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Porphyromonas gingivalis-Specific Immunoglobulin G Prevents *P. gingivalis*-Elicited Oral Bone Loss in a Murine Model

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Active immunization with *Porphyromonas gingivalis* whole-cell preparations has been shown to prevent *P. gingivalis* infection and oral bone loss. Employing passive antibody transfer and opsonization, we demonstrate with this study that immunization-elicited *P. gingivalis*-specific immunoglobulin G facilitates clearance of *P. gingivalis* in a subcutaneous chamber model and prevents *P. gingivalis*-elicited oral bone loss.

Epidemiological data indicate that by age 35 approximately 30% of the U.S. adult population suffers from periodontal disease (17), a chronic inflammatory disease of the hard and soft tissues supporting the teeth that in severe cases leads to tooth loss. Most cases of this disease are caused by the anaerobic bacterial pathogen *Porphyromonas gingivalis* (13, 18). Numerous studies have assessed the serum antibody profiles of patients with periodontitis. High levels of *P. gingivalis*-specific immunoglobulin G (IgG) are commonly observed (12, 19), yet a variable host response to this organism appears to exist (1, 11, 14, 16). However, despite this potent antibody response the host is apparently unable to clear this chronic infection. Notwithstanding this fact, Wilton et al. (20) reported that sera obtained from patients with periodontal disease contain antibodies that promote opsonophagocytosis of *P. gingivalis* by human neutrophils.

Previous reports suggest that vaccination affords protection from *P. gingivalis* infection (3, 5–7, 9, 10, 15). Gibson et al. (7) demonstrated that immunization of mice with heat-killed *P. gingivalis* elicited high levels of specific IgG and protected mice from subsequent oral bone loss elicited by this organism. Employing both murine subcutaneous chamber and oral challenge models, we demonstrate in this study that prevention of *P. gingivalis* infection occurs with immunization-elicited *P. gingivalis*-specific IgG.

P. gingivalis strain A7436 was used in all of our studies. This organism was initially cultivated on anaerobic blood agar plates, and bacteria were havested by centrifugation and grown in brain heart infusion (BHI) broth overnight. *P. gingivalis* organisms were collected and suspended in fresh BHI broth to a final cell density of 10^9 CFU/ml (7). Heat-killed *P. gingivalis* and formaldehyde-fixed *P. gingivalis* were prepared as described previously (7). BALB/C mice (Jackson Laboratories, Bar Harbor, Maine) were immunized subcutaneously with heat-killed *P. gingivalis* whole-organism preparations three times per week for 3 weeks, and the mice were exsanguinated. Serum was applied to a Protein G column (Amersham Pharmacia Biotech, Uppsala, Sweden) and total IgG was eluted (approximately 50 mg); the result was designated *P. gingivalis*-specific IgG. Using enzyme-linked immunosorbent assay, we observed that the titer of the *P. gingivalis*-specific IgG was 1:50,000 (data not shown). A commercially obtained mouse IgG (irrelevant IgG [IRR-IgG]; Sigma, St. Louis, Mo.) was used as an IgG control. For passive transfer studies, mice received an injection of 100 μl of *P. gingivalis*-specific IgG or IRR-IgG (1 mg/ml) in the subcutaneous chamber 24 h prior to *P. gingivalis* challenge. In addition, *P. gingivalis* was opsonized with either 100 μg of *P. gingivalis*-specific IgG or IRR-IgG per ml. The effect of opsonization on *P. gingivalis* viability was assessed by determination of CFU per milliliter and did not affect *P. gingivalis* viability.

Initially, we assessed the protective capacity of *P. gingivalis*-specific IgG by using a murine subcutaneous chamber model (4). Mice were separated into six groups ($n = 8$ mice/group). Group 1 received 0.1 ml of sterile BHI broth only (vehicle), group 2 received 0.1 ml of *P. gingivalis* in vehicle ($\sim 10^8$ CFU), groups 3 and 4 received passive transfer of *P. gingivalis*-specific IgG or IRR-IgG, respectively, and groups 5 and 6 were challenged with *P. gingivalis* opsonized with *P. gingivalis*-specific IgG or IRR-IgG. Following challenge, mice were examined daily for signs of infection, including cachexia (defined as ruffled hair), hunched bodies, weakness, and lethargy, as well as for the presence of primary and secondary lesions. Animals in group 2 exhibited signs of severe cachexia and presented with chamber lesions that eventually resulted in chamber rejection by day 10 (Table 1), while mice in group 1 appeared healthy. Passive immunization (group 3) or opsonization of *P. gingivalis* with *P. gingivalis*-specific IgG (group 5) protected mice from all signs of *P. gingivalis* infection (Table 1). In contrast, animals from groups 4 and 6 were not protected and resembled mice from group 2 (Table 1).

A sample of chamber fluid (50 μl) was collected from each animal at 1, 3, 7, and 10 days post-*P. gingivalis* challenge to assess bacterial replication and the inflammatory response. A 10-μl aliquot of each chamber fluid sample was serially 10-fold diluted in 1% peptone and plated onto anaerobic blood agar plates, and the number of CFU of *P. gingivalis* per milliliter was determined. Chamber fluid samples obtained from mice in group 2 demonstrated that *P. gingivalis* was capable of replication and persistence throughout the 10-day period (Fig. 1). Analysis of chamber fluid samples obtained from group 3 mice revealed that *P. gingivalis* was readily cleared, with no viable

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TABLE 1. Gross pathological observations of mice passively immunized or opsonized with *P. gingivalis*-specific IgG and challenged with *P. gingivalis* in a murine subcutaneous chamber model[a]

Group	Expt parameter			Result	
	Passive transfer	Opsonization	Challenge with	Lesions[b]	Cachexia[c]
1	None	None	None	None (0/8)	None
2	None	None	*P. gingivalis* A7436	++++ (2/8)	Moderate
3	*P. gingivalis*-specific IgG	None	*P. gingivalis* A7436	None (0/8)	None
4	IRR-IgG	None	*P. gingivalis* A7436	++++ (4/8)	Severe
5	None	*P. gingivalis*-specific IgG	*P. gingivalis* A7436	None (0/8)	None
6	None	IRR-IgG	*P. gingivalis* A7436	++++ (3/8)	Severe

[a] Groups of animals received vehicle only (none), 100 μg of murine *P. gingivalis*-specific IgG, or 100 μg of IRR-IgG 24 h prior to chamber challenge or were challenged with *P. gingivalis* opsonized with 100 μg of either *P. gingivalis*-specific IgG or IRR-IgG.

[b] The severity of the lesions was determined by using an arbitrary four-plus scale whereby lesions spreading from the area of challenge are considered the most severe. After 10 days, mice were observed for evidence of chamber rejection, and the number in parentheses is the number of mice with chamber rejection.

[c] Cachexia was expressed as none, moderate, or severe and was determined as a function of animal hunching, ruffled hair, and lethargy.

organisms recovered by day 7 (Fig. 1). Animals from group 5 also efficiently cleared *P. gingivalis*. Mice in groups 4 and 6 failed to clear *P. gingivalis* and resembled animals that had been challenged with *P. gingivalis* alone (Fig. 1). These data demonstrate that passive transfer of immunization-elicited *P. gingivalis*-specific IgG facilitates clearance of *P. gingivalis* in a virulence model. An additional 10-μl aliquot from each chamber fluid sample was used to determine the total inflammatory cell count by trypan blue staining. Animals from group 1 possessed relatively few inflammatory cells. By day 3, the levels of inflammatory cells present in chamber fluids of mice in group 2 were elevated and remained higher throughout the remainder of the experiment (Fig. 2). The inflammatory cell responses of mice from groups 3 and 5 were reduced by day 3 and resembled those of mice in group 1 at days 7 and 10, while mice from groups 4 and 6 possessed levels of inflammatory cells that resembled those of mice in group 2 (Fig. 2). These data support the supposition that *P. gingivalis* infection is controlled in mice that received *P. gingivalis*-specific IgG, as the cellular inflammatory response of mice challenged with *P. gingivalis* is resolved following clearance of bacteria.

We also employed a modified version of the fluorescent microscopic opsonophagocytosis assay of Kalmer et al. (8) and Cutler et al. (2) to assess the interaction of the inflammatory cells and *P. gingivalis* in the murine chamber fluid samples. In brief, a 10-μl aliquot of each chamber fluid sample was diluted to 1:50 in sterile phosphate-buffered saline; was incubated with 4′6′-diamidino-2-phenylindole (DAPI; 5 μg/ml, Sigma), propidium iodine (5 μg/ml, Sigma), and acridine orange (2.5 mM, Sigma); and was cytocentrifuged onto a glass slide. The polymorphonuclear leukocyte (PMN) viability, the ratio of the number of *P. gingivalis* organisms to the number of PMN (*P. gingivalis*/PMN), and the percentage of PMNs with at least one *P. gingivalis* organism were determined in blinded fashion.

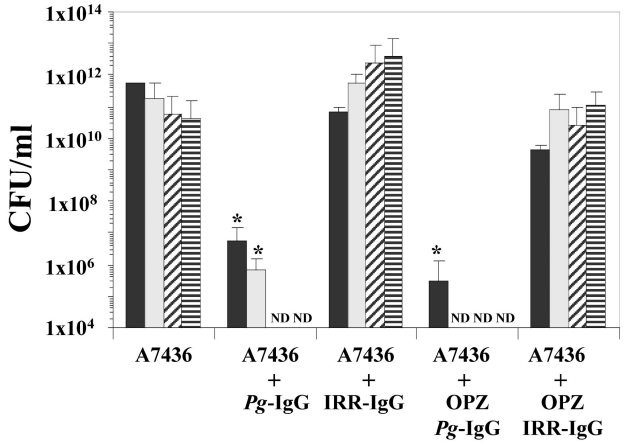

FIG. 1. Chamber fluid levels of *P. gingivalis* expressed in terms of CFU per milliliter. Chamber fluids were collected at 1 (solid bar), 3 (speckled bar), 7 (diagonal hatched bar), and 10 (horizontal hatched bar) days postchallenge from groups of mice that were challenged with *P. gingivalis* (A7436), *P. gingivalis* following passive antibody transfer of *P. gingivalis*-specific IgG (*Pg*-IgG) or IRR-IgG antibody, or *P. gingivalis* opsonized with *P. gingivalis*-specific IgG (OPZ *Pg*-IgG) or IRR-IgG (OPZ IRR-IgG). *, $P < 0.05$ compared with matched IRR-immunized groups by the Student *t* test; ND, *P. gingivalis* not detected in chamber fluid samples.

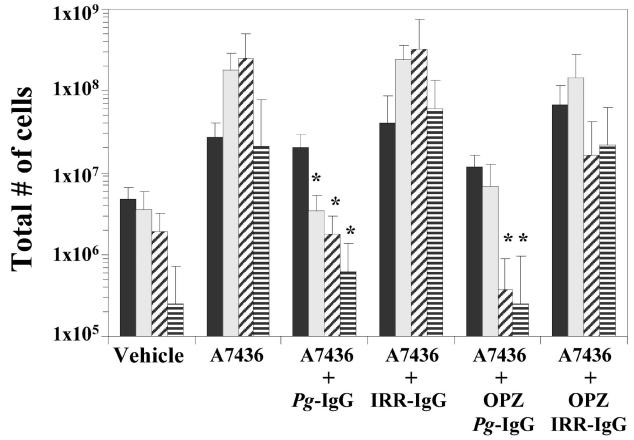

FIG. 2. Chamber fluid levels of inflammatory cells as determined by trypan blue vital dye counts. Chamber fluid samples obtained on 1 (solid bar), 3 (speckled bar), 7 (diagonal hatched bar), and 10 (horizontal hatched bar) days postchallenge from groups of mice that were challenged with *P. gingivalis* (A7436), *P. gingivalis* following passive antibody transfer of *P. gingivalis*-specific (*P. gingivalis*-specific IgG) or irrelevant (IRR-IgG) antibody, or *P. gingivalis* opsonized with *P. gingivalis*-specific IgG (OPZ *Pg*-IgG) or IRR-IgG (OPZ IRR-IgG). The samples were diluted 1:10 with trypan blue vital dye, and the total number of inflammatory cells was determined by using hemocytometer counts. *, $P < 0.05$ compared with matched IRR-immunized groups by the Student *t* test.

TABLE 2. Assessment of the interaction of *P. gingivalis* with murine inflammatory cells in murine chamber fluids by fluorescent microscopy[a]

Group	Data for day 1		Data for day 3	
	P. gingivalis/PMN[b]	% Phagocytosis[c]	*P. gingivalis*/PMN	% Phagocytosis
1	ND[d]	ND	ND	ND
2	4.81 ± 4.35	55.39 ± 42.29	3.90 ± 3.68	42.41 ± 20.45
3	0.24 ± 0.19	4.33 ± 4.91	5.62 ± 7.81	37.35 ± 27.93
4	8.08 ± 7.95	59.15 ± 30.45	2.82 ± 1.91	44.34 ± 17.41
5	7.12 ± 7.27	53.65 ± 39.66	5.66 ± 4.20	52.32 ± 14.85
6	14.81 ± 4.5	40.79 ± 44.08	1.53 ± 1.32	30.92 ± 21.94

[a] Groups of mice ($n = 8$ animals per group) were challenged subcutaneously with *P. gingivalis*, and chamber fluid samples were harvested at 1 and 3 days after challenge. Group 1 received vehicle only, group 2 was challenged with *P. gingivalis*, group 3 received *P. gingivalis*-specific IgG injection into the chamber 24 h prior to challenge, group 4 received IRR-IgG injection prior to challenge, group 5 was challenged with *P. gingivalis* opsonized with *P. gingivalis*-specific IgG, and group 6 was challenged with IRR-IgG-opsonized *P. gingivalis*.

[b] *P. gingivalis*/PMN was determined as the total number of live and dead bacteria present within at least 100 live PMNs.

[c] % Phagocytosis was determined as the number of viable PMNs that have at least one associated bacterium divided by the total number of PMNs counted, all multiplied by 100.

[d] ND, not determined.

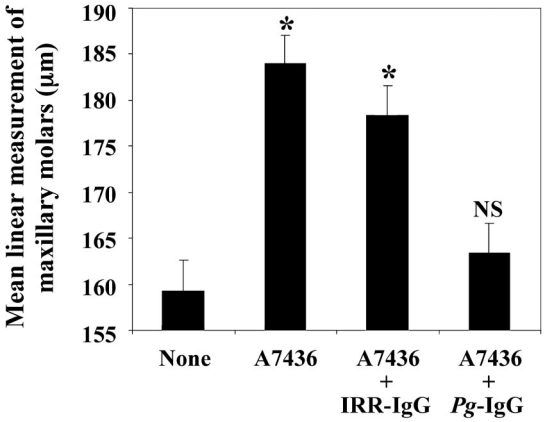

FIG. 3. Opsonization of *P. gingivalis* with *P. gingivalis*-specific IgG prior to oral challenge inhibits *P. gingivalis*-elicited oral bone loss. Groups of BALB/C mice were either unchallenged (None), were orally challenged with *P. gingivalis* (A7436), or were gavaged with *P. gingivalis* opsonized with control IRR-IgG or *P. gingivalis*-specific IgG. Linear measurements were obtained from the cementum-enamel junction to the alveolar bone crest from the maxillary molars at 14 landmark sites per mouse. *, $P < 0.05$ by the Student t test versus control mice (None); NS, not significant versus control (None).

Mice challenged with *P. gingivalis* had approximately 20% dead PMNs present in the chamber fluid samples at day 1 postchallenge. Interestingly, the percentage of dead PMNs present in chamber fluid samples of mice from groups 3 and 5 was significantly reduced compared with that of group 2 animals ($P < 0.05$, data not shown). Passive transfer (group 4) or opsonization with IRR-IgG (group 6) failed to prevent PMN death in response to *P. gingivalis* challenge, as the PMN viability results resembled those of mice in group 2.

We also recorded the number of *P. gingivalis*/PMN as well as the percentage of phagocytosis from the murine chamber fluid samples (Table 2). At day 1, we observed that approximately half of the PMNs in the chamber fluid samples of group 2 mice possessed bacteria and that each had approximately five associated *P. gingivalis* organisms. Unexpectedly, the mice from group 3 possessed fewer *P. gingivalis*/PMN and had a reduced percentage of phagocytosis compared with that of group 4 (Table 2). Opsonization of *P. gingivalis* with *P. gingivalis*-specific IgG (group 5) resulted in an increased number of *P. gingivalis*/PMN compared to that found in group 2; however, this level was only approximately half the number of *P. gingivalis*/PMN compared to that of the group 6 mice. At day 3, we observed that approximately 40% of the PMNs were actively participating in phagocytosis of *P. gingivalis*, with nearly four bacteria per PMN in group 2. Chamber fluids of the mice in group 3 presented with more bacteria within each PMN compared with results for group 2 or group 4 (Table 2). Similarly, we observed that group 5 facilitated *P. gingivalis* uptake by PMNs compared with results for the IRR-IgG opsonization group (group 6). These data support our supposition that *P. gingivalis* is actively taken up by PMNs in the presence of specific IgG.

Finally, the murine oral challenge model was employed to assess the impact of *P. gingivalis*-specific IgG opsonization on *P. gingivalis*-elicited oral bone loss (7). Eight mice were included in each group. Group 1 was not treated and served as the controls, group 2 was gavaged with 100 μl of live *P. gingivalis* in carboxymethyl cellulose, group 3 was gavaged with *P. gingivalis* opsonized with IRR-IgG, and group 4 was gavaged with *P. gingivalis* opsonized with *P. gingivalis*-specific IgG. Mice in group 2 developed significant maxillary molar bone loss compared with that of group 1 ($P < 0.05$; Fig. 3). Mice in group 4 were protected from oral bone loss ($P < 0.05$) and resembled group 1 mice ($P > 0.2$). Mice in group 3 were not protected from oral bone loss and were significantly different from mice in group 4 ($P < 0.05$; Fig. 3). These data demonstrate that opsonization of *P. gingivalis* with specific IgG inhibits *P. gingivalis*-elicited oral bone loss.

To our knowledge, passive antibody transfer studies using both prevention of infection and oral bone loss as end points have not been performed to show that immunization-elicited IgG alone mediates clearance of *P. gingivalis* in naïve animals. By using both a murine subcutaneous chamber model and an oral bone loss model, the present study demonstrates that immunization-elicited *P. gingivalis*-specific IgG prevents subsequent *P. gingivalis* infection. We are continuing to understand the contribution of immunization-elicited IgG to specific *P. gingivalis* antigens for vaccinology to prevent *P. gingivalis*-mediated periodontal disease.

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