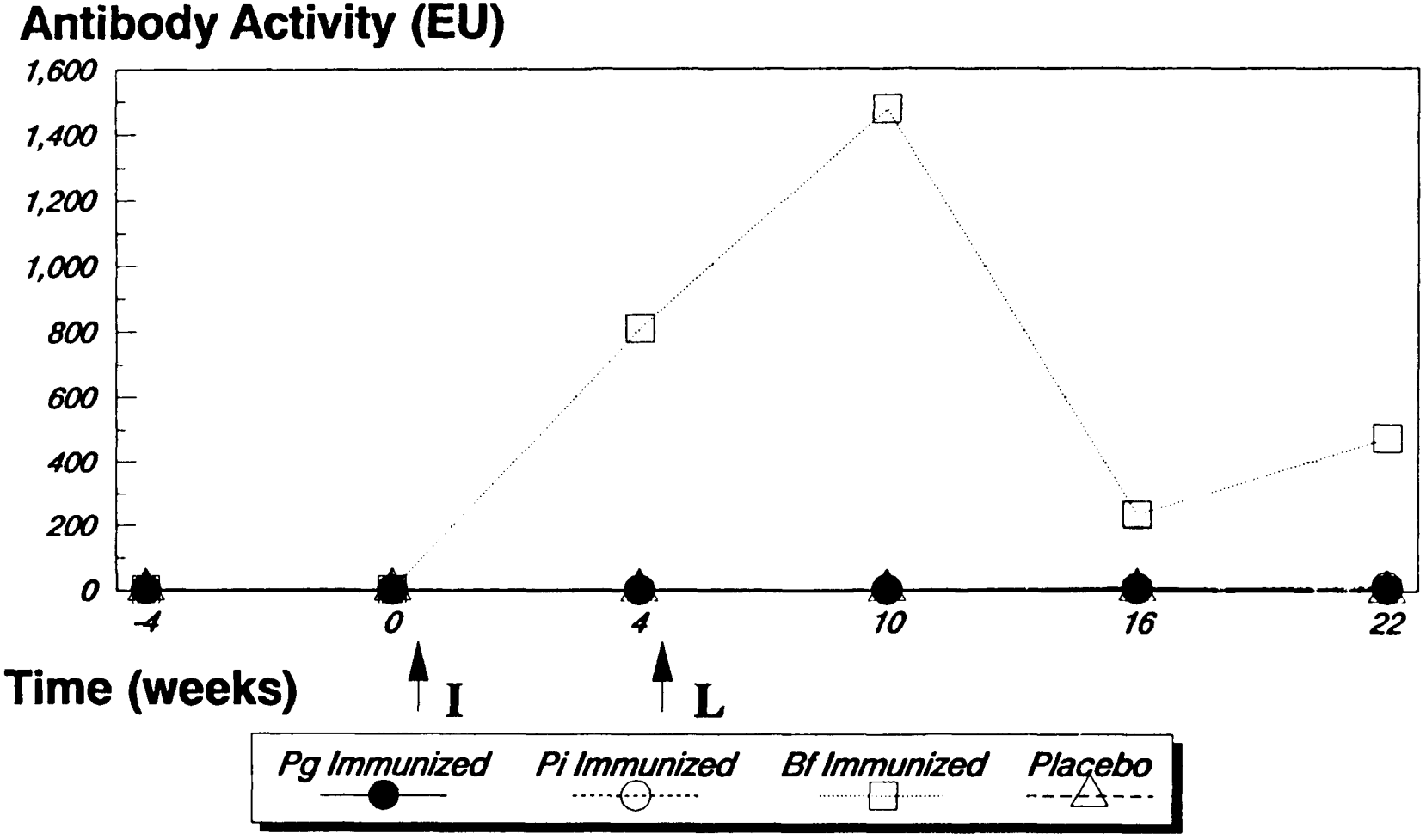
NhP IgG Antibody Response to Porphyromonas gingivalis



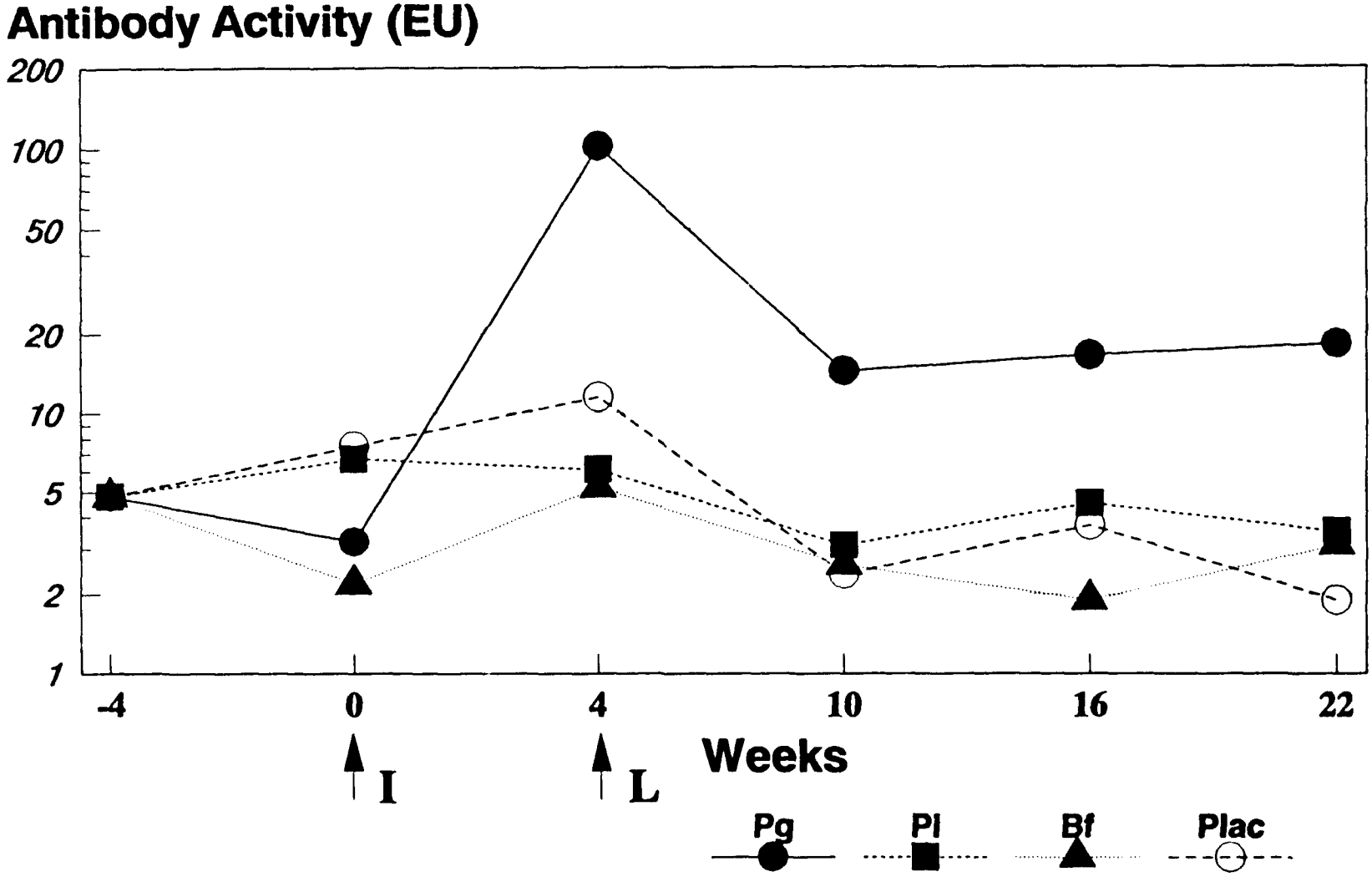
NhP IgG Antibody Response to Prevotella intermedia



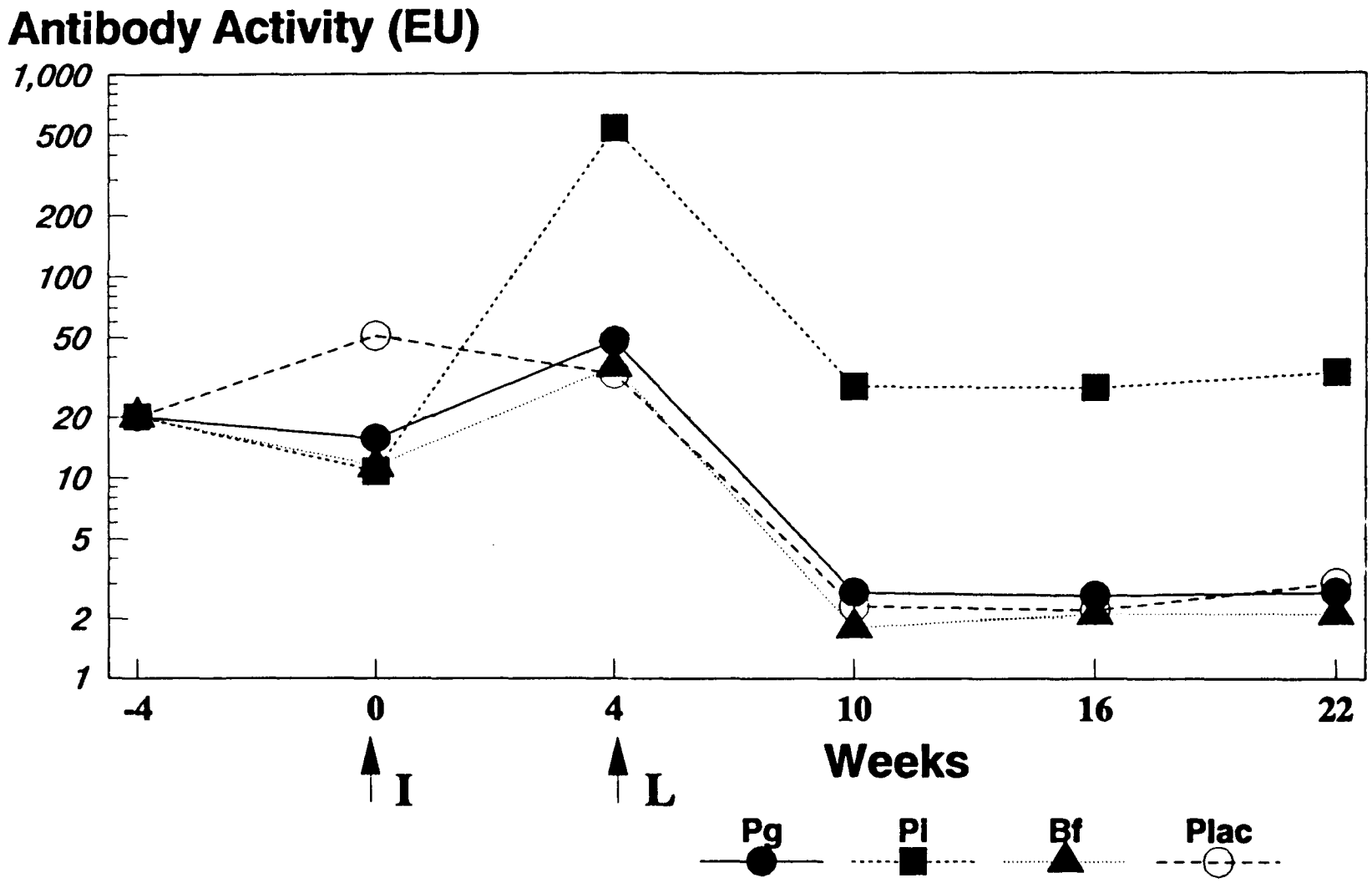
NhP IgG Antibody Response to Bacteroides fragilis



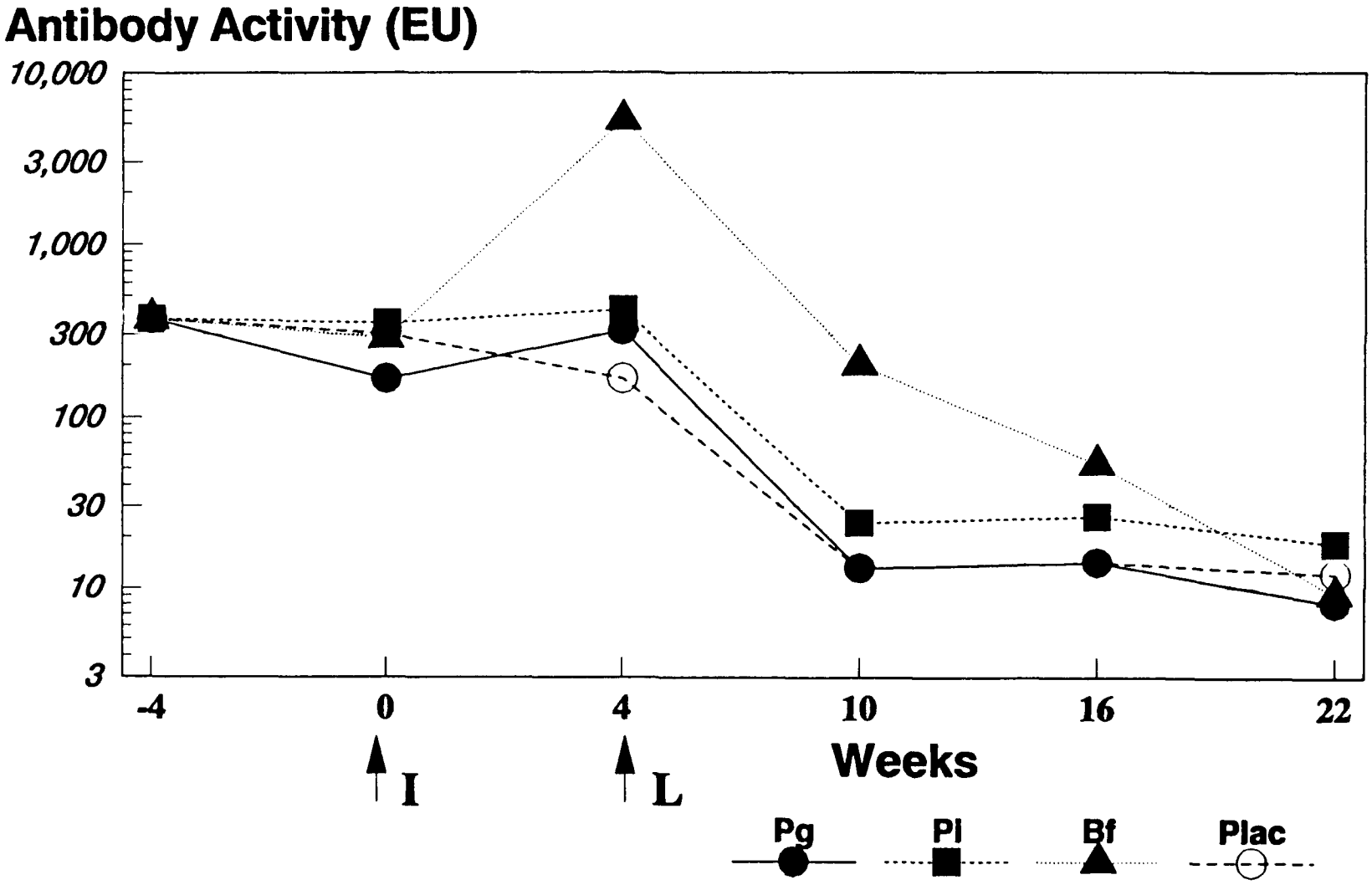
Serum IgM Antibody to P. gingivalis



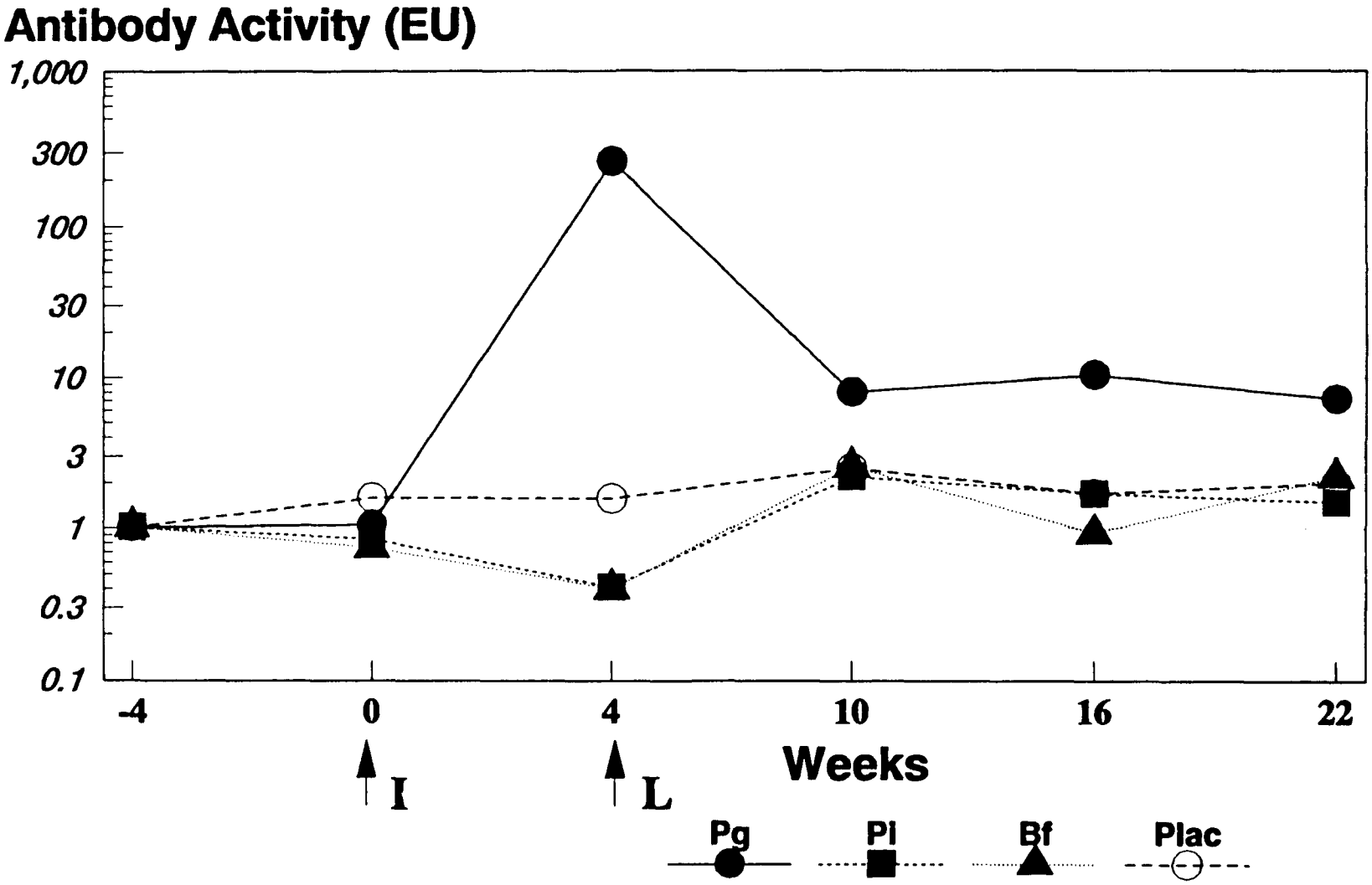
Serum IgM Antibody to P. intermedia



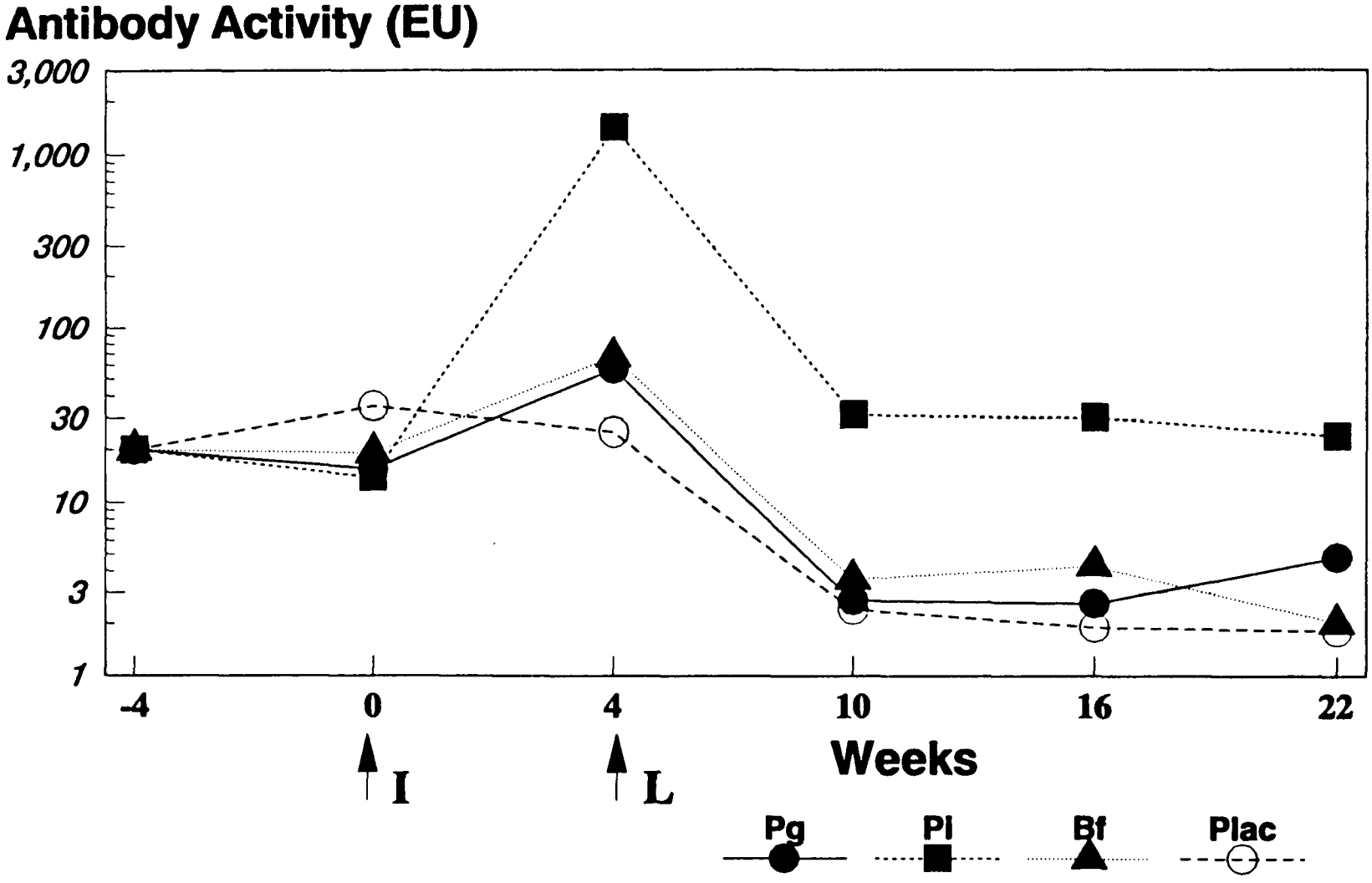
Serum IgM Antibody to B. fragilis



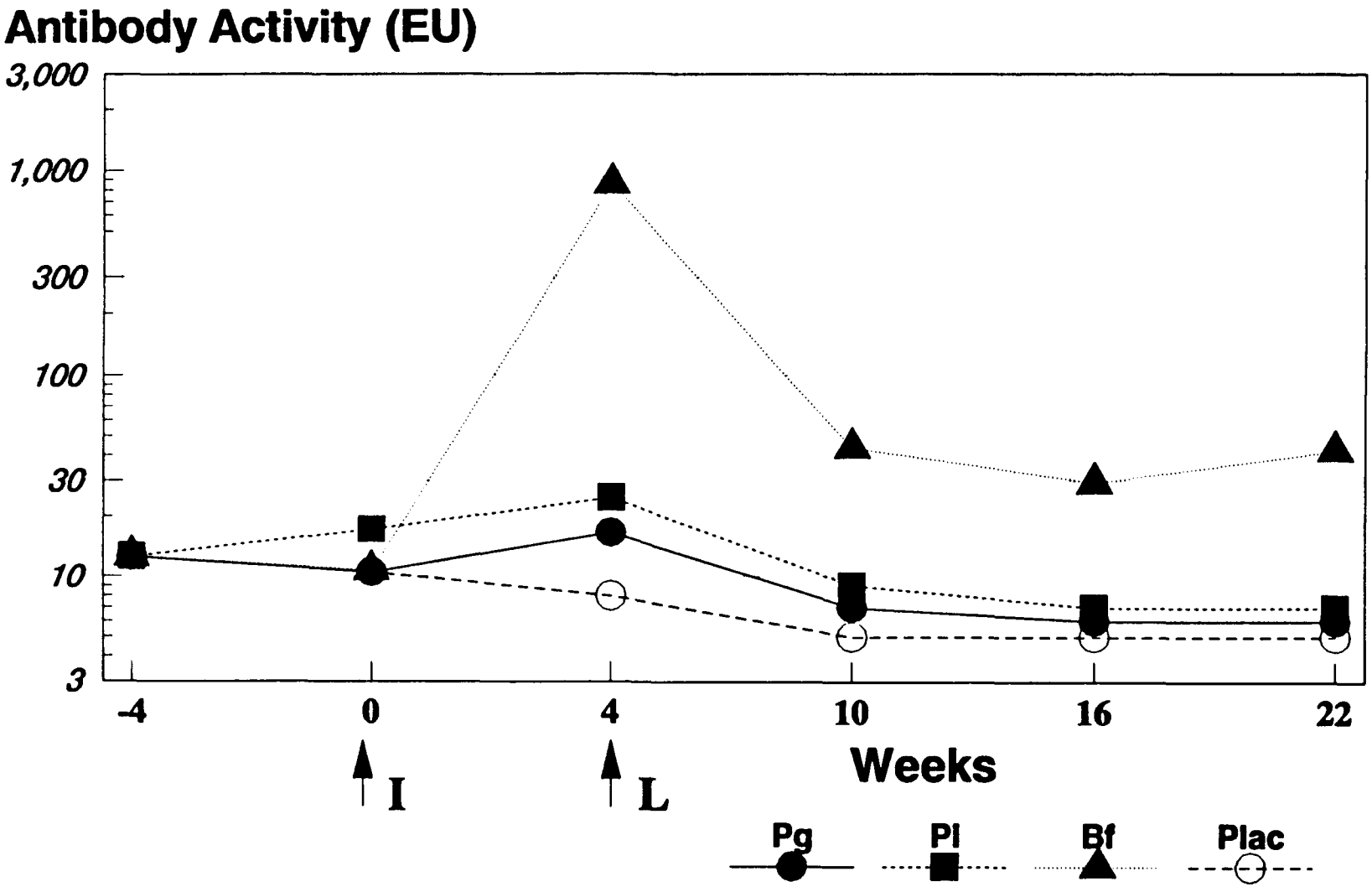
Serum IgA Antibody to P. gingivalis



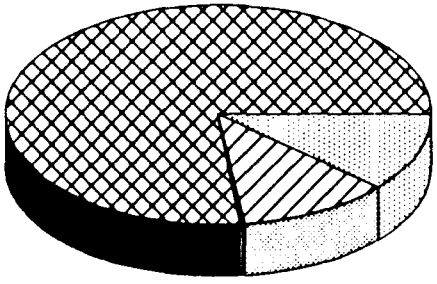
Serum IgA Antibody to P. intermedia



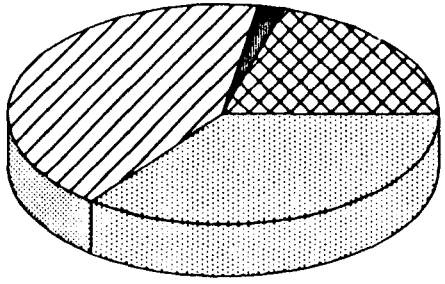
Serum IgA Antibody to B. fragilis



NhP IgG Subclass Antibody Response to Porphyromonas gingivalis



Baseline



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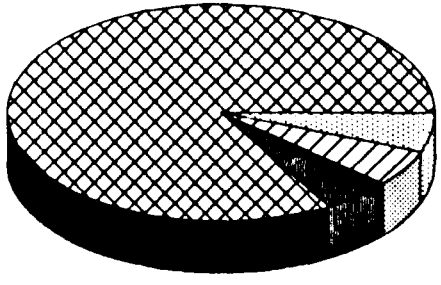

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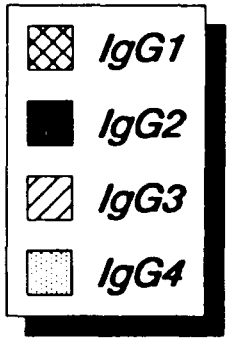
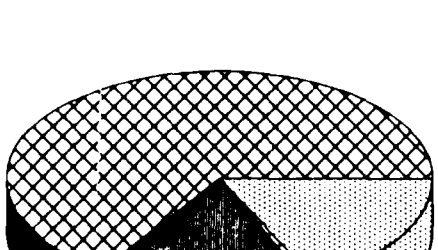


NhP IgG Subclass Antibody Response to Prevotella intermedia



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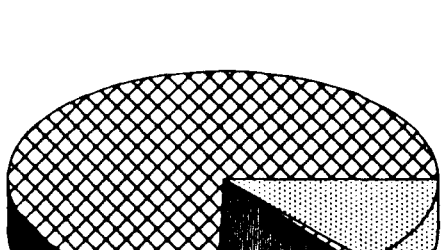

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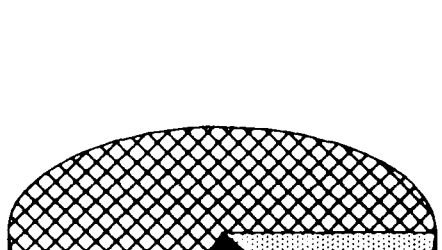

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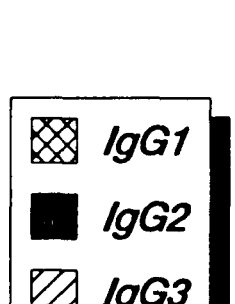
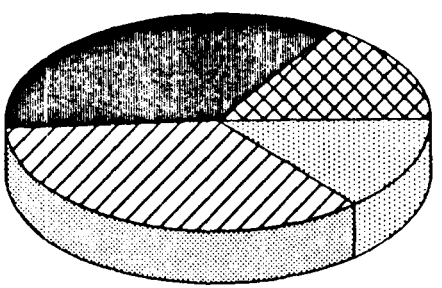


NhP IgG Subclass Antibody Response to Bacteroides fragilis



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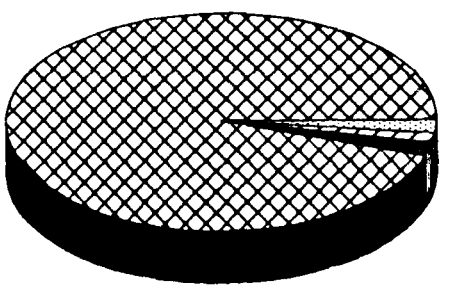
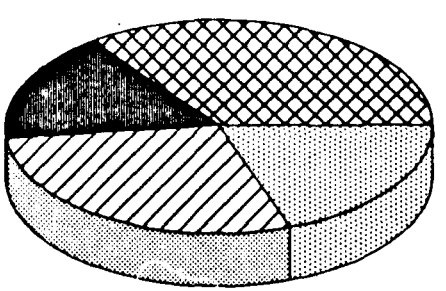
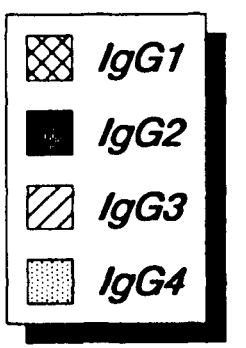


Immunization



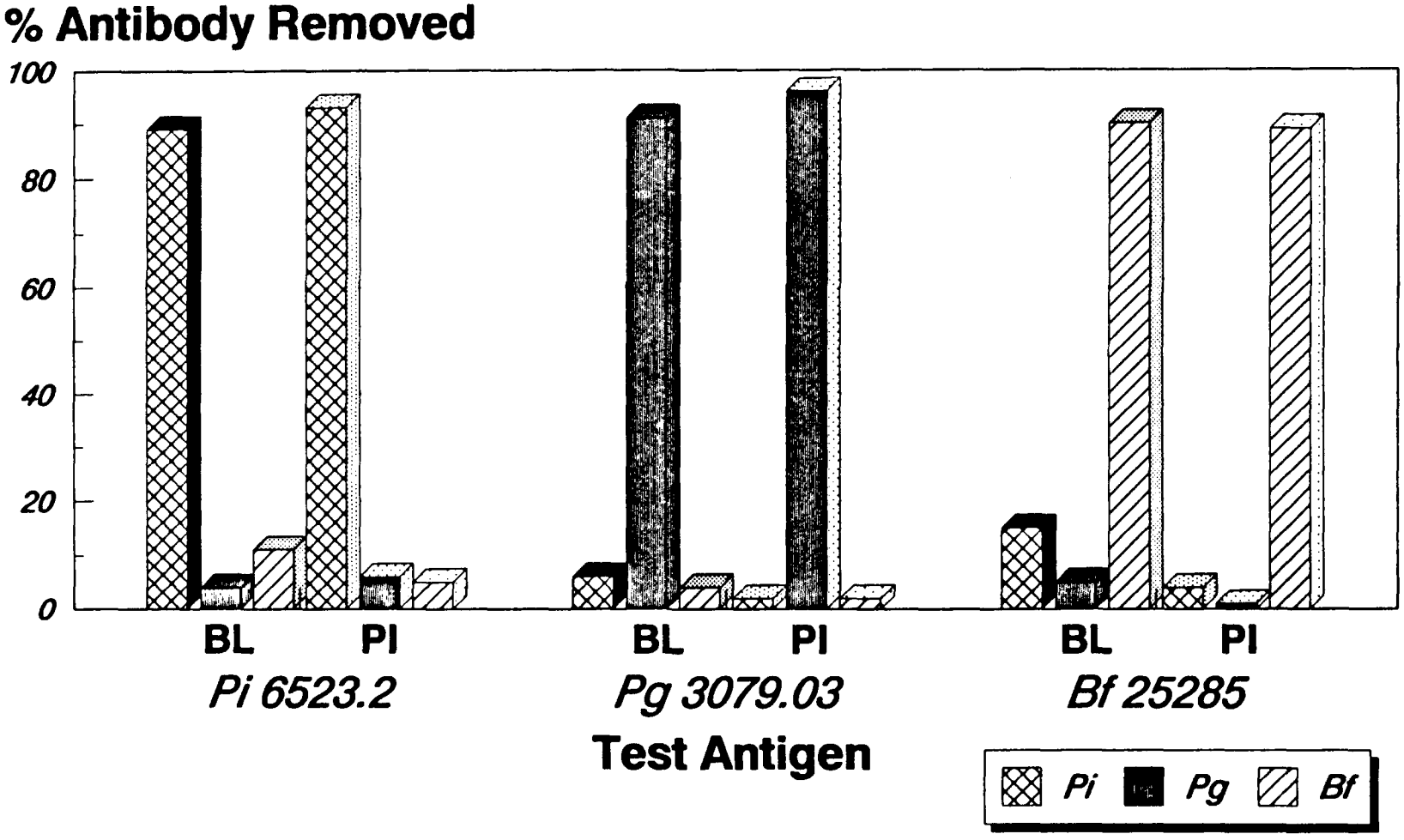
Ligation



F. Specificity of Response

Evidence for the antigenic specificity of the serum antibody elicited by parenteral immunization of Nhp with the formalin-killed bacteria is shown by three different approaches to analysis of antibody responses. First, as demonstrated in **Figures 5-7**, the IgG, IgM and IgA antibody levels to the homologous antigen was 100-1000 fold greater than antibody to either of the heterologous antigens. Second, depicted in **Figure 20** are the results of antibody adsorption studies with the bacteria. In these experiments, Nhp sera (N=5) collected at baseline and following active immunization were treated by incubation with formalin-killed bacteria of each species. The number of bacteria used for the adsorption was based upon the amount of homologous species required to remove >85% of the homologous antibody activity. Subsequently, separate aliquots of each sera were treated with identical numbers of the heterologous microorganisms. The results are expressed as % of antibody remaining after treatment when compared to unabsorbed sera from the same monkey. The data clearly demonstrate an exquisitely specific immune response in the Nhp and distinct antigenic differences among these microorganisms. Finally, correlational analyses were used to examine the relationships in the antibody isotype levels to the bacteria. The results in **Table 6** show that very few of the antibody response levels were significantly correlated. In fact, the majority of the positive correlations were among isotypes to a particular species.

Specificity of NhP Antibody Response to Test Bacteria



Correlation Coefficients of Serum Antibody Levels in NhP

	Pi IgG	Pg IgG	Bf IgG	Pi IgM	Pg IgM	Bf IgM	Pi IgA	Pg IgA	Bf IgA
Pi IgG	*1.0000* *	*0.0532*	*0.1309*	*0.1586*	*0.3797*	*0.5310*	*0.3066*	*0.2411*	*0.7222*
Pg IgG	*0.0532*	*1.0000*	*0.3225*	*0.2993*	*0.9880*	*0.6465*	*0.2993*	*0.2909*	*0.3704*
Bf IgG	*0.1309*	*0.3225*	*1.0000*	*0.7231*	*0.8361*	*0.4625*	*0.7477*	*0.9177*	*0.0283*
Pi IgM	*0.1586*	*0.6377*	*0.7231*	*1.0000*	*0.0795*	*0.0636*	*0.0006*	*0.5337*	*0.2662*
Pg IgM	*0.3797*	*0.9880*	*0.8361*	*0.0795*	*1.0000*	*0.0039*	*0.3573*	*0.4081*	*0.0880*
Bf IgM	*0.5310*	*0.6465*	*0.4625*	*0.0636*	*0.0039*	*1.0000*	*0.0800*	*0.6190*	*0.1488*
Pi IgA	*0.3066*	*0.2993*	*0.7477*	*0.0006*	*0.3573*	*0.0800*	*1.0000*	*0.2329*	*0.1251*
Pg IgA	*0.2411*	*0.2909*	*0.9177*	*0.5337*	*0.4081*	*0.6190*	*0.2329*	*1.0000*	*0.1278*
Bf IgA	*0.7222*	*0.3704*	*0.0283*	*0.2662*	*0.0880*	*0.1488*	*0.1251*	*0.1378*	*1.0000*

* *Values represent significance levels p = Spearman Rank Correlation Analysis*

IV. DISCUSSION

Mergenhagan *et al.* (1965), Evans *et al.* (1966) and Genco *et al.* (1974) have shown that antibodies in human serum are reactive with oral microorganisms. A variety of assays have been used to detect these antibodies including immunofluorescence, hemolysis reactions, bactericidal reactions, hemagglutination, and immunoprecipitation (Mashimo *et al.* 1976). In these assays, evaluation of the results is based on a certain degree of subjectiveness. Additionally, these assays are relatively insensitive. Determination of the specific isotype is not always possible even though the identification of specific antibody isotypes may be of paramount importance in interpreting the relationship between humoral antibody and periodontal disease (Ebersole *et al.* 1980).

Engvall *et al.* (1971) and Engvall and Perlmann (1972) developed the enzyme-linked immunosorbent assay as a sensitive and simple method for the quantitative determination of immunoglobulin G (IgG). Polystyrene tubes coated with human serum albumin (HSA) were incubated with rabbit anti-HSA followed by an alkaline phosphatase labelled sheep anti-rabbit IgG. The amount of alkaline phosphatase bound to the tubes was then detected using p-nitrophenylphosphate as a chromogenic substrate which is then measured in a spectrophotometrically at 400 nm. Antibodies in an experimental sera were quantitated by comparison with a standard antiserum.

To increase our understanding of the relationship of the humoral immune response to periodontal disease, sensitive methods for antibody detection and analysis are needed. The indirect enzyme-linked immunosorbent assay (ELISA) was established by Ebersole *et al.* (1980) as a sensitive assay using small quantities of sera to determine isotype specific antibody levels to oral microorganisms. They demonstrated a 5-50 fold increase of sensitivity compared

to indirect immunofluoresence or hemagglutination techniques in determining antibody reactions to *Actinobacillus actinomycetemcomitans* in human sera. The most significant advantage of the ELISA compared to passive hemagglutination is that the ELISA permits antibody quantification and isotype determination.

Subsequent studies by Mouton *et al.* (1981) and Taubman *et al.* (1982) in adult periodontitis patients showed a significantly increased serum IgG, IgM, and IgA antibody level to *P. gingivalis*. In a study examining serum IgG/M/A responses in humans to a battery of *Bacteroides* species, Patters and Kornman (1982) found a positive relationship of antibody level with disease severity. Specifically, there were significant elevations in IgG and IgA antibody to *P. gingivalis* in the periodontitis patients. High levels of antibody to *P. intermedia* were detected in both the periodontitis patients and normal subjects.

In an effort to improve the methods for describing the microflora associated with human periodontal disease, Ebersole *et al.* (1984) used an ELISA to identify black-pigmented *Bacteroides* species serologically. They used rabbit globulins containing antibody elicited by immunization with formalinized bacteria to identify the microorganisms by species in approximately 2 hours from receipt of culture plates.

Ebersole *et al.* (1986) described patterns of systemic antibody levels to *Bacteroides* species in humans and detected the presence of IgG, IgM, and IgA in all groups of subjects. Serum IgG, IgM, and IgA responses to *P. gingivalis* were increased in both frequency and level of activity in adult periodontitis and advanced destructive periodontitis patients when compared to localized juvenile periodontitis patients and normal individuals. In contrast to *P. gingivalis* responses, only advanced destructive periodontitis patients demonstrated a significantly elevated serum IgG and IgA response to *P. intermedia*. In the present study we adopted an ELISA to quantitate serum antibodies in nonhuman primates (Nhp). Previous

studies have identified the ability of antibodies directed to human immunoglobulin isotypes to react with similar molecules in the serum of Nhps (McArthur *et al.* 1989 and Nisengard, *et al.* 1989). This would be expected since most higher mammals share five distinct classes of immunoglogulin molecules which are IgG, IgA, IgM, IgE and IgD. The basic structure of all immunoglobulin molecules is similar, consisting of two identical heavy polypeptide chains and two identical light polypeptide chains linked together by disulphide bonds. Differences between classes are due to molecular differences in size, charge, amino acid composition and carbohydrate content (Roitt *et al.* 1985).

The nonhuman primates we studied demonstrated serum IgG, IgM, and IgA antibody to *P. gingivalis* and *P. intermedia* prior to immunization. The baseline antibody levels in the Nnps varied greatly; however, antibody to *P. intermedia* was increased when compared to the levels to *P. gingivalis*. This can be explained by the general preponderance of *P. intermedia* versus *P. gingivalis* in the subgingival plaque from the nonhuman primate (Kornman *et al.* 1981) Cross-sectional investigations in humans have also determined higher serum antibody levels to *P. intermedia* compared to *P. gingivalis*. Ebersole *et al.* (1986) postulated that the natural response may be a result of antigenic cross-reactions with natural serum antibody levels to *P. intermedia* resident in the gastrointestinal tract. However, a significantly elevated IgG and IgA antibody response to *P. intermedia* was detected in the advanced destructive periodontitis patients that were studied and was thought to represent additional colonization of the oral cavity with this microorganism

Several studies have demonstrated an association of *P. gingivalis* and *P. intermedia* with human periodontal lesions (Slots, 1979; Spiegel, *et al.* 1979 and Tanner *et al.* 1979). Sites severely affected by disease had a large proportion of their subgingival microflora composed of *P. gingivalis*; however, this microorganism was not detected at healthy gingival sites. Elevated *P. intermedia* subgingival levels have been demonstrated in a variety of

periodontal diseases (Slots *et al.* 1978, Spiegel *et al.* 1979, Loesche & Syed, 1978, Kornman & Loesche, 1980 and Slots & Zambon, 1982). Studies by Kornman *et al.* (1981) have demonstrated the similarity of the composition of the subgingival microbiota in non-human primates to that of humans for both gingivitis and periodontitis. Bacterial infections are frequently accompanied by an immune response that is specific for the pathogenic microorganism (Paterson, 1980). However, the role of serum antibody responses in periodontal disease is not clear. A major goal of this study is to establish the Nhp as a model to study systemic immune responses to putative periodontopathogens.

The results showed a dramatic difference in the natural systemic response to the oral versus nonoral microorganisms examined in this study. The primary serum response to both *P. gingivalis* and *P. intermedia* was IgG, while IgM antibody exceeded all other isotypes to *B. fragilis*. There does not appear to be an obvious reason for this difference in response. As a gut bacterium, *B. fragilis* may have a different relationship to the host immune system than the microorganisms colonizing the subgingival plaque. The long-term presence of IgG in the serum would indicate a more continual stimulation of the host systemic lymphoid apparatus. This is because IgG constitutes the majority of the secondary response to most antigens. After the initial antigenic challenge, some B cells switch from IgM production to IgG production and this is the basis of the change in antibody isotype from IgM in the primary response to an IgG dominated secondary response (Male, 1986). In the presence of a continual low antigen level (ie. chronic periodontitis) there may be preferential selection for stimulation of IgG producing B cells.

Grey & Kunkle (1964) were first to identify the four IgG subclasses in humans, namely IgG1, IgG2, IgG3 and IgG4. The differences between the subclasses are related to amino acid variations in the hinge region of the heavy chains. These structural differences lend to a variety of functions among the antibody subclasses in response to antigenic challenge. In adult

humans, protein antigens induce mainly an IgG1 response with minor increases in IgG3 and IgG4 levels, while IgG2 is the major fraction of total IgG produced in response to polysaccharide antigens (Hammerstrom *et al.* 1986). In conditions of chronic antigenic stimulation, IgG4 antibodies are increased and IgG4 synthesis is associated with responses to certain antigens that can lead to allergic reactions (Aalberse, 1983). Concerning specific functional capabilities, Ishizaka *et al.* (1967) determined that IgG1 and IgG3 avidly fix complement, IgG2 fixes it poorly while IgG4 does not fix complement. There are also differences in the ability of the IgG subclasses to bind Fc receptors on lymphocytes. IgG1 and IgG3 bind strongly while IgG2 and IgG4 bind relatively weakly (Froland *et al.* 1974). This is significant because stimulation of lymphocytes has been shown to result in the release of lymphokines capable of mediating tissue destruction (Mackler *et al.* 1974). Thus, determination of the proportion of IgG subclass production in response to a known antigenic agent could help define potentially pathogenic mechanisms contributing to periodontal destruction. There have been a limited number of studies concerning serum IgG subclass antibody responses. In a study of localized juvenile periodontitis (LJP) patients, Waldrop *et al.* (1981) determined IgG antibody subclass titers by radial immunodiffusion. They found an elevated total IgG antibody level in the LJP subjects; however, there were no significant differences in serum IgG subclasses compared to non-diseased patients. Farida *et al.* (1986) estimated IgG subclass antibody using polyclonal sheep anti-human antibodies. IgG1, 2 and 4 antibody levels to *P. gingivalis* were elevated in the severe periodontitis group compared to controls. The results indicated a correlation of systemic antibody to *P. gingivalis* in subjects with generalized periodontitis compared to localized juvenile periodontitis (LJP) and post-LJP subjects. Using monoclonal antibodies to human IgG subclasses in an ELISA, Schenk and Michaelson (1987) compared antibody reactivity in adult periodontitis patients to a control group. The antibody was specific for *P. gingivalis* lipopolysaccharide (LPS) and the anti-LPS response was dominated by IgG2 with moderate amounts of IgG1, IgG3 and IgG4. Compared to healthy controls, however, the adult periodontitis subjects had elevated levels of IgG1, 2

and 3 leading the authors to suggest a protective role for these antibodies against progressive disease.

To examine the IgG subclass distribution to putative periodontopathogens, Ebersole *et al.* (1985, 1989) used myeloma proteins in an ELISA to compare subclass antibody levels among LJP, rapidly progressive periodontitis (RPP), adult periodontitis (AP) and normal subjects. Elevated IgG1 and IgG3 levels to *A. actinomycetemcomitans* were found in the LJP and RPP groups and elevated IgG1, IgG2 and IgG4 responses to *P. gingivalis* were seen in the RPP and AP groups. Elevated IgG2 to *P. intermedia* was found in the disease groups, while IgG3 was not detected in any of the serum samples. The authors suggested that the dramatic differences in IgG subclass responses to the different periodontal disease-associated microorganisms resulted from the variability of dominant antigens associated with the bacteria and that the difference of immune responses demonstrates the variety of possible host mechanisms when challenged by these bacteria.

Using immunoelectrophoresis, Terry & Fahey (1964) demonstrated the similarity of IgG subclasses in humans compared to rhesus monkeys. After immunization with polyclonal IgG from human serum, three precipitin arcs were suggested by the authors to be three groups of antibodies (subclasses) common to both humans and the monkeys demonstrating protein sequence homology between species. However, there is only minimal information in the literature concerning the identification and presence of IgG subclasses in *M. fascicularis*. Thus, currently the existence and functional capabilities of these molecules remains to be elucidated. Investigation of immunoglobulin G subclasses in this study revealed that nearly 75% of natural IgG in the Nhps was comprised of IgG1 to *P. gingivalis* and *P. intermedia*, while IgG3 (62%) and IgG2 (33%) predominated to *B. fragilis*. Almost no IgG2 antibody to *P. gingivalis* was detected at baseline or after immunization. IgG2 is generally in response to polysaccharide antigens and *P. gingivalis* contains both a lipopolysaccharide (LPS) and a

capsular polysaccharide. The LPS antigen (endotoxin) of *P. gingivalis* may thus be allowed to trigger more destructive pathways of inflammation since it is not effectively neutralized by antibody. Likewise, a potential lack of response to the capsular antigen may preclude effective phagocytosis of this microorganism. In humans, a deficiency in IgG2 antibody response to pneumococcal capsular polysaccharide antigen has been associated with susceptibility to pneumonia in patients immunized with pneumococcal vaccine compared to healthy control subjects (Herer *et al.* 1990). The IgG subclass response to *P. intermedia* was primarily IgG1; however, in this case substantially greater levels of IgG2 antibody were present. *B. fragilis* also represents a microorganism that produces a polysaccharide capsule. The Nhp appeared to produce an IgG2 response to this microorganism. Thus, it may be suggested that the capsular polysaccharide of *B. gingivalis* is not particularly immunogenic in the Nhp.

Active immunization is the stimulation of a protective immune response within a host. An immunizing antigen frequently induces protection by the formation of neutralizing antibodies and vaccines in current use include both live vaccines and inactivated (killed) vaccines (Ebersole, 1988). In this study, formalin-killed *Porphyromonas gingivalis*, *Prevotella intermedia* and *Bacteroides gingivalis* were used as immunogens. The bacteria were killed because of their virulent potential and the possibility of causing systemic infection in the Nhps. Additionally, the formalin fixation allows antibody-antigen binding resulting in immune stimulation and the production of specific antibodies to the bacteria. The monkeys were immunizied with a series of three subcutaneous injections. A significant increase in antibody of all isotypes resulted from active immunization with the formalin-killed bacteria. In general, the oral bacteria (*P. gingivalis* and *P. intermedia*) elicited primarily an IgG response. Although *B. fragilis* also induced an IgG response, there was a dramatic increase in IgM to this microorganism. These results may be due to differences in the principal types of antigens on these microorganisms recognized by the nonhuman primate. The ability to respond to a particular antigen varies with genetic makeup. Within a given species, the ability to respond is

inherited as an autosomal dominant trait and this may explain individual antibody response variations (Goodman, 1987). Additionally, each of the bacteria elicited a marked increase in serum IgA. Studies have shown elevated IgA levels to *P. gingivalis* in human periodontitis patients compared to normal subjects, which indicates that antigens from this microorganism have the ability to elicit an IgA response (Taubman *et al.* 1982). IgA antibody may exert a local influence in areas of bacterial infection by reducing the magnitude of the inflammatory response as a result of its ability to bind antigen without activating the complement system (Griffiss and Jarvis, 1987). This is in contrast to IgG and IgM which both activate complement leading to an amplification of inflammation. In addition, complement is able to engage other effector systems resulting in the release of histamine from mast cells, direct migration of leukocytes, and release of lysosomal constituents from phagocytes (Cooper, 1987). It is therefore conceivable that an immunologic response dominated by IgA at the gingival site may significantly limit the destruction initiated by pathogenic microorganisms.

In general, antibody responses to each of the microorganisms reached their highest levels immediately following the active immunization. However, the temporal dynamics of the antibody response was somewhat varied between the bacterial antigens. Levels of antibody to *P. gingivalis* remained elevated throughout the study, while the antibody response to both *P. intermedia* and *B. fragilis* declined during the phase of ligature-induced periodontitis. This finding may have been due to an increased presence of *P. gingivalis* in the subgingival plaque of the ligated sites providing low levels of antigen which, through an anamnestic-type of response, induced the elevated antibody levels. This immunologic memory can be explained by clonal expansion of antibody-specific B cells after primary antigen contact. Then, upon secondary contact with the immunogen a larger population of cells are available to respond. Characteristics of the anamnestic response are a shorter lag time, a higher level of immune reactivity to the antigen and a longer duration of immune response. Characterization of the immune response after active immunization in the nonhuman primate has demonstrated a

substantial increase in antibody response which was sustained for several weeks. Additionally, the response was highly specific. These findings formed the basis for a continuation of this study by other researchers. Hypotheses are being tested concerning the ability of the serum antibody to affect the colonization and emergence of the specific microorganisms and the subsequent effect on the pathogenesis and progression of periodontal disease.

Nisengard *et al.* (1989) and McArthur *et al.* (1989) have also studied the effect of active immunization on ligature-induced periodontitis in monkeys. Evidence from these studies suggest that the induced humoral immune response after immunization with formalinized *B. macacae* and *P. gingivalis*, respectively was correlated with a reduction in the numbers of homologous bacteria found subgingivally. However, in general, these studies did not effectively relate the presence of antibody to alterations in the clinical characteristics of ligature-induced disease progression in these nonhuman primate models. Thus, further studies will be required to document the functional capabilities of these antibodies.

V. SUMMARY AND CONCLUSIONS

Serum antibodies of the IgG, IgM and IgA isotypes are detected in nonhuman primates that react with *P. gingivalis, P. intermedia,* and *B. fragilis.* Considerable variability in the natural antibody levels exists among the population of Nhp and the antibody levels among the animals appear to be unrelated to each other.

Mouse monoclonal antibodies to human IgG subclasses react with antibody molecules in serum from NhP and they show the presence of IgG1, IgG2, IgG3, and IgG4 subclass antibodies to these microorganisms.

Specificity studies demonstrate highly specific antibodies to *P. gingivalis, P. intermedia* and *B. fragilis* in the NhP.

Significant increases in IgG, IgM, and IgA antibody were noted following active immunization with each of these microorganisms that still maintained a high specificity for each of the microorganisms. These high levels of antibody persisted for 13-30 weeks post-immunization.

The majority of IgG antibody to *P. intermedia* and *B. fragilis* was of the IgG1 subclass with some IgG2 and IgG4, while antibody to *P. gingivalis* was comprised of IgG1, IgG3, and IgG4.

Little change in antibody level was detected following ligation in the immunized animals. These findings detail methodologies for analysis of NhP antibody responses to oral microorganisms and indicate the ability to induce and monitor an active immune response in

the NhP. Subsequent correlations can be derived among the immunologic, microbiologic, and clinical parameters of this disease model. These findings will lead to studies aimed at determining how to manipulate the immune system to accentuate the beneficial aspects directed at limiting progressive periodontitis.

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