Selected Characteristics of Pathogenic and Nonpathogenic Strains of
Bacteroides gingivalis

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Strains of Bacteroides gingivalis were compared for the presence of properties associated with pathogenicity.
Some strains were infectious in pure culture in an in vivo model (guinea pig), and all but one of these were more
collagenolytic than those which failed to cause lesions in guinea pigs. However, other factors seem to be
necessary for the induction of an infection in this animal model.

Gram-negative anaerobic bacteria may play an important role in the development of periodontal diseases. Bacteroides
gingivalis has been associated with adult chronic periodontitis (21, 22). Several studies have described the role this
species plays in various types of experimental infections (4, 9, 17). Indeed, the inclusion of B. gingivalis in several
different mixed-culture inocula resulted in the development of necrotic lesions, organisms from which were transmissible.
In contrast, omission of B. gingivalis from the mixed-culture inoculum failed to induce lesions. On the other hand,
a limited number of B. gingivalis strains have been pathogenic when injected into guinea pigs or mice in pure culture
(6, 18). The pathogenic potential of this species may result from the presence of a variety of virulence factors (16).
The goal of our study was to determine whether it was possible to detect differences among some putative virulence factors
diversely exhibited by B. gingivalis strains.

A total of 14 human strains of B. gingivalis were tested (Table 1). All were grown in a Trypticase (BBL Microbiol-
ogy Systems)-yeast extract medium described by Sawyer et al. (15); the requirement for the two growth factors (hemin
and vitamin K₁) was determined. In all cases, the cultures were grown in an anaerobic chamber at 37°C to late exponen-
tial phase, concentrated by centrifugation (10,000 × g, 15 min), and suspended in phosphate-buffered saline supple-
mented with sodium thioglycollate (0.05%) to a concentration corresponding, after a dilution of 1:10, to a McFarland no. 10
standard. At this time, viable counts were done on Todd-
Hewitt agar supplemented with hemin and vitamin K₁.

Dilutions of this bacterial suspension were made in the modified phosphate-buffered saline, and 0.5 ml was then
injected into the groin of each of three Hartley guinea pigs
(weight, 180 to 220 g). The animals were examined daily for
2 weeks, and the lesions were inspected and characterized as
follows: --, no infection; +, localized abscess, less than 2 cm
in diameter; ++, localized abscess, 2 cm or more in diam-
eter; ++++, necrotic abscess, not fatal; +++++, massive
infection resulting in the death of the animal in less than 3
days. Only strains which exhibited ++ or greater reaction
were considered virulent. We verified that abscess fluid
could induce similar lesions by injecting material aspirated
from a lesion into a second animal. Guinea pigs injected with
killed B. gingivalis or un inoculated medium were used as
controls.

The collagenolytic activity of the strains was determined by
the method of Gisslow and McBride (3). The collagenase
assay consisted of mixing 100 μl of bacteria (ca. 2 × 10⁹
cells) with 200 μl of [¹⁴C]collagen solution plus 100 μl of
buffer (5 mM CaCl₂, 50 mM Tris [pH 7.2]) and 100 μl of
cysteine (5 mM in buffer). The assays were done in
microtubes incubated at 37 or 25°C. To limit the action of
nonspecific proteases on the labeled collagen, the incubation
period was limited to 2 h (8). At the end of the incubation
period, 100 μl of the assay mixture was transferred to a
second microtube containing 50 μl of HCl (2 N) and 50 μl of
phosphotungstic acid (0.04 N). The microtubes were agitated
with a vortex mixer and left at room temperature for 10 min
before centrifugation. The supernatant (100 μl) was counted
by liquid scintillation spectrometry. Nonspecific protease
activity found in culture supernatants (4-day culture) was
determined by measuring activity against Azocoll (Sigma
Chemical Co.) as already described (9). The stability of the
nonspecific proteolytic activity associated with the superna-
tant was evaluated by using a 13-day culture or by heating at
55°C for 30 min (7).

The cytotoxic activity of B. gingivalis culture superna-
tants was measured against Vero cells (5). The eucaryotic
cells were grown for 2 days, and the medium was replaced
by bacterial filtrates which had their pH adjusted to 7.2.
The plates were incubated for 2 days in air containing 5% CO₂
at 37°C and 98% humidity. After the incubation period,
the liquid was decanted, and the Vero cells were fixed with
methanol and subsequently stained with 10% Giemsa.
Cytotoxic activity of culture supernatants was also mea-
sured against guinea pig leukocytes by the methods
described by Gadeberg and Örskov (2). Polyclonal antibody
gel electrophoresis of soluble proteins was done by the methods
and procedures described by Moore et al. (11); this
technique allows the detection of only major differences between
the bacterial strains.

Virulence assays showed that 6 of 14 strains (W83,
BH18/10, 22B4, RB46D-1, RB24M-2, and RB22D-1) were
pathogenic in pure culture (Table 2). All the infections were
transmissible to a second animal. The minimum number of
viable bacteria required to cause the infections ranged from
4 × 10⁶ to 7 × 10⁸/ml (Table 2). The induced lesions were
similar for the six pathogenic strains and were characterized
by a necrotic abscess of up to 20 ml of exudate containing
altered erythrocytes, some leukocytes, and large quantities
of bacteria. The other B. gingivalis strains always gave
negative results (or, at most, a slight redness at the injection
site), even when inoculated in great numbers. Strain 19A4,

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for example, was injected at a concentration which was 200 times greater than that of strain BH18/10 without detectable effect.

The relative collagenolytic and proteolytic activities of all strains are also shown in Table 2. Except for 22B4, the pathogenic strains had a higher collagenolytic activity than the nonvirulent strains. The nonvirulent proteolytic activity of certain avirulent strains was also higher than that found for the pathogenic strains, but no correlation could be established between protease activity and infectivity. In contrast with the results obtained for Bacteroides nodosus (7), it was impossible to distinguish the pathogenic strains from the nonpathogenic strains of B. gingivalis in terms of the stability of the proteolytic enzymes. Cytotoxicity experiments revealed that all B. gingivalis strains had the same effect on Vero cells. The affected cells exhibited long filamentous tendrils and a thickening of the cellular membranes. In extreme cases, the cells were rounded and often free in the medium. On the other hand, culture supernatants of all strains were not toxic to leukocytes as measured by trypan blue exclusion.

Nutritional studies revealed no perfect correlation between the requirement for hemin or vitamin K₁ and virulence. The requirement for vitamin K₁ differs within the species B. gingivalis. The growth of pathogenic strain W83 was independent of the presence of vitamin K₁. Strain W50, used in the study of McKee et al. (10), is also infective in pure culture and does not require vitamin K₁. Analysis of polyacrylamide (8.5%) gel electrophoresis patterns indicated that, except for minor variations in the median section of the gel, the protein profiles were similar. No correlation for the presence of bands and the ability to cause monoinfection in the guinea pig could be established.

B. gingivalis is frequently isolated from patients with advanced chronic periodontitis. It seems that the pathogenicity of this species varies from strain to strain. In fact, some strains can infect an animal model when injected in pure culture, whereas other B. gingivalis strains require the presence of helper species to produce an infection. In this study, several strains were highly infectious upon pure-culture inoculation. The pathogenicity of these strains was not related to the source of isolation, because some virulent strains have been isolated from infected sites and some have been isolated from healthy periodontal sites. We also found that the strains from different sites of the same subject were similar with respect to virulence, as well as vitamin K₁ requirement. These observations are in accordance with the studies of Notten et al. (13). Their findings indicated that in the mouth of an individual, one B. gingivalis antibiotic type predominates and that different patients harbor different B. gingivalis antibiotypes.

The number of in vitro transfers did not seem to influence the pathogenicity of strains, suggesting the stability of the virulence factor(s). Kastelien et al. (6) have already demonstrated the pathogenicity of strain W83 in guinea pigs. Our results confirm theirs in that it took at least 10⁸ CFU/ml to infect the animal. Strain BH18/10, which has already been shown to exhibit a large quantity of extracellular polysaccharide material (G. H. Bowden and A. H. Holthuis, J. Dent. Res. 62:179, abstr. no. 89, 1983), and the freshly isolated RB46D-1 and RB22D-1 were the three strains which produced infection with the lowest number of CFU per milliliter.

The virulence of certain microorganisms can be related to their proteolytic activity; for example, Entamoeba histolytica (12) and Aeromonas salmonicida (14). The importance of proteolytic activity in black-pigmented Bacteroides species was recently emphasized. van Steenbergen and de Graaff (19), using different species, concluded that organisms which had the lowest proteolytic activity were also the least virulent in an animal model, whereas B. gingivalis (highest proteolytic activity) was the most virulent of black-pigmented Bacteroides species. Another study (M. E. Neiders, P. Chen, H. S. Reynolds, H. Suido, J. J. Zambon, and R. J. Genco, J. Dent. Res. 65:208, abstr. no. 351, 1986) showed that some B. gingivalis strains which were lethal in a mouse model exhibited a higher capacity to degrade synthetic and native substrates. Our results indicate that B. gingivalis strains can be separated into two groups. Pathogenic strains (except 22B4) demonstrated high collagenolytic and proteolytic activities, whereas nonpathogenic strains showed lower collagenolytic activity but a high proteolytic activity. McKee et al. (10) demonstrated that the virulence of B. gingivalis W50 was closely related to the availability of hemin. A strong proteolytic activity could thus allow the

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**TABLE 1. B. gingivalis strains and sources**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>No. of in vitro transfers&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>W83</td>
<td>Clinical specimen</td>
<td>&gt;30</td>
</tr>
<tr>
<td>BH18/10</td>
<td>Human periodontal pocket</td>
<td>&gt;30</td>
</tr>
<tr>
<td>33277</td>
<td>Human gingival sulcus</td>
<td>&gt;30</td>
</tr>
<tr>
<td>381</td>
<td>Human periodontal pocket</td>
<td>&gt;30</td>
</tr>
<tr>
<td>6/26</td>
<td>Human periodontal pocket</td>
<td>&gt;30</td>
</tr>
<tr>
<td>23A4</td>
<td>Human periodontal pocket</td>
<td>15</td>
</tr>
<tr>
<td>23B4</td>
<td>Human gingival sulcus</td>
<td>15</td>
</tr>
<tr>
<td>19A4</td>
<td>Human periodontal pocket</td>
<td>15</td>
</tr>
<tr>
<td>LB13D-3</td>
<td>Human periodontal pocket</td>
<td>5</td>
</tr>
<tr>
<td>RB22D-1, RB24M-2, RB46D-1</td>
<td>Human periodontal pocket</td>
<td>5</td>
</tr>
<tr>
<td>HW11D-5, HW24D-1</td>
<td>Human periodontal pocket</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Strains listed together were from the same patient.

<sup>b</sup> Approximate number of transfers on solid media from isolation to injection.

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**TABLE 2. Characteristics of B. gingivalis strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Conc&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Infectivity</th>
<th>Requirement</th>
<th>Collagenolytic activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Proteolytic activity&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB22D-1</td>
<td>4.0 × 10⁸</td>
<td>+++</td>
<td>Hemin</td>
<td>42</td>
<td>1.16</td>
</tr>
<tr>
<td>RB24M-2</td>
<td>6.5 × 10⁸</td>
<td>+++</td>
<td>Vitamin K₁</td>
<td>49</td>
<td>0.47</td>
</tr>
<tr>
<td>RB46D-1</td>
<td>5.0 × 10⁸</td>
<td>+++</td>
<td>-</td>
<td>42</td>
<td>0.38</td>
</tr>
<tr>
<td>W83</td>
<td>4.5 × 10⁸</td>
<td>+++</td>
<td>-</td>
<td>45</td>
<td>0.97</td>
</tr>
<tr>
<td>BH18/10</td>
<td>5.0 × 10⁹</td>
<td>+++</td>
<td>+</td>
<td>31</td>
<td>0.81</td>
</tr>
<tr>
<td>22B4</td>
<td>7.0 × 10⁹</td>
<td>+++</td>
<td>+</td>
<td>28</td>
<td>1.30</td>
</tr>
<tr>
<td>381</td>
<td>7.0 × 10⁹</td>
<td>+</td>
<td>+</td>
<td>18</td>
<td>1.13</td>
</tr>
<tr>
<td>HW24D-1</td>
<td>4.0 × 10⁹</td>
<td>+</td>
<td>+</td>
<td>19</td>
<td>1.14</td>
</tr>
<tr>
<td>19A4</td>
<td>1.0 × 10¹</td>
<td>+</td>
<td>+</td>
<td>20</td>
<td>1.34</td>
</tr>
<tr>
<td>33277</td>
<td>1.6 × 10¹</td>
<td>+</td>
<td>+</td>
<td>28</td>
<td>1.33</td>
</tr>
<tr>
<td>23A4</td>
<td>1.2 × 10¹</td>
<td>+</td>
<td>-</td>
<td>14</td>
<td>0.57</td>
</tr>
<tr>
<td>6/26</td>
<td>7.0 × 10⁰</td>
<td>+</td>
<td>+</td>
<td>22</td>
<td>0.91</td>
</tr>
<tr>
<td>LB13D-3</td>
<td>1.5 × 10⁰</td>
<td>+</td>
<td>+</td>
<td>30</td>
<td>0.61</td>
</tr>
<tr>
<td>HW11D-5</td>
<td>1.0 × 10⁰</td>
<td>+</td>
<td>+</td>
<td>17</td>
<td>1.35</td>
</tr>
</tbody>
</table>

<sup>a</sup> Minimum number of cells needed to infect in the case of a necrotic infection or maximum number of cells tested in the case of negative or light abscess formation.

<sup>b</sup> Expressed as percentage of total radioactivity added in the assay (2 h); bacterial cells from early stationary phase. Under identical conditions, commercial collagenase degraded 55% of the labeled substrate in 2 h.

<sup>c</sup> Optical density at 520 nm (4 h); supernatants from a 4-day culture.
release of heme from iron-transporting plasma proteins (1) and subsequently favor the development of the infection.

Other factors could be responsible for the initiation of an experimental infection. van Steenbergen et al. (20) showed that strain W83 was more resistant to phagocytosis than were nonvirulent strains. More recently, the presence of two serogroups within B. gingivalis was proposed (J. G. Fisher, J. J. Zambon, P. Chen, and R. J. Genco, J. Dent. Res. 65:816, abstr. no. 817, 1986). Serogroup A strains were less virulent than serogroup B strains, which were lethal in a mouse model. Our pathogenic strains would probably fall within serogroup B of Fisher et al. However, in contrast with our results, their pathogenic strains (serogroup B) were always isolated from patients with severe periodontitis, whereas their serogroup A strains (avirulent) were found in sites of healthy subjects.

Our study thus confirmed the presence of two distinct groups within the B. gingivalis species: virulent and avirulent strains. Although virulence factors responsible for pathogenicity may be produced to some degree by all B. gingivalis strains, the amount or level of activity of these factors found in vivo could determine whether a strain is pathogenic in pure culture.

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LITERATURE CITED


