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Virulence of *Wolinella recta* in a Murine Abscess Model

L. KESAVALU, S. C. HOLT, R. R. CRAWLEY, R. BORINSKI, AND J. L. EBERSOLE*

Department of Periodontics, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

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The virulence of *Wolinella recta* isolates was studied in an experimental animal model by using monoinfection of BALB/c mice. Infection with clinical isolates of *W. recta* 576 and *W. recta* 234 induced dry, flat, depressed gangrenous necrotic skin lesions, whereas *W. recta* ATCC 33238 failed to induce a similar lesion. Histological examination of the skin lesion 72 h postinfection revealed coagulation necrosis of the epidermis, subcutis and cutaneous truncus muscle, with marked exudation of serum proteins and neutrophils. Virulence-modulating agents such as dexamethasone, galactosamine, hydrazine sulfate, and dextran microcarrier beads were used in conjunction with *W. recta* infection. Dexamethasone, hydrazine sulfate, and dextran beads enhanced the infectivity and pathogenicity of *W. recta* for lesion formation and tissue destruction compared with what was found in untreated control mice. Galactosamine sensitization enhanced the virulence potential of *W. recta* to such an extent that a lethal outcome was observed. Laboratory passage of clinical isolates demonstrated a decreased virulence in high-passage strains, which correlated with the minimal virulence observed in the extensively passaged *W. recta* ATCC 33238. Serum immunoglobulin G (IgG) and IgM responses were detected in the serum of infected animals, and cross-reacting antibody indicated variation in the antigenic makeup of various *W. recta* strains. Enhanced IgG antibody responses were observed following the secondary challenge. Mice with acquired antibody response to initial infection remained susceptible to lesion formation with subsequent challenge, but the size of the lesion was significantly reduced, indicating partial protection. Serum IgG and IgM antibody levels were significantly increased by active immunization when compared with levels in mice which had recovered from infection. The immunization significantly decreased the lesion size; however, even these high levels of antibody failed to abrogate the lesion induction.

Periodontitis is a tissue-destructive disease that is initiated by microorganisms colonizing the subgingival plaque (48). Members of the subgingival plaque ecology have been shown to be capable of direct tissue toxicity (21, 45), as well as to elicit a group of host mediators that have the ability to amplify local destructive inflammatory responses (39, 41). Early investigations of the microbiota suggested that the extent of the bacterial burden was related to the level and severity of the tissue breakdown; however, a large number of studies have revealed that the actual progression of periodontal disease was associated with a small number of resident microorganisms which comprised a "pathogenic subgingival plaque" (8, 52). Members of this pathogenic subgingival microbiota include *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Bacteroides* (*Prevotella*) *intermedia*, and *Wolinella recta* (9, 46, 59). Additionally, studies of periodontitis populations have suggested the possible importance of *Fusobacterium nucleatum*, *Eikenella corrodens*, and *Capnocytophaga* species in disease progression (29, 32, 35). While these microbiological studies have identified probable pathogens within the subgingival plaque, there are very few investigations which have examined the pathogenic potential and mechanisms of virulence of these putative periodontopathogens.

Wolinella species are gram-negative, anaerobic, asaccharolytic motile rods that have been isolated from necrotic root canal infections (19) and periodontal lesions in patients manifesting inflammatory bowel disease (55), and they have also been isolated in significant numbers from progressing, destructive periodontal lesions (9). Several *W. recta* species have been characterized by serological methods (53), by sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles (50), and by growth characteristics in complex media and growth stimulation by formate and fumarate (16). More recently, Gillespie et al. (17) have chemically and biologically characterized the lipopolysaccharide (LPS) molecule of *W. recta* ATCC 33238. Kennell et al. (25) have also characterized outer membrane proteins and associated structures from *W. recta* ATCC 33238 and from several human periodontitis isolates. Lai and colleagues (28), Dokland et al. (7), and Borinski and Holt (4) have examined the structural characteristics of fresh clinical isolates of *W. recta*, and they compared these to the in vitro long-maintained *W. recta* ATCC 33238. Borinski and Holt (4) observed an inverse relationship between the presence of an S, or supplementary surface, layer and fibroblast adherence. Recently, several studies have begun to examine the potential virulence capabilities of clinical isolates and of *W. recta* ATCC 33238, with special emphasis on the role of the cell surface in virulence (1, 2). These studies have demonstrated that *W. recta* strains (especially freshly isolated strains) were resistant to HL-60 phagocytosis (1) and were unable to infect human gingival fibroblasts (2). It therefore appears likely that it is the outer membrane and an associated S layer (4, 7) which provides the *W. recta* strains with their ability to resist phagocytic events. Armitage and Holt (1) have also determined that *W. recta* cells elaborate molecule(s) into the growth medium which are toxic for HL-60 cells.

While various models of pathogenicity exist (42), very few have been shown to be useful in describing the events in the progression of periodontal disease. The nonhuman primate has, for example, been used extensively for studies of ligature-induced periodontitis (22, 27); however, even this model has not allowed direct assessment of the in vivo role of the putative periodontal microbiota in the disease process.

* Corresponding author.

A number of investigations (47) have used a murine model to examine the mechanisms of tissue destruction and infectivity elicited by selected eubacteria considered to be major contributors to a variety of diseases, including periodontal disease. Included in these studies have been the use of virulence enhancing agents (i.e., galactosamine (15, 26), hydrazine sulfate (44), and corticosteroids (3, 20, 38)) which are known to enhance the expression of eubacterial virulence. To date, there is essentially no information available that establishes the capacity of *W. recta* to manifest in vivo pathogenicity and the conditions under which it does so. This report elucidates for the first time the characteristics of tissue destruction caused by *W. recta* clinical isolates in mice, defines the attributes of the acquired immune response to the microorganism, and describes the ability of active immunization to abrogate the infection and resulting tissue damage.

MATERIALS AND METHODS

Animals. BALB/c (Charles River) female mice 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 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.

Microorganisms. The *W. recta* strains used in these studies were *W. recta* ATCC 33238, *W. recta* P07057-6.2 (called 576 in this report; human clinical isolate) and *W. recta* P03023-4.2 (called 234 in this report; human clinical isolate). The human isolates were obtained from primary cultivation plates of subgingival plaque samples from periodontitis sites. Care was taken to record the passage number of the strains, since Borinski and Holt (4) have determined that in vitro passage of *W. recta* cells has a significant effect on their interaction with host cells. All cells were routinely cultivated on 24- to 48-h prereduced Trypticase soy agar plates supplemented with 5% sheep blood (ETSA) in an anaerobic chamber (85% N_2, 5% CO_2, and 10% H_2). Important to the virulence results presented here was the fact that, for the most part, all manipulations were carried out under anaerobic conditions, i.e., in the anaerobic chamber. Cells were harvested aseptically from the surface of the ETSA plates with a sterile cotton swab soaked in reduced transport fluid (49); the cell mass was immediately resuspended in reduced transport fluid and mixed completely, and aliquots were removed from the chamber for an estimation of total counts by using a Petroff-Hausser counting chamber. An aliquot of the cell suspension was diluted and plated on ETSA for estimating viable counts and verifying purity. Appropriate dilutions of this suspension were made and distributed into anaerobic gas-filled vacuum vials under anaerobic conditions. Mice were injected within 15 to 30 min of sample preparation. For the determination of sample sterility and the number of *W. recta* cells injected, an aliquot of the bacterial suspension was plated onto ETSA plates immediately following the challenge injection.

Virulence model. To examine the virulence capacity of the various *W. recta* strains, mice were challenged subcutaneously (s.c.) on the posterior dorsolateral surface. Prior to challenge, the area to be injected was shaved and swabbed with 70% ethanol, and 0.2 ml of the bacterial suspension was injected into a single site with a 26-gauge needle. Following bacterial challenge, the animals were examined at approximately 6 h and at least once daily for 15 days. Any animal determined to be moribund was euthanized by CO_2 asphyxiation and was catalogued as a death. Lesions developing in the mice were scored as (i) lateral nodule, which is described as a demarcated hard subcutaneous lump, (ii) dry, flat, depressed gangrenous necrosis of the skin, or (iii) death.

A variety of murine virulence models was examined in these studies to analyze the pathogenic potential of *W. recta* and included the following. (i) Control, in which untreated BALB/c mice were challenged at 6 to 12 weeks of age. (ii) Decreased neutrophil capacity (20), in which BALB/c mice were pretreated for 3 days with dexamethasone (DEX; 40 μg per animal; Elkins-Sinn, Inc., Cherry Hill, N.J.) prior to challenge. (iii) Endotoxin sensitivity (15, 26), in which BALB/c mice were administered D[+]-galactosamine (Sigma) intraperitoneally (i.p.) (700 mg/kg of body weight) at a single dosage that was simultaneous with the challenge with *W. recta* ATCC 33238 or 234. (iv) Endotoxin sensitivity (44), in which BALB/c mice were treated with hydrazine sulfate (Sigma) administered i.p. (50 mg/kg) 6 h prior to challenge with *W. recta* ATCC 33238 or 234. (v) Abscess formation (14), in which BALB/c mice were administered microcarrier beads. Cytodex-1 microcarrier beads (Sigma) were suspended in phosphate-buffered saline (PBS; 50 ml/g) overnight and autoclaved at 120°C. The supernatant fluid was decanted from the sedimented microcarrier, equal volumes of packed microcarriers and bacterial suspension (*W. recta* ATCC 33238 or *W. recta* 234) were mixed, and 0.2 ml of the mixture was injected s.c. on the posterior dorsolateral area for determination of abscess formation.

Immunization. For immunization, *W. recta* 576 and 234 were grown in mycoplasma-formate-fumarate broth for 48 h as described by Gillespie and Holt (16). The cells were harvested by centrifugation (7,000 × *g*), and the pellets were washed with sterile PBS (0.02 M phosphate, pH 7.4). The cells were formalin killed by overnight treatment with 0.5% buffered formal saline (13). The killed *W. recta* cells were washed and stored at 4°C in PBS. An aliquot of this suspension was removed, and total counts were determined by using a Petroff-Hausser counting chamber. The sterility of the formalinized antigen preparation was determined by plating it on ETSA. Groups of mice were immunized by the s.c. injection into the nape of the neck of 0.1 ml of a solution of 10^9 *W. recta* 576 or 234 cells emulsified in incomplete Freund's adjuvant. A booster immunization of an identical preparation was administered 10 days later.

Antibody analysis. Blood for serum samples was collected from the mice by retroorbital access, either under ether anesthesia or following cervical dislocation. Immunoglobulin G (IgG) and IgM antibody in the mouse sera was determined by using an enzyme-linked immunosorbent assay (ELISA) (10). Briefly, intact formalin-killed bacteria in carbonate buffer (pH 9.6) were coated onto microtiter plates (Immulon II; Dynatech Inc.). Following incubation with dilutions of the mouse serum (1:500 and 1:5,000 for IgG and 1:100 and 1:1,000 for IgM), the level of antibody activity was determined by development with a goat anti-mouse IgG or anti-mouse IgM antibody conjugated with horseradish peroxidase (Cappell, West Chester, Pa.). Conversion of orthophenylenediamine (Sigma) was determined spectrophotometrically at 490 nm (MR650; Dynatech Inc.), and each antibody level is expressed as the optical density multiplied by the dilution of the serum that was tested in the ELISA.

Histopathology. At intervals of 3, 5, and 7 days after local challenge with *W. recta* 234 (low passage [$n = 6$]), mice were killed by CO_2 asphyxiation. The skin with s.c. tissues containing necrotic lesions was excised from the posterior

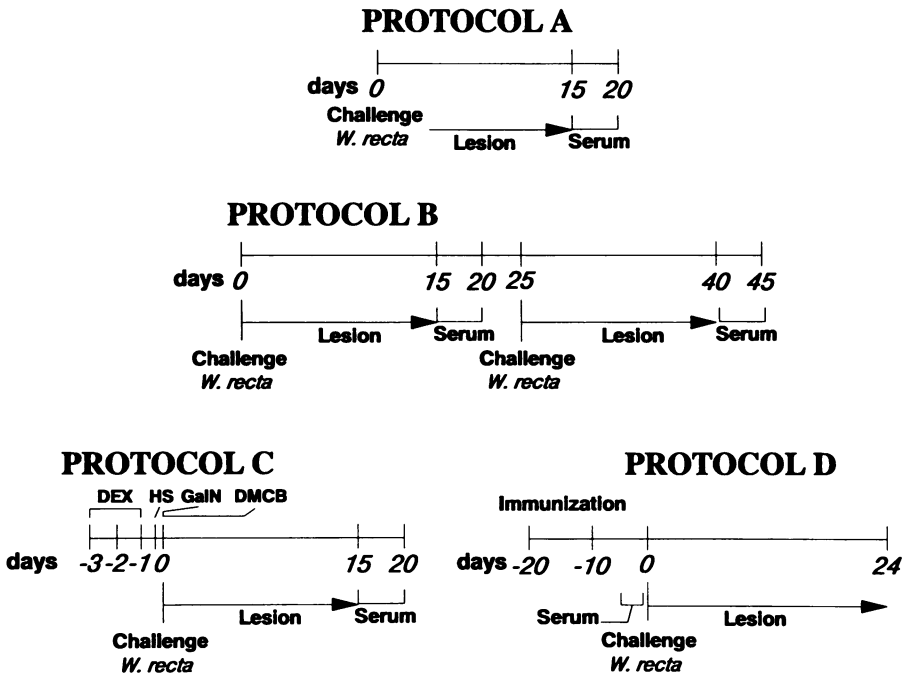

FIG. 1. Experimental protocols utilized to explore the virulence characteristics of *W. recta*. Protocol A describes a single infectious s.c. challenge with *W. recta* and monitoring for lesions through 15 days. Serum was collected to examine antibody responses within 5 days of the termination of the lesion-monitoring protocol. Protocol B describes an infectious challenge and monitoring for lesions. Following the healing of the lesion, serum is sampled for antibody and the animals are challenged with another infectious dose of *W. recta*. Lesions are monitored for 15 days, and serum is collected as described in the text. Protocol C describes experiments that are used to examine the effects of various pharmacologic agents on the virulence of *W. recta*. DEX is administered for 3 days prior to challenge, hydrazine sulfate treatment is 6 h prior to challenge, galactosamine (GalN) is administered simultaneous to challenge, and the dextran microcarrier beads (DMCB) are injected after being mixed with the bacteria. Lesions are monitored for 15 days. Protocol D describes immunoprotection experiments whereby mice are immunized with killed *W. recta* cells at 20 and 10 days prior to challenge. Serum is collected for antibody studies, and the mice are challenged s.c. with an infectious dose. Lesions were monitored through 24 days.

dorsal surface of the mouse and fixed in 10% neutral buffered formalin for at least 24 h at room temperature. After fixation, tissue sections were incised through the center of the skin lesion and embedded in paraffin. Six-micrometer sections were prepared and stained with hematoxylin and eosin or with Gram stain. The sections were examined by light microscopy for the presence and proportion of inflammatory cells and bacteria. Internal organs such as the liver, spleen, and superficial inguinal lymph nodes were also examined for gross pathologic alterations, and they were also prepared for histological examination.

RESULTS

Virulence of *W. recta* in the murine model. *W. recta* ATCC 33238, 576, and 234 were used to challenge normal BALB/c mice at doses of 10^8 to 10^{11} cells (Fig. 1, protocol A). As seen in Table 1, the untreated mice were completely refractory to tissue destruction by *W. recta* 33238; however, at very high (10^{11}) doses, *W. recta* 576 and 234 induced short periods of hypothermia, hair ruffling, and lethargy. Each of the *W. recta* clinical isolates studied elicited local lesions which developed by 24 to 72 h, progressing up to 50 to 150 mm^2 in area and resolving by 14 days (Fig. 2 and Table 1). In contrast, mice that were pretreated with DEX (to compromise neutrophil functions) exhibited localized lesions following challenge with each of the *W. recta* strains (Table 1). The dynamics of lesion development in the BALB/c mice injected s.c. with *W. recta* 33238, 576, and 234 demonstrated that, generally, lesion onset occurred by 24 to 48 h postchallenge, with lesion regression and resolution occurring by 14 days. At all *W. recta* doses studied, there was no spreading, lethal reaction in the DEX-treated mice. The size of the s.c. lesions also indicated a graded difference in virulence, such that *W. recta* 234 $\geq$ 576 $\gg$ ATCC 33238. Control mice treated only with DEX exhibited no local or spreading lesions at any time throughout these studies.

Similar experiments (Fig. 1, protocol C) with BALB/c mice treated with galactosamine, hydrazine sulfate, or Cytodex-1 beads were performed (Table 2). Galactosamine treatment significantly increased the ability of *W. recta* 234 to cause a lethal outcome. In comparison, *W. recta* ATCC 33238 produced no death and only minimal generalized effects of ruffled hair and hypothermia when injected simultaneously with galactosamine. Additionally, *W. recta* 234 induced a local lesion in one animal that was exacerbated compared with what was observed with normal mice. Hydrazine sulfate had a similar effect on lesion formation in mice that were challenged with *W. recta* ATCC 33238 and 234. Both strains produced localized lesions of approximately the same diameter. Interestingly, the clinical isolate produced an earlier onset of lesions that were of somewhat greater size. In each case, these lesions healed after 13 to 15 days. An s.c. injection of Cytodex beads resulted in the amplification of a purulent abscess in the BALB/c mice that were challenged with *W. recta* ATCC 33238. The localized nodule and abscess were noted by 72 h and generally healed

by 14 days. In contrast, *W. recta* 234 continued to elicit necrotic skin lesions in all mice, similar in size to those found on DEX-treated mice.

Various studies have suggested that in vitro cultivation of bacteria can minimize their virulence capabilities (23, 57). Therefore, we examined the effect of in vitro passage on the virulence potential (lesion formation and size) of the *W. recta* strains. Lesion size was directly related to in vitro passage of the *W. recta* strains (Fig. 3). Both low-passage (6 to 9 passages) clinical isolates of *W. recta* (strains 576 and 234) displayed significantly greater virulence than high-passage (15 to 17 passages) strains. The very-high-passage strain ATCC 33238 showed minimal virulence as measured by lesion diameter.

Histopathology of *W. recta* lesions. The general pathology of lesion formation following local challenge with *W. recta* was determined by gross observation and description of major internal organs (Table 3). Even in the absence of localized lesions, challenge with *W. recta* 234 led to gross liver and spleen abnormalities. Macroscopic methods did not permit a determination of definitive differences that could be attributed to variation in the dosage of the challenge.

Tissue sections of the skin lesions were examined on the basis of semiquantitative estimates of inflammatory cells and the presence of bacteria in the tissue. Within 72 h postinfection, histological examination of the skin lesion revealed coagulation necrosis of the epidermis, as well as of the subcutis and cutaneous truncus muscle. There was marked exudation of serum proteins and neutrophils beneath the necrotic area (Fig. 4). In addition to neutrophils, numerous bacteria were present in the exudate. Further, superficial myositis of underlying skeletal muscle and formation of granulation tissue at the periphery of the lesion were observed. No inflammatory lesions were observed in the tissues of the internal organs (liver and lymph nodes). However, reactive lymphoid germinal centers and extramedullary hemopoiesis was observed in the spleen. After 5 days of infection, there was increased granulation tissue underlying the skin lesion and around the periphery. The exudate contained large numbers of bacteria. Hyperplastic follicles and extramedullary hemopoiesis in the spleen and diffuse hyperplasia in lymph nodes was noted. The lesions became ulcerated by the 7th day, leaving a bed of granulation tissue with scanty exudate containing neutrophils and mononuclear leukocytes (Table 3).

Host antibody response to infection. IgG and IgM antibody levels in the BALB/c mice challenged with 10^8 to 10^{11} cells of *W. recta* 576 or 234 were determined 15 days after challenge (Fig. 1, protocol A). A homologous IgG and IgM antibody response was consistently detected with 10^9 to 10^{11} bacteria (Fig. 5). While there was definite evidence for cross-reactive antibody in the mouse sera (Fig. 5), the results supported a variation in the antigenic makeup of the various *W. recta* strains on the basis of significant differences in the antibody levels in the homologous and heterologous strains.

Other groups of BALB/c mice were challenged with 10^{11} *W. recta* 576 cells, which elicited local lesions and an acquired antibody response as described above. Sixteen days after the challenge, following the healing of the lesions, the mice were again challenged on the contralateral dorsolateral surface of the back with either 10^{11} *W. recta* 576 cells or 10^{11} *W. recta* 234 cells (Fig. 1, protocol B). As seen in Fig. 6, secondary challenge with *W. recta* 576 or *W. recta* 234 significantly increased the serum IgG antibody response when compared with that following the initial infection (Fig. 5). IgM levels for the two challenge regimens were similar;

TABLE 1. Clinical course of infection of *W. recta* 576, 234, and ATCC 33238 in normal and DEX treated mice

Group	*W. recta* strains[a]	Treatment[b]	Lesion onset (h)	Primary lesion symptoms	Lesion size (mm^2) ± SD	Healing (days)[c]	P^d
I	33238	N		Ruffled hair, lethargy, hypothermia (48-72 h)	0 (5/5)[e]		
II	576	N	40 (7/7)[e]	Localized, flat, dry, depressed, gangrenous necrosis of skin	63.4 ± 7.7 (7/7)	14	0.001 (I)
III	234	N	24 (4/10), 48 (4/10), 72 (2/10),	Same as for group II	108.4 ± 20.3 (10/10)	14	0.001 (I), 0.152 (II)
IV	33238	DEX[f]	72 (5/5)	Same as for group II	48.4 ± 9.0 (5/5)	14	0.001 (I)
V	576	DEX	24-48 (5/5)	Same as for group II	150.0 ± 24.7 (5/5)	14 (3/5), 16 (2/5)	0.012 (IV), 0.012 (II)
VI	234	DEX	24-48 (4/4)	Same as for group II	213.5 ± 21.5 (4/4)	15	0.018 (IV), 0.022 (III), 0.164 (V)

[a] *W. recta* strains ATCC 33238, 576 and 234 were harvested from the surface of ETSA plates and administered s.c. at 10^{11} cells per mouse in posterior dorsolateral surface.
[b] N, normal or untreated mice. DEX, dexamethasone treatment (2 mg/kg) i.p. for 3 days prior to challenge.
[c] No cachexia and/or death was noted in any of the animals.
[d] Statistical difference with group given in parentheses was determined by comparison of presence or absence of lesions or by size of lesion by using either Fisher's exact test or Mann-Whitney-Wilcoxon rank analysis.
[e] Number of positive mice per total number of mice challenged.
[f] Control mice treated only with DEX exhibited no local or spreading lesions at any interval during the study.

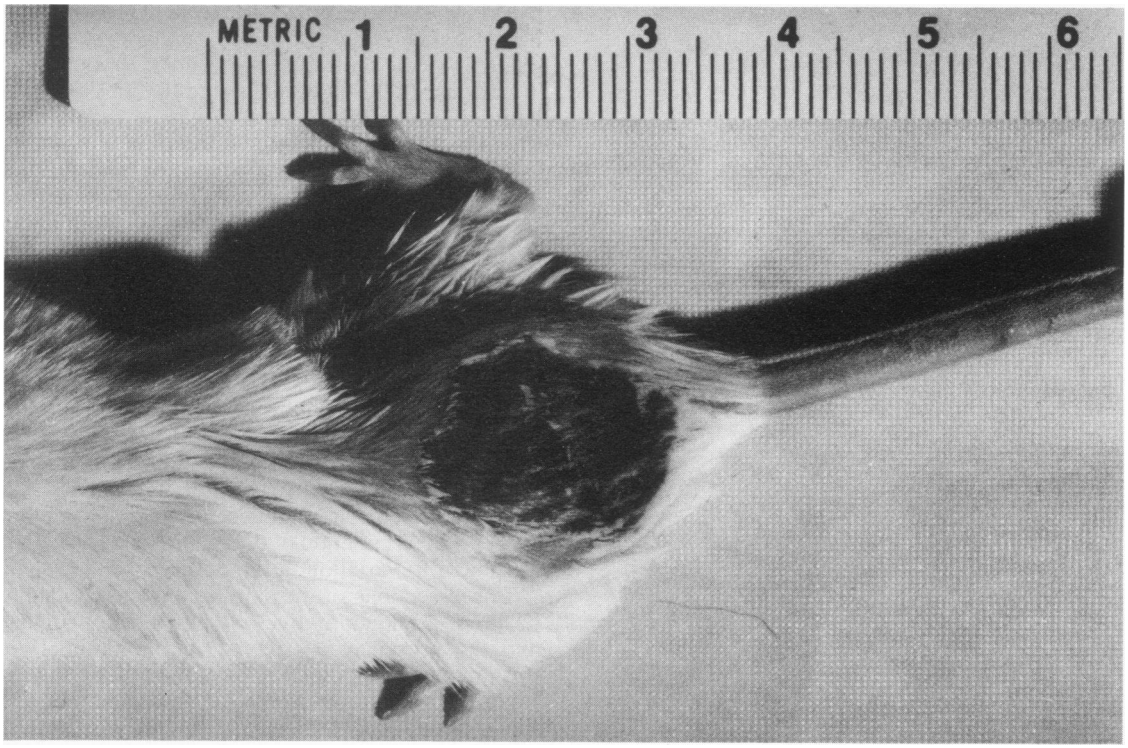

FIG. 2. Gross skin lesion resulting from s.c. inoculation of a mouse on the dorsolateral surface with 10^{11} *W. recta* 576 cells. At 3 days postinfection, the mouse developed a localized, dry, gangrenous necrotic skin lesion approximately 270 mm^2 in area.

however, they were significantly lower than the IgG response. The IgG antibody response to the heterologous 234 strain was approximately fourfold higher than it was for the challenge with 576. A secondary challenge with the heterologous strain elicited an antibody response to 576 that was intermediate between those elicited by a single and a duplicate challenge with 576. Likewise, the secondary challenge with 234 induced an antibody response to 234 that was significantly higher than those induced by a single challenge with 234 or by duplicate infections with 576. These findings indicate that antigenic cross-reactivity exists among the clinical *W. recta* isolates; however, these microorganisms may express antigens which are unique to themselves. This was most clearly seen following challenge with *W. recta* 576, which produced a much greater difference in antibody level between the homologous and heterologous strains than did challenge with *W. recta* 234. Furthermore, serum antibody elicited by infection with either 576 or 234 showed cross-reactivity to ATCC 33238; however, in each case the homologous reaction was dramatically and significantly greater (Fig. 6). Interestingly, the levels of IgG and IgM antibody produced against *W. recta* ATCC 33238 were significantly

TABLE 2. Virulence expression of *W. recta* in mice treated with galactosamine, hydrazine sulfate, or dextran beads

W. recta strain	Treatment	Lesion onset (h)	Primary lesion symptoms	Lesion size (mm^2)[a,b]	Healing (days)[b]	Cachexia or death[b]
33238	GalN[c]		Ruffled hair, lethargy, hypothermia	0 (0/5)	(0/5)	No
234	GalN	48	Localized, flat, dry gangrenous necrotic lesion	83 (1/10)	15	Minimal (9/10)
33238	HS[d]	72	Same as 234 above	52.1 ± 13.6 (4/5)	15	No
234	HS	48	Same as 234 above	89.5 ± 11.1 (5/5)	13	No
33238	DMCB[e]	72	Localized nodule, purulent lesion	59.3 ± 10.3 (5/5)	14 (4/5), closed abscess (1/5)	No
234	DMCB	48	Localized, flat, dry gangrenous necrotic lesion	103.5 ± 19.0 (5/5)	11 (1/5), 14 (4/5)	No

[a] The mean ± the standard deviation.
[b] Values in parentheses are numbers of positive mice per total mice challenged.
[c] Galactosamine (GalN; 700 mg/kg) was injected i.p. and 10^{11} *W. recta* cells were injected s.c. in the posterior dorsolateral side.
[d] Mice were taken off feed, and hydrazine sulfate (HS) was injected i.p. (50 mg/kg). Six hours later, mice were challenged with 10^{11} *W. recta* cells, and mice were placed back on feed.
[e] Cytodex-1 microcarrier beads (DMCB) were sterilized, packed, and mixed with an equal volume of *W. recta* before s.c. injection in mice.

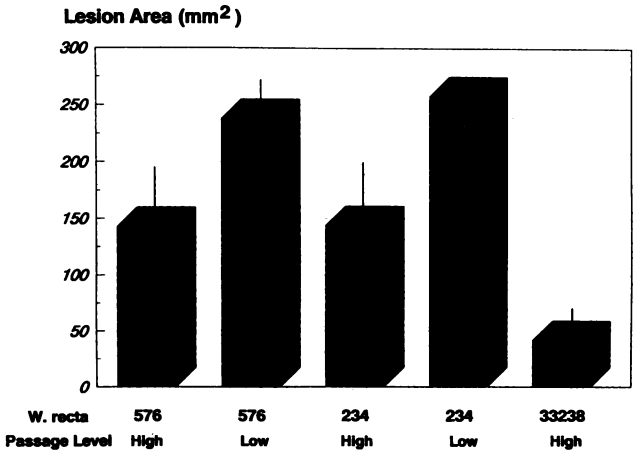

FIG. 3. Effect of in vitro passage on *W. recta* infectivity and pathogenicity. Lesions are formed following s.c. injection of 10^{11} *W. recta* 576, 234, or ATCC 33238 cells. Low-passage bacteria are those that have undergone 6 to 9 passages, while high-passage bacteria have undergone in vitro cultivation for 15 to 17 passages. The passage number of *W. recta* ATCC 33238 is unknown, but it has been routinely cultured in the laboratory for over 3 years. The bars denote the mean areas of the lesions scored at peak size (~5 to 9 days postchallenge) and represent the results of groups of 5 to 9 mice. The vertical lines denote 1 standard deviation from the mean.

lower than those produced by the two clinical isolates (Fig. 6; compare 33238 and 576 or 234). These differences might be due to an enhanced antigenicity of the S layer found covering the clinical isolates, a greater antigenic load because of this layer, or possibly a weaker antigenic presentation for ATCC 33238.

The comparative abilities of the cross-reactive and the homologous antibodies to provide protection against subsequent challenge were investigated (Fig. 1, protocol B). Groups of mice that had recovered from a primary challenge with *W. recta* 576 and demonstrated serum antibody to this microorganism were infected (10^{11} cells) with either *W. recta* 576 or *W. recta* 234 (Table 4). As is apparent in Table 4, antibody elicited to *W. recta* 576 was unable to eliminate lesion formation by this strain; however, the size of the lesions was significantly decreased in mice with high antibody levels compared with the size in normal controls. In contrast, while some antibody induced by *W. recta* 576 cross-reacted with *W. recta* 234, this antibody appeared to have no impact on lesion formation by 234.

Immunoprotection to infectious challenge. Mice were actively immunized by s.c. injection of formalin-killed *W. recta* 576 or *W. recta* 234 (Fig. 1, protocol D). The resulting IgG and IgM antibody response to these strains was significantly greater than the IgG and IgM response in normal mice or in mice that had been challenged with an infectious dose of the *W. recta* strains (Fig. 7). Similar to what was found with previous analyses of postinfection antibody reactions, cross-reactive antibodies were induced by the immunization protocol; however, unique antibody specificities were elicited by the homologous sensitization. Similar to what is seen in Fig. 6, there was very little antigenic cross-reactivity of *W. recta* 576 and *W. recta* 234 with *W. recta* 33238, as demonstrated by the low level of cross-reactive antibody produced.

Mice immunized with *W. recta* 576 or *W. recta* 234 were challenged with an infectious dose (i.e., 10^{11} cells) of the homologous *W. recta* strain to determine the ability of the antibodies formed after the primary immunization to ameliorate lesion formation (Fig. 1, protocol D). As seen in Table 5, mice actively immunized with the two *W. recta* strains and then challenged with an infectious dose of the homologous strain showed a significantly decreased induction in the size of the local lesion. Since lesions were formed in immunized animals (although they were significantly smaller), it was clear that the immune response was not capable of completely abrogating lesion formation. Similarly, mice that were actively immunized with *W. recta* 234 and then sensitized with galactosamine injected simultaneously with a challenge of an infectious dose of *W. recta* 234 showed complete protection against the lethal effect of the microorganism (compare the galactosamine treatments reported in Tables 2 and 5).

DISCUSSION

W. recta is one of a number of microorganisms that colonize the subgingival plaque and has been associated with progressing periodontal disease (9, 50, 51). *W. recta* strains have also been shown to produce an LPS that expresses a variety of biological activities, including mitogenicity and induction of inflammatory mediators (17). Gillespie et al. (17) characterized the LPS from *W. recta* and demonstrated its ability to elicit high levels of interleukin-1 and prostaglandin E_2 from monocytes and macrophages. Armitage and Holt (1) have demonstrated that *W. recta* is resistant to interaction with the neutrophil-like cell line, HL-60, which could be considered an important mechanism for evasion of host resistance. The presence of a surface protein structure external to the outer membrane (S layer) has been shown to be involved in the evasion of host defenses (4, 7). *W. recta* also produces an arylsulfatase, an enzyme which has been shown to degrade connective tissue matrix in other systems (58). Therefore, *W. recta* strains probably play an important

TABLE 3. Characteristics of *W. recta* 234 lesions in BALB/c mice[a]

Day[a]	Gross lesions[b]				Histopathology[c]						
	Skin	Liver	Spleen	Lymph node	PMN	MONO	BACT	Skin	Liver	Spleen	Lymph node
3	DGNL (3–6 mm)	—	E	—	+++	+	+	CN	–	EMH	–
5	DGNL (10–12 mm)	E	E	—	+++	+	+	G	EMH	EMH	DH
7	DGNL (10–15 mm)	E	E	E	+	+	–	G	EMH	EMH, HP	HP

[a] Number denotes days postinfection with *W. recta* 234.
[b] DGNL denotes a dry, gangrenous, necrotic skin lesion. E, enlarged; —, normal.
[c] PMN, polymorphonuclear leukocytes; MONO, mononuclear lymphoid cells. +++ indicates uniform dense population of cells, ++ indicates less cellular density, and + indicates only scattered cells in the tissue section. BACT denotes the presence (+) or absence (–) of *Wolinella*-like bacteria in the section. CN, coagulation necrosis; G, granulation tissue formation; EMH, extramedullary hemopoiesis; DH, diffuse hyperplasia; HP, hyperplastic follicles.

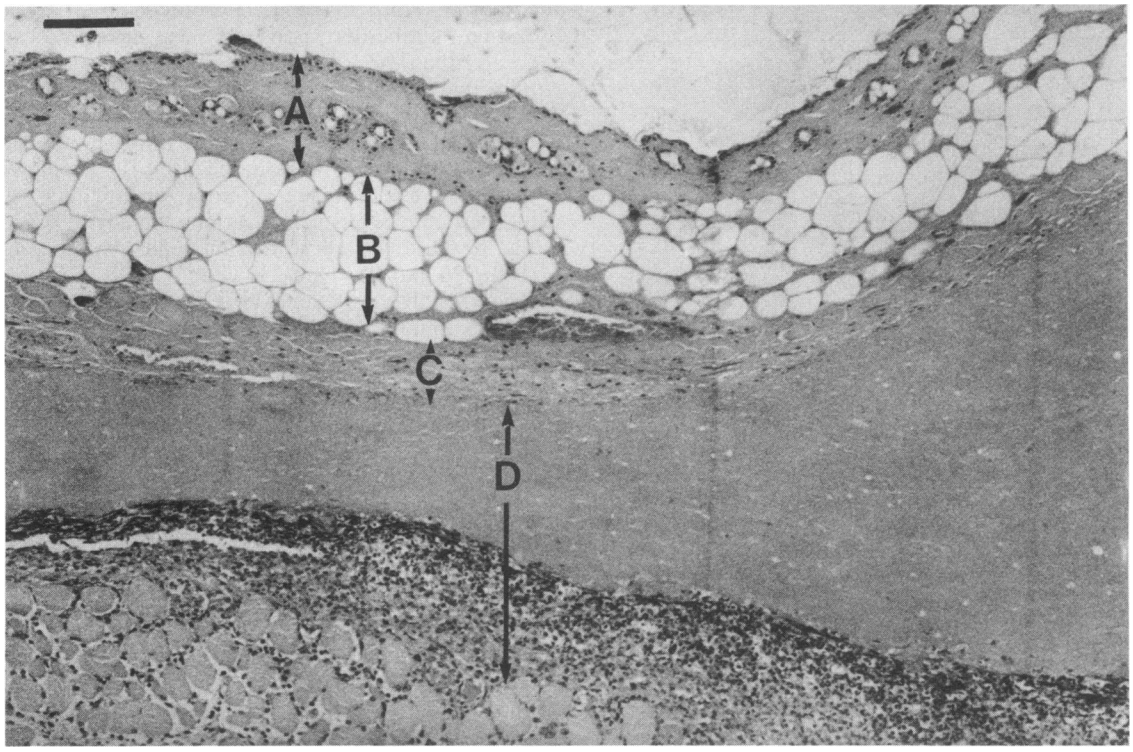

FIG. 4. Histological section of mouse skin 3 days after being inoculated s.c. with 10^{11} *W. recta* 576 cells. Note necrosis of epidermis (A), s.c. tissues (B), and cutaneous truncus muscle (C). A zone of exudate composed of serum proteins and neutrophils (D) is also noted. Magnification, ×118. Bar, 10 μm.

role in the variety of inflammatory responses of periodontal disease. The study presented here is the first to report tissue destruction caused by infection with *W. recta*.

While murine models have been employed for studies of numerous prokaryotic components thought to be involved in virulence (for example, see reference 47), it is only recently that they have been shown to be useful in studies of the virulence of several of the oral pathogens (e.g., *P. gingivalis*). These latter studies demonstrated the virulence capacities of *P. gingivalis*; however, these murine studies have indicated that there exists a variation in the pathogenicity of *P. gingivalis* strains, although the components causing these differences have not been delineated (18, 31, 36, 56). Specifically, *P. gingivalis* has been examined in both the mouse and guinea pig and demonstrated to elicit a spreading inflammatory infection (phlegmonous abscess) resulting in death, while *P. intermedia* produced only a localized abscess (6, 18, 24, 30, 31, 36, 56). A limited number of studies have also indicated that serum antibody to the microorganism can interfere with the pathogenic process (6). There is also some evidence to support the view that *P. gingivalis* virulence is regulated by hemin (5, 30). In these latter studies, Bramanti and Holt (5) have demonstrated that hemin has a significant effect on *P. gingivalis* virulence in a murine model; high levels of hemin (5.0 μg/ml) produced cells significantly less virulent than cells grown under hemin-limited conditions (0.5 μg/ml). Recently, we have also utilized the murine model to examine the virulence of *A. actinomycetemcomitans* (12). There was a dramatic difference in *P. gingivalis* and *A. actinomycetemcomitans* virulence capacity, as well as substantial variation in the characteristics of the lesions induced by these microorganisms. The association between *W. recta* virulence in the murine model presented in this study and in vitro growth of the *W. recta* strains was significant. *W. recta* ATCC 33238, which had been cultivated in the laboratory for at least 25 passages, was minimally virulent in normal BALB/c mice, while two *W. recta* clinical isolates which had minimal in vitro passages (<8) were capable of inducing local lesions in these animals. This study also demonstrated that in vitro cultivation of the clinical isolates for long periods of time resulted in a decrease in virulence. Similar results with other microorganisms, in which a maintenance of virulence requires periodic in vivo passage, have been demonstrated (23, 57). With certain microorganisms, this change in virulence has been related to structural changes, including capsule formation (40) or the expression of pili (43). Currently, we have not clearly elucidated similar changes in *W. recta* that relate to a change in virulence, although the presence and loss of an S layer (4) is consistent with the alterations in pathogenicity. Borinski and Holt (4) have demonstrated that similar low-passage *W. recta* clinical isolates adhered poorly to human gingival fibroblasts, while their corresponding high-passage strains and *W. recta* ATCC 33238 adhered very well to these fibroblasts. These authors have also observed that the low-passage human clinical isolates of *W. recta* contained a 150-kDa S layer, while both the high-passage clinical isolates and ATCC 33238 were either devoid of this protein or it was present in very low amounts.

Galactosamine (15, 26), hydrazine sulfate (44), corticosteroids (20), and abscess-promoting agents (i.e., soft agar and dextran microcarrier beads) (14) that can enhance the expression of bacterial virulence were used to investigate potential host responses that are critical for resistance

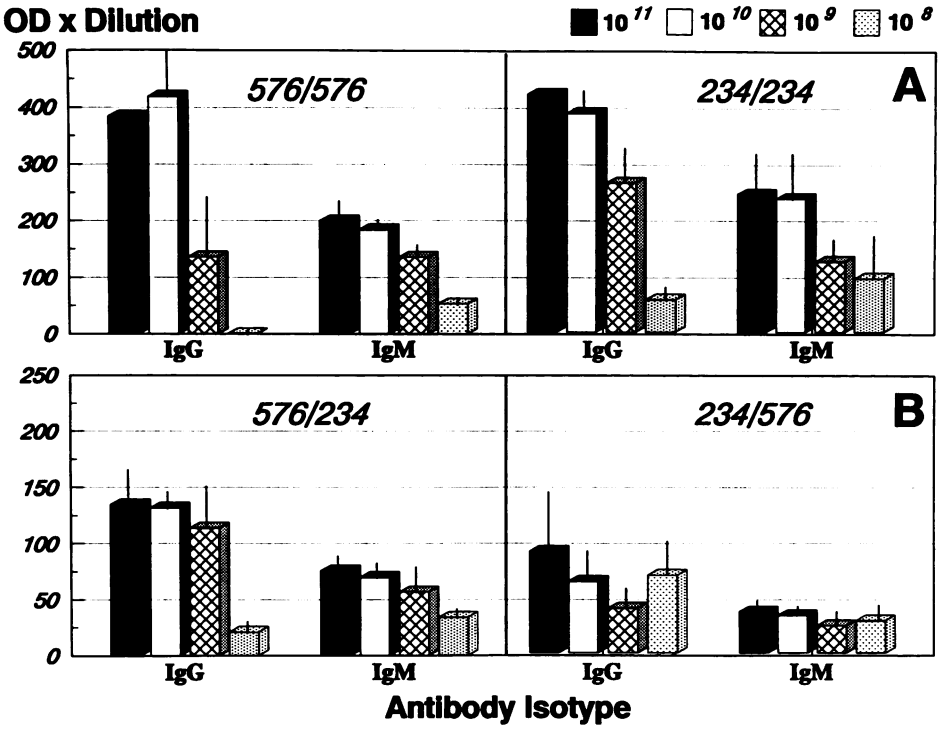

FIG. 5. Primary serum IgG and IgM antibody response following challenge with 10^8 to 10^{11} *W. recta* 576 cells (A) and 234 cells (B). Groups of 5 to 7 mice were challenged with various doses of each strain following 3 days of DEX treatment (protocol C). Blood was collected at 2 to 3 weeks postinfection, and antibody levels were determined against each homologous (576/576 and 234/234) and heterologous (576/234 and 234/576) strain. The bars denote the group means, and the vertical lines denote 1 standard deviation. The antibody levels are expressed as the optical density at 490 nm multiplied by the dilution of the serum (1:500 for IgG and 1:100 for IgM) that was tested.

against bacterial infection. Corticosteroids, such as DEX, have been utilized to minimize inflammatory responses and suppress granulocyte function (20). The results described in the studies utilizing *W. recta* demonstrated that DEX treatment significantly enhanced its virulence. Although spreading lethal infections were not observed with *W. recta*, the treatment enhanced local lesion development, suggesting that neutrophils were important components in resistance to tissue destruction by the microorganism. Galanos and colleagues (15) have employed the galactosamine treatment model to demonstrate the toxicity of LPS. When injected simultaneously with LPS, galactosamine appears to abrogate reticuloendothelial system function in the liver, which is critical for LPS detoxification. This treatment has been shown to yield an animal that is exquisitely sensitive to endotoxin activity as a result of the galactosamine effects on hepatic uridine nucleotide depletion and the inhibition of RNA and protein biosynthesis (15, 26). Galactosamine treatment significantly increased the virulence of *W. recta* and allowed expression of a lethal pathogenicity. Thus, while these results are not proof, they do suggest that the *W. recta* LPS has significant endotoxic capabilities that were expressed in vivo. Pretreatment with hydrazine sulfate has been shown to modulate endotoxin-mediated death in mice by stimulating the synthesis of hepatic phosphoenolpyruvate carboxykinase (44), which decreases dramatically in LPS-induced hypoglycemia (34). When administered prior to LPS challenge, hydrazine sulfate, which specifically inhibits gluconeogenesis (54), protects mice against lethal endotoxemia, resulting in an alteration of the gross characteristics and size of the *W. recta*-induced lesions. While it was clear that hydrazine sulfate treatment altered the size of the local lesions, these changes did not reach the level of statistical significance. Therefore, these results suggest that while LPS activity may be important in systemic changes resulting in death, the local lesions associated with *W. recta* infection are not exclusively LPS related. Finally, Cytodex-1 beads were utilized in conjunction with *W. recta* challenge to examine abscess formation (14). This agent successfully enhanced the development of a purulent abscess with *W. recta* ATCC 33238, which was minimally virulent under normal circumstances. In contrast, *W. recta* 234 continued to produce a dry, gangrenous necrotic skin lesion that was generally unaffected by the Cytodex beads. Therefore, it appears that the virulence components of *W. recta* are not of a type that have the capacity to stimulate the induction of purulent abscesses.

Histopathological studies of the *W. recta*-induced lesions demonstrated a gangrenous skin necrosis resulting from the infection. The lesions are populated by infiltrated polymorphonuclear leukocytes, and *W. recta*-like bacteria could be identified in the lesions early in their development. Even at later stages of the lesion progression, mononuclear cells were not observed to be a major component of the host response to this infection. This contrasts to lesions produced by *P. gingivalis* (18, 24, 56), in which either a spreading, phlegmonous lesion occurred or a progression of polymorphonuclear leukocytes to mononuclear cells in local purulent lesions was detected (36). In comparison, *A. actinomycetemcomitans* either caused a lethal infection that was not associated with an external lesion or produced a localized purulent abscess with continual bacterial presence following

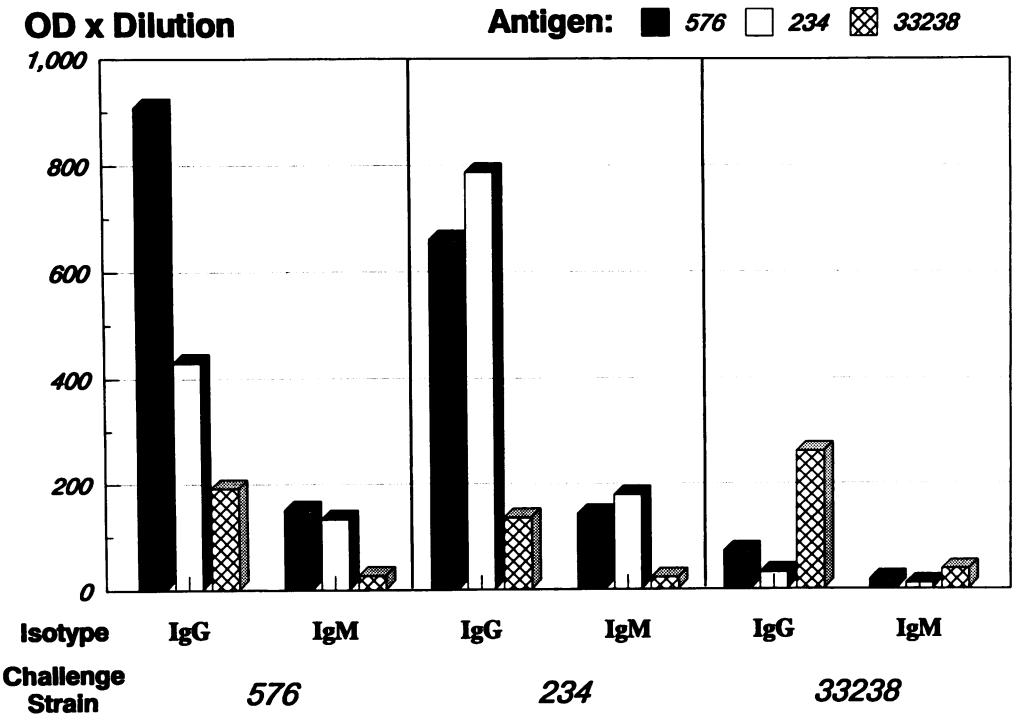

FIG. 6. Serum IgG and IgM antibody responses to *W. recta* 576, 234, or ATCC 33238 following challenge with 10^{11} cells of *W. recta* 576, 234, or ATCC 33238 (protocol B). Blood was collected at 2 to 3 weeks following the second challenge, and antibody levels were determined against each homologous and heterologous strain. The bars denote the group means (n = 5 to 7), and the standard deviation of the mean was always less than 10%. The antibody levels are expressed as the optical density at 490 nm multiplied by the dilution of the serum (1:500 for IgG and 1:100 for IgM) that was tested.

DEX treatment (12). These findings describe substantially different expressions of virulence and destruction generated by these periodontopathogens in the mouse model. Consequently, it may be suggested that local tissue destruction in human periodontitis, which has been presented as histologically similar among patients, is a consequence of very different mechanisms used by various microorganisms. Thus, approaches aimed at interfering with these virulence components may have to incorporate quite different strategies.

One approach to abrogate bacterial infections that has been routinely explored is the use of active immunization to enhance host resistance. Previous studies have indicated that the induction or transfer of antibodies to *P. gingivalis* in both mice (6, 11) and nonhuman primates (33, 37) can interfere with tissue destruction, colonization, and emergence. The studies presented here have shown that the challenge of mice with *W. recta* elicits a serum IgG and IgM antibody response. The response acquired following recovery from the infection provided some protection against a

TABLE 4. Virulence of homologous and heterologous *W. recta* in BALB/c mice with serum antibody elicited by previous infection

Group	*W. recta* strain[a]	Antibody level	Lesion onset (h)[b]	Primary lesion	Lesion size (mm^2) ± SD[b]	Healing (days)[c]	P[d]
I	576	High[e]	48	Lateral nodule; closed abscess not expressed through skin	97.3 ± 10.3 (4/4)	16	0.042 (II), 0.008 (III)
II	576	Low	48	Localized, flat, dry, depressed, gangrenous necrosis of skin	137.0 ± 47.9 (5/5)	15	0.285 (III)
III	234	High or low	24 (2/9), 48 (7/9)	Same as for group II	187.1 ± 18.5 (9/9)	16	

[a] All mice were initially challenged with *W. recta* 576 and recovered from localized lesion with the production of serum antibody. Localized lesion size in unimmunized mice is described in Table 1.

[b] Values in parentheses are numbers of positive mice per total number of mice challenged.

[c] No cachexia or death in any of the animals was noted.

[d] Statistical difference with group given in parentheses was determined by comparison of the sizes of the lesions by using the Mann-Whitney-Wilcoxon rank analysis.

[e] High IgG antibody was defined as a mean optical density times serum dilution equal to 424 ± 105; the value for low IgG antibody is 97 ± 14. Antibody reactivities to strain 234 were grouped together because there was no difference in outcome associated with antibody level.

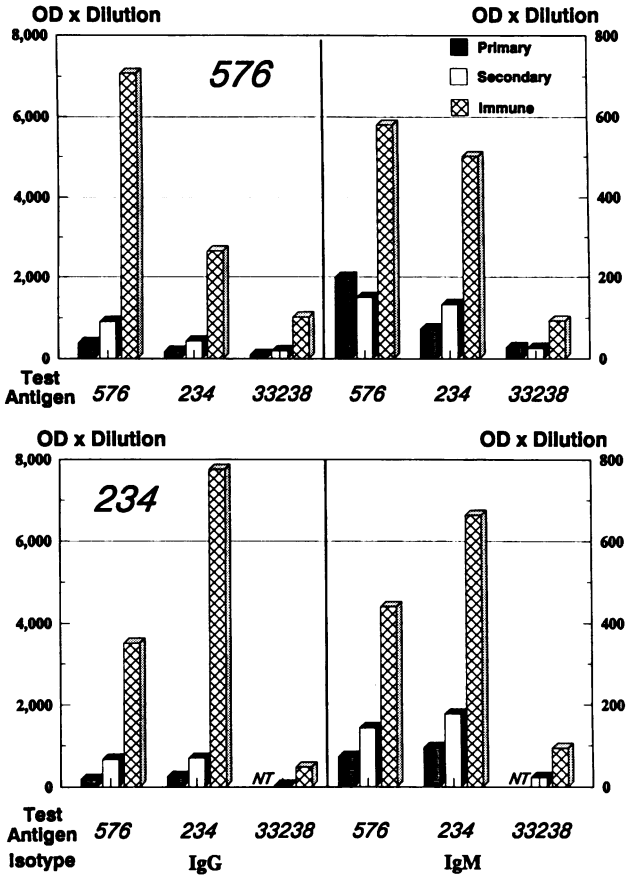

FIG. 7. Serum IgG and IgM antibody responses to *W. recta* after active immunization with *W. recta* 576 (top panel) or 234 (bottom panel). Antibody to *W. recta* 576, 234, or ATCC 33238 (test antigen) was measured in serum collected according to protocol D. The bars denote the group means (n = 5 to 12), and the standard deviation of the mean was always less than 10%. The antibody levels are expressed as the optical density at 490 nm multiplied by the dilution of the serum (1:500 and 1:5,000 for IgG, and 1:100 and 1:1,000 for IgM) that was tested. Primary and secondary denote responses following one or two infectious challenges (10^{11} cells) with the appropriate strain. Immune denotes two injections of formalin-killed *W. recta*. NT, not tested.

second *W. recta* challenge, which was manifested by a smaller lesion. Interestingly, however, immunization did not prevent lesion formation. Active immunization of mice with *W. recta* induced an IgG antibody level that was 50-fold greater than those levels achieved following infection. Previous studies of the serological properties of *W. recta* (53) have indicated that antigens on this microorganism were quite unique compared with antigens on other gram-negative asaccharolytic bacteria from the oral cavity. Rabbit antibody to *W. recta* did show some cross-reactivity with *Wolinella curva* and *Wolinella succinogenes* (53). However, examination of numerous human clinical isolates of *W. recta* with rabbit antisera did not demonstrate major serological diversity within the *W. recta* strains. The results of the current studies suggest that *W. recta* strains can exhibit some antigenic diversity. While both infection and active immunization elicited cross-reacting antibodies among the *W. recta* strains in the BALB/c mice, there was significant evidence for unique antigens on the individual *W. recta* cells to which the mice responded. Additionally, we found that antibodies induced by the homologous strain were substantially more protective than those produced following heterologous challenge. Thus, these findings indicate that *W. recta* may express some type- or group-specific antigens that are crucial for protective immune responses. Further studies are required to identify these antigens. In this regard, the results presented in Fig. 6 indicate that *W. recta* ATCC 33238 appeared to be significantly less antigenic than either of the clinical isolates. It is tempting to speculate that the higher antigenicity of the clinical *W. recta* isolates is related to the S-layer protein that surrounds the outer membrane of the cells (4, 7). It is also possible that the long-term cultivation of *W. recta* ATCC 33238 has selected for microorganisms that have a reduced composition of outer membrane proteins. In either case, ATCC 33238 would provide less antigenic challenge to the mice.

In conclusion, we have demonstrated the usefulness of a murine model for a description of pathogenic processes associated with the periodontal pathogen *W. recta*. The results support the continued use of this model in the assessment of critical virulence components produced by *W. recta* that are necessary for tissue destruction. Additionally, immune responses that were characterized by the presence

TABLE 5. Virulence of homologous *W. recta* in BALB/c mice with serum antibody elicited by active immunization[a]

Immune strain	*W. recta* strain[b]	Treatment[c]	Lesion onset (h)	Primary lesion	Lesion size (mm^2) ± SD[d]	Healing (days)	P^e
	576	DEX	40	Localized, flat, dry, depressed, gangrenous necrosis of skin	150.0 ± 24.7 (7/7)	14	0.002
576	576	DEX	48	Same as above	68.3 ± 6.3 (12/12)	22	
	234	DEX	24–72	Same as above	238.5 ± 23.0 (5/5)	14	0.007
234	234	DEX	48	Same as above	54.4 ± 7.6 (8/8)	20–24	
234	234	GalN	48	Same as above	41.6 ± 5.7 (4/4)	15	0.001
	234	GalN	18	Lethal challenge; liver, spleen, lymph nodes, enlarged and hemorrhagic	(14/15)		

[a] Three groups of mice (n = 4 to 12) were actively immunized twice with 10^9 formalin-killed *W. recta* 576 or 234 cells in incomplete Freund's adjuvant by s.c. injection in the nape of the neck.

[b] Groups of actively immunized and nonimmunized animals were challenged s.c. in the posterior dorsolateral side with 10^{11} *W. recta* cells.

[c] DEX denotes pretreatment with dexamethasone and GalN denotes i.p. injection of galactosamine simultaneous with challenge.

[d] Number of positive mice per total number of mice challenged is given in parentheses.

[e] Statistical differences for pairs were determined by comparison of the sizes of the lesions by using Mann-Whitney-Wilcoxon rank analysis and of lethality by Fisher's exact test.

of serum antibody were shown to lessen the destruction and indicate that the quality and specificity of these antibodies could be examined to focus attention on crucial host-parasite interactions with this microorganism.

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