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Environmental Modulation of Oral Treponeme Virulence in a Murine Model

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This investigation examined the effects of environmental alteration on the virulence of the oral treponemes *Treponema denticola* and *Treponema pectinovorum*. The environmental effects were assessed by using a model of localized inflammatory abscesses in mice. In vitro growth of *T. denticola* and *T. pectinovorum* as a function of modification of the cysteine concentration significantly enhanced abscess formation and size. In contrast, growth of *T. denticola* or *T. pectinovorum* under iron-limiting conditions (e.g., dipyridyl chelation) had no effect on abscess induction in comparison to that when the strains were grown under normal iron conditions. In vivo modulation of the microenvironment at the focus of infection with Cytodex beads demonstrated that increasing the local inflammation had no effect on lesion induction or size. In vivo studies involved the determination of the effects of increased systemic iron availability (e.g., iron dextran or phenylhydrazine) on the induction, kinetics, and size of lesions. *T. denticola* induced significantly larger lesions in mice with iron pretreatment and demonstrated systemic manifestations of the infectious challenge and an accompanying spreading lesion with phenylhydrazine pretreatment (e.g., increases in circulating free hemoglobin). In contrast, *T. pectinovorum* virulence was minimally affected by this in vivo treatment to increase iron availability. *T. denticola* virulence, as evaluated by lesion size, was increased additively by in vivo iron availability, and cysteine modified growth of the microorganism. Additionally, galactosamine sensitized mice to a lethal outcome following infection with both *T. denticola* and *T. pectinovorum*, suggesting an endotoxin-like activity in these treponemes. These findings demonstrated the ability to modify the virulence capacity of *T. denticola* and *T. pectinovorum* by environmental conditions which can be evaluated by using in vivo murine models.

Alterations in the growth environment of a bacterium, such as changes in carbon, carbohydrate, and nitrogen source, growth temperature, and iron concentration, have been shown to alter the expression of a variety of virulence factors (13, 15, 32, 48). These environmental alterations, or stress responses, also result from placing the bacterium in adverse growth environments, such as conditions of starvation, nutrient limitation, pH shift, heat shock, or change in oxygen tension. With few exceptions, all bacteria, whether living free or associated with a host, such as the periodontopathogenic bacteria, must be able to respond to these signals or cues. Adjustment to these environmental changes permits them to survive and multiply in this changing environment (11, 13, 34–36). Oral pathogens are an excellent example of bacteria that survive, grow, and multiply in a complex and rapidly changing environment. With potentially hundreds of different bacterial species occupying a periodontal niche, producing many metabolic end products and associated physicochemical changes, these bacteria are highly adapted for this niche. We have noted that hemin limitation increases the expression of new outer membrane proteins (2, 27) by *Porphyromonas gingivalis*. Additionally, various studies have shown that hemin limitation has a significant effect on *P. gingivalis* virulence in mice (22, 31, 33). Moreover, we have noted that environmental growth conditions alter the virulence potential (24) and in vitro S-layer expression of *Campylobacter rectus* (38).

Ultimately, to understand the disease caused by any pathogen, its virulence potential in an in vivo environment must be examined (20, 45). In vivo studies provide a means to address the role of the host and selected host-associated environmental factors on specific manifestations of disease progression. Recent work in our laboratory has shown that the murine abscess model is capable of establishing a role for the trypsin-like protease activity in *P. gingivalis* pathogenesis (25). Although we have some knowledge of the in vitro elaboration of putative treponeme virulence factors (i.e., hemolysin, trypsin-like enzyme, major outer sheath proteins, and lipopolysaccharide [LPS]-like molecules), we still have very little understanding of their functional role in vivo or regulation by environmental factors such as iron (28, 41, 52). The oral treponemes *Treponema denticola*, *Treponema socranskii*, *Treponema pectinovorum*, and *Treponema vincentii* have been implicated as etiological agents of severe periodontal disease in adults (19, 30, 37, 44, 46). Among the oral treponemes, *T. denticola* is the predominant one identified within the gingival crevice and subgingival ecology of the developing periodontal pocket and has been the most extensively studied. In vitro studies have revealed that oral treponemes elaborate a variety of proteolytic enzymes, hemolysins, esterases, collagenase, fibrinolytic enzymes, iminopeptidases, phospholipase C, hyaluronic acid, and chondroitin sulfate-degrading enzymes. The functional significance of these virulence factors has not been clearly elucidated in vivo. There are no data documenting the role of iron as a modulator of oral treponeme virulence. The murine abscess model provides the wherewithal to study the role of putative virulence factors of the oral treponemes in tissue destruction. Previously, we employed this model to explore variations in virulence among the oral treponemes, as well as the contribution of a trypsin-like protease to the virulence of *T. denticola* and *T. pectinovorum* strains (26).

The objectives of this study were to determine the role of

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TABLE 1. Species and strains of oral treponemes used in this study and their sources

Species	Strain	Source of isolate	Source[a]	Reference
T. denticola	ATCC 35404	Human with periodontitis	ATCC	5
	GM-1	Human with periodontitis	AW	43
T. pectinovorum	ATCC 33768	Subgingival plaque	EPG	46
	S1	Human with HIV[b] and periodontitis	SW	49

[a] ATCC, American Type Culture Collection, Rockville, Md.; AW, Aaron Weinberg, University of Texas Health Science Center at San Antonio; EPG, E. Peter Greenberg, University of Iowa; SW, Stephen Walker, University of Texas Health Science Center at San Antonio.
[b] HIV, human immunodeficiency virus.

iron, cysteine (as a sulfhydryl reducing agent), local inflammation, and endotoxin in the pathogenesis of *T. denticola* and *T. pectinovorum* in a murine abscess model. The hypotheses tested included the following. (i) Alterations in iron availability, in vitro or in vivo, modify the virulence of *T. denticola* and *T. pectinovorum*. (ii) Cysteine, which significantly enhances in vitro growth of the treponemes, increases the virulence of the species. (iii) Existing localized inflammation enhances treponeme virulence. (iv) Both *T. denticola* and *T. pectinovorum* demonstrate toxic activities in mice which had been sensitized for evaluation of endotoxin-induced lethality.

MATERIALS AND METHODS

Bacterial strains. The *Treponema* spp. used and their sources are given in Table 1. *T. denticola* strains were grown in GM-1 broth (1) in a Coy anaerobic chamber in an atmosphere of 85% N_2, 5% CO_2, and 10% H_2 for 72 h at 37°C. *T. pectinovorum* strains were grown in GM-1 broth supplemented with 0.3% pectin (Sigma) for 24 to 72 h (49, 50). All manipulations were carried out under anaerobic conditions to ensure maximum cell viability. Culture purity was determined by dark-field and phase-contrast microscopy. Viability was estimated by the degree of motility and the presence or absence of spherical bodies of the treponemes. Growth in liquid media was estimated at an optical density of 660 nm. Log-phase cultures were harvested by centrifugation (9,000 × *g* for 10 min), and pellets were resuspended in the same medium under anaerobic conditions. An aliquot of the culture was removed from the chamber, and 10-fold dilutions were made for estimating total counts with a Petroff-Hausser bacterial counting chamber. After allowing sufficient time for treponemes to settle in the counting chamber, the cells were enumerated, with spherical bodies excluded.

Mice. ICR (Harlan Sprague-Dawley Inc., Indianapolis, Ind.) female mice, aged 8 to 12 weeks old, were utilized. The animals were housed in isolator cages in an American Association for Accreditation of Laboratory Animal Care-accredited animal facility at the University of Texas Health Science Center at San Antonio and were provided with autoclaved TEKLAD chow (Harlan Sprague-Dawley Inc., Madison, Wis.) and water ad libitum. BALB/c mice were used for testing the lethality of the *T. denticola* GM-1 strain, and ICR mice were used in all other experiments.

Murine virulence model. The murine abscess model (23, 24, 26) was used to examine the virulence capacity of the oral treponeme strains. For determination of virulence and abscess-forming capability, appropriate dilutions of the treponemes were made under anaerobic conditions and mixed with the virulence-modulating agents. Mice were injected subcutaneously (s.c.) or intraperitoneally (i.p.) within 15 to 30 min of sample preparation. After challenge, the animals were monitored daily for symptoms of infection. Progression of lesion formation for up to 3 weeks and virulence were scored on the basis of (i) onset and characteristic of lesion, (ii) size of localized abscess and/or necrotic skin lesion, (iii) size of spreading exudative hemorrhagic lesion, and (iv) death. Subcutaneous abscess and necrotic skin lesion sizes (length and width) were measured daily for 15 days with a caliper gauge, and the area was determined and expressed in square millimeters. The maximum abscess size was noted for each animal, and the mean per group was calculated. Three experimental models were used.

(i) Bacterial growth modulation. The cysteine concentration was altered such that *T. denticola* and *T. pectinovorum* were grown in GM-1 medium (1, 49, 50) supplemented with 6, 18, or 36 mM cysteine. Logarithmic-phase broth cultures were harvested by centrifugation and resuspended in GM-1 medium (with or without cysteine) to a final concentration of 2.5×10^{11} treponemes per ml. GM-1 medium supplemented with 6, 18, or 36 mM cysteine alone was injected into groups of animals to serve as controls.

Iron-limiting GM-1 medium was produced by incubating the medium with 0.1 mM 2,2′-dipyridyl (BPD; Sigma) for 48 h (9). This BPD level was noted previously to deplete iron levels in the GM-1 medium by >90% and to decrease the growth of both of the treponemes (unpublished observations). This iron-chelated medium was then inoculated with *T. denticola* and *T. pectinovorum*. Logarithmic-phase GM-1 broth cultures (approximately 72 h of growth) with or without BPD were harvested by centrifugation and resuspended in GM-1 medium with or without 0.1 mM BPD to a final concentration of 2.5×10^{11} treponemes per ml. The treponemes were injected s.c. in mice. GM-1 medium with or without 0.1 mM BPD was injected into other animals to serve as controls.

(ii) Bacterial environment modulation in vivo. Alterations in *T. denticola* virulence were assessed by the extent of abscess formation following coinoculation of mice with treponemes and Cytodex 1 dextran microcarrier beads essentially as described by Ford et al. (16). The beads induce a localized inflammatory response and can increase bacterial pathogenicity. Cytodex 1 microcarrier beads (Sigma) were suspended in phosphate-buffered saline (50 ml/g) overnight and autoclaved at 120°C for 10 min. The sterile supernatant fluid was decanted from the sedimented microcarrier beads, equal volumes of packed microcarrier beads and an appropriate concentration of *T. denticola* were mixed, and 0.2 ml of the mixture was injected s.c. into the posterior dorsolateral surface of each mouse for determination of abscess formation. Sterile packed Cytodex 1 beads alone were injected s.c. into a group of five animals as a negative control.

The effects of D-[+]-galactosamine (GalN) on the susceptibility of mice to lethal toxicity (17) following challenge with *T. denticola* or *T. pectinovorum* were also studied. *T. denticola* GM-1 and ATCC 35404 and *T. pectinovorum* S1 and ATCC 33768 were grown as described above and harvested by centrifugation, and an appropriate concentration of cells was suspended in sterile GalN (20 mg/animal; Aldrich Chemicals) and injected i.p. into mice. Control mice received GalN only, and other groups of animals received similar concentrations of treponemes alone. Symptoms of infection and death were recorded for 4 days.

(iii) Host environment modulation. To evaluate the effect of iron pretreatment of mice on the virulence of *T. denticola* and *T. pectinovorum*, mice were administered iron dextran (ferric hydroxide dextran complex; Sigma) i.p. at 5 mg per animal 3 h prior to (41) treponeme challenge. Alternatively, administration of phenylhydrazine (Sigma) to mice elicits a rapid lysis of circulating erythrocytes with a resulting substantial increase in the levels of free hemoglobin in plasma (21). This host alteration lasts for approximately 4 days, at which time the blood levels of hemoglobin return to normal. Phenylhydrazine (2 mg/animal) was administered i.p. 3 h prior to *T. denticola* or *T. pectinovorum* challenge. Groups of animals without iron or phenylhydrazine pretreatment were also infected with the treponemes to serve as untreated controls; mice injected with iron dextran or phenylhydrazine alone served as treatment controls.

Statistical analyses. Statistical differences in lesion size between various groups were determined by use of a Wilcoxon-Mann-Whitney U test (Minitab, State College, Pa.).

RESULTS

In vitro environmental modulation of virulence. The ability of cysteine to enhance the virulence of *T. denticola* and *T. pectinovorum*, as determined by the production of soft tissue lesions in mice following s.c. challenge, was evaluated (Fig. 1). We have previously described the characteristics of the induction, progression, and gross and histologic characteristics of these lesions (26). *T. denticola* and *T. pectinovorum* grown in 18 mM cysteine induced significantly ($P < 0.01$) larger local abscesses than bacteria grown in 6 mM cysteine when a challenge dose of 10^{10} was used. Also, *T. denticola* grown in 36 mM cysteine induced significantly ($P < 0.01$) larger abscesses than bacteria grown in 6 mM cysteine, although the lesion size was similar to that of bacteria grown in 18 mM cysteine (data not shown). Similar onsets and similar lesion characteristics were elicited by both treponemes, irrespective of the availability of cysteine during in vitro cultivation. As we had previously noted, there was no mortality induced by s.c. challenge infection with these treponemes under any of the conditions.

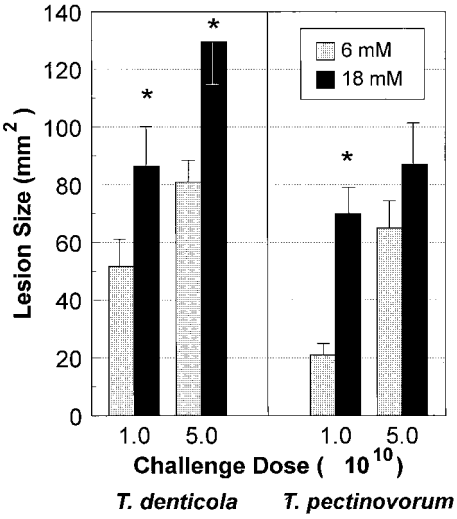

FIG. 1. Effect of growth of *T. denticola* ATCC 35404 and *T. pectinovorum* ATCC 33768 in cysteine-containing GM-1 medium on virulence in a murine abscess model. The bars denote the mean abscess size in square millimeters, and the error bars represent 1 standard deviation derived from groups of five mice. NT, not tested. *, significantly different at $P < 0.01$ when results for treponemes grown in 18 and 6 mM cysteine are compared. The treponeme-free negative control injected with cysteine in GM-1 medium demonstrated neither toxicity nor any lesion at the site of injection (data not shown).

We had noted previously that cultivation of various oral microorganisms under iron-depleted conditions decreased their virulence in this murine model (22). In contrast, the capacity of *T. denticola* and *T. pectinovorum* grown in 0.1 mM BPD (iron-depleted conditions) to induce abscess formation was not significantly affected in comparison to that of control treponemes cultivated under normal iron conditions (data not shown).

In vivo bacterial environment modulation. Injection of the inflammation-stimulating Cytodex 1 beads into the dorsal surface of mice induced a closed sterile abscess of approximately 80 mm^2 (Fig. 2). The abscess persisted for approximately 14 days and then resolved. A similar s.c. injection of *T. denticola* alone at a dose of 10^9 bacteria/site produced a small abscess of approximately 28 mm^2. In contrast to the response induced by the Cytodex 1 beads (i.e., a closed sterile abscess), the abscess induced by *T. denticola* was expressed through the skin and healed more slowly (i.e., 2 to 3 weeks). When 10^8 or 10^9 *T. denticola* bacteria were combined with the Cytodex 1 beads, the abscess formed was significantly larger ($P < 0.05$) than that resulting from *T. denticola* injection alone (Fig. 2). Interestingly, while the abscess was larger (110 to 150 mm^2) in the *T. denticola*-Cytodex 1 bead-injected animals than in the animals injected with *T. denticola* or Cytodex 1 beads alone, the lesion from the combined injection appeared to be the result of an additive virulence outcome rather than a synergistic effect. Nevertheless, this local abscess persisted for more than 3 weeks and did not break through the skin, as was noted with *T. denticola* alone.

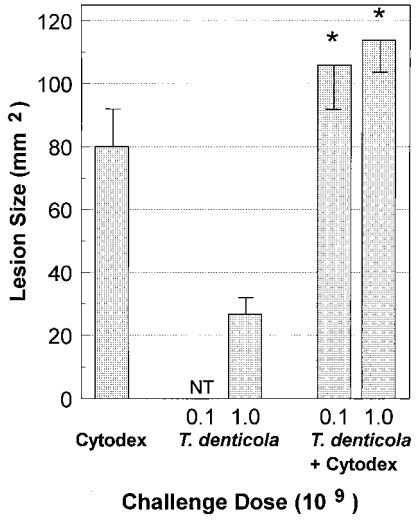

FIG. 2. Effect of sterile Cytodex 1 microcarrier bead-induced abscess on the virulence of *T. denticola* ATCC 35404. Mice were challenged with 10^8 or 10^9 bacteria as described in Materials and Methods. NT, not tested. The bars represent the mean abscess size in square millimeters, and the error bars represent 1 standard deviation derived from groups of five mice.

TABLE 2. GalN effects on lethality of treponeme infection in mice

Treponeme strain[a]	GalN[b]	Treponeme dose	Lethality[c]
T. denticola GM-1	+	10^7	1/5
	+	10^8	1/5
	+	10^9	4/4
	+	10^{10}	5/5
	−	10^9	0/5
	−	10^{10}	0/5
T. denticola 35404	+	10^9	4/5
	+	10^{10}	5/5
	−	10^9	0/5
	−	10^{10}	0/5
T. pectinovorum S1	+	10^7	0/5
	+	10^8	0/5
	+	10^9	8/10
	+	10^{10}	10/10
	−	10^7	0/5
	−	10^8	0/5
	−	10^9	0/5
	−	10^{10}	0/5
T. pectinovorum 33768	+	10^9	5/5
	+	10^{10}	5/5
	−	10^9	0/5
	−	10^{10}	0/5
None	+	−	0/5

[a] *T. denticola* GM-1 was tested in BALB/c mice; all other strains were tested in ICR mice and provided comparable results irrespective of the mouse strain used.

[b] GalN was mixed with an appropriate concentration of treponemes and administered i.p. to groups of mice. Control animals received either GalN or an appropriate concentration of treponemes i.p.

[c] Number of animals that died/total number of animals infected.

GalN sensitization increases the susceptibility and sensitivity of mice to the lethal effects of gram-negative bacteria and endotoxin (LPS) (17). In this study, GalN was mixed with different concentrations of *T. denticola* and *T. pectinovorum* and administered i.p. to groups of mice (Table 2). The results demonstrated that *T. denticola* GM-1 and ATCC 35404 were uniformly lethal at doses of 10^{10} or 10^9 bacteria. Similarly, *T. pectinovorum* S1 and ATCC 33768 caused 80 to 100% lethality at similar doses. Below a dose of 10^9 bacteria, minimal toxicity was noted with the treponemes. GalN or treponemes injected separately in control animals had no lethal effect at any dose tested.

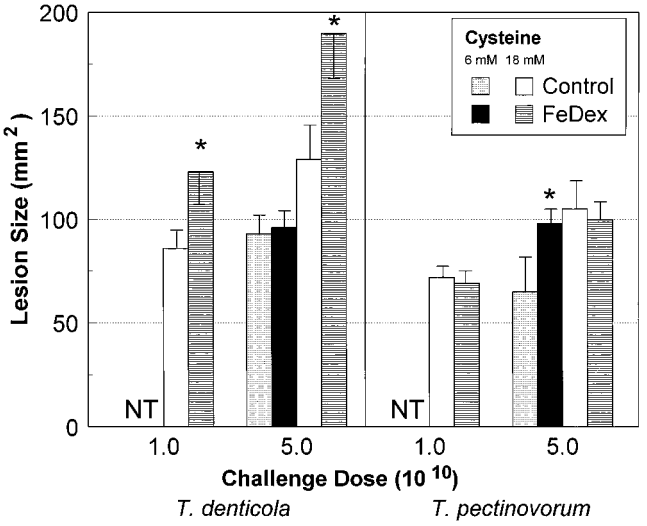

FIG. 3. Effect of administration of iron dextran (FeDex) to mice on the virulence of *T. denticola* ATCC 35404 and *T. pectinovorum* ATCC 33768 cultivated in medium with cysteine at various concentrations (6 or 18 mM). Animals were challenged with 1×10^{10} or 5×10^{10} bacteria as described in Materials and Methods. The bars represent the maximum mean lesion size in square millimeters, and the error bars represent 1 standard deviation derived from groups of five mice. NT, not tested. *, significantly different ($P < 0.01$) when results of growth in 18 mM cysteine with host iron pretreatment and growth in 18 mM cysteine alone are compared. Mice injected with iron dextran alone demonstrated neither toxicity nor any lesion at the site of injection (data not shown).

Host environment modulation. The in vivo administration of iron-containing compounds (e.g., iron dextran, hemin, hemoglobin, or transferrin) has been shown to enhance the virulence of many bacterial pathogens (28, 42, 51, 52), although these studies have generally emphasized microorganisms which cause systemic infections. Mice pretreated with iron dextran and infected with *T. denticola* (grown in 18 mM cysteine) induced at least 50% larger lesions ($P < 0.025$) than untreated *T. denticola*-challenged mice (Fig. 3). No abscess or symptoms of toxicity were observed in control mice that received iron dextran alone. In contrast, iron dextran had no effect on the virulence of infection with *T. pectinovorum* (grown in 18 mM cysteine) in comparison to that in untreated control animals (Fig. 3).

The experimental design also enabled us to evaluate the capacity of *T. denticola* and *T. pectinovorum* to utilize multiple virulence-enhancing environmental signals in the murine lesion model. As such, the treponemes were cultured in 6 or 18 mM cysteine (Fig. 3) and subsequently used to challenge mice which had been pretreated with iron dextran to increase in vivo iron availability. The results demonstrated that the environmental conditions, that is, elevated cysteine and iron pretreatment, contributed additively to the resulting lesion size of *T. denticola*. Interestingly, under growth conditions with lower cysteine concentrations, iron pretreatment had no effect on the virulence of *T. denticola* but enhanced *T. pectinovorum* virulence significantly (Fig. 3).

In an experiment with a similar design, we noted that *T. denticola* and *T. pectinovorum* cultivated in 6 mM cysteine were significantly ($P < 0.01$) more virulent in mice treated with phenylhydrazine than in untreated mice (Fig. 4). As interesting, *T. denticola* caused an exudative necrotic spreading lesion in the abdomen, in addition to inducing a local abscess. This spreading lesion appeared to be initiated by a spirochetemia based upon the clinical presentation of the mice, which demonstrated gross manifestations (i.e., ruffled fur, body posture, weight loss, or change in eating behavior) of infection for up to 6 days postinfection. In contrast, phenylhydrazine treatment had no significant effect on s.c. abscess formation by *T. denticola* cultured in 18 mM cysteine (Fig. 4). None of the phenylhydrazine-treated control animals died nor demonstrated any acute toxic symptoms to the treatment except for a brief (<24-h) period of malaise with ruffled hair.

DISCUSSION

In vitro studies of environmental regulation of bacterial virulence have provided a number of important observations regarding strategies used by pathogens to respond and survive under changing environmental conditions in vivo. However, recent evidence, including sophisticated in vivo expression technologies (20, 45), have demonstrated the importance of the in vivo environment in delineating significant virulence determinants which contribute to the host-parasite interactions resulting in manifestations of disease. An in vivo expression technology vector (pPGIVET) has been constructed and used to determine the expression of hemagglutinin genes *hagB* and *hagC* of *P. gingivalis* during an infectious process in mice (30). Thus, these genetic approaches to evaluate the virulence mechanisms of oral microorganisms have been initiated, but the capabilities of these approaches are still quite limited. The experiments described in this report examine the oral treponemes *T. denticola* and *T. pectinovorum* as members of the putative periodontopathic microbiota in the subgingival sulcus (30, 44, 46). In particular, we used more classic approaches to specifically evaluate the ability of in vitro and in vivo environmental conditions to modify the virulence capacity of oral treponemes by using a murine model (14, 23, 24).

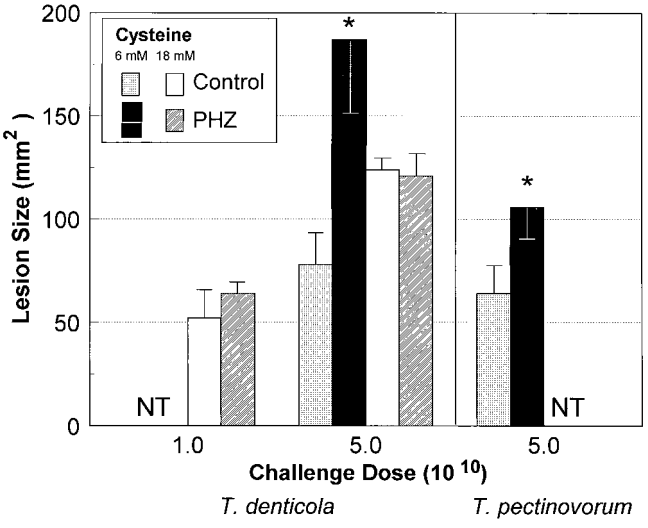

FIG. 4. Effect of administration of phenylhydrazine (PHZ) to mice on the virulence of *T. denticola* ATCC 35404 and *T. pectinovorum* ATCC 33768 cultivated in medium with cysteine at various concentrations (6 or 18 mM). Animals were challenged with 1×10^{10} or 5×10^{10} bacteria as described in Materials and Methods. The bars represent the maximum mean lesion size in square millimeters, and the error bars represent 1 standard deviation derived from groups of five mice. The stacked bars for PHZ treatment and *T. denticola* challenge represent the localized abscess (solid bar) and total (spreading necrotic lesion plus local abscess) (horizontally lined bar) lesion sizes in the mice. NT, not tested. *, significantly different ($P < 0.01$) from results for PHZ treatment of control infected mice. Mice injected with PHZ alone demonstrated neither toxicity nor any lesion at the site of injection except for a brief (<24-h) period of malaise with ruffled hair (data not shown).

Our initial investigation expanded previous in vitro observations from studies using cysteine (9) or iron levels (3, 4, 7) to modulate the growth characteristics of oral microorganisms. These have been suggested to result in the up- and down-regulation of selected surface and cytoplasmic molecules that aid in the ability of the bacteria to utilize alternative mechanisms for acquisition of critical nutrients and cofactors. We have noted that cysteine can stimulate or enhance enzyme activities of *P. gingivalis*, although high levels of this sulfhydryl-containing amino acid can actually abrogate the virulence of *P. gingivalis* (unpublished observation). Cysteine has also been identified as a protease activator, although it had been shown previously to exhibit a minimal effect on a number of *T. denticola* enzyme functions in vitro (26). However, previous studies in our laboratory have clearly shown that cell-associated hemolytic and hemoxidative activities of *T. denticola* are cysteine dependent (6). Further studies of these biologic activities described the isolation and characterization of a 46-kDa protein from *T. denticola*, referred to as cystalysin, with hemolytic activity different from that of complement and classical hemolysin (8). Its preferred substrate compound was cysteine with a sulfur atom adjacent to both a β-methylene and an α-amino group (9). The cystalysin exhibited its enzymatic activity by degrading cysteine, yielding H_2S and pyruvate, which increased the growth of the bacteria and caused the hemolytic activity (9). In contrast, *T. pectinovorum* does not express a cystalysin activity. We hypothesized that cysteine would significantly increase the in vivo virulence of *T. denticola*, via these cystalysin activities. The results showed that, in fact, the virulence of *T. denticola* was significantly increased by growth in media with elevated levels of cysteine. However, this amino acid also appeared to modify the characteristics of *T. pectinovorum* and increased its lesion-inducing capacity. Thus, while the *T. denticola* cystalysin may be a virulence factor affected by the cysteine, it appears that the oral treponemes may have multiple mechanisms for interacting with this molecule in the environment.

Previous studies have clearly shown that environmental iron has a significant regulatory effect on the growth (3) and virulence expression (22) of *P. gingivalis*. We have noted that cultivation of oral treponemes under iron-limited conditions alters outer membrane protein profiles (7), enzymatic activities (9), and hemolytic activities (8). Recently, we have observed that *T. denticola* and *T. pectinovorum* have a minimal capacity to survive under continued iron-depleted conditions in vitro (unpublished observations), in contrast to the ability of *P. gingivalis* to survive and grow for three to seven passages in media devoid of iron (unpublished data). Thus, we hypothesized that depleting the environment of iron would have a profound effect on virulence expression by the oral treponemes. Although iron is essential for the in vitro growth of oral treponemes and appears to alter putative virulence characteristics, including toxins, cytotoxins, and hemolysins (6), depletion of this molecule during a single in vitro passage had a negligible impact on in vivo virulence. These results suggest that the treponemes may be less affected by the dramatic fluctuations in environmental iron in the gingival sulcus and, as such, are less dependent upon the local availability of iron for maintenance of their virulence capability.

Iron is a necessary factor for the survival and growth of prokaryotes, which is clearly evident in the numerous evolutionary strategies that have been developed by bacteria to acquire iron from the natural environment (51). Various studies have begun to contribute to a clearer understanding of mechanisms by which oral anaerobes acquire iron from the environment (3, 4, 18), particularly with respect to *P. gingivalis*. We have recently reported preliminary observations concerning the accession of iron in vivo by *P. gingivalis* (22). Additionally, we have determined that *P. gingivalis* virulence is enhanced by pretreatment of mice with iron dextran, hemin, and transferrin (unpublished data). This has been suggested to result from increased iron availability and altered regulation of virulence determinants. However, very little is known concerning the characteristics of iron acquisition and utilization by oral treponemes. To this end, we assessed iron utilization by *T. denticola* and *T. pectinovorum* in vivo in mice. Specifically, numerous studies have demonstrated that iron loading of mice significantly increases the virulence of systemic pathogens, such as *Vibrio vulnificus* (21), *Escherichia coli* (48), and *Yersinia enterocolitica* (41), although this model has never been used to examine localized, tissue-destructive pathogens. In our study, iron dextran administration significantly enhanced the virulence (abscess size) of *T. denticola*. Similarly, phenylhydrazine pretreatment of mice, which has been reported to induce hemoglobinemia in mice, significantly enhanced lesion formation by *T. denticola*. The phenylhydrazine pretreatment also substantially altered the virulence characteristics of *T. denticola* either by a direct effect of the increased iron on bacterial virulence mechanisms or by altering host resistance and allowing the *T. denticola* to spread from the infection site. Both iron dextran and phenylhydrazine pretreatments appeared to have less of an effect on the virulence of *T. pectinovorum* in vivo. One interpretation of these findings is that the alterations were at the level of the microorganism rather than the host, although further studies will be required to validate this concept. Additionally, the minimal effect of host iron changes on *T. pectinovorum* suggested that this microorganism either requires less iron for in vivo survival and virulence expression or does not express the capacity to utilize either of these host iron sources but acquires its iron by other mechanisms. Again, these options need to be evaluated in future studies.

Extensive inflammation, which enhances delivery of soluble and cellular serum components to the sulcular environment, results in dramatic changes in the milieu of the sulcus, from health to periodontitis. Extensive data have shown that the oral treponemes emerge as dominant members of the oral ecology at gingival sites with increased inflammation (19, 37). This change in the inflammatory environment may be expected to result in alterations in a variety of local cues which require bacterial responsiveness to the milieu. Thus, we tested the synergistic effect of cysteine and iron, based upon the findings that cysteine and in vivo iron individually enhance *T. denticola* virulence. We noted that cysteine and iron contributed additively to the virulence of *T. denticola*. In contrast, these two environmental cues appeared to act independently with respect to the virulence of *T. pectinovorum*. These findings suggest that the two signals may be acting upon different aspects of each microorganism's survival and virulence strategies as expressed in this murine model.

It has also not been determined whether the increase in treponemes associated with gingivitis and periodontitis (19, 30, 37, 44) represents the treponemes contributing to the induction of an inflammatory environment or if their increase in the plaque results from the creation of a more favorable ecological niche at the inflammation sites. Thus, we hypothesized that locally induced inflammation would create a more favorable environment and increase the ability of *T. denticola* to induce tissue-destructive lesions, which could be evaluated in a murine model. We had previously reported that Cytodex 1 microcarrier beads, which create a localized inflammatory milieu in vivo, enhance the infectivity and pathogenicity of *C. rectus* as assessed by the murine abscess model (24). We evaluated the

ability to affect treponeme virulence by altering the local in vivo environment of the bacteria. The abscess-promoting agent Cytodex microcarrier beads was evaluated for increasing local inflammation and for potentially providing a more favorable environment for the treponemes to establish an infectious focus. The results indicate that within the limitations of this model, local inflammation appears to have a minimal effect on virulence expression by treponemes. Of potential importance is that this model may be used to explore other modifications of the local in vivo environment and to determine their effect on treponeme virulence.

The LPS from gram-negative bacteria is capable of eliciting a wide range of biological responses and is implicated in the pathogenesis of a variety of diseases. Members of the genus *Treponema* have an outer envelope resembling that of gram-negative bacteria. Several reports have described an LPS-like component in *T. denticola* (10, 29, 53, 54), *Serpulina* (*Treponema*) *hyodysenteriae* (39, 40), and the nonpathogenic Reiter treponeme (12). Injection of GalN makes mice increasingly susceptible to the lethal effects of endotoxin (17). This model has been suggested to be a quantitative measure of the endotoxicity of LPS, particularly as related to the toxic activity of the lipid A portion of the molecule (47). We have recently identified an LPS-like macromolecule isolated from the outer sheath of *T. pectinovorum* (unpublished data). Thus, we hypothesized that LPS-like macromolecules could contribute to the pathogenicity of oral treponemes and evaluated this biological activity in a galactosamine sensitization model in mice. The results demonstrated a general lack of toxicity of both treponemes when injected i.p. into normal mice. However, both treponemes were lethal for mice that were concomitantly treated with GalN and elicited systemic changes normally associated with endotoxic activities. The majority of GalN-sensitized animals died within 8 h of bacterial challenge, consistent with an LPS-mediated effect (17). The systemic manifestations of endotoxemia have been identified as resulting from the ability of LPS to activate host cells to produce circulating levels of cytokines, such as interleukin 1β, interleukin 6, and tumor necrosis factor alpha, which are the direct effectors of endotoxic shock and death. Therefore, while this study did not identify cytokines in the murine serum, nor specifically identify the capacity of the LPS to affect host responses, the results suggest that examination of the factor(s) that elicited the lethal outcome would be important in understanding treponeme-mediated pathogenicity.

The findings in this study clearly demonstrate the ability to modify the virulence of oral treponemes by altering the in vitro and in vivo environments to which these bacteria are subjected. This model can be manipulated to evaluate the contribution of putative virulence components of oral treponemes within the host.

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