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Immunochemical Investigations of Cell Surface Antigens

of Anaerobic Bacteria

ANNUAL REPORT

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Abstract of Progress since January 15, 1977

ANNUAL REPORT
(progress since report of January 15, 1977)

A. Background

In the past 6 months, several important studies have been completed which
will guide future studies on vaccine development to prevent infections due to
anaerobic bacteria. First it would be helpful to summarize briefly the progress
of these investigations over the past 3½ years.

Our studies have focused on the immunochemical characterization of
Bacteroides fragilis and Bacteroides melaninogenicus

1. Bacteroides fragilis

The outer membrane of gram-negative bacteria is immunologically the
most important structure of the cell because of its accessibility to host defense
mechanisms. By electron micrographic studies, it has been shown that Bacteroides
fragilis has a cell envelope typical of gram-negative bacteria. The outer mem-
brane of this envelope was found to consist of protein, two polysaccharides
bound to lipid in various degrees, and loosely bound lipid. The protein component
of the outer membrane had a distinct pattern when studied by polyacrylamide gel
electrophoresis. Similar peptide band patterns were seen in all strains of
Bacteroides fragilis subspecies fragilis studied, but this pattern was distinct
from the pattern found in the outer membranes of other subspecies. The carbohy-
drates and the protein components were both associated with subspecies-specific
immunoprecipitins. We have proposed that this antigenic complex represents
the outer membrane of B. fragilis in its native state and that the immunologically
important antigens of this organism are associated with this complex.

Bacterial lipopolysaccharides extracted from Bacteroides fragilis, sub-
species fragilis lacked 2-keto-3-deoxyoctonate and heptose, sugars which make
up part of the inner core of most bacterial endotoxins. Over 98% of the lipid
portion of this material could be removed easily with chloroform-methanol and
alcohol, a finding which indicates a loose association between the polysaccharide
and lipid moieties. The lipopolysaccharides caused gelation of limulus lysate
at a concentration significantly higher than that for the endotoxin of Salmonella
typhi. None of the extracts was lethal in 10-day-old chick embryos at doses
of larger than 200 ug per egg, whereas the endotoxin of Neisseria meningitidis
was lethal at a dose of 1.2 ug per egg. The local Shwartzman reaction could
not be induced by levels of B. fragilis endotoxin of up to 1,000 ug per rabbit,
whereas a (control) endotoxin of S. typhi induced this phenomenon at a level
of 3 ug per rabbit. Intact oxygen-killed B. fragilis failed to provoke the
the local Shwartzman reaction at dose of 2,500 ug. These results indicate
that B. fragilis has a lipopolysaccharide different from that of most gram-
negative bacteria. Although it retains some of the chemical biologic properties
of classical endotoxin, it seems to lack others. This observation may have
significant clinical implications, in view of the unique spectrum of disease
caused by these bacteria.

A large-molecular weight capsular polysaccharide was isolated from strains
of Bacteroides fragilis subspecies fragilis. By means of electron microscopy
and staining with ruthenium red, the thick polysaccharide capsule was also
visualized. With use of a radioactive antigen-binding assay, antibody to this
capsular polysaccharide was demonstrated in antisera prepared in rabbits to
each of eight strains of B. fragilis fragilis. Antibody of similar specificity
was not found in antisera prepared to Bacteroides melaninogenicus or to strains
of Bacteroides fragilis subspecies vulgatus and Bacteroides fragilis subspecies
distasonis; such antibody was found in antisera to only one of two strains of

culdocentesis showed significantly greater changes in antibody concentration than corresponding sera obtained from women with gonococcal or non-gonococcal pelvic inflammatory disease when B. fragilis ss. fragilis was not isolated from the cul-de-sac.

These data support the bacteriologic evidence that B. fragilis is more important an etiologic agent in acute non-gonococcal than in gonococcal pelvic inflammatory disease. B. fragilis plays a significant role in acute pelvic inflammatory disease and can be involved early in the infectious process.

2. Bacteroides Melaninogenicus

Morphologic study of Bacteroides melaninogenicus subspecies asaccharo-lyticus by electron microscopy disclosed the presence of a capsule and a cell wall structure otherwise typical of a gram-negative organism. An outer membrane complex was isolated with use of gentle methods. Relative purity of the preparation was confirmed by electron microscopy and by the formation of a single band in a sucrose density gradient. Gel chromatography was used for separation of the major components of the membrane. Antigenicity of the first component, a protein-polysaccharide complex, was demonstrated by agar gel diffusion. Further purification of this fraction showed antigenicity of the polysaccharide component, which cross-reacted with antiserum to another strain of the same subspecies. This component probably represents the capsular antigen and may prove to be the basis for serogrouping. The second membrane fraction differed chemically from the first fraction and represents the lipo-polysaccharide component of the outer membrane. Notably, this component lacks 2-keto-3-deoxyoctonate, one of the backbone suguars of aerobic, gram-negative lipopolysaccharides, as does B. fragilis.

B. Introduction

In the past 6 months, our investigations have been directed toward potential vaccine development using the capsular polysaccharide from B. fragilis in a rat model of intra-abdominal abscess formation.

As reviewed previously, one difference between the various subspecies of B. fragilis is that B. fragilis ss. fragilis contains a capsule which is seldom found in the strains of the other subspecies.

Previous studies have employed a model of intra-abdominal sepsis in which a complex fecal inoculum containing obligate anaerobes and facultative bacterial species was implanted into rats (1-4). Initial observations revealed a biphasic disease. The first phase was characterized by an acute peritonitis in all animals and a 43% mortality within the first four days. E. coli and enterococci were the predominant isolates from the peritoneal exudates and E. coli was found in 90% of blood cultures. Recipients surviving this initial phase uniformly developed intra-abdominal abscesses by the seventh post-opera-tive day. This second stage revealed a shift in the predominant microbes at the infected site, with B. fragilis and Fusobacterium being the numerically dominant isolates in the abscesses. Subsequent experiments suggested that microbial synergy between a facultative species and an anaerobic species was responsible for abscess formation. In particular, an unencapsulated strain of B. fragilis failed to produce abscesses when implanted alone, but 87-100% of animals receiving a combination of this B. fragilis strain and E. coli, or B. fragilis and enterococci, developed typical loculated purulent collections. The pathogenic potential of encapsulated and unencapsulated B. fragilis strains were compared using this same rat model of intra-abdominal abscess formation. It was found that implantation of encapsulated B. fragilis alone resulted in abscesses in most recipients, whereas unencapsulated strains seldom pro-duced this effect unless combined with another organism (4). Subsequent studies suggested that the abscess potentiating ability of encapsulated

B. fragilis was related to the capsular polysaccharide. Implantation of
200 ug of purified capsular material alone produced abscesses in the majority
of animals tested (4).

The rat model data has been detailed in prior annual reports and is
already published (see C.V. in proposal).

C. Progress towards vaccine development using the rat intraabdominal
sepsis model

We have studied the capacity of capsular polysaccharide - vaccine
induced antibody to protect animals from intra-abdominal abscess formation.
The protocol used for these studies is outlined in Table 1.
After initial serum was obtained from 5 wk old Wistar rats, the animals
were vaccinated three times a week for two weeks and given a booster in the
third week. Four weeks after the initial vaccine was administered the
animals were challenged intraperitoneally with various test microbes. Serum
was also obtained at this time. One week later the animals were sacrificed
and studied. Both histologic and gross examination of abscesses was done
and abscess material was cultured.

Table 1: Experimental Design - Protocol for Immunization of Rats.

Day	Procedure
0	bleed, immunize
3	immunize
5	immunize
9	immunize
11	immunize
13	immunize
20	immunize
28	bleed and challenge
35	sacrifice

Animals were vaccinated with one of three regimens. (Table 2)

Table 2: Experimental Design - Vaccine Constituents.

Immunize with:

1. 10 µg B. fragilis capsular polysaccharide (0.1ml) IM, or

2. 10 µg B. fragilis capsular polysaccharide (0.1ml) + 10 µg methylated
    bovine serum albumin (0.1ml) + 0.2ml complete Freund's adjuvant
    IM on 0, 9 day, and

    10 µg B. fragilis capsular polysaccharide (0.1ml) +
    10 µg methylated bovine serum albumin (0.1ml)
    IM on the remainder of the days, or

3. 30 ug B. fragilis outer membrane IM.

The first group received 10 ug of capsular polysaccharide on each day of the outlined protocol (Table 1). A second group received 10 ug polysaccharide combined with an equal quantity of methylated serum bovine albumin in an equivalent volume of complete Freund's adjuvant on the first day of each of the first two weeks of immunization. On all other days the animals received 10 ug methylated bovine serum albumin combined with 10 ug capsule. The third group of rats received the outer membrane of strain 23745 B. fragilis ss. fragilis, the strain from which the capsular material was obtained. This outer membrane contains capsular polysaccharide, distinct membrane proteins (which have been shown to be immunologically active), lipopolysaccharide, and loosely bound lipid.

Table 3: Experimental Design - Challenge

On the 28th day after beginning immunizations challenge with:

1. Sterile cecal contents
2. BaSO$_4$
3. Test microbes

Intraperitoneal insertion in rats

Sacrifice at 35 days after beginning immunization

At the time of challenge after serum was obtained for antibody measurement, antibody to the capsular antigen was quantitated in the radioactive antigen binding assay, described in the principal investigator's past work on this subject (5,6). A gelatin capsule, which contained 10% barium sulfate, sterile cecal contents and various test microbes standardized to give 10^8 total bacteria, was inserted intraperitoneally (Table 3). The combination had been shown previously to induce abscesses in animals; however, the microbes by themselves without barium sulfate or sterile cecal contents do not induce abscesses nor do sterile cecal contents and barium sulfate without appropriate microbes. The only thing varied in these studies were the various microbes that were inoculated into the rat peritoneum and the vaccine. Seven days after implantation the animals were sacrificed and bacteriologic and anatomic studies were performed on the abscess material.

In the non-immunized central groups (Table 4) all rats had very low concentrations of antibody at the time of challenge. 90% of those animals given an unencapsulated B. fragilis strain with an enterococcus developed abscesses and no encapsulated B. fragilis were recovered from these abscesses. Greater than 80% of rats given either of 2 strains of encapsulated B. fragilis, either alone or with an enterococcus developed culture positive abscesses. 25-40% of rats implanted with a microbially complex mixture of rat cecal contents died, and all survivors developed abscesses from which B. fragilis was recovered from 7 to 17.

Table 4: Non-Immunized Controls.

Challenge	Mean ± SD Antibody Concentration (at challenge) (ug/ml)	Abscess in Survivors	B. fragilis in Abscesses
Unencapsulated B. fragilis & Enterococcus	0.44 ± .04	9/10	9/9
Encapsulated B. fragilis (23745) & Enterococcus	0.53 ± .08	8/10	8/8
Encapsulated B. fragilis (26783)	0.77 ± .36	9/10	9/9
Encapsulated B. fragilis (26783) & Enterococcus	0.57 ± .14	9/10	9/9
CECAL (I-8)	0.42 ± .59	10/10	2/4
CECAL (I-10)	0.71 ± .33	11/13	5/13

Rats immunized with capsule alone, without adjuvants, are shown in Table 5. Generally, capsular polysaccharides are rather poorly immunogenic in laboratory animals. However, only 4/9 animals challenged with the unencapsulated strain developed abscesses and 10/26 rats challenged with either of two encapsulated strains developed abscesses. B. fragilis could be recovered from some of these. All animals challenged with the cecal contents developed abscesses, but encapsulated B. fragilis was absent from these cultures. These cecal inocula contained 1-2 logs fewer B. fragilis than the implants with one or 2 species.

Table 5: Capsule Immunization.

Challenge	Preimmune Antibody Concentration Mean ± SD	Challenge Antibody Concentration Mean ± SD	Survivors with Abscesses	Culture of B. fragilis
Unencapsulated B. fragilis & Enterococcus	2.57 ± 0.79	6.82 ± 2.70	4/9	0/4
Encapsulated B. fragilis (23745) & Enterococcus	2.88 ± 1.58	8.60 ± 3.23	3/8	2/3
Encapsulated B. fragilis (26783)	1.27 ± 0.98	7.20 ± 2.15	2/9	1/2
Encapsulated B. fragilis (26783) & Enterococcus	1.19 ± 0.74	5.93 ± 3.27	5/9	5/5
CECAL (I-8)	1.04 ± 0.53	6.93 ± 3.50	6/6	0/6

Rats immunized with the capsule + MBSA and Freund's as adjuvants developed much higher levels of antipolysaccharide antibodies (Table 6). These levels were 3-10 times higher than those achieved if the capsule alone was used as the immunogen.

3/9 animals challenged with the unencapsulated strain plus the enterococcus developed abscesses. 2/10 animals challenged with an encapsulated strain (not the vaccine strain) developed abscesses. Again, all animals implanted with the cecal contents developed abscesses. However, in these groups encapsulated B. fragilis was not cultured from the abscesses.

Table 6: Immunization with Capsule + MBSA + Freunds (complete).

Challenge	Preimmune Antibody Concentration Mean ± SD	Challenge Antibody Concentration Mean ± SD	Survivors with Abscesses	Culture of B. fragilis
Unencapsulated B. fragilis & Enterococcus	0.78 ± 0.38	1.71 ± 5.8	3/9	0/3
Encapsulated B. fragilis (26783)	1.38 ± 0.95	51.7 ± 7.44	2/10	0/2
CECAL (I-10)	0.66 ± 0.22	20.7 ± 6.8	9/9	0/9

Rats immunized with outer membrane (Table 7) responded reasonably well immunologically to the capsular portion of the membrane and in addition developed precipitins to other, non-capsular antigens, particularly outer membrane proteins and lipopolysaccharide. Only 1/9 animals challenged with the unencapsulated strain plus the enterococcus developed abscesses. 1/10 animals implanted with the heterologous encapsulated strain developed abscesses and 5/7 animals challenged with the cecal contents developed abscesses. Again in this latter group no abscesses contained encapsulated B. fragilis.

Table 7: Immunization with Outer Membrane.

Challenge	Preimmune Antibody Concentration Mean ± SD	Challenge Antibody Concentration Mean ± SD	Survivors with Abscesses	Culture of B. fragilis
Unencapsulated B. fragilis & Enterococcus	0.32 ± .06	6.52 ± 3.27	1/9	0/1
Encapsulated B. fragilis (26783)	0.31 ± .06	9.15 ± 5.13	1/10	0/1
CECAL (I-10)	0.31 ± .04	22.9 ± 12.7	5/7	0/5

Statistical estimates of the significance of these data were done by chi square analysis with a Yates correction (Table 8). With regard to the protective effect of various immunogens on abscess formation, immunization with the capsule alone, significantly protected only against challenges with flora containing encapsulated B. fragilis. Protection against challenge with the unencapsulated strain was found to be not statistically significant. In animals immunized with either capsule combined with adjuvants or outer membrane, there was significant protection against challenges with either encapsulated or unencapsulated strains. The outer membrane contains multiple antigenic determinants which may be cross reactive between the encapsulated and unencapsulated strains. Indeed if animals are immunized to high enough levels with capsule and adjuvant, antibody to some of these cross-reacting determinants are present in sufficient quantities on the capsule to protect against unencapsulated strains. It is possible that these are shared carbo-hydrate determinants between the lipopolysaccharide of the unencapsulated strain and the capsular polysaccharide. No significant protection to abscess formation was afforded by any vaccine to challenge with the mixed cecal contents.

Table 8: Chi Square Analysis of Effect of Immunization on Abscess Formation.

| Immunized with: | | | |
| Challenge (Control No. with Abscesses/Total) | P valve (No. with Abscesses/Total Tested) | | |
	Capsule	Capsule + MBSA + Freunds	Outer Membrane
Unencapsulated B. fragilis & Enterococcus (9/10)	Less than .1 (4/9)	Less than .05 (3/9)	Less than .01 (1/9)
Encapsulated B. fragilis & Enterococcus (26/30)	Less than .001 (10/26)	Less than .001 (2/10)	Less than .001 (1/10)
CECAL (23/23)	NS* (6/6)	NS (9/9)	NS (5/7)

* Not significant

However, encapsulated B. fragilis was largely eliminated from abscesses developing in all immunized animals challenged with cecal contents.

Table 9: Effect of Immunization on Recovery of B. fragilis ss. fragilis from Abscesses in Animals Receiving Cecal Contents.

No. with B. fragilis/Total	
Control	7/17
Immunized	0/20

$P = .01$

In summary, we have shown that highly significant protection from abscess formation can be offered in animals challenged in this model (intraperitoneally) with encapsulated B. fragilis, if these animals are immunized with the capsular polysaccharide or outer membrane. Protection to challenge with unencapsulated

B. fragilis + enterococci was best in animals immunized with outer membrane but also significant if the rats were immunized with capsule and adjuvants.

No protection to abscesses was afforded in animals challenged with cecal contents. However, abscesses from all immunized groups challenged with cecal contents failed to grow encapsulated B. fragilis, demonstrating the specificity of the vaccine antigen.

Intraabdominal sepsis and abscess formation is obviously results from a complex set of interactions between multiple bacteria. However, successful immunization with the capsule of B. fragilis could well protect against the formation of abscesses due to these bacteria, and thereby possibly eliminate the pathogen most frequently isolated from abscesses in intraabdominal sepsis.

D.   Adherence of Bacteroides fragilis to Peritoneal Mesothelial Cells.

This section contains studies of adherence of Bacteroides fragilis to mesothelial cells, a possible contributing factor to the pathogenic potential of these bacteria.

Our interest in this group of organisms has been expanded, based on our observations (reviewed above) with the Wistar rat model of intra-abdominal sepsis. The results of these studies, which have been summarized previously, indicate that encapsulated B. fragilis produced abscesses in 19 to 20 animals following intraperitoneal challenge. By contrast, unencapsulated strains failed to produce any detectable lesions.

The adherence studies were designed to examine adherence as a possible mechanism of increased virulence of encapsulated B. fragilis in this rat model.

Inocula consisted of 18 hour BHI broth cultures of the test organisms which were harvested by centrifugation, washed once in phosphate buffer and diluted to give a final concentraion of approximately 10^8 colony forming units per ml. Small plexiglass plates with 8 mm holes were attached to the peritoneal mesothelium of anesthesized Wistar rats. Each chamber was filled with 0.25 ml of the cell suspension. After incubation in vivo for 30 minutes, the suspension was aspirated, the wells were washed once with phosphate buffer and a tissue biopsy was obtained using a 4 mm diameter dermal punch. Each biopsy was washed 6 times in phosphate buffer and then placed in an anaerobic glove box. The tissue was homogenized and aliquots were plated on brucella-base blood agar to determine viable cell counts. Results were expressed as $\log_{10}$ colony forming units per biopsy.

The apparatus used for the in vivo adherence assay consisted of plexiglass plates, held with clamps so that peritoneal mesothelium formed the bottom of the well. There were six wells so that different bacterial strains could be tested simultaneously. This permitted the use of each animal as its own control. All procedures were conducted using aseptic techniques.

Results with two groups of organisms are summarized in Table 10. One group represents 20 experiments with eight different strains of encapsulated B. fragilis. The second group represents 17 experiments using 8 strains of unencapsulated organisms. Included in the latter were representatives of all 5 subspecies of B. fragilis other than ss fragilis. No important differences were noted between strains within these two groups; therefore, the results for each group are combined. It was found that the encapsulated B. fragilis had a mean concentration of approximately 1000 bacterial cells per biopsy while the unencapsulated strains had a mean concentration of 12 bacterial cells per biopsy. A statistical analysis using Student's T test shows that the difference between these two groups is significant (p less than 0.001).

Table 10: Adherence: Results

Bacteroides species Tested	Mean Inoculum Size ($\log_{10}$ CFU/ml $\pm$ 1 SEM)	Mean Adherence ($\log_{10}$ CFU/biopsy $\pm$ 1 SEM)
B. fragilis	8.02 ± 0.10	3.00 ± 0.07
B. distasonis B. vulgatus B. ovatus B. thetaiotaomicron B. other	7.98 ± 0.12	$1.07 \pm 0.25*$

* P less than 0.001 compare to B. fragilis.

Scanning electron microscopy was also performed as another method to assess bacterial adherence in this in vivo model.
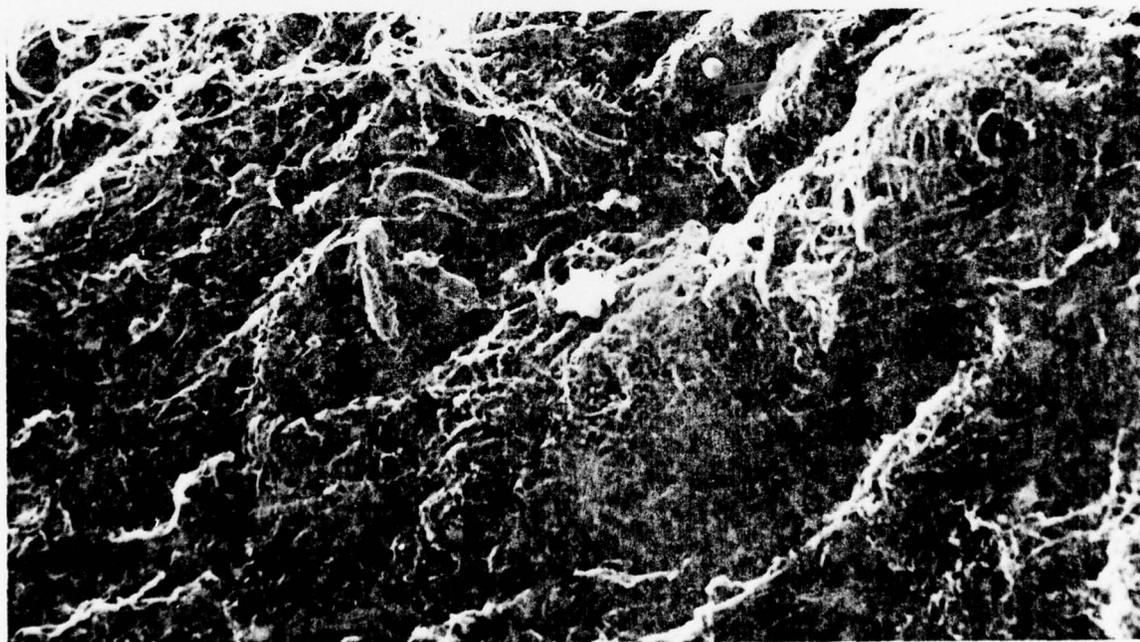
The results showed that encapsulated B. fragilis were readily apparent on the mesothelial surface in most fields, whereas unencapsulated strains proved extremely difficult to detect at all. Illustrated here is a representative photograph with encapsulated B. fragilis showing organisms on the mesothelial surface. It was noted that these organisms are far more prevalent than their unencapsulated counterparts, nevertheless they are quite scarce. On the basis of cell counts and biopsy surface area it is estimated that there were approximately 80 organisms per square mm of mesothelial surface. These electron microscopic photographs tend to corroborate this estimate.

Additional studies were done to examine certain variables which might influence adherence by encapsulated B. fragilis. One consideration was pili since these structures are considered important for surface attachment by other organisms, especially aerobic gram-negative bacilli and Neisseria. Shown here is a micrograph of negatively stained encapsulated B. fragilis strain which was used in our previous adherence assay. This and all other strains of B. fragilis tested failed to show pili. (Fig. 2).

The final experiments concerned possible methods to reduce adherence with cell wall components of encapsulated B. fragilis. First, the animals were immunized with either the entire outer membrane or the capsular polysaccharide prior to the in vivo adherence assay. Despite high levels of anticapsular antibody in the immunized animals there was no change in adherence results compared to non-immunized controls.

The mesothelial tissue was then preincubated with 200 ug of purified B. fragilis capsular polysaccharide. After 30 minutes the well was washed and the standard adherence assay was performed using encapsulated B. fragilis. Results showed that preincubation of the tissue with capsular polysaccharide reduced counts to $10^{2.2}$ bacteria/biopsy. This represents a reduction approximately 90% compared to controls and this difference is significant by the Student T test (p less than 0.05).

This in vivo adherence assay has shown that encapsulated B. fragilis have a greater propensity to attach to mesothelial cells than unencapsulated strains. The increased adherence does not appear to be related to pili since these structures were not noted in the bacterial strains tested. Pretreatment of mesothelial cells with capsular polysaccharide decreased adherence. This suggests that the capsular polysaccharide or associated material is responsible for the increased adherence.

The failure to decrease adherence by immunizing with capsule would indicate that the protection found in the rat model of intra-abdominal sepsis after immunization with capsule is due to a different mechanism. This mechanism may be enhanced opsonophagocytosis.

II Bacteroides melaninogenicus

Immunochemical and Biologic Studies of the Lipopolysaccharide of Bacteroides melaninogenicus subspecies asaccharolyticus.

A. Materials and Methods

Isolation of the Lipopolysaccharide from the Native Complex (EDTA-extracted LPS)

Lyophilized outer membrane extracted as described previously (7) was solubilized in 2 ml of a buffer (0.5% NaD) containing 0.05 M glycine, 0.001 M EDTA, and 0.5% sodium deoxycholate, adjusted to pH 9.5 with NaOH. The pH of the suspension was raised to 11 with 1 N NaOH when necessary to clarify the suspension and was then lowered to pH 9 with 1 N NCL where it remained clear. The sample was immediately chromatographed on a 1.6 x 82 cm column of Sephadex G-100 (Pharmacia, Uppsala, Sweden) equilibrated with 0.5% NaD buffer and 2 ml fractions were collected, as indicated previously (7).

The void volume (V_o) outer membrane material (Pool 1, greater than 10^5 daltons) was collected and concentrated to a volume of 5 ml with use of an ultrafiltration cell (Amicon Corp., Lexington, MA) with a PM-20 membrane. Pool 1 material was removed from the residual deoxycholate buffer by precipitating the concentrate with 0.2 M NaCl and 4 volumes of absolute ethanol. After repeating the alcohol precipitation, the precipitated material was suspended in water and lyophilized. The remainder of the column eluant (Pool 2) was separated most efficiently from the deoxycholate buffer by cold alcohol precipitation (80% ethanol) without first concentrating the pool to a small volume.

In one experiment, following extraction and purification of the outer membrane, intrinsically labeled with ^{3}H-acetate Pool 2 material, after lyophilization, was re-suspended in 0.05 M Tris buffer, pH 7.3, and chromatographed on a 2.5 x 80 cm Sepharose 4-B column (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer.

Lipopolysaccharide Extraction by the Phenol-Water Method

A modification of the procedure described by Westphal was used (8). Pelleted whole bacteria were suspended to a concentration of 20 mg/ml (wet weight). Eleven volumes of 88% hot phenol (68 C) were added to nine volumes of bacterial suspension, and the mixture was shaken vigously for five minutes in a 68 C water bath. The two phases were separated ovenight at 4 C in a separatory funnel. The aqueous phase was removed and the phenol phase was re-extracted with nine volumes of water. The aqueous phases were then combined and the residual phenol was removed by repeated extraction with ether. The residual ether was evaporated from the aqueous phase by a stream of warm air. The aqueous phase was then concentrated in an ultrafiltration cell (Amicon Corp., Lexington, MA) using a PM-30 membrane. The concentrated material was precipitated overnight with four volumes of absolute ethanol in 0.2 M NaCl at 4 C. The precipitate was removed by centrifugation at 10,000 g for fifteen minutes. The pellet was then suspended in Tris buffer, pH 7.7, and incubated with DNAse and RNAse for 24 hrs at 37 C. The enzyme treatment was repeated once. The pH was adjusted to 8 and the material was then treated with Pronase (Calbiochem, San Diego, CA), 1mg/ml, overnight at 37 C. The Pronase treatment was repeated once. Following enzyme treatment, the sample was chromatographed on a 2.5 x 80 cm column of Sepharose 4-B, equilibrated with 0.05 M Tris buffer. The void volume fraction was collected, concentrated on a PM-30 membrane, alcohol precipitated, and lyophilized. In some experiments, the phenol-water extracted LPS was further purified by ultracentrifugation (100,000 g x 1 hr) of the material after enzyme treatment. The pellet was then suspended in water and lyophilized.

Chemical Determinations

Chemical studies were performed as described previously (5,7,9).

Fatty Acid Determination by Gas-Liquid Chromatography

One mg of specimen was dissolved in 1 ml absolute ethanol. 15% KOH was added. The sample was heated at 80 C for 3 hrs. The pH was then lowered to 2 with HCl and the sample was extracted three times with n-hexane. The hexane portion of the sample was then evaporated with dry nitrogen at 45 C. The residue was then methylated using BF_3-methanol. The conditions of methylation were 56 C for 30 min. One ml of distilled water was added to stop the reaction. The specimen was again extracted three times with n-hexane and dried with nitrogen. An aliquot of the specimen was used for GLC. Separation was made using 10% diethylene glycol succinate on 100-120 mesh Chromsorb AW. A 2 m long x 3 mm internal diameter column was used. The column temperature was 190 C. The injection port was 220 C and the detector was kept at 220 C. Flow rate was 20 ml/min. Peaks were detected using a flame ionization detector at 10^{-9} amps. The signal was integrated using a Shimadzu ITG-4X Digital Integrator and the % composition was determined using a comparison of microvolt-sec of each peak compared to the total.

Tests for Biologic Activity

Dermal Shwartzman tests were performed on weanling New Zealand White **rabbits.** Various concentrations of each antigen, diluted to 0.2 ml with

sterile 0.15 M NaCl were injected intradermally into the shaved abdomen of each rabbit. After 24 hr, a 0.4 ml challenge dose was given intravenously. Hemorrhage or necrosis at 6 hr and 24 hr was read as a positive reaction (10).

Limulus lysate assays were performed graciously by Dr. M. Johns (Boston University Hospital, Boston, MA) according to the method of Levin (11).

Chick embryo toxicity tests were done as described by Smith and Thomas (12), as modified by Finkelstein (13). 10-day old chick embryos (Spafac Inc., Norwich, CT) were given 0.2 ml intravenous injections of various concentrations of antigen into a vein in the chorioallantoic membrane, after a window was cut in the shell over the vein. The window was sealed with tape and the eggs incubated for 24 hr at 39 C. Mortality was determined after 24 hr and 50% lethal doses (LD_{50}) were calculated according to the method of Reed and Muench (14). Purified outer membrane protein from Group B Neisseria meningitidis and the capsular polysaccharide of Group C N. meningitidis (neither containing detectable endotoxin), were used to determine the range of endotoxin-specific chick embryo toxicity.

Serologic Methods

Rabbit antisera to whole organisms were prepared as detailed previously. All antisera were stored at -20 C prior to use.

The double diffusion method of Ouchterlony was used (15) and precipitin patterns were read after incubation at room temperature for 24-48 hr.

Indirect hemagglutination (IHA) was performed in U-well plastic trays (Microtiter, Cooke Engineering Co., Alexandria, VA). Sheep red blood cells (SRBC) were stored at 4 C in Alsever's solution (Scott Laboratories, Inc., Fiskeville, RI), and diluted to 2% after several washings with neutral PBS prior to each experiment. The SRBC were sensitized by incubating 1 ml of 2% SRBC with 1 ml of antigen at 37 C for 30 min. The optimal antigen concentration was determined to be 100 ug. The test was performed by adding 0.025 ml antigen-SRBC suspension to 0.025 ml of serial dilutions of antiserum. Hemagglutination patterns were read after incubation of the trays for 2 hr at 37 C, and again after overnight incubation at room temperature. All washings, dilutions, and suspensions were done with neutral PBS (Grand Island Biological Co., Grand Island, NY).

Hemagglutination inhibition (HAI) was performed in U-well plastic trays. Four hemagglutinating (HA) units of antiserum were used in the test. This serum concentration was defined as the highest hemagglutinating titer achieved by the homologous antigen divided by four. Four HA units of antiserum were added to various concentrations of inhibitor and incubated at 37 C for 30 min. An equal volume (0.05 ml) of antigen-RBC suspension was then added to each well and the mixture was incubated at 37 C for 2 hr, after which hemagglutination patterns were read.

B. Results

Chemical Composition of the Lipopolysaccharide

The EDTA-extracted outer membrane preparation, when chromatographed on Sephadex G-100 using 0.5% sodium deoxycholate (an endotoxin disaggregating substance) in the running buffer, demonstrated two major fractions (5,7,16). The void volume material (Pool 1, greater than 10^5 daltons) was found to be a protein-polysaccharide complex with 26% loosely bound (chloroform:methanol extractable) lipid. This fraction, as detailed previously (7), contains the capsular antigen. The second fraction, Pool 2, eluted at a point con-

sistent with a molecular size of 12,000 daltons, the monomeric unit of dis-
aggregated bacterial LPS. This fraction consisted of 32% carbohydrates with
less than 5% protein, and had 62% loosely-bound lipid by dry weight analysis.
This second fraction was re-chromatographed on Sepharose 4-B in Tris buffer
after removal of the sodium deoxycholate (Figure 3).

Fig. 3: Sepharose 4B-column elution profile of EDTA-extracted LPS, labeled
with ^{3}H-acetate, using Tris as the running buffer.
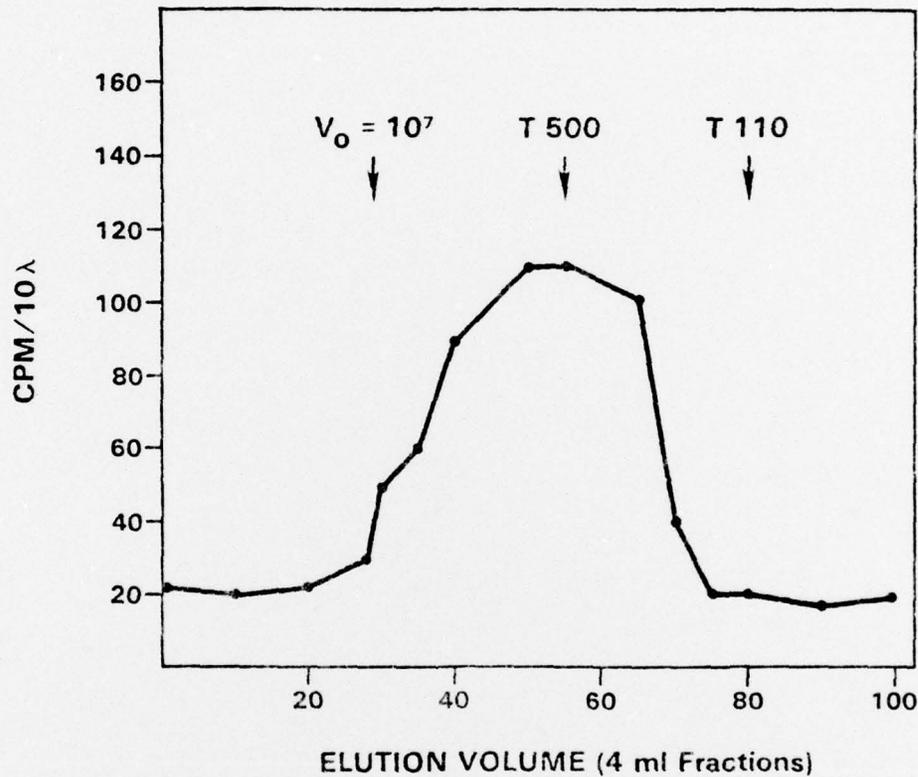
The material eluted slightly beyond the void volume (V_o = 5 x 10^6 daltons),
demonstrating reaggregation. This indicated that the second peak was an ag-
gregated lipopolysaccharide. The LPS extracted with aqueous phenol from the
same strain, intrinsically labeled with ^{3}H-acetate, eluted at the same K_{av} as
the aggregated second pool. This extract, when chromatographed on Sephadex
G-100 (V_o = greater than 10^5 daltons) demonstrated partial disaggregation and
a rather broad peak in the presence of the disaggregating buffer (Figure 4).

Fig. 4: Sephadex G-100 column elution profile of a phenol-water-extracted LPS labeled with ^{3}H-acetate.

ELUTION VOLUME (2 ml Fractions)
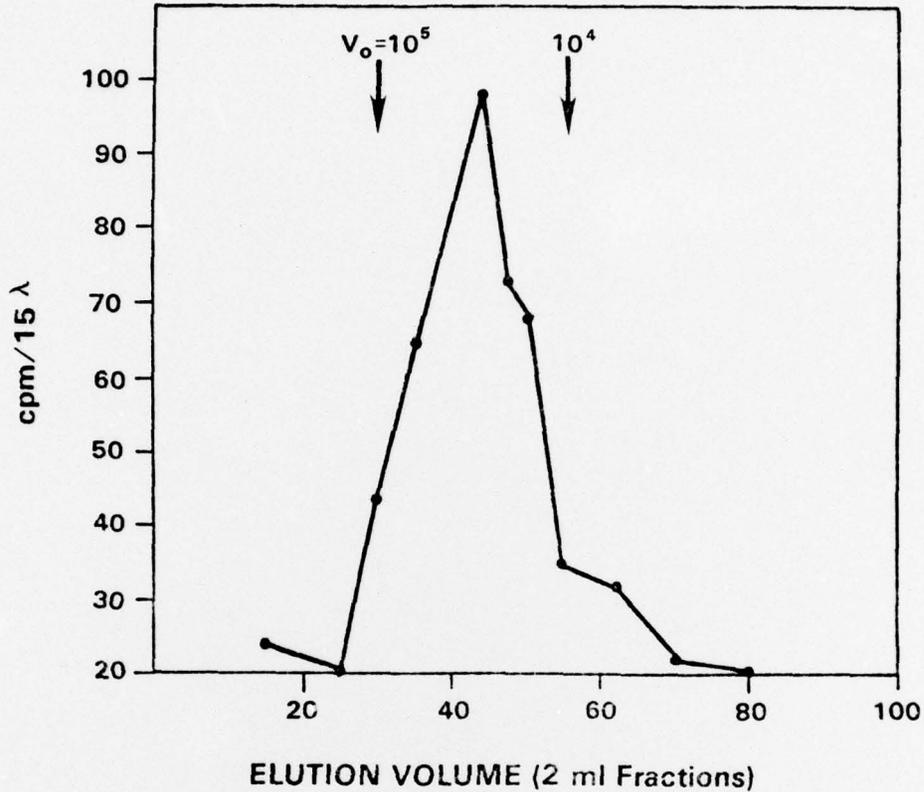
Estimates of its molecular size ranged between 10^4 daltons and 10^6 daltons. Thus, both the EDTA-extracted LPS and the phenol-water extracted LPS displayed the capacity to disaggregate in the presence of an LPS disaggregating medium and to reaggregate in its absence, and thereby demonstrated physico-chemical similarity.

Gas-liquid chromatographic analysis of both the EDTA and the phenol-water LPS preparations of several strains of B. melaninogenicus subspecies asaccharolyticus revealed the presence of glucose, galactose, and glucosamine (Table 11).

Table 11: Gas-liquid Chromatographic Analysis of Sugars.

| | Strain 382 | | Strain 376 | Strain 536 |
	EDTA-LPS	ϕ-H_2O-LPS	EDTA-LPS	ϕ-H_2O-LPS
Glucose	32*	32.8	19.3	11
Galactose	7	12.3	14.8	28.9
Glucosamine	11	35.3	16.5	28
Rhamnose	4			5.8
Mannose	9	20.5	13.8	4.7

*Numbers represent the percentages of total identifiable sugars.

Small amounts of mannose (9%) were found in the EDTA-extracted LPS of strain 382, and rhamnose was detected in the EDTA extract of strain 382 (4%) and the phenol-water extract of strain 536 (5.8%). 4-20% of the chromatographed material from all three strains tested had retention times similar to that of a peak tentatively identified as a heptose. Definitive identification of this peak, however, requires confirmation by mass spectrometry. However, colorimetric determinations of heptose showed it to be absent in all of the LPS preparations. Similarly, no KDO was found in any of the preparations.

A unique pattern of fatty acids was detected by gas-liquid chromatography (Table 12).

Table 12: Gas-liquid Chromatographic Analysis of Fatty Acids.

	Strain 382 EDTA-LPS	Strain 376 EDTA-LPS	Strain 536 ϕ-H_2O-LPS
C-12*	-	5**	-
C-14	-	10	<5
C-16	62.5	35-40	34.2
C-16:1	13	20	10.1
C-18	9.3	15	21.1
C-18:1	10	10	8.0
Unknown A	<5	5	<5
Unknown B	<5	<5	8

* Refers to number of carbons in the chain; number following colon refers to the site of a double bond.

**Numbers refer to approximate percentages of total fatty acid measured.

The predominant fatty acids were stearic (18 Carbon) and palmitic (16 Carbon). Two unknown fatty acids which may be odd-changed were detected. Notably, no β-OH myristic acid was detected. This fatty acid is found commonly among the lipolpolysaccharide of Salmonella and other facultative gram-negative bacteria (17).

Several preparations of LPS from two strains of B. melaninogenicus were tested in the Limulus lysate assay (Table 13).

Table 13:   Results of Limulus Lysate Test.

Preparation	Concentration (μg/ml)*
EDTA-LPS, Strain 382	5×10^3
ϕ-H$_2$O-LPS, Strain 382	4.8
Outer membrane, Strain 536	4.8
ϕ-H$_2$O-LPS, Strain 536	4.8
H$_2$O	no gelation
ϕ-H$_2$O-LPS, S. typhi 0:901	0.15

* Figures refer to concentration of individual preparations
  necessary to gel limulus lysate.

The LPS of Salmonella typhi 0:901 (phenol-water extract) gelled the Limulus lysate at a concentration of 0.15 μg/ml, whereas the LPS preparations of two strains of B. melaninogenicus gelled the lysate in a range between 4.8 μg/ml and 5×10^3 μg/ml of LPS, a minimum of thirty-fold less activity than the LPS from S. typhi.

The same two strains of B. melaninogenicus were tested for induction of dermal Shwartzman reactions in weanling rabbits (Table 14).

Table 14: Comparison of the capacity of various endotoxin (LPS) preparations to elicit the dermal Shwartzman reaction in groups of rabbits.

Preparation tested	Dose (µg)		positive / total	comment
	prepar-ative	provo-cative	tests / tested	
Salmonella typhi 0:901 endotoxin	50	10	4/4	Positive tests
	25	10	4/4	manifested by
	5	10	0/4	hemorrhage or necrosis
	2.5	10	0/4	
	0.5	10	0/4	
Phenol-water extracted LPS, strain 382	500	250	0/3	
	100	250	0/3	
	25	250	0/3	
	5	250	0/3	
EDTA extracted LPS, strain 382	500	250	0/3*	* 1 rabbit had an
	200	250	0/3	area of hyperemia
	50	250	0/3	without hemorrhage
	5	250	0/3	or necrosis at 500 µg
Phenol-water extracted LPS, strain 536	1000	250	0/3	
	500	250	0/4	
	100	250	0/4	
	25	250	0/4	
	5	250	0/4	
EDTA extracted LPS, strain 536	500	250	0/3	
	100	250	0/3	
	5	250	0/3	

S. typhi 0:901 LPS was used as a positive control, and demonstrated a 50% endpoint (hemorrhage or necrosis after 5 hr) at a concentration of 12.5 µg/ml. The LPS preparations from strains of B. melaninogenicus, both the EDTA and the phenol-water extracts, gave no positive reaction in doses up to 1000 µg.

Chick embryo lethality was assessed with LPS preparations from two strains of B. melaninogenicus and compared with that of S. minnesota S:218. Results are summarized in Table 15.

Table 15: Chick embryo lethality of LPS from Bacteroides melaninogenicus subspecies asaccharolyticus.

Preparation	Dose
Strain 382, outer membrane	400 µg
Strain 382, Pool 1*	100 µg
Strain 382, EDTA-LPS	>300 µg
Strain 382, ϕ-H$_2$O-LPS	45 µg
Strain 536, outer membrane	150 µg
Strain 536, Pool 1	50 µg
Strain 536, EDTA-LPS	100 µg
Strain 536, ϕ-H$_2$O-LPS	28 µg
(pos. control) S. minn. S218, ϕ-H$_2$O-LPS	<1 µg
Group B, N. mening. protein**	<100 µg
Group C, N. mening. polysaccharide	>100 µg

* Pool 1 refers to the outer membrane fraction eluted from a Sephadex G-100 column at the void volume, which is distinct from the LPS fraction.

**The preparation from N. meningitidis are outer membrane antigens which have been purified and are distinct from the LPS fractions.

The 50% lethal dose (LD$_{50}$) of S. minnesota was found to be <1 µg. The LD$_{50}$ of the EDTA-extracted LPS and the phenol-water LPS of strain 382 were >300 µg and 45 µg, respectively. Purified outer membrane and Pool 1 (protein-polysaccharide complex) from strain 382 as well as an outer membrane protein from Group B Neisseria meningitidis and a capsular polysaccharide from Group C N. meningitidis were tested in order to determine the range of endotoxin specific chick embryo lethality. These four preparations were all found to have an LD$_{50}$ of 50 µg or greater, within the range found for both B. melaninogenicus LPS preparations. These effective killing concentrations were all 30-100 fold greater than that of the positive control (S. minnesota) and were thereby considered endotoxin non-specific reactions. Both the EDTA-extracted LPS and the phenol-water extracted LPS from strain 536 were also tested for chick embryo lethality. The EDTA-extracted LPS had an LD$_{50}$ of 100 µg and that of the phenol-water LPS was 28 µg. Comparison to whole outer membrane and the Pool 1 fraction from strain 536, which had LD 50's of 150 µg and 50 µg, respectively, revealed that the 50% lethal dose for the LPS extracts of strain 536 was clearly in a endotoxin non-specific range.

Serologic Activity

A previous study has shown the presence of two major outer membrane fractions in B. melaninogenicus when the EDTA extraction was performed followed by gel chromatography with Sephadex G-100 in the presence of sodium deoxycholate. When the void volume fraction (Pool 1) was tested by agar gel diffusion with homologous antiserum to whole organisms, two precipitin lines were formed. After digestion of Pool 1 with Pronase, a proteolytic enzyme, one precipitin line remained. This remaining line was shown to be formed by an antigen that

is very likely to be a capsular polysaccharide. When the capsular antigen from strain 536 (Figure 5) was tested by Ouchterlony double diffusion in agar against homologous antiserum to whole organisms, a single precipitin line was formed. The phenol-water extracted LPS from the same strain, partially purified by column chromatography ("crude" phenol-water LPS), showed a line of identity with the capsule when tested by double diffusion. After the phenol-water LPS was further purified by ultracentrifugation ("purified" phenol-water LPS), the precipitin line, although still present, was barely visible. No precipitin line was formed when the EDTA-extracted LPS from the same strain was tested by gel diffusion.

Fig. 5: Double diffusion in agar with rabbit antiserum to strain 536 in center well. Outer wells contain: A = capsular antigen, strain 536; B = "crude" ϕ-H$_2$O-LPS, strain 536; C = "purified" ϕ-H$_2$O-LPS, strain 536; D = EDTA-LPS, strain 536. Antigen concentrations in wells A and B are 3 mg/ml. Antigen concentrations in wells C and D are 6 mg/ml.
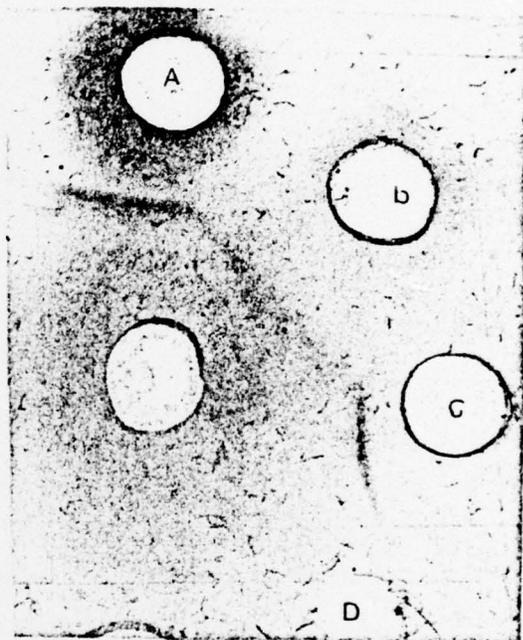
In order to study further the relationship between the LPS antigens and the capsular antigen, indirect hemagglutination (IHA) was performed. A single strain, 536, was selected for analysis. Sheep red blood cells (SRBC) sensitized with any of the four antigens (capsule, EDTA-extracted LPS, "crude" phenol-water LPS, "purified" phenol-water LPS) showed hemagglutination with hyperimmune rabbit antiserum prepared to the homologous strain. The highest titer was found with capsular antigen-sensitized SRBC which hemagglutinated at a 1:64 serum dilution; SRBC sensitized with any of the three LPS preparations hemagglutinated with the serum diluted to 1:8 - 1:16. SRBC sensitized with these antigens did not demonstrate hemagglutinating activity with antiserum prepared to heterologous antigens. Hemagglutination inhibition (HAI) experiments (Table 16) revealed that SRBC sensitized with capsular antigen were inhibited by both the capsular polysaccharide and by the "crude" phenol-water LPS at an amount of 0.6 µg, but not inhibited by the EDTA-LPS and inhibited only at a very high concentration (25 µg) by the "purified" phenol-water LPS.

Table 16: Hemagglutination inhibition of capsular antigen from <u>B. melaninogenicus</u> (Strain 536).

Inhibitor	Lowest Dose Producing Inhibition
Capsule	<0.3 µg
EDTA-LPS	no inhibition (>25 µg)
"crude" ϕ-H$_2$O-LPS	>0.6 µg
"purified" ϕ-H$_2$O-LPS	25 µg

HAI was done using "crude" pheno-water LPS sensitized SRBC (Table 17).

Table 17: Hemagglutination inhibition of "crude" ϕ-H$_2$O-LPS from <u>B. melaninogenicus</u> (Strain 536).

Inhibitor	Lowest Dose Producing Inhibition
"crude" ϕ-H$_2$O-LPS	>0.6 µg
Capsule	no inhibition (>25 µg)
EDTA-LPS	no inhibition (>25 µg)
"purified" ϕ-H$_2$O-LPS	no inhibition (>25 µg)

Only the "crude" phenol-water LPS showed inhibition of these sensitized SRBC (>0.6 µg). No inhibition of hemagglutination of "crude" phenol-water LPS sensitized SRBC was noted by the "purified" phenol-water LPS, the EDTA-LPS, or by the capsule. To test the hypothesis that the "crude" phenol-water LPS contained both LPS and capsular determinants, capsule and "purified" phenol-water LPS were added together to determine their combined capacity to inhibit hemagglutination by "crude" phenol-water LPS sensitized SRBC. At a serum dilution 1:8, this combination of antigens showed hemagglutination inhibition at 3 µg; at 1:16 serum dilution, inhibition was accomplished at >0.3 µg. To determine whether the EDTA-LPS and the "purified" phenol-water LPS were identical, HAI was performed using "purified" phenol-water as the SRBC sensitizing antigen and the EDTA-extracted LPS as inhibitor. HAI was noted at >0.3 µg, suggesting that the two LPS preparations are antigenically identical.

C. Summary of LPS Studies

Lipopolysaccharide (LPS) was isolated from the outer membrane complex of <u>Bacteroides melaninogenicus</u> by gel chromatography using sodium deoxycholate (NaD), an endotoxin disaggregating detergent in the running buffer. Reaggregation occurred after removal of residual NaD. LPS was also prepared by the phenol-water method for comparison. The LPS was composed of loosely-bound lipid (62%) and carbohydrate, with less than 5% protein. Glucose, galactose, and glucosamine were the major sugars, as detected by gas-liquid chromatography (GLC). KDO and heptose were found to be absent using colorimetric tests. Fatty acid analysis by GLC disclosed the notable absence of B-OH myristic acid. Biologic activity as measured by dermal Shwartzman, Limulus lysate, and chick embryo lethality tests in comparison with LPS from <u>Salmonella</u> <u>typhi</u>. Hemagglutination inhibition tests demonstrated that the "crude" phenol-water LPS contained capsular material in addition to LPS, which was partially removed by ultracentrifugation, and that the "purified" phenol-water LPS was apparently identical to the EDTA-extracted LPS.

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Three groups of rats were immunized i.m. with the capsular polysaccharide (sss) of Bacteroides fragilis ss. fragilis (B.F.F.) alone, or with adjuvant (MBSA + complete Freund's), or as part of the intact outer membrane (o.m.) of these bacteria. Protection correlated with the level of anti-sss antibody measured in a quantitative radioactive binding assay. These studies demonstrated that significant protection can be afforded against abscess development due to B. fragilis strains by immunization with the capsular

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20. Abstract

polysaccharide of B.F.F. and that protection can be enhanced either with adjuvants or the adjuvant effect of the outer membrane (p <.05).

The ability of the encapsulated species, B. fragilis to adhere to rat peritoneal mesothelium was compared to the adherence of unencapsulated strains of Bacteroides. It was found that B. fragilis adhered significantly better ($10^{3.00}$ CFU/biopsy) to mesothelial tissue than unencapsulated species of Bacteroides ($10^{1.07}$ CFU/biopsy). Rats immunized with the capsular polysaccharide did not demonstrate decreased adherence; however, pre-incubation of the wells 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.

Lipopolysaccharide (LPS) was isolated from the outer membrane complex of Bacteroides melaninogenicus. The LPS OF B. melaninogenicus lacks two of the core sugars characteristic of aerobic Gram-negative endotoxins, has a unique fatty acid composition, and displays low biologic potency. These findings may explain the rarity of septic shock in patients infected with this organism.