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Porphyromonas gingivalis Virulence in Mice: Induction of Immunity to Bacterial Components

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Selected cell envelope components of *Porphyromonas gingivalis* were tested in a BALB/c mouse model in an attempt to elucidate further the outer membrane components of this putative oral pathogen that might be considered as virulence factors in host tissue destruction. Lipopolysaccharide (LPS), outer membrane, and outer membrane vesicles of *P. gingivalis* W50, ATCC 53977, and ATCC 33277 were selected to examine an immunological approach for interference with progressing tissue destruction. Mice were actively immunized with heat-killed (H-K) or Formalin-killed (F-K) whole cells or with the outer membrane fraction, LPS, or outer membrane vesicles of the invasive strain *P. gingivalis* W50. The induction of invasive spreading lesions with tissue destruction and lethality were compared among different immunization groups in normal, dexamethasone-treated (dexamethasone alters neutrophil function at the inflammatory site), and galactosamine-sensitized (galactosamine sensitization increases endotoxin sensitivity) mice after challenge infection with the homologous strain (W50) and heterologous strains (ATCC 53977 and ATCC 33277). Enzyme-linked immunosorbent assay analyses revealed significantly elevated immunoglobulin G and M antibody responses after immunization with H-K or F-K cells or the outer membrane fraction compared with those of nonimmunized mice. The killed whole-cell vaccines provided significantly greater protection against challenge infection in normal mice (decreased lesion size and death) than did either the outer membrane fraction or LPS immunization. The lesion development observed in dexamethasone-pretreated mice was significantly enhanced compared with that of normal mice after challenge with *P. gingivalis*. Immunization with *P. gingivalis* W50 provided less protection against heterologous challenge infection with *P. gingivalis* ATCC 53977; however, some species-specific antigens were recognized and induced protective immunity. Only viable *P. gingivalis* induced a spreading lesion in normal, dexamethasone-treated, or galactosamine-sensitized mice; F-K or H-K bacteria did not induce lesions. The F-K and outer membrane vesicle immunization offered greater protection from lesion induction than did the H-K immunogen after challenge infection simultaneous with galactosamine sensitization. The H-K cell challenge with galactosamine sensitization produced 100% mortality without lesion induction, suggesting that LPS or LPS-associated outer membrane molecules were functioning like endotoxin. Likewise, *P. gingivalis* W50 LPS (1 μg per animal) administered intravenously produced 80% mortality in galactosamine-sensitized mice. In contrast to the effects of immunization on lesion development, immunization with H-K or F-K cells or LPS provided no protection against intravenous challenge with LPS; 100% of the mice died from acute endotoxin toxicity. These findings suggest that the murine model will be useful in examining the tissue-destructive components of *P. gingivalis*.

Porphyromonas gingivalis, which is frequently implicated in chronic and severe adult periodontitis (40), possesses a variety of virulence factors that may contribute to the destructive events of periodontitis (18, 19, 29). In addition to a large number of proteolytic enzymes, *P. gingivalis* elaborates a lipopolysaccharide (LPS) that affects both immune and nonimmune mammalian cells (14, 18, 21) and outer membrane blebs (i.e., outer membrane vesicles [OMVs]), which contain, in addition to LPS and outer membrane proteins, other toxic molecules (i.e., proteolytic enzymes and porins) that affect the integrity of the periodontium (18, 28, 29, 39).

Although a large number of reports have described significant elevations in levels of specific serum immunoglobulin G (IgG) antibody to *P. gingivalis* infection in patients with adult and advanced destructive periodontitis and in the gingival crevicular fluids from these patients (9, 11, 27, 32, 40), only limited information has been published related to the functional activity of these antibody molecules to these antigens.

Since functional studies in humans are difficult to nearly impossible to perform, several laboratories have explored the efficacy of animal models in the study of periodontal disease progression. Taubman et al. (44) and Wyss and Guggenheim (49) employed a rat model to examine the pathogenesis of several of the putative periodontal pathogens, whereas Holt and coworkers (20) studied the ability of *P. gingivalis* to initiate progressive periodontitis in primates. The primate model provides excellent information on the longitudinal progression of periodontal disease (26); however, it is limited in its usefulness for studies of *P. gingivalis* virulence factors and their in vivo participation in tissue destruction. Van Steenbergen et al. (48), Grenier and Mayrand (17), and Kastelein et al. (22) established several of the virulence characteristics of a variety of *P. gingivalis* strains in the guinea pig and mouse abscess models. We and several other groups (10, 13, 22, 31, 36, 41, 48) also described virulence characteristics of *P. gingivalis* strains in similar rodent models. However, although we have an excellent knowledge of the in vitro elaboration of *P. gingivalis* viru-

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lence factors (i.e., enzymes, LPS, outer membrane proteins), we still have very little understanding of their activity in in vivo models of tissue destruction and inflammation. The murine model will provide the wherewithal to study the role of these putative virulence factors in tissue destruction and the use of active immunization to modify the course of bacterial infections by these virulence factors (2, 18). Several of these studies have already suggested that active immunization with killed *P. gingivalis* can minimize the progression of tissue destruction in mice in a species-specific fashion. Chen et al. (5), for example, demonstrated that lithium diiodosalicylate extracts from *P. gingivalis* interfere with the spreading lesions associated with infectious challenge with strains of the species. While the studies of Chen and colleagues (5, 6) have described the activity of whole cells or extracts of *P. gingivalis* in the murine model and the action of specific antibody to whole cells of *P. gingivalis* in modulating virulence potential, there are no studies that have described the role of isolated *P. gingivalis* virulence components (outer membrane fraction [OMF], OMV, and LPS) in modulating the progression of *P. gingivalis* virulence in mice. The aim of this study incorporates a variety of experimental designs that are selected to test the following individual and specific hypotheses: (i) various immunogens derived from *P. gingivalis* elicit serum antibodies and can afford protection from tissue destruction and/or lethality in a murine abscess model; (ii) immune responses elicited by active immunization with these immunogens are most effective in the presence of normal neutrophil function; (iii) immune protection provided by active immunization exhibits some cross-reaction among *P. gingivalis* strains; (iv) viable bacteria are required to initiate progressing tissue destruction in the murine abscess model; and (v) LPS is a critical component of *P. gingivalis* for eliciting tissue destruction and/or lethality in the murine abscess model.

MATERIALS AND METHODS

Microorganisms. The *P. gingivalis* strains used in these studies were W50 (ATCC 53978), ATCC 33277, and A7A1-28 (ATCC 53977). All cells were routinely cultivated for 24 to 48 h on prereduced Trypticase soy agar plates enriched with 5% (vol/vol) sheep blood in an anaerobic chamber (85% N_2, 5% CO_2, 10% H_2).

The *P. gingivalis* strains used as immunogens were grown in batch cultures in 2.1% (wt/vol) mycoplasma broth base (BBL, Becton Dickinson, Cockeysville, Md.) supplemented with 5 μg of hemin per ml and 1 μg of menadione per ml at 37°C for 48 h in the anaerobic chamber. The cells were harvested by centrifugation at 10,000 × *g* for 30 min at 4°C, and the pellet was washed with sterile phosphate-buffered saline (PBS) (0.02 M phosphate, pH 7.4). The washed cells were resuspended in sterile PBS to a concentration of 5 × 10^{10} cells per ml in a Petroff-Hausser counting chamber. The resulting suspension was separated into two aliquots; one aliquot was treated overnight with 0.5% (vol/vol) buffered formal saline (Formalin-killed [F-K] cells), and the other was heated at 80°C for 10 min (heat-killed [H-K] cells). Both preparations were stored at 4°C. Aliquots of the two suspensions were removed, and the total counts, purity, and sterility were determined.

Isolation of LPS and cell envelope. (i) LPS. LPS was extracted from *P. gingivalis* W50 and W83 by the cold $MgCl_2$-ethanol precipitation method of Darveau and Hancock (7). Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis and protein determination with the BCA assay kit (Pierce Chemical Co., Rockford, Ill.) revealed <1% contaminating protein in the LPS preparation. Previous results from our laboratory have demonstrated a virtual identity between LPS (4), outer membranes (23), and antigenic components from *P. gingivalis* W50 and W83, suggesting a substantial homology. We therefore felt justified in using *P. gingivalis* W83 LPS in the studies described in this report.

(ii) Cell envelope fraction. A cell envelope fraction was isolated from *P. gingivalis* W50 by mechanical disruption (3). Whole cells were sedimented by centrifugation at 15,000 × *g* for 20 min and washed twice with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4). The pellet was resuspended to a final concentration of 1 g of wet weight in 3 ml of 10 mM HEPES. These cells were mixed with 50 mg each of DNase and RNase per ml, and 50 mM $MgCl_2$ and 2 mM sodium phosphate-tosyl-L-lysine choloromethylketone. The cells were immediately broken by repeated disruption in a cold French pressure cell (SLM Instruments, Inc., Urbana, Ill.) at 17,000 lb/in^2 until >90% cell breakage was observed under phase microscopy of wet-mount preparations. After whole cells and debris were removed by centrifugation at 5,000 × *g* for 20 min at 4°C, the supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C to obtain a cell envelope fraction. The cell envelope fraction was resuspended in 20 ml of 1% (wt/vol) sodium *N*-lauryl sarcosinate (Sigma Chemical Co., St. Louis, Mo.) for each g (wet weight) of the original bacterial pellet and mixed gently for 30 min at room temperature. The resulting preparation was again centrifuged at 100,000 × *g* for 1 h at 4°C to concentrate the OMF. This OMF was washed once with 10 mM HEPES and further solubilized in 2% (wt/vol) sodium lauryl sulfate by sonication at ice bath temperature. The resulting solubilized OMF was adjusted to a protein content of 2 mg/ml (BCA assay) and stored at −70°C until used.

Preparation of OMVs. OMVs were prepared from *P. gingivalis* W50 by the method of Grenier and Mayrand (16) by using $(NH_4)_2SO_4$ (50% [wt/vol] saturation of the culture fluid at 4°C) after sedimentation of the whole cells at 16,000 × *g* for 30 min. The OMVs were resuspended in sterile PBS and stored at −70°C until used. The protein content was determined by using the BCA assay.

Animals. The BALB/c female mice (Charles River Laboratories, Willmington, Mass.) used in these studies were generally 6 to 12 weeks old when they were tested for bacterial virulence. The animals were housed in isolator cages in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited biohazard facility at the University of Texas Health Science Center at San Antonio and were provided autoclaved TEKLAD chow (Sprague-Dawley Co., Madison, Wis.) and water ad libitum.

Murine virulence model. (i) Experimental groups. The BALB/c mice were randomly separated into the following groups for the virulence studies: (i) control, untreated (placebo) mice challenged with viable *P. gingivalis* W50, ATCC 33277, or ATCC 53977; (ii) mice pretreated with dexamethasone (DEX) (40 μg per animal; Elkins-Sinn, Inc., Cherry Hill, N.J.) (12, 35, 37) 3 days before challenge (this treatment has been shown to decrease neutrophil infiltration into sites of inflammatory lesions); and (iii) mice sensitized with D-[+]-galactosamine (GalN; 700 mg/kg of body weight in a single intraperitoneal dose; Sigma) (15, 24, 45) and

simultaneously challenged with subcutaneous (s.c.) *P. gingivalis*.

(ii) Preparation of *P. gingivalis* cells for challenge. All *P. gingivalis* strain manipulations were carried out under anaerobic conditions in a Coy anaerobic chamber to ensure maximum cell viability. Cells were grown on plates for approximately 3 to 4 days and harvested aseptically with sterile cotton swabs soaked in reduced transport fluid (42). The cell mass was immediately resuspended in reduced transport fluid, and aliquots were removed from the chamber for estimation of total counts in a Petroff-Hausser counting chamber and determination of viable counts and purity. For mouse inoculation, appropriate dilutions of this suspension were made and distributed into anaerobic gas-filled sealed vacuum vials under anaerobic conditions. Mice were injected within 15 to 30 min of sample preparation. The sample sterility and the number of *P. gingivalis* cells injected were determined by plating.

(iii) Animal challenge. Animals were challenged s.c. on the posterior dorsolateral surface with 0.2 ml of the original bacterial suspension. After the challenge, the animals were examined for lesion formation at approximately 6 h and at least once daily for 15 days for visible signs of general systemic infection. Lesion size (length and width) was measured with a caliper, and the area was determined and expressed in square millimeters. Any moribund animal was euthanized by CO_2 asphyxiation and was catalogued as a death. Clinical symptoms of infections developing in the mice were scored as (i) spreading infection or phlegmonous abscess with pus and exudate in the flank, ventral side of abdomen, and thoracic cavity; (ii) localized abscess or necrosis of skin and subcutaneous muscles at the site of injection; or (iii) death.

The lethal activity of LPS from *P. gingivalis* W83 was determined in preliminary studies by administering 1.0 and 0.1 μg per animal intravenously, simultaneous with intraperitoneal GalN sensitization. This resulted in an 80 and 0% mortality, respectively (data not shown). The results of these findings were used to examine the ability of the antibody to protect against at least 5 times the lethal toxicity of LPS from this microorganism.

Immunization protocol. Mice were immunized by s.c. injection into the nape of the neck with 0.1 ml of 10^9 F-K or H-K *P. gingivalis* W50 cells emulsified in incomplete Freund adjuvant (IFA). OMV and OMF (100 μg of protein) and 25 μg (dry weight) of LPS were emulsified in IFA and administered into groups of mice. The control placebo mice received IFA emulsified with sterile PBS (pH 7.2). A booster immunization was administered 10 days later.

Antibody analysis. Blood for serum samples was collected from the mice by retroorbital access either under ether anesthesia or after cervical dislocation. IgG and IgM antibodies in the mouse sera were determined by using the enzyme-linked immunosorbent assay (ELISA) procedure of Ebersole et al. (8). The antibody level is expressed as the optical density multiplied by the dilution of the serum that was tested in the ELISA.

Statistical analyses. Statistical differences in lesion size with various challenge doses, effects of immunization after challenge infection, and death in different groups were determined by using the Fisher exact test. Differences in serum antibody levels and lesion size were compared by using the Kruskal-Wallis and Wilcoxon-Mann-Whitney U tests. Nonparametric statistical analyses were used to compare the ranks of the data among groups. These procedures were employed because of the inherent variation in the characteristics of the spreading lesions as well as the technical limitations in accurately measuring a nongeometric lesion spreading over an animal's body. Means and standard deviations are presented in the figures.

RESULTS

Murine immune response to F-K, H-K, OMF, and LPS preparations. The IgG and IgM immune responses to F-K-, H-K-, OMF-, and LPS-immunized mice are seen in Fig. 1. Immunization with both F-K and H-K whole cells and OMF elicited significant ($P < 0.001$) IgG and IgM responses to the F-K and H-K antigens compared with those of placebo (IFA)-injected mice. The level of antibodies produced by the H-K whole-cell antigen was almost a 50% higher than that produced by the F-K whole-cell antigen. Both LPS and OMF elicited IgG and IgM responses; however, both responses were significantly lower than those elicited by the H-K and F-K antigens. LPS immonization induced IgG and IgM antibodies that reacted with the F-K and H-K antigens (Fig. 1).

Virulence of *P. gingivalis* after active immunization. As reported previously by us (10), the dynamics of lesion development in s.c. injected BALB/c mice included the formation of recognizable lesions at approximately 18 h and death, when it occurred, by 2 to 3 days postchallenge. In surviving mice, lesion regression and resolution occurred by 15 days. Therefore, this time frame was utilized in the experiments described in this report.

Normal BALB/c mice immunized with H-K or F-K whole cells, OMF, LPS, or placebo were examined for immune responses that ameliorated lesion formation (Fig. 2). At 2 weeks postimmunization, the animals were challenged with 5×10^{10}, 2×10^{10}, or 1×10^{10} *P. gingivalis* W50 cells. Within 18 h after *P. gingivalis* W50 challenge, all placebo (IFA)-immunized control animals developed a dose-dependent spreading *P. gingivalis* lesion, particularly at *P. gingivalis* doses of 5×10^{10} and 2×10^{10} (Fig. 2). This lesion spread laterally and ulcerated the entire abdomen and was accompanied by severe cachexia; death routinely followed. In contrast, H-K and F-K whole-cell-immunized animals exhibited a moderate spreading lesion by 18 to 72 h with minimal cachexia (data not shown). Compared with placebo-immunized mice, both H-K and F-K whole-cell-immunized animals showed a highly significant decrease in induction of a spreading lesion at all challenge doses. Lesion formation was not observed in 20% of the H-K-immunized animals challenged with 2×10^{10} *P. gingivalis* whole cells. Also apparent was that the killed whole-cell vaccines (F-K, H-K) provided better protection (i.e., smaller lesion size and lower percentage of death; see below) than did immunization with either OMF or LPS. However, the OMF- and LPS-immunized animals did show a significant decrease in the induction of the spreading lesion compared with that of the placebo-treated animals (Fig. 2).

The effect of immunization with *P. gingivalis* H-K and F-K cells, LPS, and OMF on BALB/c mice with altered neutrophil capabilities (DEX treatment) is shown in Fig. 3. Compared with placebo immunization at the highest *P. gingivalis* W50 challenge dose (5×10^{10}), active immunization with H-K or F-K cells or LPS did not significantly reduce the size of the spreading lesion. However, H-K-, F-K-, and LPS-immunized animals challenged with 2×10^{10} *P. gingivalis* W50 cells showed a significantly decreased lesion size ($P < 0.05$). OMF immunization decreased the lesion size uniformly ($P < 0.05$) at all challenge doses. DEX

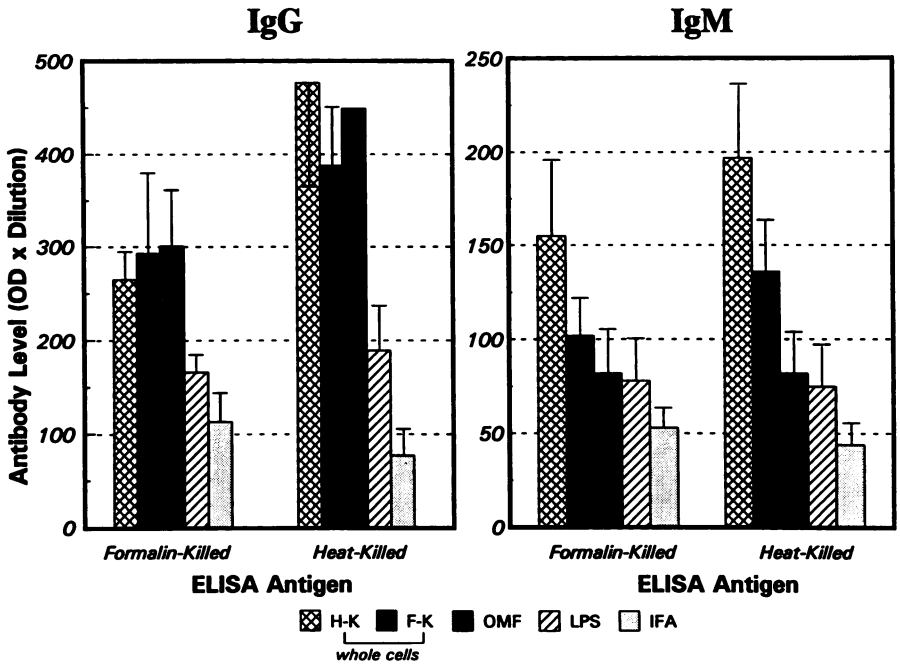

FIG. 1. IgG and IgM antibody titers in BALB/c mice immunized with F-K and H-K *P. gingivalis* W50 whole cells, OMF, and LPS and with a placebo (IFA). ELISA antigen denotes the F-K and H-K bacteria attached to microtiter plates for determining antibody levels. Each bar represents the mean antibody titer, and the vertical lines denote standard deviations from the means. The antibody levels are expressed as the optical density (OD) at 490 nm multiplied by the dilution of the serum.

also had a minimal effect on lesion size in the OMF- and LPS-immunized mice compared with that in placebo animals (Fig. 4). H-K and F-K whole-cell immunization therefore afforded less protection in the DEX-treated animals than the placebo (IFA)-immunized animals. OMF and LPS immunization, on the other hand, appeared to have a similar effect in the presence or absence of normal neutrophil capacity (i.e., with or without DEX). Placebo-treated animals with and without DEX treatment developed large spreading lesions after challenge with 5×10^{10} *P. gingivalis* W50 cells; 100% of the mice died (Fig. 4). F-K and H-K whole-cell immunization reduced the lethal effects of this high *P. gingivalis* challenge by 60% in the DEX-treated group (two of five animals died), whereas lethal effects were completely

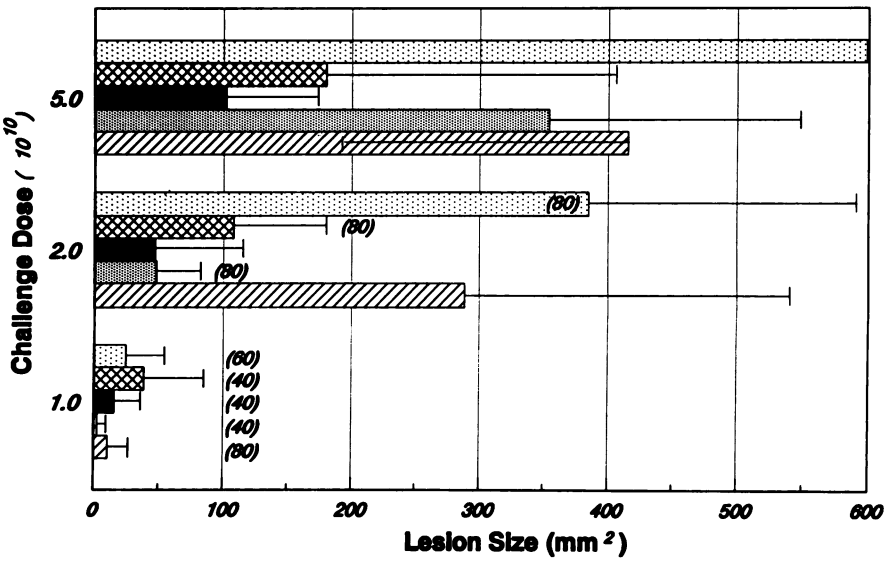

FIG. 2. Effect on lesion size of active immunization of BALB/c mice with (reading from top to bottom) IFA (placebo), H-K and F-K cells, OMF, and LPS. Mice were challenged s.c. with 5×10^{10}, 2×10^{10}, and 1×10^{10} *P. gingivalis* W50 cells. Bars denote group mean lesion sizes, and the horizontal lines denote standard deviations from the means. The numbers within the parentheses indicate the percentage of animals in each group that formed lesions if <100%. Unless otherwise indicated, five mice per group were used in all other experiments in Fig. 2 through 7.

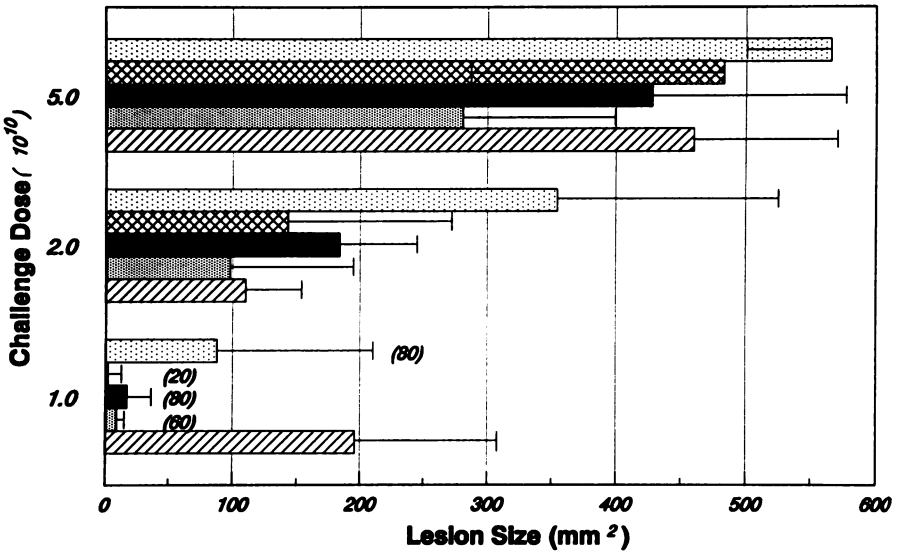

FIG. 3. Effect of active immunization of DEX-treated BALB/c mice with H-K and F-K cells, OMF, LPS, and IFA (placebo) followed by an s.c. challenge with 5×10^{10}, 2×10^{10}, and 1×10^{10} *P. gingivalis* cells on lesion size. All mice were pretreated with DEX (2 mg/kg of body weight) intraperitoneally for 3 days before the challenge infection. The lesion size was measured as described in the text. Bars denote the group mean lesion sizes, and horizontal lines denote the standard deviations from the means. The numbers in parentheses and the bars are as in Fig. 2. See Materials and Methods for the DEX protocol.

eliminated in normal animals. The OMF- and LPS-immunized normal and DEX-treated animals also showed a significant decrease in mortality (i.e., 60 and 40%, respectively) when challenged with 5×10^{10} *P. gingivalis* W50 cells. H-K and F-K *P. gingivalis* W50 whole-cell immunization significantly protected DEX-treated animals from death, with 60% survival compared to 100% death in normal IFA-injected animals and DEX-treated IFA-injected animals.

Immune cross-protection studies. The effect of heterologous challenge with *P. gingivalis* ATCC 53977 and ATCC 33277 on lesion development in mice immunized with H-K and F-K *P. gingivalis* W50 whole cells, LPS, and OMVs and placebo-immunized BALB/c mice is shown in Fig. 5. Challenge of the placebo-treated mice with heterologous *P. gingivalis* ATCC 53977 or homologous *P. gingivalis* W50 at 2×10^{10} viable cells per ml induced lesions of approximately the same sizes; in mice challenged with *P. gingivalis* ATCC 33277, the lesion size was significantly smaller. Immunization with *P. gingivalis* W50 H-K, F-K, and LPS antigens resulted in a significant protection of the animals from lesion formation (as measured by lesion size) after challenge with *P. gingivalis* W50 or the heterologous strain *P. gingivalis*

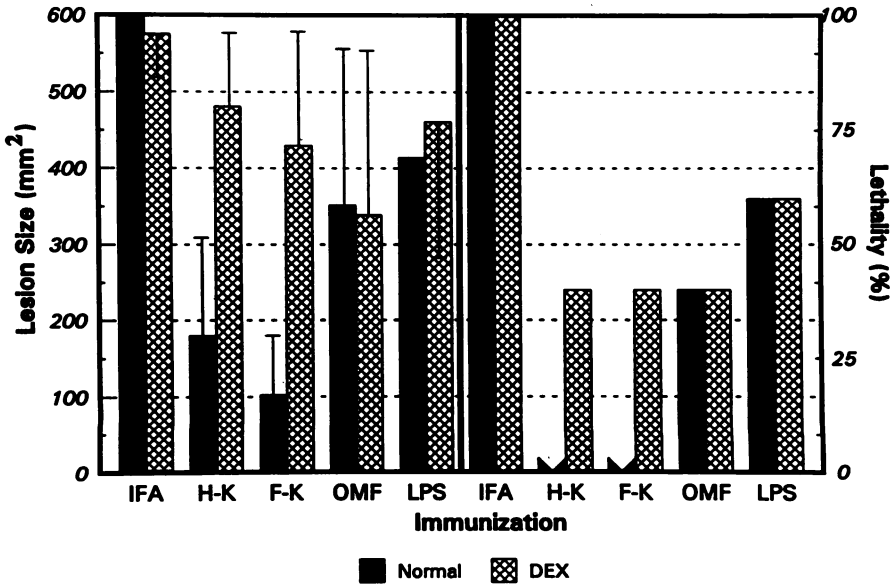

FIG. 4. Effect of active immunization of normal and DEX-treated BALB/c mice with H-K and F-K cells, OMF, LPS, and IFA (placebo) on lesion size and lethality. Both normal and DEX-treated mice were challenged with 5×10^{10} *P. gingivalis* W50 cells. The bars denote the group mean lesion sizes and the percent lethality. The vertical lines denote standard deviations from the means.

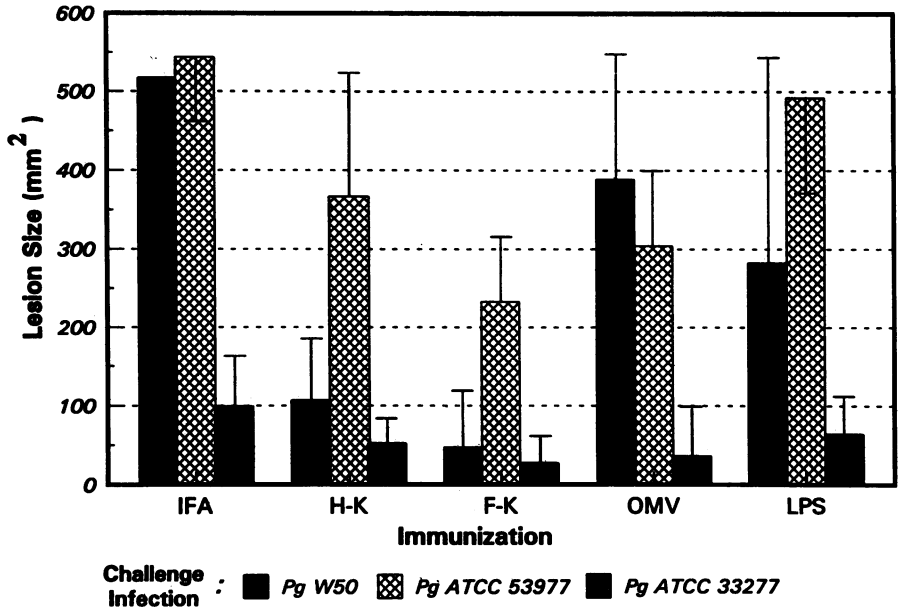

FIG. 5. Effect of homologous (*P. gingivalis* W50) and heterologous (*P. gingivalis* ATCC 53977 and ATCC 33277) challenge (2×10^{10} cells) on lesion size in BALB/c mice actively immunized with H-K and F-K cells, OMVs, and LPS from *P. gingivalis* W50. The bars denote the group mean lesion sizes, and the vertical lines denote standard deviations from the means.

ATCC 33277. Although immunization with H-K *P. gingivalis* W50 protected the *P. gingivalis* ATCC 53977-challenged animals from lesion formation, it only resulted in a 30% reduction in lesion size. The lesions induced by heterologous challenge with *P. gingivalis* ATCC 53977 were significantly larger than those induced by homologous challenge with *P. gingivalis* W50 in H-K-, F-K-, and LPS-immunized mice. Immunization with the *P. gingivalis* W50 OMV also provided protection from lesion formation induced by *P. gingivalis* W50 and ATCC 53977 (IFA-injected animals were used as controls). *P. gingivalis* ATCC 33277 challenge had essentially no effect; however, this strain also produced a very small lesion in the placebo-immunized animals. *P. gingivalis* W50 LPS immunization afforded only partial protection in mice challenged with homologous *P. gingivalis* W50; however, it did not protect animals challenged with *P. gingivalis* ATCC 53977 or ATCC 33277 from lesion formation. Immunization of mice with *P. gingivalis* W50 H-K and F-K cells also resulted in a significant decrease in lesion size in mice challenged with *P. gingivalis* ATCC 33277 (Fig. 5). The mortality associated with homologous or heterologous *P. gingivalis* challenge is seen in Table 1. *P. gingivalis* infection indicated a graded difference in the degree of cross-immune protection, such that F-K = OMV > H-K > LPS, after heterologous *P. gingivalis* ATCC 53977 challenge infection; the relative immune protection to homologous *P. gingivalis* W50 challenge infection was F-K = H-K > OMV > LPS (Table 1).

TABLE 1. Comparison of lethality in homologous and heterologous challenge infection in immunized BALB/c mice

Immunogen[a]	Lethality[b] of challenge infection with *P. gingivalis* strain:	
	W50	ATCC 53977
F-K cells	0/5 (0)	0/5 (0)
H-K cells	0/5 (0)	1/5 (20)
OMV	2/5 (40)	0/5 (0)
LPS	7/8 (83.3)	3/5 (60)
IFA	4/5 (80)	5/5 (100)

[a] All immunogen preparations were derived from *P. gingivalis* W50.
[b] Values denote the number dead/total number of mice, with the percentage of dead mice in parentheses.

Effect of challenge with viable or killed *P. gingivalis* W50 in normal, DEX-treated, and GalN-sensitized mice. Nonimmunized animals treated with DEX or sensitized with GalN were challenged with 2×10^{10} viable *P. gingivalis* W50 cells; spreading lesions formed in the normal, DEX-treated, and GalN-sensitized mice (Fig. 6). H-K *P. gingivalis* W50 challenge with simultaneous GalN sensitization also resulted in 100% lethality by 18 h without lesion formation, indicating that one component of the H-K antigen is a heat-stable endotoxin. In contrast, injection of H-K *P. gingivalis* W50 induced no morbidity or mortality in normal and DEX-treated mice. Challenge with 2×10^{10} F-K *P. gingivalis* W50 cells resulted in neither the formation of spreading lesion nor death in any of the animal groups (Fig. 6), suggesting that Formalin modified or altered the lethal activity of the *P. gingivalis* W50 LPS or other contributing bacterial components.

Immune protection in GalN-sensitized mice. Since the above findings suggested that the *P. gingivalis* W50 LPS contributed to mouse lethality, mice were actively immunized with *P. gingivalis* W50 H-K and F-K whole cells, OMV, or LPS to determine the ability of these antigens to induce immunological interference with the LPS-mediated toxicity (Fig. 7). Both IgG and IgM antibodies were produced against the *P. gingivalis* W50 LPS (data not shown). Both F-K- and OMV-immunized animals had significantly greater ($P < 0.025$) protection from lesion development than did the H-K-immunized animals after challenge with viable *P. gingivalis* W50. The protection provided by immunization with the *P. gingivalis* W50 LPS in mice challenged with viable *P. gingivalis* W50 was significantly less than that

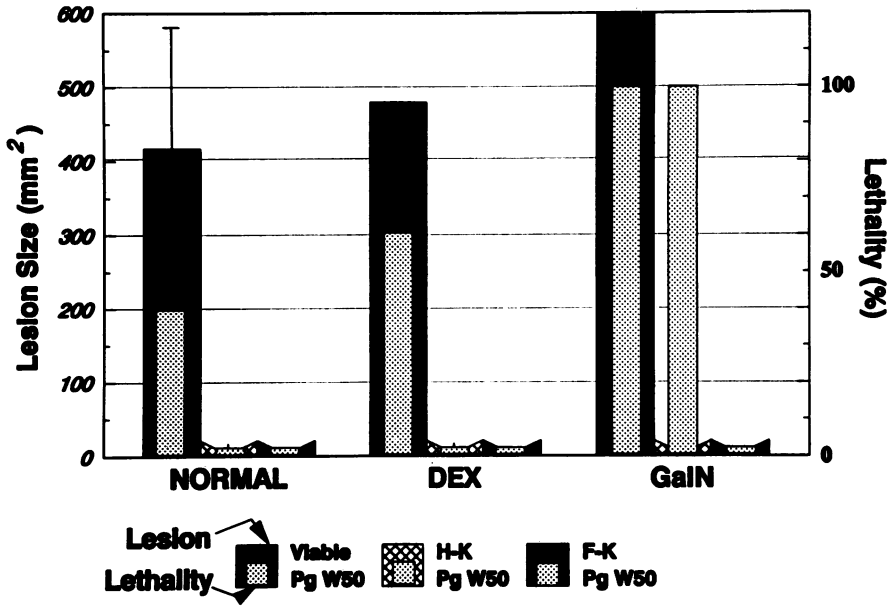

FIG. 6. Effect of active immunization with H-K, F-K, and viable *P. gingivalis* W50 on lesion formation and lethality in normal, DEX-treated, and GalN-sensitized BALB/c mice. The challenge dose was 2×10^{10} cells. The bars denote group mean lesion sizes. The vertical lines denote the standard deviations from the means. See Materials and Methods for DEX and GalN protocols. The stippled bars (▩) denote lethality.

provided by any of the other immunogens. When similarly immunized animals were challenged with five times (5 μg per animal) the concentration of a normal lethal dose of *P. gingivalis* W50 LPS, OMV was the only antigen that partially protected (60% mortality) mice against LPS challenge (Fig. 7).

DISCUSSION

Even though numerous studies (40) have established the participation of a small number of microorganisms in human periodontal disease (18, 40), there are only a few studies that have explored the longitudinal events (i.e., clinical, microbiological, immunological) that occur during disease progression in humans. To more accurately document the characteristics of disease progression, investigators have used several animal models (10, 13, 20, 22, 48). The most prominent model is the primate model pioneered by Kornman et al. (26) and extended by Holt et al. (20). McArthur et al. (30) and Nisengard and coworkers (34) also provided evidence that the primate is an important animal model for the study of periodontal disease progression.

Although it has been verified that the primate is an excellent system for the study of disease progression, it has been difficult to design useful experiments with these animals in an attempt to understand the in vivo pathogenic activity of several of the putative periodontal pathogens (18, 19). The advances in understanding the in vivo pathogenesis (i.e., virulence) of these oral microorganisms (i.e., *P. gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Wolinella recta*) have emerged from the rodent model (10, 22, 29, 43, 49). These murine models have been used to measure the effects of these periodontal disease-related bacteria on rodent morbidity and mortality. Several of these models have also studied the effects of LPS, OMVs, and outer membranes on virulence. These in vivo mouse-periodontal pathogen interactions were also observed by several investigators who studied the mitogenic effects of these bacteria. In addition to activating murine macrophages, lymphocytes, and other immune system components, *P. gingivalis* produces significant amounts of a large variety of proteolytic enzymes (25). We know very little of the elaboration of these proteolytic-hydrolytic molecules in the in vivo environment of the rodent. Since *P. gingivalis* produces spreading, phlegmonous lesions in the rodent model (22, 48) and since, depending upon the numbers of pathogens and lesion integrity, the outcome can be fatal, it is essential to understand the contribution of this important oral bacterium to tissue and bone destruction. Hence, it is important to study the role of these microorganisms in the production of these extensive, phlegmonous lesions in *P. gingivalis*-infected mice. It is not possible to translate the findings in the rodent to the progression of human periodontal disease. All that is possible is an understanding of the high virulence of these bacteria in a rodent host.

In addition to the study presented here, there are only two other published studies that have investigated the ability of actively acquired immune responses to *P. gingivalis* or selected surface components to interfere with *P. gingivalis* pathogenesis (i.e., abscess formation) in this rodent model (5, 6). These latter studies utilized immunogens restricted to pasteurized *P. gingivalis* 381 and A7A1-28, *Prevotella intermedia*, and a lithium diiodosalicylate cell surface extract and the LPS from *P. gingivalis* A7A1-28 (ATCC 53977). Although the lithium diiodosalicylate cell surface extract provided strain-specific immune protection, its complex nature was not useful in determining which components within it were the effective immunogens. The present study more clearly detailed several of the potential components of the *P. gingivalis* surface that might be involved in tissue destruction and lethal outcome associated with *P. gingivalis* infections. We compared the potential of F-K or H-K whole cells, LPS, OMF, and OMV to protect against the tissue destructive and lethal events of *P. gingivalis* infection. Parenteral

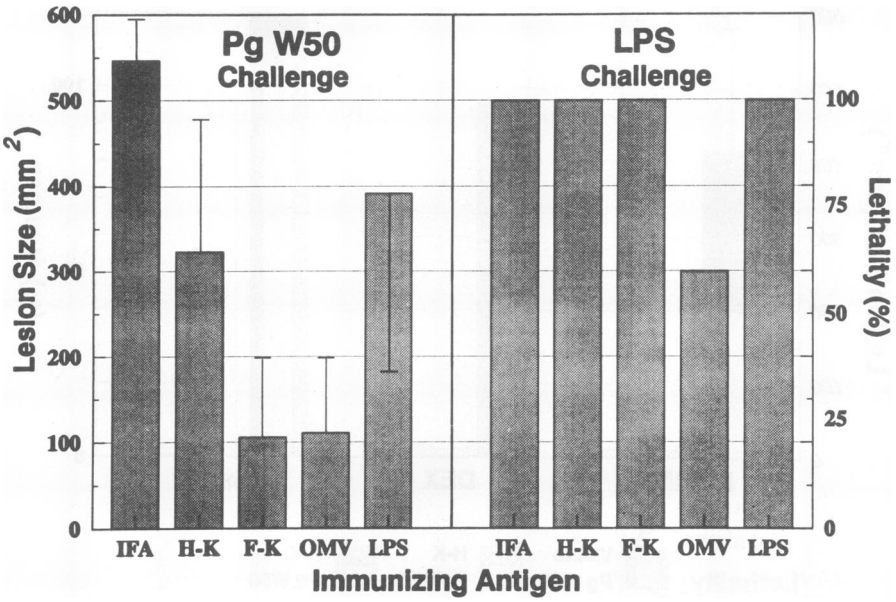

FIG. 7. Immune protection against challenge infection with *P. gingivalis* W50 and W83 LPS challenge in GalN-sensitized BALB/c mice. The mice were actively immunized with H-K and F-K cells, OMV, LPS, and IFA (placebo) and challenged with either an s.c. injection of 2×10^{10} viable *P. gingivalis* W50 cells or an intravenous injection of 5 μg of LPS per animal. Both animal groups were sensitized with GalN as described in Materials and Methods. The bars denote mean lesion sizes, and the vertical lines denote standard deviations from the means.

immunization with F-K and H-K *P. gingivalis* whole cells elicited a classical elevation in serum IgG and IgM antibodies that reacted with intact bacteria. Since the H-K antigen reacted with more antibody molecules than did the F-K antigen, it is possible that the differences in protection were due to differences in antigenic composition or to an increased antigen epitope expression present in the H-K whole-cell test antigen.

Although cross-sectional immunological studies of sera from periodontitis patients have demonstrated high titers of antibody to *P. gingivalis* LPS (38), fimbriae (50), and outer membrane proteins (1), there are no data indicating that the antibodies to these specific *P. gingivalis* surface molecules are actually protective by interfering with or neutralizing the activity of these components in vivo. In fact, our observations revealed that the *P. gingivalis* W50 immunogens used in our experiments (F-K and H-K whole cells, LPS, OMF) only partially protected the animals from lesion formation by the homologous *P. gingivalis* strain. These observations suggest that surface molecules other than LPS and outer membrane proteins (i.e., proteolytic enzymes) are involved in lesion formation. Kesavalu et al. (25) have preliminary evidence that trypsin-deficient mutants of *P. gingivalis* produced only small localized lesions, unlike the spreading lesions elicited by the parental wild-type strain. Immunization with F-K *P. gingivalis* did provide the greatest protection from lesion formation by this pathogen (Fig. 2 and 4). Previous findings in other infection models have suggested that this type of vaccine (i.e., F-K whole cells) can elicit an efficient protective immune response (2). The H-K *P. gingivalis* immunogen was not as effective as the F-K immunogen in ameliorating lesion initiation (compare Fig. 2 and 4), suggesting that heating of the whole cells modified or altered protective protein epitopes and permitted the presentation of only, for example, heat-stable polysaccharides of the *P. gingivalis* capsule or the O-polysaccharide of the LPS for antibody induction. These latter molecules were not involved in lesion formation, since antibody production against them was not protective. These findings are also consistent with the previous observations that LPS is not a vital component in the production of lesions by *P. gingivalis* (5).

Previous studies suggested that the immune protection afforded by immunization with *P. gingivalis* whole cells was strain specific (6). Other reports also described a variability in lesion formation by different *P. gingivalis* strains (17, 22, 33, 47). The results presented in this report demonstrate that *P. gingivalis* ATCC 33277, ATCC 53977, and W50 are in fact very different in their ability to induce a lesion in BALB/c mice. The lesions produced by *P. gingivalis* ATCC 53977 and W50 in mice were significantly larger than those produced by *P. gingivalis* 33277. Previous immunologic protection studies (5, 6) are in agreement with our observations that active immunization with *P. gingivalis* strains or their components produced significantly greater immunological protection in the homologous system. However, although the homologous immunization system produced the greatest protection against lesion formation and death, our observations demonstrated clear immunologic cross-reactivity, because lesions caused by *P. gingivalis* ATCC 53977 or ATCC 33277 were diminished in size after heterologous challenge compared with those of the placebo-injected group (Fig. 5). Thus, although the *P. gingivalis* strains used in this study represent three different serotypes (13), it appears that species-specific antigens are also present and that inhibition of these components by an antibody can result in decreased disease expression.

Antibodies protect the host against microbial and viral pathogenesis by inhibiting the ability of the invading organism to enter the host and successfully colonize it (30, 34). Antibodies also protect the host by fixing complement, neutralizing toxins, opsonizing invading bacteria, and providing a significant advantage for cell-mediated events (i.e., phagocytosis). Neutrophils are therefore essential to the

host in interfering with disease progression (46). However, whether neutrophil function is dependent upon antibodies (opsonization) has not been delineated in the rodent model of *P. gingivalis* virulence. Although we clearly showed that the immune response to *P. gingivalis* whole cells or cell surface components was related to antibody elicited in these animals and provided a measure of protection, it was not clear how this antibody functioned in host protection. We addressed the question of the role of the neutrophil in protection against lesion formation and lethality by lowering the neutrophil population infiltrating the local lesion by treating the animals with DEX (12, 35). Although the antibody to any of these immunogens decreased both lesion size and lethal outcome in the absence of normal neutrophil levels at the site of infection, after treatment with DEX the virulence of the *P. gingivalis* strains was significantly enhanced (Fig. 6). Thus, it appears that in this model system the antibody is functioning primarily as an opsonin to bind to bacteria or their products and enable phagocytic destruction of the virulence components. The DEX reduction of the local neutrophil content in the presence of sufficient antibody strongly suggests an opsonic effect of the elicited antibody.

Finally, the question of whether live (viable) or dead (i.e., F-K, H-K) *P. gingivalis* cells were required for lesion formation and lethality and the endotoxicity of the *P. gingivalis* LPS was also addressed in these studies. Comparison of normal and DEX-treated mice convincingly demonstrated that lesion formation and lethality required viable *P. gingivalis* (Fig. 6). Similarly, the endotoxic potential of the *P. gingivalis* LPS was examined in mice by utilizing the GalN sensitization model of Galanos et al. (15, 24), which permits LPSs of even very low endotoxicity to be studied in an exquisitely sensitive in vivo system. We were able to demonstrate that simultaneous injection of GalN with LPS or whole cells of *P. gingivalis* W50 enhanced the morbidity caused by these agents in mice (Fig. 7). Additionally, active immunization with H-K and F-K *P. gingivalis* and OMVs significantly ameliorated lesion progression. In contrast, immunization with LPS had only a minimal effect on lesion size, whereas GalN sensitization of mice intravenously challenged with LPS provided no protection against lethal events induced by the LPS (Fig. 7). Thus, it appears that in GalN-sensitized mice LPS is not a critical component for spreading lesion progression; however, it is critical in lethality in this murine model. Therefore, the *P. gingivalis* LPS does possess endotoxic activity. The formation of lesions in all of the immunized animals (although the lesions were significantly smaller) in this study indicated clearly that the elicited immune response with all the antigens tested was not capable of completely abrogating lesion formation but could eliminate mortality. Thus, it would appear that these two disease outcomes are regulated by different pathogenic mechanisms of this microorganism.

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