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A Novel Mouse Model To Study the Virulence of and Host Response to *Porphyromonas* (*Bacteroides*) *gingivalis*

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We describe here the development of a mouse subcutaneous chamber model that allows for the examination of host-parasite interactions as well as the determination of gross pathology with *Porphyromonas* (*Bacteroides*) *gingivalis* challenge. When inoculated into stainless-steel chambers implanted subcutaneously in female BALB/c mice, *P. gingivalis* W83, W50, and A7436 (10^8 to 10^{10} CFU) caused cachexia, ruffling, general erythema and phlegmonous, ulcerated, necrotic lesions, and death. *P. gingivalis* W50/BEI, HG405, and 33277 (10^{10} CFU) produced localized abscesses in the mouse chamber model with rejection of chambers at the injection site. Analysis of chamber fluid from 33277-, HG405-, and W50/BEI-infected mice by cytocentrifugation revealed inflammatory cell debris, polymorphonuclear leukocytes, and high numbers of dead bacteria. In contrast, fluid from A7436-, W50-, and W83-infected mice revealed infiltration predominantly of polymorphonuclear leukocytes and live bacteria. Bacteria were found primarily associated with polymorphonuclear leukocytes in the fluid from W50-, HG405-, and W83-infected mice but not from A7436-infected mice. Viable isolates were recoverable from the chamber fluid through day 3 for W50/BEI, day 5 for 33277, day 6 for HG405, day 7 for W50, day 14 for W83, and day 26 for A7436. All strains induced a systemic immunoglobulin G response in serum and chamber fluid samples. The use of this model will allow us to examine the virulence of *P. gingivalis* as defined by the interaction of host response to localized infection with *P. gingivalis*.

One of the most frequently isolated microorganisms from progressive adult periodontitis lesions appears to be the black-pigmented *Bacteroides* species and, in particular, *Porphyromonas* (*Bacteroides*) *gingivalis* (32). Recently, Holt et al. (13) demonstrated the progression of periodontal lesions in nonhuman primates with the implantation of *P. gingivalis* alone, indicating that *P. gingivalis* is capable of functioning as a primary pathogen in periodontal disease. Although much is known about host factors in periodontal disease, little is known about the in situ interaction of these factors with bacterial components and how they relate to the progression of disease. Unmasking the complex interactions among bacterial growth conditions, bacterial products, host factors, and disease progression requires a suitable in vivo model.

The animal models currently used are models in which the specific clinical features of periodontal diseases can be demonstrated or those in which the growth of specific etiological agents can be assessed. A murine abscess model (MAM) has been used extensively to examine the virulence properties of *P. gingivalis* strains (4, 5, 15, 18, 19, 20–22, 30, 38, 39). A similar model that uses guinea pigs has also been used to examine ecological relationships of mixed anaerobic infections involving *P. gingivalis* (9, 15, 17, 37). On the basis of the pathology observed following inoculation in the abscess models, strains of *P. gingivalis* have been classified as invasive or noninvasive (38). At high inocula ($\geq 10^{10}$ CFU), the noninvasive strains produce a localized abscess at the site of injection, while invasive strains spread to distant sites and may produce septicemia and death. Chen et al. (4, 5) have used the MAM to examine the effects of immunization on infection induced by *P. gingivalis* in mice. McKee et al. (19) have found that colonial variants of *P. gingivalis* W50 which were weak pigmenters were less virulent in the MAM and that limitation of hemin during in vitro growth decreased the virulence of *P. gingivalis* (18). Although these studies have provided valuable information on the pathogenic potential of this organism, the results obtained are limited due to the inability to monitor the production and interactions of localized host factors throughout the course of infection.

Assessment of host responses to localized infections can be performed quite easily with the use of subcutaneous (s.c.) chambers implanted in mice or guinea pigs. s.c. chambers have been used previously as a model for studying the immunogenicity and strain diversity of *Neisseria gonorrhoeae* in mice (41), as well as the pathogenesis of foreign body infections in guinea pigs (42). Sundquist et al. (36) have reported the growth of *P. gingivalis* and *Bacteroides intermedius* in s.c. implanted Teflon cages in guinea pigs. These investigators were unable to detect any C3 protein in complement in fluid from cages infected with *P. gingivalis* W83. This strain fails to accumulate C3 during opsonization by serum due to its proteolytic capacity (28, 29). Recently, Dahlen and Slots (6) examined the interactions between *P. gingivalis* and systemic antibodies in tissue cages implanted in the backs of New Zealand White rabbits. These authors evaluated infectivity according to clinical signs and bacterial counts within cages, but they did not examine cage fluid for local host inflammatory cells.

Animal models that use s.c. chambers allow continual access to the chamber contents throughout the course of infection for microbiological, immunological, and cytological examination. This allows for the longitudinal examination of the specific host factors, both cellular and soluble, that are produced locally in response to bacterial challenge during the course of infection. In addition, one can easily

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sample in vivo grown bacteria and assess the modulation of potential virulence factors by this specific growth environment.

We report here the growth of *P. gingivalis* in s.c. stainless-steel chambers implanted in mice. Unlike earlier studies in guinea pigs or rabbits (6, 36), we have longitudinally monitored the chamber contents by bacterial culturing and cytocentrifugation and staining for bacterial and host cells. This allowed a determination of the composition of host cell types in response to bacterial challenge, as well as an assessment of specific host cell-bacterium interactions. This has provided us with a model for examining the modulation of virulence as defined by the interaction of host-specific responses to localized infection with *P. gingivalis*.

MATERIALS AND METHODS

Bacterial strains. The *P. gingivalis* strains studied included strains W83 and HG405 obtained from A. van Winkelhoff, Vrije Universiteit, Amsterdam, The Netherlands; strains W50 and W50/BEI obtained from P. Marsh, PHLS Center for Applied Microbiology and Research, Salisbury, Wiltshire, England; strain 33277 obtained from the American Type Tissue Collection, Rockville, Md.; and strain A7436 (our own periodontal isolate). Strain A7436 was initially isolated from a refractory case of periodontitis and was extensively characterized by V. R. Dowell, Anaerobic Microbiology Laboratory, Centers for Disease Control, Atlanta, Ga.

P. gingivalis was typically grown on anaerobic blood agar plates (Remel, Lenexa, Kans.) in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, Mich.) with 85% N_2, 5% H_2, and 10% CO_2. After incubation at 37°C for 2 to 3 days, the bacterial cells were inoculated into Schaedler broth (Difco Laboratories, Detroit, Mich.) and grown for 1 to 2 days until the culture reached an A_{660} of 1.2 as read on an LKB spectrophotometer. This corresponded to approximately 10^9 CFU/ml. *P. gingivalis* 33277 grew poorly in Schaedler broth and thus was grown on anaerobic blood agar plates (for 48 h) and resuspended in Schaedler broth to an A_{660} of 1.2.

Cultures were concentrated by centrifugation at 10,000 × *g* (10 min at room temperature) and resuspended in 1/10, 1/100, 1/1,000, or 1/10,000 of the original volume in prereduced Schaedler broth. To obtain diluted inocula, the initial culture was diluted 1/10, 1/100, or 1/1,000 in prereduced Schaedler broth. Viable bacterial cell counts were determined immediately prior to inoculation by serial dilution.

Experimental animals. Female BALB/c mice approximately 8 weeks old obtained from Sasco, Omaha, Nebr., or Charles River Laboratory, Wilmington, Mass., were used in these studies. Coil-shaped s.c. chambers were prepared from 0.5-mm stainless-steel wire and surgically implanted in the s.c. tissue of the dorsolumbar region of each mouse. At least 10 days elapsed before the chambers were inoculated with *P. gingivalis*. During this period, the outer incision healed completely, and the chambers became encapsulated by a thin vascularized layer of fibrous connective tissue and gradually filled with approximately 0.5 ml of light-colored transudate. Ten days after implantation, chambers were inoculated with 0.1 ml of a suspension of *P. gingivalis* in prereduced Schaedler broth. Control mice were inoculated with Schaedler broth only. Mice were examined daily for size and consistency of primary or secondary lesions and health status. Severe cachexia was defined as ruffled hair, hunched bodies, and weight loss. Chamber fluid was aseptically removed from each implanted chamber with a hypodermic needle (25 gauge) and syringe at 1 to 7, 14, and 26 days postinoculation for bacteriological culture and microscopic examination. All surviving animals were sacrificed 30 to 40 days postinoculation, and serum was separated from blood obtained by cardiac puncture.

Female BALB/c mice approximately 8 weeks of age were also used for the MAM. These animals were inoculated s.c. with 0.1 ml of a suspension of *P. gingivalis* in prereduced Schaedler broth and examined daily as described previously (5). Surviving animals were sacrificed 30 to 40 days postinoculation, and serum was obtained as described above.

Chamber fluid analysis: microbiological. Aliquots of fluid from each chamber were streaked for isolation onto anaerobic blood agar plates and cultured at 37°C for 7 days under anaerobic conditions as described above. *P. gingivalis* was identified by standard techniques used in our laboratory as described before (12). Cultivable bacterial counts were obtained by serially diluting chamber fluid in Schaedler broth and spin plating onto anaerobic blood agar plates.

Host response. A combination of fluorescent dyes, cytocentrifugation, and fixation (14) was used to examine the association of polymorphonuclear leukocytes (PMNs) and *P. gingivalis* in mouse chamber fluid. A 1:50 dilution of each chamber sample was made in Hanks balanced salt solution, and a 30-μl sample was incubated with 3 μl of propidium iodide (5 μg/ml) for 5 min at room temperature. To this was added 7 μl of acridine orange (2.5 mM), and the resulting samples were centrifuged in a Cytospin apparatus (Shandon Inc., Pittsburgh, Pa.) for 3 min at 850 rpm. Cytocentrifugation induces spreading and flattening of PMNs such that plasmic volumes fall within a single plane of focus, which allows for more rapid and accurate interpretations than with wet mounts. Following cytocentrifugation, specimens were fixed and coverslips were mounted with cyanoacrylate. Slides were visualized on a Zeiss fluorescence microscope: propidium iodide excitation, 546 ± 6 nm, and emission, >590 nm; acridine orange excitation, 450 to 490 nm, and emission, >520 nm. Membrane topography was also visualized with differential interference contrast (Nomarski) optics.

Antibody response. Specific immunoglobulin G (IgG) to *P. gingivalis* whole cells was quantitated from both chamber fluids and sera for each group of mice. IgG specific for *P. gingivalis* whole cells was assayed by a modification of an enzyme-linked immunosorbent assay (ELISA) described by Ebersole et al. (8), and results were read with a V_{max} kinetic photometer (Molecular Devices) at 450 nm. An aliquot of serum from each group of mice (inoculated with different *P. gingivalis* strains) was pooled and used as a positive standard and run on each plate. ELISA units were expressed as the (OD_{450} sample/OD_{450} positive control) × 100, where OD_{450} is optical density at 450 nm.

RESULTS

Virulence of *P. gingivalis* in the MAM and the MCM. The primary objective of these studies was to compare the mouse s.c. chamber model (MCM) for the growth of *P. gingivalis* with the traditionally used MAM. Our initial experiments were therefore designed to compare the response of well-characterized *P. gingivalis* strains in the MCM to that in the MAM. In addition, we wished to characterize the virulence of the previously uncharacterized strain, A7436. At a high inoculum (5 × 10^{10} CFU), *P. gingivalis* A7436 was highly infectious in the MAM, producing ulcerated necrotic lesions

TABLE 1. Pathological course of *P. gingivalis* infection in the MAM[a] or the MCM[b]

Strain and inoculum (CFU)	Primary lesion[c]		Abdominal lesion[d]		Cachexia	Death[e]	
	Day(s)	No. with lesions/total	Day(s)	No. with secondary lesions/total		Day(s)	No. of deaths/total
A7436[a]							
5×10^{10}		0/5	2 & 3	5/5	Severe	3	5/5
5×10^{9}		0/3	2	1/3	Mild		0/3
10^{8}		0/5		0/5	No		0/5
A7436[b]							
3×10^{9}	1	4/4[f]	1 & 2	4/4	Severe	2	4/4
5×10^{8}	1	4/4[f]	1 & 2	4/4	Severe	3	4/4
9×10^{7}	7–14	4/4[f]		0/4	Mild		0/4
HK	7	1/4[f]		0/4	Mild		0/4
W83[a]							
4×10^{10}		0/3	2	3/3	Severe	4	2/3
5×10^{8}		0/5		0/5	No		0/5
W83[b]							
5×10^{8}	1–7	3/5[f]	1 & 2	5/5	Severe	2	2/5
2×10^{8}	1–3	3/3[f]		0/3	Moderate		0/3
5×10^{6}	1–5	3/4[f]		0/4	Mild		0/4
HK	3–7	1/2[f]		0/2	Mild		0/2
W50[b]							
3×10^{9}	7	3/5[f]	1–3	4/5	Severe	7	2/5
8×10^{8}	1	4/4[f]		0/4	Moderate		0/4
2×10^{7}	1	1/4[f]		0/4	Mild		0/4
HK	1	4/4[f]		0/4	Mild		0/4
W50/BEI[a]							
5×10^{10}	3	3/4		0/4	Mild		0/4
5×10^{8}		0/5		0/5	No		0/5
W50/BEI[b]							
3×10^{9}	1	3/4[g]		0/4	Mild		0/4
4×10^{8}	1	4/4[g]		0/4	Mild		0/4
10^{7}	1–7	4/4[g]		0/4	Mild		0/4
HK	1	4/4[f]		0/4	Mild		0/4
33277[b]							
8×10^{11}	1–7	5/6[g]		0/6	Mild		0/6
HK	2	2/5[f]		0/5	Mild		0/5
HG405[b]							
9×10^{12}	1–7	4/4[g]		0/4	Mild		0/4
HK	2	3/5[f]		0/5	Mild		0/5

[a] Pathological course observed following s.c. injection into the mice as described for the MAM (see Materials and Methods).
[b] Pathological course observed following injection of live or heat-killed (HK) *P. gingivalis* directly into s.c. chambers as described for the MCM (see Materials and Methods).
[c] Days postinoculation when lesion at injection site appeared, and number of mice with lesions/total mice in each group. The abscesses averaged 2.0 by 1.0 cm in size.
[d] Days postinoculation when secondary lesion on the ventral abdomen appeared, and number of mice with secondary lesions/total mice in each group. Secondary lesions ranged in size from 2.0 by 3.0 to 3.0 by 4.0 cm.
[e] Days postinoculation when mice died, and number of deaths/total number of mice in each group.
[f] Lesions were resolved within 10 days.
[g] Lesions resulted in the rejection of chambers on days 7 to 14.

on the abdomens of mice, with death of all mice on day 3 (Table 1). In contrast with an inoculum of 5×10^{9} CFU, only one mouse developed an abdominal lesion which was resolved by day 7, and with an inoculum of 10^{8} CFU no pathology was observed. At a high inoculum (10^{10} CFU), *P. gingivalis* W83 (characterized previously as virulent) in the MAM produced ulcerated necrotic lesions on the abdomen of mice, with death of all mice within 7 days (Table 1). Strain W50/BEI (characterized previously as avirulent in the MAM) at high inocula (e.g., 10^{10} CFU) in the MAM produced localized abscesses at the site of injection which resolved within 3 weeks (Table 1). A lower inoculum of either strain W50/BEI or W83 (5×10^{8} CFU) did not result in any apparent pathology.

As observed in the MAM, *P. gingivalis* A7436 was virulent in the MCM; however, we found that a lower inoculum of A7436 (5×10^{8} CFU) in the MCM caused similar pathology as observed in the MAM (at an inoculum of 5 ×

10^{10} CFU). Strain A7436 in the MCM at an inoculum of 3×10^9 or 5×10^8 CFU caused severe cachexia with secondary ulcerated necrotic lesions on the ventral abdomen of mice and death of all animals on day 2 to 3 (Table 1). At a lower inoculum of A7436 (9×10^7 CFU), only primary lesions were evident with some cachexia.

Injection of *P. gingivalis* W83 at an inoculum of 5×10^8 CFU into chambers produced secondary ulcerated lesions on the abdominal surface of mice and death of two of five mice within 24 h postinoculation (Table 1). All surviving animals rejected their chambers 3 weeks postinoculation; however, when a lower inoculum (10^8 CFU) was used, only primary lesions were observed at the site of injection (top end of the chamber). Our data suggest that *P. gingivalis* W50 was not as virulent as strain W83 in the MCM and correlate with the results obtained by van Steenbergen et al. (38) and others (22) who used the MAM. At an inoculum of 3×10^9 CFU, W50 caused abdominal lesions in four of five animals, with death of two of five mice on day 7 (Table 1); however, a lower inoculum (8×10^8 CFU) of *P. gingivalis* W50 caused only moderate cachexia and localized primary lesions.

Inoculation of *P. gingivalis* W50/BEI (3×10^9 or 4×10^8 CFU), 33277 (8×10^{11} CFU), or HG405 (9×10^{12} CFU) into chambers resulted in the production of localized abscesses at the site of injection (Table 1). These mice appeared healthy otherwise with only mild cachexia. Lesions were resolved in 3 weeks for mice inoculated with 4×10^8 CFU of strain W50/BEI with chambers intact; however, a high inoculum of strain W50/BEI (3×10^9 CFU), 33277 (8×10^{11} CFU), or HG405 (9×10^{12} CFU) resulted in the rejection of chambers 7 to 14 days postinoculation.

Histopathological examination of the spleens of mice infected with *P. gingivalis* A7436 or W83 revealed areas of lymphoid hyperplasia. In addition, the subcutis and underlying musculature were diffusely infiltrated with numerous macrophages and neutrophils (data not shown).

Unlike the results obtained in the MAM, all virulent strains of *P. gingivalis* examined in this study with the MCM caused localized lesions at the site of injection at low inocula. These localized lesions were resolved within 10 days for strains A7436, W50, and W83; however, the lesions produced by the avirulent *P. gingivalis* W50/BEI, HG405, and 33277 at high inocula resulted in the rejection of chambers by day 14. Inoculation of heat-killed *P. gingivalis* A7436, W50, W83, HG405, 33277, or W50/BEI into chambers caused only mild cachexia with localized lesions at the site of injection (Table 1). These lesions did not result in chamber rejection and were resolved within 10 days postinoculation.

In preliminary experiments we have observed a difference in the pathology observed in mice which have been inoculated with *P. gingivalis* grown in different growth media prior to inoculation into chambers (data not shown). These results are the subject of a project in progress in our laboratory.

Analyses of chamber fluid for viable bacteria. Chamber fluid was cultured throughout the course of the experiments to correlate bacterial growth within chambers with the pathology observed in mice. Inoculation of *P. gingivalis* A7436 (3×10^8 CFU) into s.c. chambers resulted in the growth of these organisms for up to 26 days postinoculation, with chamber rejection at 33 days postinoculation (Table 2). A lower inoculum (7×10^6 CFU) of *P. gingivalis* A7436 resulted in detectable growth within chambers up to day 3. Inoculation of *P. gingivalis* W83 (2×10^8 CFU) resulted in the establishment of this strain within s.c. chambers for up to 14 days (Table 2). Injection of a lower inoculum (5×10^6) of strain W83 resulted in detectable growth in chambers for only 2 days. At an inoculum of 8×10^8 CFU, *P. gingivalis* W50 was cultured from chamber fluid to day 7. In contrast, strain W50/BEI was not detected past 3 days even at a high inoculum (4×10^8 CFU). *P. gingivalis* 33277 (8×10^{11} CFU) was detected in chamber fluid up to day 5. Strain HG405 (9×10^{12} CFU) was cultured from chamber fluid to day 6, after which chambers were rejected (day 7). Blood sampled 24 h after inoculation with *P. gingivalis* also demonstrated the presence of these *P. gingivalis* strains after microbiological culture (data not shown).

We also quantitated the number of viable cells of *P. gingivalis* sampled from chamber fluid at various times after inoculation. At high inocula *P. gingivalis* A7436, W83, and W50 increased in numbers relative to the initial inoculum through the course of the experiment, indicating that bacterial multiplication was occurring within chambers (data not shown). In contrast, the number of CFUs obtained from chamber fluid of mice inoculated with W50/BEI never reached that of the initial inoculum (data not shown), suggesting that strain W50/BEI was not multiplying within chambers.

In the course of our culturing experiments, we observed the presence of bacteria other than *P. gingivalis* in chamber fluids sampled from mice inoculated with virulent strains of *P. gingivalis* in the apparent absence of any communication of the chamber with the external environment (data not shown).

Analysis of chamber fluid by fluorescence microscopy. A combination of fluorescent dyes, cytocentrifugation, and

TABLE 2. *P. gingivalis* cultured from chamber fluid

Strain and inoculum (CFU)[a]	No. of mice from which *P. gingivalis* was cultured/total no. sampled on given day postinoculation							
	1	2	3	5	7	14	17	26
A7436								
3×10^8	4/4	3/4	3/4	3/3	2/3	2/2	2/2	1/1[b]
7×10^6	4/4	2/4	2/4	0/4				
W83								
2×10^8	3/3	2/2	2/2	ND[c]	2/2	1/1	0/1	
5×10^6	3/3	1/3	0/3					
W50								
8×10^8	4/4	4/4	4/4	4/4	1/4	0/4		
2×10^7	3/4	0/4						
W50/BEI								
4×10^8	4/4	4/4	3/4	0/4				
10^7	4/4	0/4						
33277								
8×10^{11}	6/6	6/6	6/6	2/6	0/6			
HG405								
9×10^{12}	4/4	4/4	4/4	4/4	4/4[b]			
SB	0/4							

[a] Data are not presented for higher inocula of *P. gingivalis* A7436 (3×10^9 CFU), W83 (5×10^8 CFU), and W50 (3×10^9) since these inocula resulted in death of mice. However, *P. gingivalis* was cultured from chamber fluid samples obtained 1 day prior to the death of these mice. SB, Schaedler broth only.

[b] Chambers were rejected following this sample day; therefore, chamber fluid could no longer be sampled.

[c] ND, Not determined.

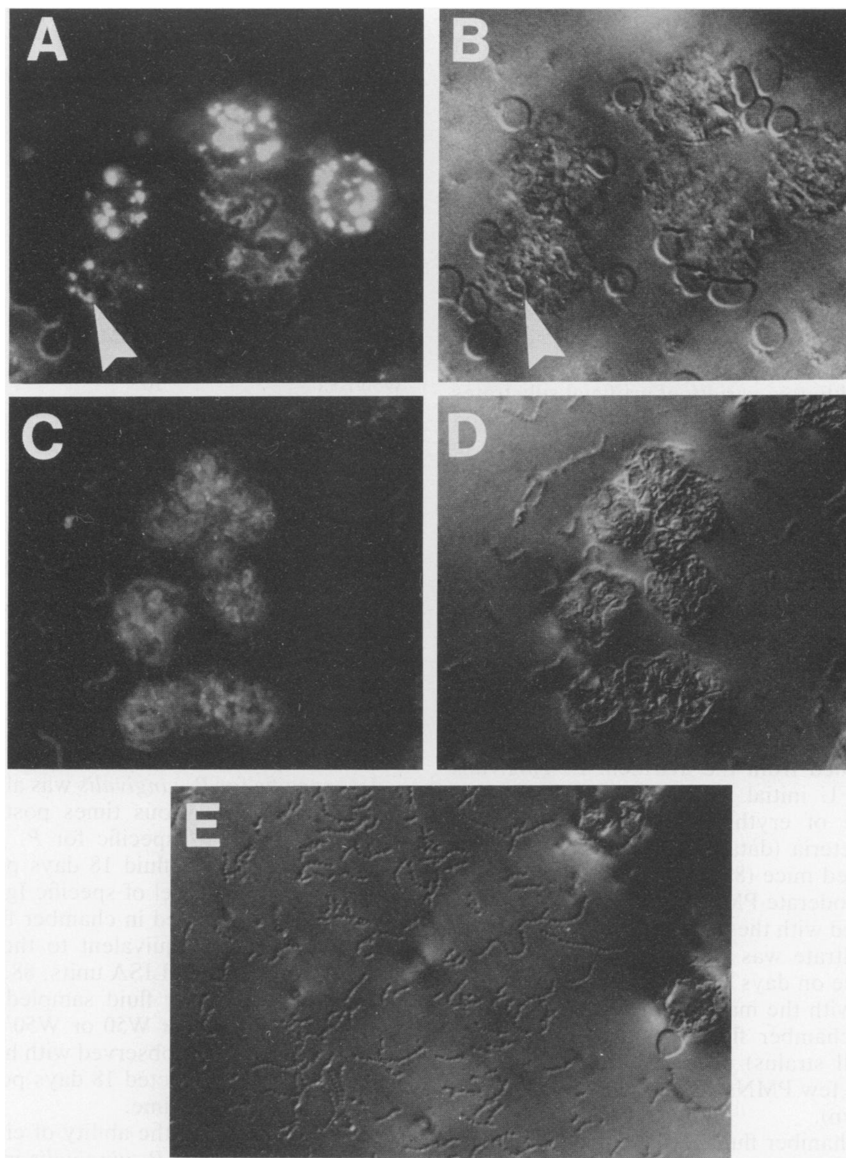

FIG. 1. Chamber fluid samples from *P. gingivalis*-infected mice. Chamber fluid was removed from mice 1 day postinoculation with *P. gingivalis* A7436 or 2 days postinoculation with strain W83, diluted 1:50 in Hanks balanced salt solution, and prepared for staining by cytocentrifugation as described in Materials and Methods. (A and B) The same microscopic slide and field of chamber fluid sampled from *P. gingivalis* W83-infected mice; (C, D, and E) the same microscopic slide of chamber fluid sampled from *P. gingivalis* A7436-infected mice. Panels C and D are the same microscopic field. Panel E is from a different microscopic field. Panels A and C were viewed by epi-illumination UV microscopy and panels B, D, and E were viewed by differential interference contrast optics. Arrowheads are pointing to bacteria within the PMN.

fixation was used to examine host cells and *P. gingivalis* in mouse chamber fluid. Typically, when we examined chamber fluid from W83-infected mice (2×10^8 CFU initial inoculum), we observed a heavy PMN infiltrate 2 to 5 days postinoculation, with the majority of bacteria PMN associated (Fig. 1A). Figure 1A and B shows photographs of the same microscopic field viewed by epi-illumination UV microscopy or by differential interference contrast optics, respectively. PMN granules and bacteria were evident within PMNs, although the differentiation of the two was difficult to capture in the black-and-white photograph (Fig. 1A). However, PMN granules and bacteria were easily differentiated on the basis of fluorescence when slides were viewed in the microscope, which allowed for the distinction of acridine orange and propidium iodide emissions. Chamber fluid sampled from mice inoculated with *P. gingivalis* W50 (8×10^8 CFU initial inoculum) at day 3 also exhibited a heavy PMN infiltrate with bacteria primarily cell associated; however, few PMNs were observed in chamber fluid sampled prior to 3 days postinoculation (data not shown). Many PMNs and erythrocytes were evident in chamber fluid sampled 6 days postinoculation with strain W50 (data not shown).

We also observed a heavy PMN infiltrate in chamber fluid from A7436-infected mice; however, in contrast to that observed with W83 and W50, the chamber fluid from A7436-

infected mice revealed a heavy PMN response on day 1 and primarily extracellular live bacteria not associated with PMNs (Fig. 1C and D). Figure 1E is a photograph of the same microscopic slide pictured in Fig. 1C and D, but a different field. This field is typical of many fields viewed from this slide in which the extracellular bacteria in chamber fluid sampled from A7436 were growing in chains. The bacteria in these chamber fluid samples were confirmed to be *P. gingivalis* by Gram strain, in vitro culture characteristics, and standard biochemical tests. The analysis of PMNs depicted in Fig. 1B, D, and E also allowed for the assessment of the state of activation of these PMNs based on the membrane topography. Interestingly, PMNs from leukocyte adhesion patients which are defective in phagocytosis have been reported to adhere weakly to a variety of artificial substrates (2). In contrast, normal PMNs which have been primed and are undergoing active phagocytosis have been observed to be "sticky" (2, 3); thus, they would adhere to the microscopic slide and appear flatter as a consequence of the cytocentrifugation procedure. Cytocentrifugation induces spreading of PMNs such that plasmic volumes fall within a single plane of focus, and differential interference contrast optics allows for the visualization of membrane topography. The PMNs in Fig. 1B appeared flatter when compared with those in Fig. 1D and E which appear to have more dimension. The contrast between these cells is consistent with the observed cell association in Fig. 1B and the absence of bacteria associated with the PMNs in Fig. 1D and E.

Chamber fluid obtained from the avirulent *P. gingivalis* W50/BEI (4×10^8 CFU initial inoculum) on days 1 to 3 revealed the presence of erythrocytes, a minimal PMN response, and few bacteria (data not shown). In chamber fluid from 33277-infected mice (8×10^{11} CFU initial inoculum), we observed a moderate PMN response on day 2 with most bacteria associated with these PMNs (data not shown). A moderate PMN infiltrate was also observed in chamber fluid sampled from mice on days 1 to 3 postinoculation with *P. gingivalis* HG405, with the majority of bacteria found in clumps. Analysis of chamber fluid inoculated with heat-killed *P. gingivalis* (all strains) on day 1 revealed mainly dead bacteria and very few PMNs. By day 3 no PMNs were evident (data not shown).

We also examined chamber fluid 26 days postinoculation with *P. gingivalis* A7436 and W83. Chamber fluid from A7436-infected mice revealed live bacteria both intra- and extracellularly. In contrast, chamber fluid from W83-infected mice revealed mainly cellular debris and erythrocytes with no visible bacteria (data not shown).

Serum IgG response to *P. gingivalis*. We also measured the systemic antibody response induced in mice by injection with *P. gingivalis* W83, W50/BEI, A7436, HG405, W50, and 33277 in chambers. All strains examined induced a high IgG response as measured 30 days postinoculation which also correlated with the inoculum used (Table 3). *P. gingivalis* A7436 was the only strain which induced a detectable IgG response at an inoculum of 10^6 CFU. Interestingly, injection of heat-killed cells (10^8 CFU) gave an IgG response similar to that of live bacteria (10^8 CFU) for all strains examined. Inoculation of *P. gingivalis* W83 or W50/BEI induced a higher IgG response in the MCM than was observed in the MAM with an inoculum 10-fold less (Table 3). Since *P. gingivalis* A7436 killed all mice within 3 days at inocula $>10^8$ CFU, we could not make a similar comparison with this strain. An IgG response was also detected in the sera obtained from mice inoculated with *P. gingivalis* W50 (3×10^9 CFU), HG405 (9×10^{12} CFU), and 33277 (8×10^{11} CFU); ELISA units were 84.98 ± 7.0, 79.30 ± 6.37, and 79.0 ± 18.0, respectively.

TABLE 3. Systemic IgG response to *P. gingivalis* whole cells[a]

Strain inoculated	Inoculum (CFU)	ELISA units (mean ± SD)[b]
A7436	10^8	117.37 ± 2.46
	10^8 HK[c]	88.15 ± 10.87
	10^6	31.02 ± 5.76
	10^{10} MAM	151.23 ± 28.48
W83	10^9	262.25 ± 21.45
	10^8	67.66 ± 21.48
	10^8 HK	65.98 ± 8.1
	10^6	6.49 ± 3.48
	10^{10} MAM	109.36 ± 24.01
W50/BEI	10^9	199.23 ± 7.78
	10^8	72.15 ± 11.56
	10^8 HK	90.72 ± 11.02
	10^6	3.81 ± 0.29
	10^{10} MAM	96.93 ± 6.57

[a] *P. gingivalis* was inoculated in mice into s.c. chambers (MCM), or s.c. (MAM), and 30 days postinoculation serum was separated from blood obtained by cardiac puncture. Serum samples were tested against the homologous strain.

[b] ELISA units = (sample V_{max} in OD/min)/(positive pool V_{max} in OD/min) × 100, where OD is optical density.

[c] HK, Heat-killed.

IgG specific for *P. gingivalis* was also assessed in chamber fluid samples at various times postinoculation with heat-killed bacteria. IgG specific for *P. gingivalis* was initially detected in chamber fluid 18 days postinoculation (ELISA units, 38.67). The level of specific IgG increased with time, and the levels detected in chamber fluid on day 30 (ELISA units, 85.87) were equivalent to those observed in serum sampled on day 30 (ELISA units, 88.15). IgG specific for *P. gingivalis* in chamber fluid sampled from mice inoculated with live *P. gingivalis* W50 or W50/BEI was also detected (data not shown). As observed with heat-killed strain A7436, specific IgG was detected 18 days postinoculation, and this level increased with time.

We also examined the ability of either serum or chamber fluid IgG to opsonize *P. gingivalis* in an in vitro assay with human PMNs. Both serum and chamber fluid obtained from *P. gingivalis*-infected mice (strains A7436, W83, and W50/BEI) were opsonic for the homologous *P. gingivalis* strain when examined in this assay (data not shown).

DISCUSSION

In this report we describe the development of a novel mouse model for the in vivo growth of *P. gingivalis*. When avirulent and virulent organisms were inoculated into s.c. chambers, similar tissue responses were induced as when these strains were inoculated directly into the s.c. tissues of mice. *P. gingivalis* W50/BEI, 33277, and HG405 caused the formation of abscesses around chambers, while *P. gingivalis* A7436, W83, and W50 produced rapidly spreading purulent infections. *P. gingivalis* was cultured directly from s.c. chambers throughout the course of the infection, and culture results were correlated with the resulting pathology observed in mice.

All strains of *P. gingivalis* examined induced a systemic IgG response to *P. gingivalis* whole cells that was detected in both serum and chamber fluid. Both chamber fluid and

serum antibodies were opsonic for *P. gingivalis* when used with human PMNs in our in vitro phagocytosis assay. We have also observed this antibody to be protective against invasion (but not growth of *P. gingivalis*) following subsequent challenge with homologous and heterologous virulent strains of *P. gingivalis* (unpublished data).

We found that fewer numbers of organisms were required for the establishment of an infection in the MCM as well as the induction of an IgG response. In addition, inoculation of *P. gingivalis* into s.c. chambers resulted in a course of pathology that was observed much sooner than that observed in the MAM; thus, this model may be a more sensitive alternative to the MAM. The use of mice in this report, as opposed to guinea pigs or rabbits (6, 36), has proven advantageous with respect to the ease of sampling from these small animal species. The use of mice in future studies will also allow us to employ genetically defined, inbred strains of mice and should enable us to examine genetically determined, host-mediated responses to *P. gingivalis* infection.

Growth of *P. gingivalis* in s.c. chambers exposes bacterial cells to antimicrobial mechanisms of the host such as phagocytic cells, antibodies, and complement and may more closely reflect the infectious process involved in the formation of a periodontal lesion. The net effect of the host response to organisms causing periodontal infections is the localization of tissue destruction to the periodontium and protection from extensive local or systemic infection. The site of tissue destruction in the severe periodontal lesion is characterized by an influx of "primed PMNs" in humans (26) and in most animal models for periodontitis (24). The role of PMNs in periodontitis is thought to be principally one of host protection. Although most adult periodontitis patients mount a humoral response to *P. gingivalis*, the antibody produced is apparently ineffective at limiting continued disease progression (1).

Slots and Genco (33) have described four stages in the pathogenesis of human periodontal disease: colonization, invasion, destruction, and healing. These four stages were also evident in our MCM with avirulent versus virulent *P. gingivalis* strains differentiated on the basis of their ability to invade from the initial site of inoculation to distant sites. *P. gingivalis* produces a number of factors that may be responsible for its pathogenicity including enzymes and cytotoxic metabolites which may damage host tissues directly, the induction of an inflammatory response which could damage host tissues indirectly, and the evasion of certain host defenses by capsule formation and protease production.

Although other reports (8, 21, 38) with the MAM have found PMNs to be the primary cell type in lesions, the assessment of host cell response by cytocentrifugation of chamber fluid in our model allowed us to examine specifically the association of PMNs and different *P. gingivalis* strains in vivo. A majority of the bacteria in early chamber fluid samples from *P. gingivalis* W83- and W50-infected mice were found associated with PMNs. In contrast, bacteria in chamber fluid samples from A7436-infected mice appeared to be primarily extracellular and not associated with PMNs. *P. gingivalis* A7436 was shown to persist in vivo for 26 days postinoculation as assessed by microbiological culture. We also have observed *P. gingivalis* A7436 to be highly resistant to phagocytosis in an in vitro assay system (5a). The persistence of strain A7436 in vivo, its apparent evasion of the PMN in vivo, and its resistance to phagocytosis in vitro thus appear to be related to its virulence and the subsequent pathology observed in mice.

A common feature of chamber fluid sampled from mice infected with the less virulent strains was the presence of dead bacteria and the appearance of cellular debris. This cellular debris may be indicative of the walling off of the infection with the formation of a primary abscess at the site of injection. Although avirulent strains 33277 and W50/BEI caused chamber rejection, microbiological sampling prior to this revealed no cultivable organisms. The avirulent strain, HG405, was cultured until the point of chamber rejection; however, we did not examine these mice post-chamber rejection for the presence of viable bacteria. Injection of *P. gingivalis* HG405 into s.c. chambers also induced a moderate PMN response; thus, factors other than a simple influx of PMNs may be responsible for the virulent phenotype. When heat-killed organisms were inoculated into chambers, an influx of PMNs was not observed, nor were chambers rejected; thus, viable organisms appear to be required for these host responses.

van Steenbergen et al. (38) suggested that differences in virulence defined by the MAM were partly due to differences in phagocytosis and killing of *P. gingivalis*. PMN phagocytosis of virulent *P. gingivalis* strains has been demonstrated to be dependent on opsonization with specific antibody, and some strains which are resistant to phagocytosis in vitro have been described (5a). Strains of *P. gingivalis* known to be resistant to serum killing in the absence of antibody have also been described (23). The ability to monitor PMNs, complement, and antibody in vivo by the use of this model will be invaluable to our understanding of the evasion by *P. gingivalis* of these host defense mechanisms.

Efficient phagocytosis of certain strains of *P. gingivalis* in vitro by human PMNs has been associated with the presence of an electron-dense layer (35) and may be related to their nonpathogenic potential in the guinea pig abscess model (37). Encapsulated *P. gingivalis* strains are virulent in the MAM, while nonencapsulated strains are not (25), and immunization with purified polysaccharide antigen from *P. gingivalis* has been shown to protect mice from subsequent challenge with *P. gingivalis* in the MAM (30). Virulent *P. gingivalis* W50 possesses a capsular layer on its external cell surface, whereas this layer is absent in the avirulent strain W50/BEI (11, 16). It has been suggested that the presence of a capsule could account for the difference in phagocytosis of these organisms and, thus, the difference in their pathogenic potential in vivo; however, *P. gingivalis* W50 was clearly associated with PMNs as observed in chamber fluid, so the presence of a capsule in and of itself may not be sufficient for the resistance to phagocytosis. van Steenbergen et al. (39) concluded that *Bacteroides* strains which had the lowest proteolytic activity were also the least virulent in an animal model. Sundquist et al. (36), employing Teflon cages implanted s.c. in guinea pigs, found that the fluid of cages inoculated with *P. gingivalis* W83 had a high proteolytic activity with no complement C3 present and only traces of immunoglobulins. This strain requires complement in activating PMNs and these authors suggested that the degradation of C3 by this strain could be responsible for its pathogenic potential. Schenkein (28, 29) has reported that certain pathogenic strains of *P. gingivalis* (including strain W83) fail to accumulate C3 during opsonization by serum due to their proteolytic capacity. However, the results presented here clearly demonstrate the association of PMNs and *P. gingivalis* W83 in vivo as detected by fluorescent staining and cytocentrifugation. We did not examine chamber fluid from W83-infected mice for C3 and, thus, cannot speculate on its involvement in phagocytosis in our model.

In addition to the degradation of complement and antibody which is required for efficient phagocytosis, the enzymes produced by *P. gingivalis* may also play a role in tissue destruction during periodontal diseases. Grenier and Mayrand (10) have observed that pathogenic strains of *P. gingivalis* (as assessed in a guinea pig abscess model) have a higher collagenolytic activity than nonvirulent strains, as well as a high nonspecific proteolytic activity. However, the specific influence of proteolytic enzymes on invasiveness is still speculative. Strain W50 has been shown to produce over threefold more trypsinlike activity than the avirulent variant, W50/BEI (31, 34). Although one report by Neiders et al. (21) showed that some *P. gingivalis* strains which were lethal in the MAM exhibited a higher capacity to degrade synthetic and native substrates, in later reports (20, 22) these authors could not detect differences in enzyme production between invasive and noninvasive strains of *P. gingivalis*.

Current studies in our laboratory are aimed at understanding the virulence factors produced by *P. gingivalis* in vivo and the responses of the host to these factors. These host-parasite relations are obviously important to the disease process. The variations observed with different strains of *P. gingivalis* suggest that the MCM may serve as a valuable tool for the characterization of clinical isolates and for the delineation of potential virulence factors that may prove useful for immunological intervention in *P. gingivalis*-mediated periodontal disease.

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