Bactericidal Activity of a Granule Extract from Human Polymorphonuclear Leukocytes Against Bacteroides Species

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The microbicidal activity of an acetate extract of human polymorphonuclear leukocyte granules was tested against Bacteroides fragilis, Bacteroides vulgatus, Bacteroides distasonis and Bacteroides thetaiotaomicron. All strains tested were killed by the extract, and there were no significant differences between the different Bacteroides species.

The importance of Bacteroides species in a wide range of serious infections is now unequivocally established, and the prominence of Bacteroides fragilis as a cause of suppuration and sepsis suggests that this species of Bacteroides possesses unique virulence factors. However, the attributes of virulence constitute a set of complex issues; and very little is known of the mechanisms by which obligate anaerobes are dealt with by polymorphonuclear leukocytes (PMN). PMNs maintain a formidable defense system against bacterial invaders. After phagocytosis, bacteria are exposed to potent antimicrobial mechanisms. These mechanisms are broadly divided into two categories: the oxygen-dependent microbicidal systems and those not requiring oxygen for activity (13). Within an abscess, low oxygen tension restricts killing by PMN to the latter mechanisms; this may be the reason for reduced killing of certain facultative bacteria under anaerobic conditions (8, 15, 16, 17, 22, 26). However, the killing of anaerobic bacteria, including Bacteroides species, is not impaired (3, 8, 15).

The purpose of the present study was to assess the effectiveness of the PMN oxygen-independent bactericidal activity against several species of Bacteroides and, as animal-passaged B. fragilis has been reported to be more resistant to opsonophagocytic killing than laboratory-passaged strains (23), to determine whether this finding could be explained by increased resistance of B. fragilis to intracellular killing mechanisms.

The use of a neutrophil granule extract permitted direct study of the interaction of bacteria and oxygen-independent microbicidal systems, without interference of respiratory burst products and complexities inherent in the interpretation of phagocytic studies.

PMN were obtained from citrated venous blood of healthy donors by the method of Boyum, modified for large volumes (4). The final cell suspension contained 95% PMN. The cells were suspended in ice-cold sucrose (0.34 M) and homogenized to greater than 85% disruption in a Teflon glass homogenizer. The granule fraction was obtained by differential centrifugation, and an acetate extract was prepared from the granules as described by Rest et al. (21). A 10^5-PMN amount yielded ca. 1 µg of granule protein. Portions (0.4 ml) were stored at −70°C and thawed immediately before use.

Bacteroides strains were isolated from clinical material, and identification to the species level was carried out according to established criteria (7). The strains were maintained in an anaerobic glove box on blood agar and subcultured every 6 to 8 weeks. For several strains, multiple subculture was avoided by storing primary isolates in skim milk at −70°C. Bactericidal assays were carried out in sterile Linbro tissue culture trays (Flow Laboratories, Inc., Hamden, Conn.). The bacteria were grown to the logarithmic phase in Schaedler broth and diluted through anaerobic saline solution; 2 × 10^6 to 4 × 10^6 bacteria were inoculated into 0.1 ml of aerobic granule extract dilutions. These dilutions were prepared in tryptone-saline solution, pH 5.0. The tissue culture trays were transferred into the anaerobic glove box and incubated for 2 h at 37°C. After incubation, duplicate 20-µl portions were spread onto blood agar plates. This procedure was carried out aerobically. The plates were incubated anaerobically for 48 h, and the number of CFU (viable bacteria) were determined using an automatic colony counter (model 880; Artek Systems Corp., Farmingdale, N.Y.). In some experiments, the time of exposure of bacteria to oxygen was minimized by carrying out all procedures under strictly anaerobic conditions, except for the final transfer onto blood agar.
plates. Because similar results were obtained under either condition, the data presented are those obtained from experiments with less control of oxygen exposure. Between four and eight determinations of the 50% lethal dose (LD₅₀) and the 90% lethal dose (LD₉₀) of the extract against each strain were carried out. The mean values for each species are given in Table 1. All strains were killed by the extract. The LD₅₀ and LD₉₀ values for the species were not significantly different (P > 0.05). To determine whether the bacterial populations were uniformly sensitive to the granule extract, the LD₅₀/LD₉₀ ratio was calculated. A value of 1.8 indicates total uniformity of sensitivity between the first 50% and the next 40% of the population. The calculated values for the LD₅₀/LD₉₀ ratio given in Table 1 indicate that the four species of Bacteroides all consist of uniformly sensitive populations of bacteria, with no large subpopulations of bacteria which have increased resistance.

An analysis of variance in LD₅₀ values was carried out, both between species and within each species, and the results are given in Table 2. The within-species analysis demonstrates that individual strains differ significantly from each other (p < 0.001). However, the between-species analysis shows that the mean values for the four species were not significantly different (p > 0.05). This indicates that the variation in sensitivity to the granule extract between the strains of any one species of Bacteroides is as large as the variation between the four species tested.

The polysaccharide capsular material of B. fragilis has been reported to be lost with in vitro passage (12) with commensurate loss of virulence (23). To determine whether fresh clinical isolates of abscess-borne B. fragilis were more resistant to the PMN granule extract, we tested three such strains after no more than two passages on blood agar. Their mean LD₅₀ values were 5.2, 5.3, and 4.2, and their LD₅₀/LD₉₀ ratios were 1.94, 1.76, and 1.99, respectively. These values are not significantly different from the pool values for B. fragilis (Table 1). All of these strains produced capsules after growth in Schaedler broth, as detected by the India ink wet-mount technique.

The presence of capsular polysaccharide has been associated with the increased virulence of B. fragilis (18, 19). The great majority of B. fragilis strains are capable of synthesizing a capsular polysaccharide, whereas non-B. fragilis species possess such a capsule less frequently (1, 11, 14). The results of this study indicate that encapsulation of B. fragilis may not confer upon this species increased resistance to oxygen-independent killing mechanisms of human PMN.

Facultative gram-negative bacteria are also killed by granule extracts (6, 21, 24, 25). Their susceptibility to oxygen-independent killing mechanisms of PMN varies greatly. The LD₉₀ of clinical isolates of Escherichia coli range from 5 to 100 µg/ml (unpublished data), whereas the range for B. fragilis, given the same granule extract, is 3.3 to 8.7 µg/ml. This variation among facultative gram-negative strains of bacteria has been attributed to the structural organization of the bacterial cell surface, in particular, the lipopolysaccharide chemotype (6, 21, 24), and to differences in binding of the bactericidal proteins to the cell surface (25). With the granule extract as a probe for structural variability, the data suggest that there may be little variation among clinical isolates of Bacteroides, both between strains within species and between different species, in cell wall structures associated with conferring resistance to intracellular oxygen-dependent killing mechanisms.

The importance of the oxygen-dependent bactericidal system of PMNs is well established (2, 5, 13). Chronic granulomatous disease is a clinical disorder of this bactericidal system in which

<p>| TABLE 1. Killing of Bacteroides species by granule extract |
|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Species</th>
<th>Number of strains</th>
<th>Mean LD₅₀</th>
<th>Mean LD₉₀</th>
<th>LD₅₀/LD₉₀ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. fragilis</td>
<td>25</td>
<td>5.6</td>
<td>9.9</td>
<td>1.76</td>
</tr>
<tr>
<td>B. vulgatus</td>
<td>5</td>
<td>4.7</td>
<td>9.1</td>
<td>1.93</td>
</tr>
<tr>
<td>B. distasonis</td>
<td>7</td>
<td>6.4</td>
<td>11</td>
<td>1.71</td>
</tr>
<tr>
<td>B. thetataomicron</td>
<td>8</td>
<td>5.2</td>
<td>10.1</td>
<td>1.94</td>
</tr>
</tbody>
</table>

*Granule extract (µg extract protein/ml) required to kill 50% or 90% of the bacterial inoculum (2 × 10⁴ to 4 × 10⁴ bacteria) after 2 h of incubation. Four to eight determinations were carried out for each strain.

<p>| TABLE 2. An analysis of variance in LD₅₀ of Bacteroides species |
|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Species</th>
<th>n* squares of LD₅₀</th>
<th>Variance ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-species comparison</td>
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<td></td>
</tr>
<tr>
<td>B. fragilis</td>
<td>25</td>
<td>7.0</td>
<td>3.5</td>
</tr>
<tr>
<td>B. vulgatus</td>
<td>5</td>
<td>10.7</td>
<td>6.4</td>
</tr>
<tr>
<td>B. distasonis</td>
<td>7</td>
<td>8.0</td>
<td>4.3</td>
</tr>
<tr>
<td>B. thetataomicron</td>
<td>8</td>
<td>14.9</td>
<td>7.3</td>
</tr>
<tr>
<td>Pool</td>
<td>45</td>
<td>9.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Between-species comparison</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroides spp.</td>
<td>4</td>
<td>15.4</td>
<td>1.73</td>
</tr>
</tbody>
</table>

*For within-species comparison, n = number of strains; for between-species comparison, n = number of species.
PMNs are unable to mount a respiratory burst (2, 5, 9, 10) and are defective in intracellular killing of a variety of microorganisms (2, 9, 10, 20). However, the microorganisms capable of causing infections in chronic granulomatous disease patients appear to be predominantly facultative anaerobic bacteria and fungi. In a survey over the period from 1968 to 1976 and encompassing 125 chronic granulomatous disease patients from whom reasonable information in infecting organisms was available, 253 bacterial and fungal isolates were described, none of which were obligately anaerobic (10).

Our finding that Bacteroides species are killed by the oxygen-independent antibacterial mechanisms of PMN provides an explanation for this clinical observation.

LITERATURE CITED