Adherence of *Bacteroides fragilis* In Vivo

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The ability of the encapsulated species *Bacteroides fragilis* to adhere to rat peritoneal mesothelium was compared to the adherence of unencapsulated strains of *Bacteroides* (*B. distasonis*, *B. vulgatus*, *B. thetaiotaomicron*, *B. ovatus*, and *B. "other"*). Adherence was assayed by attaching plexiglass plates containing 8-mm holes to the peritoneal mesothelium of anesthetized rats. Cell suspension (0.25 ml) was incubated in each well, after which the suspension was aspirated, and a 4-mm punch biopsy was removed. Viable organisms adhering to the biopsy specimen were enumerated by plate count following washing of the biopsy tissue. It was found that *B. fragilis* adhered significantly better to mesothelial tissue (10^6-10^7 colony-forming units per biopsy) than unencapsulated species of *Bacteroides* (10^1-10^2 colony-forming units per biopsy). This effect was not due to differential oxygen sensitivity of the various inocula. Immunization of rats with capsular polysaccharide did not demonstrate decrease in the adherence of *B. fragilis*; however, preincubation of the mesothelium with purified capsular polysaccharide resulted in a substantial reduction in adherence. These results indicate that *B. fragilis* adheres to rat peritoneal mesothelium better than unencapsulated species and suggests that the capsular polysaccharide of *B. fragilis* plays some role in this increased adherence.

*Bacteroides fragilis* is the predominant organism found in intra-abdominal sepsis, and it is the most common blood culture isolate in patients with anaerobic bacteremia (6). Although the prevalence of this organism in certain types of infection was established 80 years ago, little has been known about its pathogenic properties.

Recent studies have shown that some strains of *B. fragilis* have a polysaccharide capsule (9, 11). It was also noted that many of the organisms classified as *B. fragilis* had minor differences in biochemical reactions which led to subspecies designations, i.e., *B. fragilis* subsp. *fragilis*, *B. fragilis* subsp. *ovatus*, *B. fragilis* subsp. *vulgatus*, *B. fragilis* subsp. *distasonis*, *B. fragilis* subsp. *thetaiotaomicron*, and subsp. "other." Recent deoxyribonucleic acid homology analyses indicate major differences in guanine plus cytosine ratios of these subspecies, leading to a reclassification of the former subspecies into distinct species (1). Relevant to our work with the capsular polysaccharide is the observation that only one of the five former subspecies of *B. fragilis* harbors a readily detectable capsule. This organism is *B. fragilis* subsp. *fragilis* or, according to the new classification scheme, *B. fragilis*. It is of interest that 70 to 80% of clinical isolates of all the organisms formerly classified as *B. fragilis* are this encapsulated species (17). Experimental animal work has shown that the capsule of *B. fragilis* plays a distinctive role in abscess formation in a rat model of intra-abdominal sepsis (15). Thus, encapsulated *B. fragilis* clearly stands apart from the other former subspecies in terms of pathogenic potential by both clinical data and experimental animal studies.

For an organism to invade tissue, it must first adhere to a surface cell. Previous investigations have shown that selective bacterial attachment to certain cells may represent a virulence factor (2–5, 7). The present study was designed to study adherence to peritoneal mesothelial cells by encapsulated *B. fragilis* compared with the other former subspecies in this group.

**MATERIALS AND METHODS**

**Cultures.** A total of 14 strains of *Bacteroides* were used for these studies. Cultures were obtained from the stock culture collection of the Infectious Disease Research Laboratory, Boston Veterans Administration Hospital, and included *B. fragilis* (8), *B. distasonis* (2), *B. vulgatus* (1), *B. thetaiotaomicron* (1), *B. ovatus* (1), and *Bacteroides* "other" (1). All cultures were maintained in the lyophilized state. Prior to use, each strain was reconstituted in brain heart infusion broth (Scott Laboratories, Fiskeville, R.I.) and then subcultured onto Brucella base blood agar containing...
hemin (5 μg/ml) and menadione (10 μg/ml) (BMB). Inocula for assay were prepared by growing each test strain for 18 h at 37°C in prereduced brain heart infusion broth supplemented with hemin and menadione. Following incubation, the cells were pelleted by centrifugation at 10,000 × g for 30 min at room temperature, washed once in phosphate-buffered saline (PBS), centrifuged, and resuspended in PBS to give a final concentration of approximately 10^6 colony-forming units (CFU)/ml as determined spectrophotometrically at 550 nm according to prior viable cell density versus optical density curves. Inocula were also assayed before and after use for viable cell density by placing a portion of the cell suspension into an anaerobic chamber and making serial decimal dilutions, using prereduced VPI dilution salts (8). Portions of 0.1 ml of each dilution were plated on BMB and incubated for 48 h at 37°C within the anaerobic chamber. Following incubation, colonies on plates were enumerated. All counts were expressed as log_{10} CFU/ml.

Animals. Male Wistar rats (Charles River Laboratories, Wilmington, Mass.) weighing 250 to 300 g were used for all experiments. Animals were caged separately and maintained on chow (Ralston Purina, St. Louis, Mo.) and water ad libitum.

Immunization. For certain experiments, rats were immunized with purified B. fragilis capsular polysaccharide prior to use. Preparation of the purified antigen has been described elsewhere (9, 10). Immunization was accomplished by subcutaneous injection of 10 μg of capsular polysaccharide emulsified in Freund complete adjuvant containing 10 μg of methylated bovine serum albumin. Injections were made at days 1, 7, 14, and 28. Intervening injections of 10 μg of capsular polysaccharide with 10 μg of methylated bovine serum albumin were given at days 3, 5, 9, and 13. Antibody levels were assayed, using the radioactive antigen-binding assay as described by Kasper (9, 12). Antibody concentration was expressed as micrograms of antibody per milliliter of serum.

Immune sera. Immune sera were prepared to the capsular polysaccharide in rabbits as described previously (9, 11).

Adherence assay. Animals were anesthetized with 0.25 ml of nembutal (50 mg/ml), and the abdomen was shaved. An anterior midline incision was made from the base of the sternum to the pelvis. Two lateral incisions were also made at the proximal and distal ends of this midline incision. The skin was detached from the underlying musculature, and the two flaps of muscle were folded back to expose the peritoneal mesothelium. Plexiglas plates measuring 1.5 × 4 cm with three 8-mm holes were attached to each flap with clamps so that the mesothelium formed the bottom of each well. Inocula of 0.25 ml were placed into each well and incubated in situ for 90 min. Following incubation, the cell suspension was aspirated, the well was rinsed once with 0.25 ml of PBS, and a 4-mm punch biopsy was obtained, using a sterile dermal punch. The tissue biopsy was washed six times with 5 ml of PBS, using gentle shaking, and the tissue was then placed into an anaerobic chamber. The tissue was homogenized using a hand homogenizer, and portions of serial 10-fold dilutions were plated on BMB to give final dilutions of 10^-1, 10^-2, and 10^-3. Portions of 1.0 and 0.1 ml of the final wash solution were plated on BMB to detect washing efficiency. All plates were incubated at 37°C for 48 h within the anaerobic chamber. Following incubation, colonies were enumerated and identified by established criteria (8). Colony counts for tissue specimens were expressed as log_{10} CFU/biopsy, and counts for wash fluid were expressed as log_{10} CFU/ml. Any sample showing a final wash containing viable bacteria was excluded from analysis.

Electron microscopy. Selected tissue biopsy specimens were placed in 5% glutaraldehyde after the previously described washing procedure. These specimens were dehydrated in increasing concentrations of alcohol and subjected to critical-point drying. Specimens were then fixed to metal planchets, coated with platinum, and examined using a JEOL-50A scanning electron microscope (JEOL, Inc., New Bedford, Mass.). Cell cultures for negative staining were grown overnight in peptone yeast glucose broth and spun at 15,000 × g for 30 min, and the pellet was resuspended in 0.2% phosphotungstic acid buffered to pH 7.4 with 1 N potassium hydroxide. Samples were then coated onto Formvar-treated copper grids, dried, and examined using a JEM 100B electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan).

RESULTS

Adherence of Bacteroides. The adherence assay was performed a total of 20 times using eight strains of encapsulated B. fragilis and 17 times using six strains of other Bacteroides species. The latter group included B. distasonis, B. vulgatus, B. ovatus, B. thetaotaomicron, and B. "other." The results were similar for the eight encapsulated strains and for the six other strains; therefore, the data were combined for each of the two groups (Table 1).

It was found that B. fragilis adhered significantly better than the other Bacteroides, with a mean adherence of 10^4.00 CFU/biopsy versus 10^3.07 CFU/biopsy for the other Bacteroides. These differences were found to be significant at the 0.001 level (Student's t test). It should be noted that mean inocula sizes for these two groups were virtually identical (10^7.98 versus 10^8.01 CFU/ml).

Since differential sensitivity to oxygen between strains could explain the difference in adherence, the effect of oxygen on adherence was quantitated (Table 1).

| Table 1. Comparison of adherence of B. fragilis and other Bacteroides |
|---------------------------------|-----------------|-----------------|-----------------|
| Bacteroides strain              | No. of strains | No. of observations | Mean inoculum size (log_{10} CFU/ml) ± 1 SE | Mean adherence (log_{10} CFU/biopsy) ± 1 SE |
| B. fragilis                     | 8               | 20               | 8.01 ± 0.09     | 3.00 ± 0.06     |
| Other†                         | 6               | 17               | 7.98 ± 0.12     | 1.07 ± 0.25     |

* SE, Standard error.
† Includes B. distasonis, B. vulgatus, B. ovatus, B. thetaotaomicron, and B. "other."
results, a comparison of viable cell counts of inocula was made before and after use in the adherence assay. The results showed no decrease in mean viable cell density for either test group during the assay period. The mean counts at the beginning of the assay for a representative group of eight strains including B. fragilis and other Bacteroides were $10^{8.35} \pm 0.07$ CFU/ml compared with $10^{8.35} \pm 0.07$ CFU/ml at the end of the assay.

Surface adherence of microorganisms to mesothelial tissue was confirmed by scanning electron microscopy (Fig. 1). B. fragilis was readily detected, whereas cells of other strains of Bacteroides were only infrequently observed. It was noted that, although B. fragilis was far more prevalent than the other Bacteroides species on examined tissue, they were nevertheless quite scarce. On the basis of cell counts and biopsy surface area, it was estimated that there were approximately 80 bacteria per mm². Scanning electron micrographs tend to corroborate this estimate.

Inhibition of adherence. The demonstration that B. fragilis adhered to mesothelial tissue significantly better than the other strains of Bacteroides suggested that this microbe possessed a factor responsible for adherence which was not present in other Bacteroides. Efforts were therefore directed towards inhibiting adherence by immunization of animals with capsular polysaccharide prior to the adherence assay. Capsular antibody levels in these animals prior to testing were 51 to 84 µg/ml. The results (Table 2) indicate that, despite the high levels of anticapsular antibody in the sera, there was no significant reduction in adherence of B. fragilis.

Additional experiments were conducted in which B. fragilis were preincubated with rabbit hyperimmune sera to the capsular polysaccharide. These results were compared with those using preincubation with serum from the same rabbits prior to immunization. A reduction in adherence was noted with both immune and preimmune sera. Subsequent testing revealed a decrease in the viable counts of the inocula during serum incubation from $10^{8.0}$ to $10^{7.5}$ CFU/ml. These data indicate that serum bactericidal factors unrelated to anticapsular antibody were present in normal rabbit sera, making it difficult to assess the role of antibody in adherence inhibition.

Efforts were next directed towards inhibiting...
adherence in vivo by pretreatment of the mesothelium with the purified capsular polysaccharide or sugars, some of which are known to be present in the polysaccharide material. Sugars tested in these experiments were D-glucose, L-rhamnose, N-acetyl-D-mannosamine, and N-acetyl-D-galactosamine. The results (Table 3) indicate that preincubation of the tissue with 200 µg of capsular polysaccharide for 30 min prior to adding the bacterial inoculum resulted in a decreased ability of B. fragilis to adhere. Counts were reduced in these experiments from a mean of $10^{11}$ CFU/biopsy to $10^{2.2}$ CFU/biopsy, a decrease of approximately 90%. This difference was found to be significant at the 0.05 level (Student’s t test). In contrast, preincubation with 1,000 to 3,000 µg of specific sugars from the polysaccharide capsule failed to significantly reduce B. fragilis adherence.

**Negative staining.** Since adherence of some other microorganisms has been related to the presence of pili, consideration of this factor was necessary. Negatively stained cells from several B. fragilis strains were examined by transmission electron microscopy. A representative micrograph (Fig. 2) shows that no pili are present. This strain (ATCC 23745) and all others examined showed no evidence suggesting the presence of pili. A strain of Neisseria gonorrhoeae, colony type T2, was examined as a positive control.

**DISCUSSION**

The present study implicates bacterial adherence as a possible factor in the virulence of B. fragilis. The results also suggest that the ability of B. fragilis to adhere to mesothelial tissue resides in the capsular polysaccharide of this microbe. Interest in the capsular polysaccharide as a virulence factor stems from previous studies (15) in which it was shown that the presence of this material enhanced the abscess-forming capacity of Bacteroides in a model of intra-abdominal sepsis. Subsequent research (12) has also shown that immunization of rats to the capsular polysaccharide of B. fragilis prior to challenge with homologous or heterologous strains of Bac-

| TABLE 2. Results of adherence in animals immunized prior to assay with B. fragilis |
|-----------------------------------|----------------|----------------|
| Expt no. | Log_{10} CFU/ml (inoculum size)** | Log_{10} CFU/biopsy (adherence)** | Antibody (µg/ml) |
|----------|----------------|----------------|
| 1        | 8.28           | 3.00           | 51              |
| 2        | 8.11           | 2.60           | 64              |
| 3        | 8.23           | 2.78           | 61              |
| 4        | 8.76           | 3.00           | 84              |

**TABLE 3. Inhibition of adherence of B. fragilis**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Amt (µg)</th>
<th>No. of observations</th>
<th>Log_{10} CFU/biopsy (mean adherence ± SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsular polysaccharide</td>
<td>200</td>
<td>9</td>
<td>2.2 ± 0.20</td>
</tr>
<tr>
<td>α-D-glucose</td>
<td>3,000</td>
<td>2</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>α-L-rhamnose</td>
<td>3,000</td>
<td>2</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>N-acetyl-D-mannosamine</td>
<td>1,000</td>
<td>2</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>N-acetyl-D-galactosamine</td>
<td>3,000</td>
<td>2</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td></td>
<td>3.1 ± 0.2</td>
</tr>
</tbody>
</table>

* SE, Standard error.

**FIG. 2.** Electron micrograph of negatively stained B. fragilis. No pili were found on any of the examined strains (×15,000). streptococcal pyogenes adheres better to pharyngeal tissue compared with E. coli, which attaches better to bladder epithelial cells (2, 3). These observations on site specificity correlate well with clinical observations concerning the relative frequency of these organisms as infecting pathogens at the two anatomical locations. Similar observations correlating adherence and
the properties of bacteria incriminated as pathogens have been noted for piloted versus nonpiloted gonococci adhering to urethral mucosa (18) and for E. coli possessing the K-88 antigen attachment to intestinal epithelium. It is noteworthy that cell type appears to be an important factor in these adherence models. Our experiments with B. fragilis were performed with rat peritoneal mesothelium because of the documented role of this organism in intra-abdominal sepsis both clinically and in the Wistar rat model (16, 19).

The present research addressed the question of whether a bacterial outer cell membrane constituent, the capsular polysaccharide, is responsible for adherence by attempting to block attachment by preincubation with the capsular polysaccharide or by immunization of the animals to capsular polysaccharide. It was noted that animals immunized against the capsular polysaccharide of B. fragilis showed no decrease in the number of adherent microbes. In addition, pretreatment of the tissue with capsular polysaccharide resulted in a significant reduction in the number of adherent microbes recovered from tissue biopsies. These results suggest that the capsular polysaccharide is involved in adherence, but that circulating serum antibody to this material is not effective in preventing microbial adherence to mesothelium. Previous observations (13), in which immunization with capsular polysaccharide was shown to reduce the percentage of animals with abscess, suggest that the protective effect of antibody is related to other host defense factors such as opsonization or phagocytosis.

Additional experiments in which attempts were made to block adherence using specific carbohydrates known to be part of the capsular polysaccharide were ineffective. These data indicate that the tissue-binding ability of the capsular polysaccharide resides in the tertiary structure of the capsular material. It is clear that further study of this capsular polysaccharide and those of other related species will be necessary for a complete understanding of the role of the capsular polysaccharide of B. fragilis in potentially infected sites, since adherence is only one of several possible virulence factors.

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LITERATURE CITED