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Serum Antibodies to *Porphyromonas gingivalis* Block the Prostaglandin E_2 Response to Lipopolysaccharide by Mononuclear Cells

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The ability of rabbit and monkey immune sera to neutralize prostaglandin E_2 (PGE_2) production by human monocytes stimulated with lipopolysaccharide (LPS) was examined. CD14-dependent LPS activation of PGE_2 was examined under assay conditions which allowed the comparison of preimmune and immune sera. Serum obtained from rabbits immunized with formalin-fixed *Porphyromonas gingivalis* cells dramatically reduced the amount of PGE_2 produced in response to LPS obtained from three different strains of *P. gingivalis* but not that from *Escherichia coli* or *Bacteroides fragilis*. In addition, a significant reduction in the mean PGE_2 level was observed in the presence of sera from immunized but not control monkeys employed in a vaccine trial. Immune serum samples from five of nine immunized monkeys were able to reduce LPS-induced production of PGE_2 by greater than 50% compared to that in the corresponding preimmune sera. Immune monkey serum, similar to immune rabbit serum, blocked PGE_2 production in response to *P. gingivalis* LPS but not *E. coli* LPS. These data demonstrate that immunization with *P. gingivalis* whole cells can elicit an antibody response that is able to block the PGE_2 response to LPS. Neutralization of LPS-mediated inflammatory mediator production may account in part for the observed suppression of alveolar bone loss in immunized monkeys.

Periodontitis is a chronic inflammatory disease associated with a pathogenic microflora which colonizes the tooth surface and which is distinct from the normal oral flora. Associated species include *Bacteroides forsythus*, *Actinobacillus actinomycetemcomitans*, *Campylobacter rectus*, *Prevotella intermedia* (*Bacteroides intermedius*), and *Porphyromonas gingivalis* (*Bacteroides gingivalis*) (10, 16, 27). These bacteria appear to promote tissue destruction and bone resorption both directly by the production of collagenases and other proteolytic enzymes (17) and by triggering host inflammatory responses in a manner also leading to destructive processes (9, 23, 30). Bacterial cell wall components such as lipopolysaccharides (LPSs) are known to be potent stimulators of mediators of inflammation and tissue destruction such as interleukin 1β, tumor necrosis factor alpha, metalloproteinases, and prostaglandins, notably prostaglandin E_2 (PGE_2) (15, 23). PGE_2 is known to be a potent inducer of alveolar bone resorption and is found in elevated concentrations in inflamed tissue from periodontal patients. It has been implicated as important in periodontal disease progression by many studies, including clinical trials demonstrating that nonsteroidal anti-inflammatory drugs which suppress prostaglandin production can arrest disease progression (13, 18, 19).

P. gingivalis is an asaccharolytic gram-negative anaerobe that has an LPS which is different from enterobacterial type LPS both with regard to its chemical structure and to its biological activity (5, 14, 20, 21). Periodontitis patients typically display a significant antibody titer to *P. gingivalis* and to its LPS (22, 27, 34), and antibody titer and avidity may increase after treatment by scaling and root planing (3). The protective capability of these antibodies against disease is not known (4, 22). They could potentially provide protection by promoting phagocytosis, complement-dependent killing, or clearance through the reticular endothelial system (26). In addition it is also possible that such antibodies may bind to and neutralize the effects of bacterial components such as LPS. Such a mechanism has been proposed for the action of anti-LPS antibodies in septic shock models (26).

Vaccine studies with nonhuman primates have been controversial regarding protection against challenge with *P. gingivalis* in periodontitis models (7, 11, 24), and the evaluation of the functional properties of sera obtained from these studies has only recently been attempted (1, 12). In a recent study with *Macaca fascicularis* monkeys, immunization with *P. gingivalis* was able to suppress bone loss despite a statistically insignificant reduction of the pathogen in the periodontal pocket (24). This result suggested the possibility that antibodies generated during immunization might be neutralizing the ability of bacterial components to stimulate inflammatory mediators, thus directly suppressing tissue destruction even in the continued presence of the pathogen. In the present study, we demonstrate that immune sera from these monkeys as well as a control immune rabbit serum specifically inhibit the ability of *P. gingivalis* LPS to stimulate PGE_2 release by blood mononuclear cells in an in vitro assay.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* AO16 (serogroup O6), a human isolate, was provided by Marie Coyle (Harborview Medical Center Seattle, Wash.). *P. gingivalis* human strain A7-A128 was obtained from Joseph Zambon, State University of New York at Buffalo. *P. gingivalis* 5083, isolated from an *M. fascicularis* monkey, was obtained from Stanley Holt, University of Texas at San Antonio. *P. gingivalis* ATCC 33277 and *B. fragilis* ATCC 25285 were obtained from the American Type Culture Collection, Rockville, Md. Strains were examined for purity, properly identified, and stored at −70°C. Cultures were made from frozen stocks to avoid repetitive subcultures. *E. coli* AO16 was grown overnight in Trypticase soy broth at 37°C with shaking in room air. *B. fragilis* was grown on brucella blood agar and harvested by being scraped with sterile swabs into phosphate-buffered saline. *P. gingivalis* was grown anaerobically at 37°C in enriched Trypticase soy broth supplemented with cysteine, hemin, and vitamin K. Strains 33277 and A7A128 were also grown in a large batch culture by Lee

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Laboratories (Grayson, Ga.) under similar conditions and shipped as frozen cell pellets.

LPSs. LPS from *E. coli* AO16 was isolated by the hot phenol-water method (33) and further purified by digestion with nucleases, proteinase, and cycles of ultracentrifugation. *P. gingivalis* and *B. fragilis* LPSs were prepared by the cold magnesium-ethanol precipitation technique (6), followed by lipid extraction (8) and conversion to sodium salts (25). LPS preparations were subjected to gas chromatography for analysis of carbohydrates (2) and fatty acids (28) and were found to have compositions consistent with previous reports (14, 20, 29, 32) and to be devoid of phospholipids and procedure-related detergent. A_{280} and A_{260} indicated the preparations were free of detectable levels of protein and nucleic acid contamination.

Sera and antibodies. Preimmune and peak-immunoglobulin G (IgG)-titer immune sera were obtained from *M. fascicularis* monkeys immunized with *P. gingivalis* monkey isolate 5083 formalinized whole cells in the presence of Syntex adjuvant formulation M (SAF-M; Syntex Laboratories, Palo Alto, Calif.) as previously reported (24). In the study, which lasted 36 weeks, the monkeys were ligated in week 16. Immune and preimmune rabbit sera were obtained from New Zealand White female rabbits immunized with formalin-fixed *P. gingivalis* 33277 and *A. actinomycetemcomitans* Y4 formulated in SAF-M adjuvant as described previously (31). Pooled normal human serum used as a control in stimulations was from Gemini Bioproducts, Calabasas, Calif. The CD14-specific monoclonal antibody MY4 was purchased from Coulter Immunology, Hialeah, Fla. In some experiments, as noted, sera were heated at 56°C for 60 min to inactivate complement.

ELISA for serum antibodies to LPS. The enzyme-linked immunosorbent assay (ELISA) for serum antibodies to LPS was performed as follows. LPSs were coated at 10 μg/ml in pH 9.6 sodium carbonate buffer onto Immulon II plates (Dynatech) overnight at 4°C. Plates were washed three times with 0.05% Tween 20 in phosphate-buffered saline, blocked with 5% nonfat dry milk, and then incubated for 1 h with dilutions of rabbit or monkey serum in 0.1% Tween 20 and 0.1% bovine serum albumin in phosphate-buffered saline (PTB). Plates were washed, goat anti-rabbit or goat anti-human antibody conjugated with horseradish peroxidase (TAGO Immunochemicals) diluted 1:2,000 in PTB was added, and after being washed again, plates were developed with tetramethylbenzidine substrate (Genetic Systems).

Human PBMCs. Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood obtained from normal human volunteers with Lymphocyte Separation Medium (Organon Teknika) as described by the manufacturer. Stimulations were performed in 96-well polypropylene plates (Sigma) by addition of LPS and serum appropriately diluted in RPMI 1640 medium (Gibco) to always yield a final concentration of 10^6 cells per ml. After 24 h at 37°C in a 5% CO_2 atmosphere, the plates were centrifuged and the cell supernatants were collected and assayed for PGE_2 or frozen immediately at −70°C until assayed. Immune sera and the corresponding preimmune sera were preincubated with the stimulating LPS for 1 h at 37°C before addition to the PBMCs to allow antibody-antigen interaction. Since preliminary studies indicated that PBMC responsiveness varied greatly from day to day (data not shown), all comparisons were made with cells isolated on the same day from the same donor. All stimulations were performed at least in duplicate, and values are presented as means. All experiments were performed at least twice with PBMCs from more than one blood donor, and a representative experiment is presented.

PGE_2 assay. Culture supernatants were assayed for PGE_2 by an inhibition enzyme immunoassay with a kit (Biotrack EIA [enzyme immunoassay]; Amersham Life Sciences) as described by the manufacturer.

RESULTS

Serum requirement for LPS stimulation of PGE_2 in human mononuclear cells. The effect of serum on the production of PGE_2 by blood mononuclear cells in response to LPS was examined (Fig. 1). Various doses of LPS were examined with and without 1% select serum. The levels of PGE_2 stimulated by both *P. gingivalis* and *E. coli* LPS were significantly enhanced by the addition of normal human, rabbit, or monkey serum compared to those of no-serum controls. Although the maximal levels of PGE_2 induced by the two LPSs were comparable, the dose of LPS required to achieve the maximal level for *P. gingivalis* LPS (10 ng/ml) was 100 times the amount required for *E. coli* LPS (0.1 ng/ml). LPSs from *P. gingivalis* A7A128 and 5083 and from *B. fragilis* were found to have similar dose-response behavior to *P. gingivalis* 33277 LPS (data not shown). In addition, it was observed that stimulation was inhibited by the anti-CD14 monoclonal antibody MY4 for both LPS types regardless of the presence or type of serum, demonstrating a requirement for CD14.

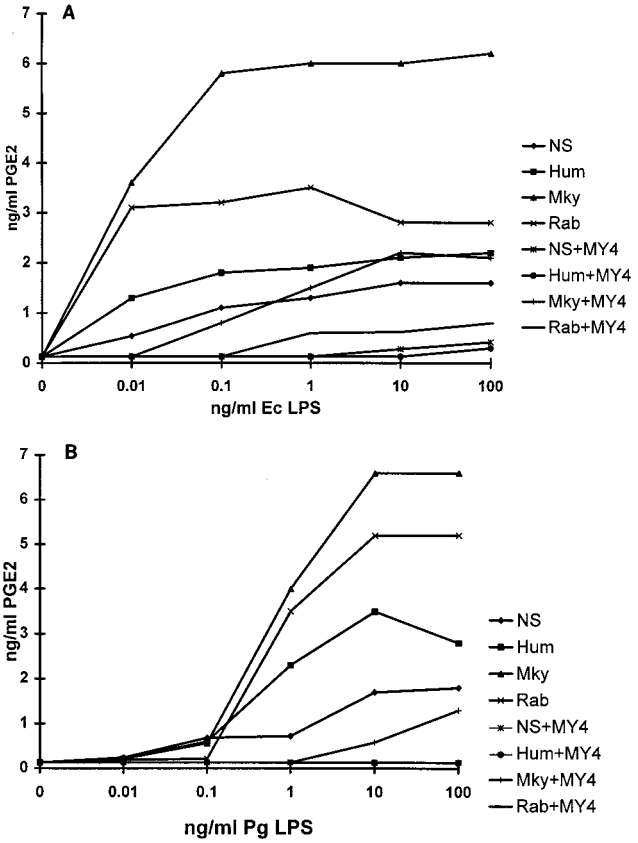

FIG. 1. PBMCs were stimulated with increasing amounts of *E. coli* (Ec) AO16 LPS (A) or *P. gingivalis* (Pg) 33277 LPS (B) in the presence or absence (NS) of 1% normal human (Hum), rabbit (Rab), or monkey (Mky) serum and the supernatant PGE_2 levels determined after 24 h. The effect of MY4 was examined by preincubation of the cells for 1 h in the presence of 10 μg of MY4 per ml before the addition of LPS and serum. Similar results were obtained from experiments performed on four separate occasions with PBMCs from different donors.

Inhibition of PGE_2 by rabbit immune sera. A control immune serum was generated by immunization of rabbits with formalinized *P. gingivalis* 33277 cells. This serum reacted strongly with *P. gingivalis* LPS but not *E. coli* LPS, as determined by ELISA with purified LPS. This immune serum and the preimmune serum from the same animal were added to PBMCs stimulated with 10 ng of LPS preparations per ml obtained from select bacteria. As shown in Fig. 2, when immune serum was compared to its corresponding preimmune serum, a substantial (84% mean) reduction in PGE_2 was observed for the three strains of *P. gingivalis*. In contrast, the PGE_2 levels were only slightly lower in the presence of immune serum when the PBMCs were stimulated with LPS from *B. fragilis* or LPS from *E. coli*, indicating that immune serum had very little neutralizing ability against LPS from either a related or unrelated nonoral bacterium while dramatically reducing the effect of LPS from three strains of *P. gingivalis*. A similarly specific inhibitory effect of the immune serum was observed on the ability of *P. gingivalis* LPS to stimulate interleukin 1β and tumor necrosis factor alpha, indicating that the inhibitory effect is not peculiar to PGE_2 production (data not shown).

The ability of the immune serum to neutralize *P. gingivalis* LPS induction of PGE_2 occurred in an identical manner if heat-inactivated serum was used, suggesting that serum antibody and not complement was responsible for the observed

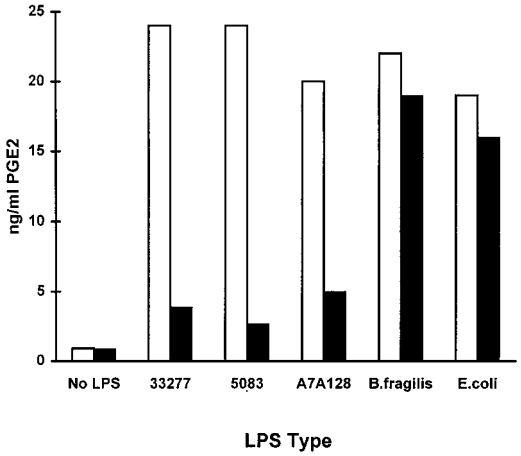

FIG. 2. Effect of immune serum from a rabbit immunized with *P. gingivalis* 33277 whole cells (solid bars) or preimmune serum from the same rabbit (open bars) on the ability of various LPS preparations (10 ng/ml) to stimulate PGE_2 in human PBMCs. Similar results were obtained from experiments performed on four separate occasions with PBMCs from different donors.

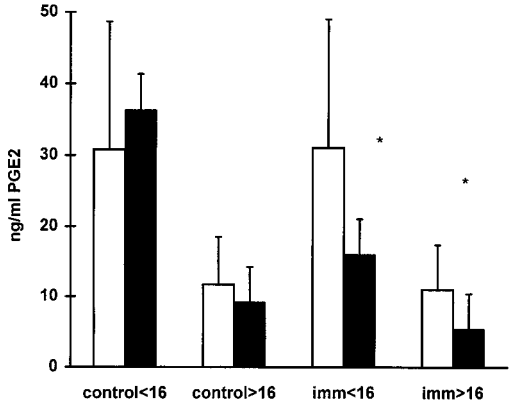

FIG. 3. Mean PGE_2 levels obtained from PBMCs stimulated in the presence of peak-IgG-titer serum samples from 10 control monkeys (nonimmunized) and 9 immunized monkeys (imm) collected before ligation (<16) or after ligation (>16). PBMCs were stimulated with 10 ng of *P. gingivalis* 5083 LPS per ml in the presence of 1% baseline serum (open bars) or peak-IgG-titer serum (solid bars) from each immune or control monkey, and the supernatants were assayed for PGE_2 after 24 h as described in the text. Stimulations in the presence of sera from each group (preligation control, postligation control, preligation immune, and postligation control) were performed as separate experiments on different days, and the results are compared with those of the appropriate baseline sera. *, immune significantly different from preimmune ($P < 0.05$).

reduction. In addition, in two separate experiments, no reduction in PGE_2 release was observed when preimmune and immune sera obtained from rabbits immunized with *A. actinomycetemcomitans* were examined, indicating that specific *P. gingivalis* antibody is required for blocking (data not shown). Although the immune sera generated against *A. actinomycetemcomitans* contained a high titer of antibody against LPS as measured by ELISA, it did not block the ability of this LPS to elicit PGE_2 release (data not shown).

Effect of immune monkey serum on LPS-stimulated PGE_2 production. *M. fascicularis* monkeys were immunized with formalinized *P. gingivalis* 5083 cells as previously reported (24). Silk ligatures were placed around teeth at week 16 in all animals to promote periodontal tissue destruction. The sera from each monkey with the highest titer to *P. gingivalis* 5083 prior to the placement of ligature, the sera with the highest titer after ligation, and the baseline (day 0) sera from both immunized and control monkeys were tested for their ability to block PGE_2 production in response to *P. gingivalis* 5083 LPS. Mean data from these experiments are shown in Fig. 3. There was a statistically significant reduction in PGE_2 production from baseline to peak titer only in the immunized animals ($P < 0.013$ for preligature, $P < 0.02$ for postligature). Even though the control animals as a group had a small observed increase in antibody titer to *P. gingivalis* after ligature placement (24), the ability of the sera from these animals to inhibit PGE_2 production was not acquired. The immunized monkeys as a group maintained their ability to neutralize PGE_2 production during the postligation period, although some individual monkeys showed increased or decreased inhibitory ability. A significant ($P < 0.05$) correlation of serum IgG levels against *P. gingivalis* 5083 LPS to low PGE_2 levels was found in the postligation immunized monkeys. No correlation with serum IgM for any group was observed.

Figure 4 shows the neutralizing ability of individual immunized monkeys prior to ligation. The amount of inhibitory effect induced by immunization varied substantially, ranging from 99% for animal 82371 to only 13% for animal 91050. The majority of monkeys demonstrated an increased ability to block PGE_2 production after immunization when the respective preimmune and immune sera were examined.

Effect of monkey sera on the ability of heterologous LPS to stimulate PGE_2. The ability to inhibit PGE_2 production in response to LPS from bacteria other than the immunization strain of *P. gingivalis* was examined for serum samples from two select monkeys. One displayed a strong inhibitory ability in the immune serum compared to the preimmune serum (82371), and the other demonstrated low levels of PGE_2 production after addition of either the preimmune or immune serum (88023 [Fig. 4]). As shown in Fig. 5, in both preimmune serum (82371) and immune serum (88023), the same pattern of specific inhibition of *P. gingivalis* LPS but not *B. fragilis* or *E. coli* LPS was observed. These data demonstrate that regardless of how neutralization activity was obtained, either in preimmune sera presumably by preexposure (88023) or by immunization (82371), there was a preferential reduction of PGE_2 production in response to *P. gingivalis* LPS but not *E. coli* LPS.

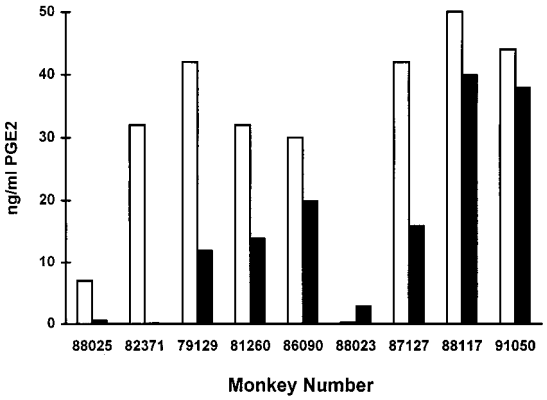

FIG. 4. Inhibition of *P. gingivalis* LPS-stimulated PGE_2 production by individual preligation monkey sera. PBMCs were stimulated in the presence of 1% preimmune (open bars) or immune (solid bars) monkey serum. Each stimulation was performed at least three times. Percent inhibition did not vary between experiments by more than 28% for any given monkey.

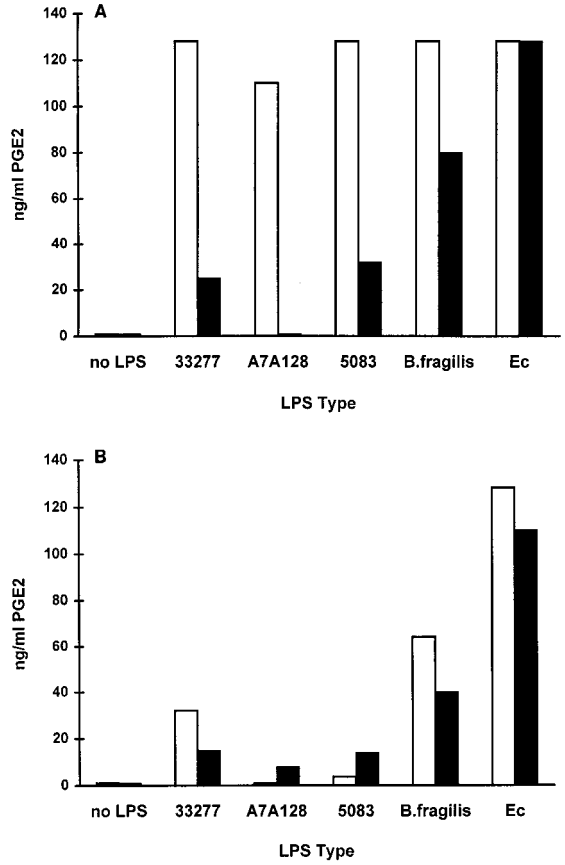

FIG. 5. Effect of sera from two monkeys, 82371 (A) and 88023 (B), immunized with *P. gingivalis* 5083 whole cells (solid bars) or the corresponding preimmune sera (open bars) on the ability of various LPS preparations to stimulate PGE_2 production in human PBMCs. Similar results were obtained in experiments performed on three additional occasions. Ec, *E. coli.*

DISCUSSION

A recent immunization study with *P. gingivalis* in *M. fascicularis* (24) suggested that antibody neutralization of LPS may play a role in alleviating the progression of periodontitis. Immunization and generation of antibody reactive with antigens of *P. gingivalis* resulted in reduced bone destruction, despite the continued presence of large numbers of the pathogen. This result indicated that nonopsonic antibodies may have participated in the attenuation of bone loss. In this study, the possibility that immunization could elicit an antibody response with the ability to neutralize *P. gingivalis* LPS and thus block the stimulation of the destructive release of inflammatory mediators such as PGE_2 was examined. In both control rabbit immune serum and serum obtained from immunized monkeys, immunization induced the ability to specifically block PGE_2 release in response to *P. gingivalis* but not *E. coli* LPS. This result is consistent with the notion of a systemic antibody response suppressing PGE_2 at sites of *P. gingivalis*-induced inflammation leading to reductions in tissue destruction and bone loss.

Specific neutralizing anti-LPS antibody was generated by immunization with formalinized whole *P. gingivalis* cells. Furthermore, both immune rabbit sera and the immunized monkey sera inhibited the production of PGE_2 in response to LPS obtained from two other strains of *P. gingivalis*. This ability to inhibit heterologous strains is in contrast to what has been previously reported in studies employing enterobacterial LPS (26). The result is important, since an effective vaccine must be able to neutralize the effects of numerous strains. Although the epitopes on LPS which are involved in the neutralization have not been determined, the observed cross-reactivity implicates those in the conserved, core-lipid A region of the molecule. Alternatively, the cross-reactivity could be the result of a more highly conserved O-polysaccharide in *P. gingivalis* LPS compared to enterobacterial LPS; however, detailed knowledge about the structure of the *P. gingivalis* O-polysaccharide is not yet available for comparison. It is also not known whether immunization with purified LPS rather than whole bacteria would result in an antibody response with similar neutralizing ability or epitope specificity.

The mechanism of immune serum neutralization of *P. gingivalis* LPS induction of PGE_2 is not known. The immune sera either may have prevented *P. gingivalis* LPS from entering the CD14 activation pathway or may have interacted with LPS after it was complexed with serum or cellular factors necessary for LPS-induced cellular activation. For example, it is possible that the immune sera prevented *P. gingivalis* LPS from binding LPS-binding protein (LBP) by inducing agglutination. This could have effectively prevented LBP from correctly presenting LPS to CD14, a step necessary for subsequent cellular activation and PGE_2 release. Alternatively, immune sera may have bound key epitopes located on the *P. gingivalis* LPS which were required for interactions with either LBP, CD14, or other monocyte cell surface components necessary for *P. gingivalis* LPS-induced PGE_2 release. Likewise, the reasons immune sera generated against *A. actinomycetemcomitans* did not neutralize the ability of this LPS to elicit PGE_2 secretion were not investigated. It is possible that alternative immunizations or other antigen preparations were required to elicit functional blocking antibody to this LPS. Additional studies to elucidate the mechanism of antibody neutralization may provide more information about vaccine preparations that result in high blocking activity.

Immunization resulted in a statistically significant reduction in the ability to support PGE_2 production for the group of nine monkeys, and reduced ability was correlated with serum IgG titer to *P. gingivalis* LPS. The monkeys did not, however, all respond to immunization in the same manner. Some of the monkeys showed large increases in neutralizing ability, while others showed minimal or no increases. Additionally, some of the monkeys had an ability to specifically inhibit *P. gingivalis* LPS-stimulated PGE_2 production before immunization. The fact that the preimmune serum from these monkeys did not neutralize PGE_2 production in response to *E. coli* LPS suggests that the reduction is likely due to prior exposure to *P. gingivalis*. Although the ability of the immune monkey sera did not correlate directly with attenuation of bone loss, it is believed that LPS neutralization may be one of many functions of induced antibody which can lead to protection, for example, by opsonization, neutralization of factors such as proteases, or blockade of *P. gingivalis* interaction with host cell types other than monocytes. It is also possible that protective blocking antibody is prominent at particular times in the development of the antibody response and was underestimated in some animals because sera collected when the activity was highest were not examined.

In summary, it has been shown that immunization can lead to an increase in the ability to neutralize the potentially destructive production of inflammatory mediators by LPS. We believe this ability may have been a factor in the reduced tissue destruction observed in the immunized animals. If a similar antibody is generated during the course of periodontal disease

progression in humans or as a result of the treatment of periodontitis patients (3), it may play a role in alleviating the disease.

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