Immunochemical Investigations of Cell Surface Antigens of Anaerobic Bacteria

Annual Report

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January 15, 1977

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-74-C-4056

Harvard Medical School
Channing Laboratory
Boston City Hospital
Boston, MA 02118

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A155 747
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) IMMUNOCHEMICAL INVESTIGATIONS OF CELL SURFACE ANTIGENS OF ANAEROBIC BACTERIA	5. TYPE OF REPORT & PERIOD COVERED Annual Report
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Denni L. Kasper, M.D.	8. CONTRACT OR GRANT NUMBER(s) DAMD17-74-C-4056
9. PERFORMING ORGANIZATION NAME AND ADDRESS Harvard Medical School Channing Laboratory Albany Street, Boston, MA 02118	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102B.3M161102BS01-00-001
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, MD 21701-5012	12. REPORT DATE January 15, 1977
	13. NUMBER OF PAGES 40
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Immunochemical; virulence; wound infections; Anaerobic bacteria; animal models; vaccines; capsules of bacteria; protection; immunity; human infections

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Isolates of Bacteroides fragilis from 83 infected patients were collected over two years. Of 103 isolates, 68% were subspecies fragilis. This was the single most common subspecies isolated from blood ~~(12/17)~~, intraabdominal

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ANNUAL REPORT
Resume of Accomplishments in the Past Year
(since last annual report of Jan. 15,1976)

In the past year we have made considerable progress toward the goals of this project. Each portion of progress is presented below.

1. Bacteroides fragilis

Bacteroides are the most frequently encountered obligately anaerobic organisms in clinical infection (1,2). These organisms have been reported to be the most common gram-negative rod, next to E. coli, isolated from all blood cultures (3). Seventy-eight percent of Bacteroides isolated from blood cultures have been identified as Bacteroides fragilis. Despite these clinical observations, relatively little has been known about the mechanisms responsible for the virulence of these bacteria. Bacteroides fragilis strains have been catergorized on the basis of sugar fermentation and indol production into five subspecies: fragilis, distasonis, thetaiotaomicron, vulgatus, and ovatus (4). It has been shown that B. fragilis ss. vulgatus and ss. thetaiotaomicron are the predominant isolates in normal feces and that ss. fragilis is a relatively minor component of fecal flora (5,6). This subspecies prevalence appears to contrast with the distribution of subspecies in clinical infection where ss. fragilis is the most commonly isolated (7,8).

A. Clinical distribution of subspecies at Boston City Hospital

Over a two year period, we subspeciated all clinical isolates of B. fragilis identified in the clinical microbiology laboratory at Boston City Hospital, and reviewed the patients' records to define more clearly the epidemiology of these subspecies.

Boston City Hospital is a 400 bed general hospital. Between April, 1974 and April, 1976, 186,994 clinical specimens were processed by the clinical microbiology laboratory under the direction of Ms. A.K. Daly. Bacteroides fragilis was isolated 103 times from 83 patients. Specimens which are routinely cultured for anaerobes, include body fluids, aspirated pus, female genital specimens, surgical specimens, and transtracheal aspirates. Specimens were transported to the laboratory in capped, air-free syringes or in gassed-out oxygen-free, medium-free collection tubes. Immediately on arrival in the laboratory, specimens were plated on prereduced, anaerobically sterilized media and anaerobic bacteria were identified by colony morphology, gram-stain characteristics, biochemical reaction and growth on selective media as outlined by the anaerobe laboratory at the Virginia Polytechnic Institute (9). All isolates of Bacteroides fragilis were subspeciated by determining acid production from trehalose, glucose, mannitol and rhamnose and indol production.

The clinical site of all Bacteroides fragilis isolates was determined by chart review. Many of the patients were seen by us. The distribution of the 103 isolates by site and subspecies is shown in Table 1.

Table 1: Distribution of Clinically Significant Isolates of B. fragilis by Site and Subspecies

Subspecies	Site						
	Blood	Intra-abdominal	Wound*	Perirectal	Pelvic	Other+	Total
Fragilis	12	11	17	11	6	8	65
Distasonis	2	5	1	1	0	0	9
Vulgatus	1	3	0	1	4	1	10
Thetaiotaomicron	2	9	2	3	0	3	19
Totals	17	28	20	16	10	12	103

*Source of 14/20 specimens was infected abdominal surgical wound
+Other sites included transtracheal aspirate, empyema, middle ear, hidradenitis, osteomyelitis, and bladder.

B. fragilis ss. fragilis was the single most common subspecies isolated from blood (12/17), intraabdominal sites (11/27), wounds (17/20), perirectal abscesses (11/16), pelvic sites (6/10) and overall (65/103). From 12 of these 103 specimens, two subspecies of B. fragilis were recovered and of these 24 subspecies isolates there were 6 ss. fragilis, 6 ss. distasonis, 9 ss. thetaiotaomicron, and 3 ss. vulgatus. Eight of the twelve specimens were from intraabdominal infections.

Of the 103 clinical isolates of B. fragilis, 16 were grown in pure culture, and 13 (81%) of these were ss. fragilis. Pure ss. fragilis isolates came from the blood in 9 instances. Eighty-seven clinical specimens from the other 67 patients were polymicrobial. Overall, these mixed infections contained an average of 2.2 aerobic/facultative and 2.8 anaerobic bacteria. Twelve of 17 blood cultures frew only B. fragilis; otherwise, in cultures isolated from other sites, mixed aerobic-anaerobic cultures were the rule and the number and type of concommitantly isolated organisms did not vary significantly among infection sites. The frequency distribution of the various strains of bacteria isolated from these mixed infections is given in Table 2.

Table 2: Other Bacteria Associated with B. fragilis in Mixed Infections

Aerobes and Facultative Aerobes	Blood	Intra-abdominal	Wound	Perirectal and pilonidal	Pelvic	Other	Total
Enterobacteraciae and Pseudomonas	3	26	12	1	3	10	(55)
Diptheroids	0	0	2	8	4	3	(17)
Other	0	0	1	0	1	1	(3)
Streptococcus and Staphylococcus aureus	0	4	6	12	8	10	(40)
Enterococcus	0	3	3	0	0	3	(9)
Staphylococcus epidermitis	1	0	6	12	2	2	(23)
Other	1	5	2	1	3	1	(13)
Anaerobes							
Peptostreptococcus	2	5	7	5	6	5	(30)
Peptococcus	1	0	4	7	6	5	(23)
B, non-fragilis and fusobacteria	1	1	5	9	4	5	(25)
Clostridia (all)	0	9	4	1	2	1	(17)
Other	0	2	3	2	3	2	(12)

The species of anaerobic bacteria which is isolated most frequently from clinical specimens is Bacteroides fragilis. It is the most common species of intestinal anaerobe isolated from bacteremia (10,11), intraabdominal infections (12), pelvic inflammatory disease (13), infections associated with appendicitis (14), wounds and abscesses (15). It is a common cause of serious infections of the female genital tract (16,17).

On the basis of their ability to ferment certain sugars and to produce indol, members of this species have been divided into 5 distinct subgroups and and "other" subgroup. The five subspecies are distasonis, fragilis, ovatus, thetaiotaomicron, and vulgatus. Of interest has been the observation that the distribution of these subspecies in clinical infections is different from their relative prevalence in normal flora. B. fragilis ss. vulgatus and ss. thetaiotaomicron are the most prevalent in normal flora (5,6), whereas B. fragilis ss.

fragilis has been isolated more frequently from clinical specimens. Subspecies fragilis was the most common subspecies isolated in 83 consecutive patients with B. fragilis infection over a 2 year period. It was the predominant subspecies overall, and in every site of infection. It also occurred in pure culture more frequently than the other subspecies. Eighty-one percent of the sixteen pure cultures were subspecies fragilis.

Interestingly, only in infections from which two B. fragilis subspecies were isolated did ss. fragilis fail to predominate. This suggests that some combinations of the subspecies distasonis, thetaiotaomicron, and vulgatus may have enhanced virulence through synergy.

B. Prevalence of encapsulated strains within B. fragilis

Bacterial strains. Reference strains 23745 (B. fragilis ss. fragilis). 8482 (ss. vulgatus), 8503 (ss. distasonis), 8492 and 12290 (ss. thetaiotaomicron) were obtained from the American Type Culture Collection, (Rockville, Md). Dr. Tracey Wilkins (Virginia Polytechnical Institute) kindly supplied 6 strains isolated by Prevo: 0435 (ss. ovatus), 4076 (ss. fragilis), 4361 (ss. fragilis), 3390 (ss. fragilis), 0476 (ss. fragilis) and 4366 (ss. fragilis). All other strains reported were clinical isolates collected at the Boston City Hospital (87 isolates), Boston Veteran's Administration Hospital (12 isolates), Wadsworth Veteran's Administration Hospital (5 isolates) and the Temple University Health Science Center (4 isolates). An additional 18 clinical isolates of aerobic bacteria and three isolates of Bacteroides melaninogenicus ss. asaccharolyticus were obtained at the Boston City Hospital.

Electron Microscopy. Thirty-six hour cultures of strains of B. fragilis grown on modified thioglycollate broth were prepared for electron microscopy, staining specifically for capsules with ruthenium red. Twenty-one strains of Bacteroides fragilis representative of the various subspecies were studied (Table 3).

Table 3: Electron Microscopic Analysis of B.fragilis strains for capsules.

Subspecies				
fragilis	distasonis	vulgatus	theitaiotaomicron	ovatus
9/9*	0/4	0^{+}/3	0/4	0/1

*No. /total tested
+The cultures of one strain contained 20% encapsulated organisms.

All but four of these strains (one of each subspecies except ovatus) were clinical isolates. All nine strains of subspecies fragilis studied showed a distinct capsule on the external surface of the outer membrane. In all cultures of this subspecies, over 98% of the organisms were found to be encapsulated. Nearly all organisms in cultures of 12 strains representative of the other subspecies failed to demonstrate this dense capsular material. However, the culture of one ss. vulgatus strain, 8482, did show dense capsular material in 20% of the organisms. Several strains of the subspecies other than fragilis demonstrated a less dense material external to the outer membrane, which was easily differentiated from the electron dense area around the B. fragilis ss. fragilis strains examined (Fig. 1).

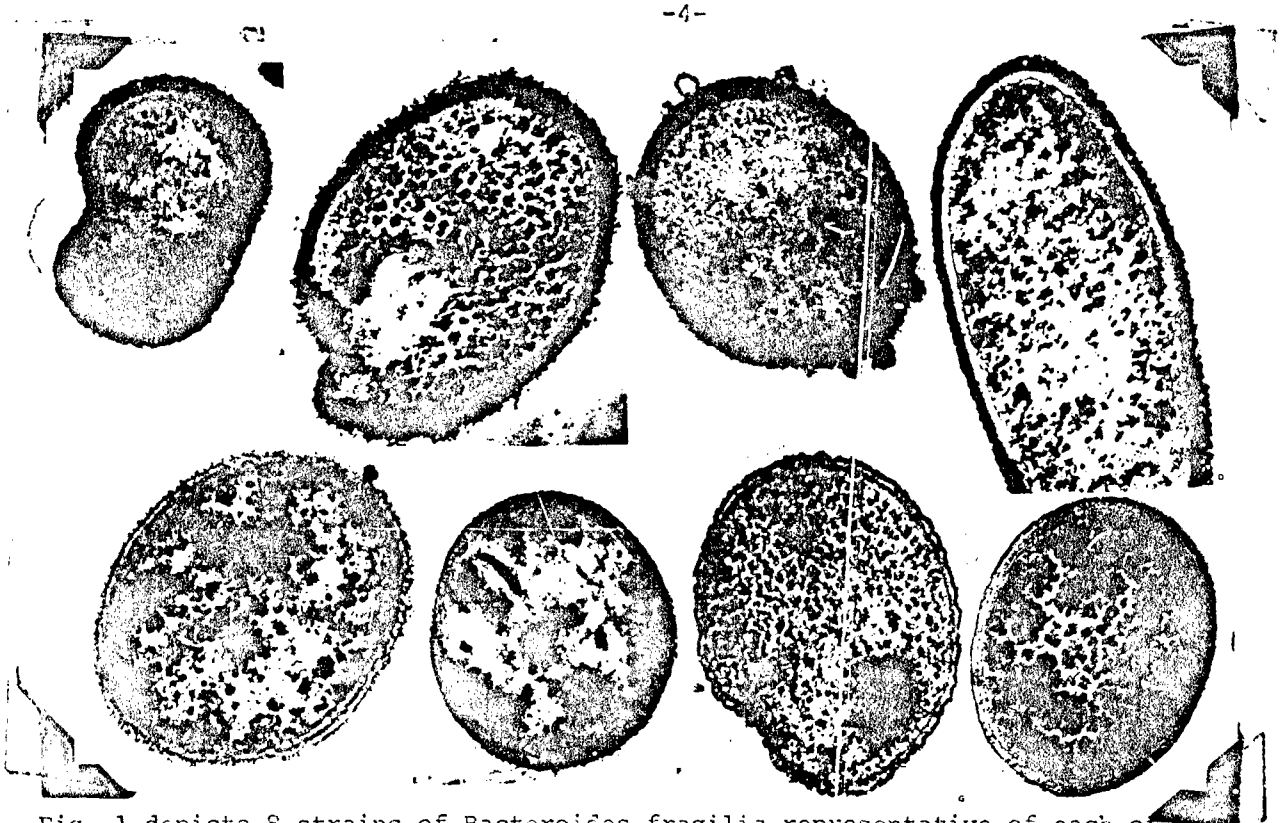
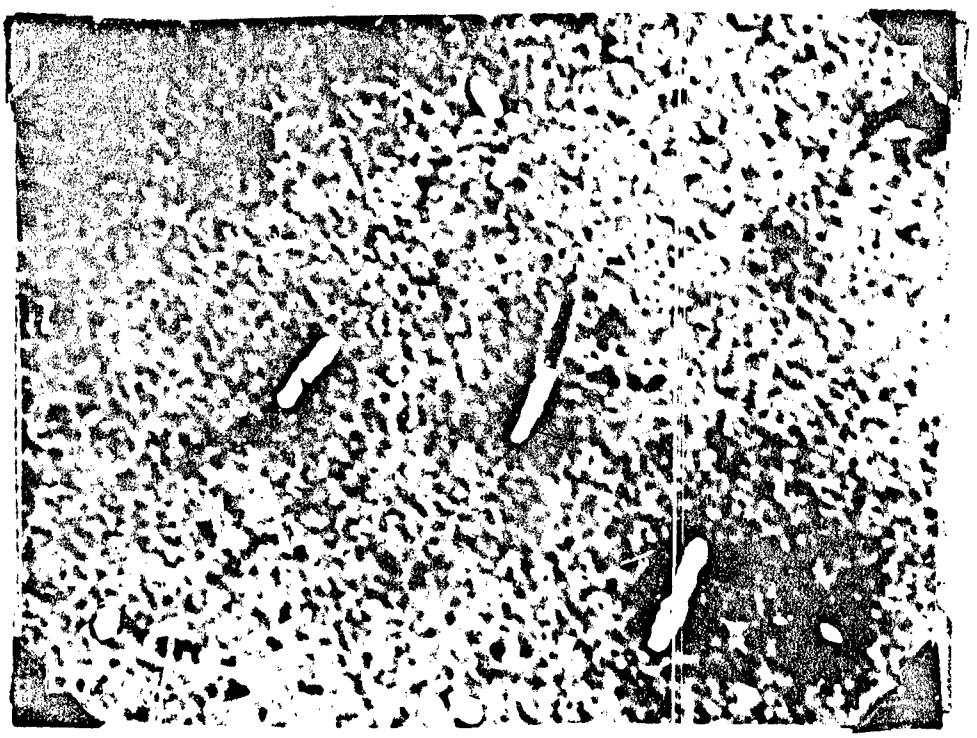


Fig. 1 depicts 8 strains of Bacteroides fragilis representative of each of the subspecies. In the top row are 4 clinical isolates of subspecies fragilis. The typical dense capsule can be seen. In the bottom row are clinical isolates of subspecies, vulgatus, ovatus, distasonis, and thetaiotaomicron. These organisms do not demonstrate this dense material.

India Ink. Twenty-two clinical isolates of B. fragilis representative of the various subspecies were examined by the india ink technique (18) for the presence of a capsule. A distinct clear zone could be seen around the encapsulated organisms (Fig. 2).



Nine of nine strains of subspecies fragilis were positive when examined using this technique while no capsules were demonstrated in 13 strains of the other subspecies. This finding supports the observation that encapsulation is most common among strains of ss. fragilis.

Quellung. Quellung reaction (19) using specific antiserum for the capsular polysaccharide of B. fragilis ss. fragilis (PS) demonstrate capsular swelling of all 10 strains of ss. fragilis. Five strains of each of the other subspecies failed to swell with this antiserum or with antiserum prepared to isolates of the homologous subspecies. The ss. fragilis strains only showed swelling in the presence of the homologous ss. fragilis PS antiserum.

Indirect Immunofluorescent assay (IFA). Strains of anaerobic bacteria were grown on blood agar plates in Gas-paks (BBL, Baltimore, MD) at 37 C. Aerobic bacteria (clinical isolates) were grown in a 37 C incubator on blood agar plates. One standard loopful of bacteria were suspended in 1 ml sterile Gey 's balanced salt solution and innoculated into 10 ml modified thioglycollate broth and incubated at 37 C overnight. Organisms were pelleted from the thioglycollate broth by centrifugation at 3000 xg for 20 minutes at 4 C in a RC-5 centrifuge (Dupont-Sorvall, Newtown, Conn). The organisms were washed once in phosphate buffered saline (PBS) pH 7.4. After repeat centrifugation, the final pellet was suspended in 0.5 ml PBS. Three ul of this suspension was added to 300 ul of PBS to make the final bacterial suspension. Teflon coated clides were prepared by dotting glass microscope slides with drops of glycerin, spraying the slide with teflon (Fluoro-glide, Chemplast, Inc. Wayne, N.J.), and rinsing with H_2O to produce wells where the glycerin dots had been. One ul of the final antigen suspension was applied to each well. The slides were air dried, gently heat fixed and rinsed with distilled H_2O to remove precipitated buffer salts. Antisera to the capsular polysaccharide antigen (PS) alone and the capsular polysaccharide-outer membrane protein complex (PPS) of Bacteroides fragilis ss. fragilis strains 23745 were made in 3-4 lb New Zealand white rabbits. Five ul of a 1:30 dilution of the PPS sera or 1:64 of the PS sera were applied to each well over the organisms. Similar dilutions of the preimmunization sera from these rabbits were used as controls. Slides were incubated for 10 minutes, rinsed in distilled H_2O and air dried.

Conjugate. Fluorescein conjugated IgG fraction goat anti-rabbit gamma globulin (Capel Laboratories, Downington, PA) was diluted (1:8) in PBS and 2 ul of conjugate was applied over each well. The slides were incubated at room temperature for 30 minutes in a moist chamber, then washed in PBS for 10 minutes, rinsed in distilled H_2O and air dried. Then a small drop of glycerol-carbonate buffer mounting fluid (1 part 0.05 M carbonate buffer, pH 9.0, and 9 parts glycerol) was placed over each well and a number 1 coverslip applied. Slides were read at 40x using a Zeiss fluorescent microscope with a mercury HB)-200 bulb. A BG 12 and heat absorption light source filter were used. Results were graded using a scale of 1+ to 4+ where "4+" designated brightly fluorescent bacteria and "1+" implied faint visibility. Strains scoring 3+ or 4+ were called fluorescent positive and strains with lesser scores were determined fluorescent negative. A positive result was recorded only if preimmunization sera obtained from the same rabbit was fluorescent negative at the test dilution.

Controls. In each experiment, a known positive strain was included and all strains were tested with immune sera (PS or PPS) and compared to preimmunization sera obtained from the immunized rabbit. If the preimmunization sera showed fluorescent at a given dilution, further dilutions were made to compare immune and preimmune sera before reading a positive result. The PS antiserum was made by sterile intravenous inoculation of the animal 3x/wk for 2 weeks with a mixture of 50 ug purified capsular polysaccharide antigen and 50/ug methylated bovine serum albumin (MBSA). WEekly foot pad injections of this mixture in complete Freund's adjuvant were given for 3 weeks. The rabbit was boosted intravenously in the third week and bled a week later. The PPS antiserum was prepared with a similar inoculation schedule but MBSA and Freund's adjuvant were not used in conjunction with the capsular polysaccharide-outer membrane protein complex. The two sera were diluted in PBS and titrated, using a preimmunization sera from these rabbits as controls, to determine an appropriate serum dilution.

115 strains of B. fragilis were examined by the indirect immunofluorescence assay. One antiserum employed was developed to a purified capsular polysaccharide outer membrane protein complex (PPS)(Table 4).

Table 4: Indirect Fluorescent Antibody Analysis of Strains of B. fragilis for outer membrane capsular polysaccharide-protein antigens and capsular polysaccharide antigens of B. fragilis ss. fragilis (ATCC# 23745).

Serum	Subspecies					
	fragilis	distasonis	vulgatus	thetaiotaomicron	ovatus	other
PPS	77/77*	0/7	0/10	1/12	0/4	0/4
PS	37/37	0/4	0/6	0/6	0/2	0/1

*No.$^{+}$/total tested

All seventy seven strains of subspecies fragilis were positive when studied using this technique. In contrast, only 1 fluorescent positive strain was found among 32 strains of other subspecies. This data indicates that the strains of subspecies fragilis almost uniformly contained shared outer membrane antigens, which are rarely found in strains of the other subspecies.

Using the PS antiserum (Table 4, all thirty-seven strains of subspecies fragilis were fluorescent positive, and none of nineteen strains of other subspecies were positive. This indicates that the capsular polysaccharide is shared among strains of subspecies fragilis. Included in the 19 strains of other subspecies was one ss. thetaiotaomicron strain that was positive with the PPS serum and negative with the PS serum. This strain most likely shared an outer membrane protein antigen with the reference strains of ss. fragilis.

Eighteen clinical isolates of aerobic bacteria were also studied using the PS antiserum in the IFA. Included were clinical isolates of Proteus mirabilis [2], Proteus rettgeri [1], Klebsiella strain [3], E. coli [5], Streptococcus pneumoniae [3] and Streptococcus faecalis [3] and group B Streptococcus [2]. All 18 isolates were fluorescence negative in the IFA. 3 strains of Bacteroides melaninogenicus were also fluroescene negative in this assay.

The prevalence of encapsulated organisms within clinical isolates of the various subspecies were studied by several techniques. Using electron microscopy, india ink staining and Quellung reactions, capsules were demonstrated only in organisms classified as subspecies fragilis. These techniques failed to demonstrate capsules on bacteria of ss. distasonis, ss. thetaiotaomicron, ss. vulgatus or ss. ovatus. Therefore, the subspecies of B. fragilis which predominated in clinical specimens were encapsulated by these criteria.

Previously, it had been demonstrated that antibody to the capsular material from a single strain of ss. fragilis could be detected in rabbit antisera prepared to other strains of subspecies fragilis. To expand this observation, we developed an indirect immunofluorescence assay to examine clinical isolates for the presence of capsular material immunologically similar to the reference strain of B. fragilis ss. fragilis (23745). All strains examined which were identified as ss. fragilis were positive in this assay, while strains of other subspecies were negative. The large number of strains examined by this rapid and simple technique would indicate that strains of ss. fragilis uniformly contain this capsular material, which is absent in strains of the other subspecies. This is phenotypic evidence supporting the genotypic differences between B. fragilis ss. fragilis and the other subspecies based on DNA homology (20). These differences may be large enough to warrent re-classification of B. fragilis fragilis as a distinct species.

The identification of B. fragilis from clinical isolates may have important therapeutic implications because of the resistance of this particular obligate anaerobe to penicillin. Considerable progress has been made in the identification of these bacteria in recent years, however, the procedures employed for species identification are expensive and time consuming. Frequently useful identification of these bacteria is not available until after this information is no longer useful to the patient's physician. The specificity, sensitivity, rapidity and simplicity of the IFA assay reported here for the identification of B. fragilis fragilis and the greater prevalence of this subspecies in clinical isolates, raises the possibility of the utilization of this system in a clinical laboratory.

C. The Animal Model of Bacterial Peritonitis- (collaborative with Drs.A.B. Onderdonk and J. Bartlett).

Rowley (21) has suggested that perhaps the thickness of the capsule of a bacterium is a major factor in its virulence. Studies of Streptococcus pneumoniae have indicated that virulence varies with the quantitative amount of polysaccharide synthesized by different strains of the same type; these data show that one of the factors concerned with the mouse virulence of these strains is probably the genetic apparatus that controls the amount of polysaccharide produced (22). It is apparent from these studies that B. fragilis has a rather thick capsular polysaccharide associated with its outer membrane. With certain organisms, particularly S. pneumoniae it has been found that although the specific capsular polysaccharide is non-toxic in the isolated state, it contributes to the virulence of the pneumococcus by acting as an anti-phagocytic agent. In a paper by Casciato, et al. (23), strains of B. fragilis fragilis were shown to be relatively resistant to serum bactericidal activity. Muschel (24) has indicated that the presence of a capsular polysaccharide may account for the resistance of an organism to the bactericidal activity of the complement-antibody system. The existence of a capsule in this bacterium may be a factor in its apparent virulence.

Previous studies of Drs. Onderdonk, Bartlett, Gorbach, et al. have employed a model of intrabdominal sepsis in which a complex fecal inoculum containing obligate anaerobes and facultative bacterial species was implanted into rats (25, 26, 27). Initial observations revealed a biphasic disease. The first phase was characterized by peritonitis in all animals and a 43% mortality. E. coli and enterococci were the predominant isolates from the peritoneal exudates and blood. Recipients surviving this initial phase uniformly developed intraabdominal abscesses by the seventh post-operative day. This second stage revealed a shift in the predominant microbes from facultative species to two anaerobes, B. fragilis and Fusobacterium. Subsequent experiments have demonstrated a microbial synergy between a facultative species, such as E. coli or enterococci, and an anaerobic species such as B. fragilis or Fusobacterium in promoting abscess formation (27). It has also been shown that E. coli was responsible for the early mortality associated with peritonitis. Neither the two facultative species combined nor the two anaerobes implanted together were capable of producing abscess in recipient animals. However, a combination of either anaerobe with either aerobe resulted in abscess formation. These studies have clearly implicated gram-negative anaerobes as being involved in the infectious process leading to abscess formation in the animal model.

As stated previously, it has been shown that B. fragilis ss. vulgatus and thetaiotaomicron are the predominant fecal isolates (5) and that B. fragilis ss. fragilis is a relatively minor component of the fecal microflora. This observation suggests that B. fragilis ss. fragilis may possess a virulence factor not present in the other subspecies which gives this microorganism an increased potential to infect human tissues.

One difference between the various subspecies of B. fragilis has been discussed. B. fragilis ss. fragilis contains a polysaccharide capsule which does not appear to be present in the other subspecies of B. fragilis.

Collaborative studies involving testing the relative ability of encapsulated and unencapsulated strains of B. fragilis to potentiate abscess formation using the previously described rat model of intraabdominal sepsis have been undertaken. In prior studies an unencapsulated strain of B. fragilis was employed. Since a synergy between facultative species and unencapsulated B. fragilis had been shown in this model for abscess formation, the ability of the various encapsulated and unencapsulated strains to promote abscess when implanted alone and in conjunction with the non-lethal, but synergistic enterococci was tested. Differences in the relative ability of the various implants to promote abscess in recipients was used as a criteria of virulence.

Microorganisms used in the model

Cultures of B. fragilis ss. fragilis (ATCC 23745, BCH 26783), B. fragilis ss. distasonis (ATCC 8503, BCH 39675), B. fragilis ss. vulgatus (BCH 2242) and B. fragilis ss. thetaiotaomicron (BCH 43172) were obtained from stock cultures at the Channing Laboratory. All BCH strains were clinical isolates. Stock cultures of enterococci (BVA 18-1) and B. fragilis ss. other (BVA 18-13) were obtained from Boston Veteran's Administration Hospital and were isolated from infected rats. Stock cultures were grown in pre-reduced peptone yeast glucose broth for 24-48 hrs. within an anaerobic chamber, frozen at -70 C and manitained in the frozen state until used. S. pneumoniae Type III was obtained at the Bacteriology Laboratory of Boston City Hospital and was maintained on blood agar plates.

Animals

Male Wistar Rats (Simonsen Laboratories, Palo Alto, California) weighing between 160-180 grams were used for all experiments. Animals were housed separately and received chow (Ralston Purina) and water ad libitum. Animals were anesthetized with an intraperitoneal injection of 0.15 cc Nembutol (50 mg/ml), shaved and prepped with iodine. A 1 cm anterior midline incision was made through the abdominal wall and peritoneum and a double gelatin capsule containing 0.5 ml of the inoculum was inserted into the pelvic region. The incisions were closed with interrupted 3.0 silk sutures, and the animals returned to separate cages. Surgical mortality in all groups was less than 1% of the implanted animals.

Inoculum

Test groups of animals received an inoculum consisting of 10% w/v barium sulfate, 50% v/v sterile cecal contents and either bacteria and/or bacterial cell components (Table 5).

Table 5:

Group	Bacteria (CFU/ml)	Inocula*	Cell component
1	Enterococcus	5×10^7	
	B. fragilis UC**	5×10^7	
2	Enterococcus	5×10^7	
	B. fragilis C***	5×10^8	
3	*B. fragilis* UC	1×10^8	
4	*B. fragilis* C	1×10^8	
5	*B. fragilis* UC	5×10^7	
	Heat-killed *B. fragilis* C		
6	Heat-killed *B. fragilis* C		
8	*B. fragilis* UC	5×10^7	*B. fragilis* C outer membrane
9	*B. fragilis* UC	5×10^7	*B. fragilis* C Protein polysaccharide
10	*B. fragilis* UC	5×10^7	*B. fragilis* C Lipopolysaccharide
11	*B. fragilis* UC	5×10^7	*B. fragilis* C Capsular polysaccharide
12	*B. fragilis* UC	5×10^7	*E. coli* 07:K1 (L):NM Capsular polysaccharide
13	*B. fragilis* UC	5×10^7	
	Heat-killed *S. pneumoniae*		
14			*B. fragilis* C Capsular polysaccharide
15			*E. coli* 07:K1 (L):NM Capsular polysaccharide
16	Heat-killed *S. pneumoniae*		

*All implants contained: 10% w/v barium sulfate and 50% w/v sterile cecal contents.
**UC = unencapsulated strain.

A total of 15 groups containing 5-60 animals each were implanted. The first two groups received a combination of enterococci and either unencapsulated *B. fragilis* (UC) or encapsulated *B. fragilis* (C). Groups 3 and 4 received viable *B. fragilis* C and UC alone, group 5 was implanted with *B. fragilis* UC combined with heat-killed *B. fragilis* C and group 6 received the heat-killed *B. fragilis* alone. Group13 animals were challenged with heat-killed *S. pneumoniae* combined with *B. fragilis* UC. An additional series of experiments were performed using cell wall components of various bacteria. Groups 8-12 received *B. fragilis* UC in combination with four cell wall fractions from *B. fragilis* C, (outer membrane, capsular polysaccharide-protein, lipopolysaccharide or capsular polysaccharide), capsular polysaccharide of *E. coli* 07:K1(L):NM. The final groups were challenged with capsular polysaccharide from *B. fragilis* C, capsular polysaccharide from *E. coli* 07:K1(L)NM or heat-killed *S. pneumoniae* Type III. The inoculum in these last three groups contained no viable bacteria.

Four control groups contained 10 animals/group were implanted with sterile cecal contents alone, barium sulfate alone, sterile cecal contents plus barium sulfate and enterococci (10^8 CFU/ml) combined with sterile cecal contents and barium sulfate.

Preparation of bacteria

Quantitative determinations of viable cell density were obtained for all bacterial strains following quick freezing of the original culture. Aliquots of each species were thawed within an anaerobic chamber, and serial 10-fold dilutions made using VPI dilution salts (9). Samples of 0.1 ml of each dilution were plated on pre-reduced Brucella-base blood Agar (BMB). Plates were incubated for 48 hours at 37 C within the anaerobic chamber and colonies counted. Viable cell density was expressed as colony forming units/milliliter. Prior to implantation, bacterial cultures were diluted to the appropriate concentration using sterile PYG broth. Implants consisted of gelatin capsules containing 0.5 ml of broth mixed with barium sulfate (10% w/v) and sterile cecal contents (50% v/v). The number of bacteria implanted were expressed as viable CFU/implant.

Preparation of cell fractions

Cell outer membranes and fractions were prepared as described above. E. coli 07:K1(L)NM capsular polysaccharide was isolated and purified by previously described techniques (28). Implants of heat-killed Streptococcus pneumoniae Type III and B. fragilis ss. fragilis (ATCC 23745) were prepared by heating aliquots of 1×10^8 CFU/ml at 80 C for 30 minutes in a water bath.

Evaluation of implant results

Autopsy of recipients of B. fragilis C or B. fragilis UC in combination with enterococci revealed similar results when autopsied at seven days (Table 6).

Table 6: Results of implanting B. fragilis UC and B. fragilis C alone and in combination with enterococci.

Group	Implant	No. tested	Abscesses	Mean ug antipolysaccharide antibody/ml Day after implant 0	5-7	12
1	B. fragilis UC + enterococcus	10	8 (80%)	3.2	3.2	3.2
2	B. fragilis C + enterococcus	10	8 (80%)	3.2	110.5	144.7
3	B. fragilis UC	60	10 (17%)	3.2	3.2	3.2
4	B. fragilis C	20	19 (95%)	3.2	59.7	242.7
5	B. fragilis UC + heat-killed B. fragilis C	20	17 (85%)	3.2	43.4	-
6	Heat-killed B. fragilis C	20	13 (65%)	3.2	61.0	-

Eight of ten animals in both groups contained intraabdominal abscesses which yielded the implanted bacterial strains on culture. On the other hand, only 10 of 60 animals (16.7%) showed intraabdominal abscess formation when given B. fragilis UC alone. Implants of B. fragilis C alone resulted in abscess formation in 19 of 20 animals, an incidence similar to that noted in the groups receiving these strains in combination with enterococci. These results showed that B. fragilis C alone had a significantly greater capacity ($p < .01$ chi square) to promote abscess formation in the animal model than the unencapsulated strains of B. fragilis.

Implants of heat-killed B. fragilis C and B. fragilis UC

In an effort to define the factors responsible for the differences in abscess formation between C and UC B. fragilis, heat-killed B. fragilis C was implanted alone and in combination with B. fragilis UC. The results indicate that heat-killed B. fragilis C in combination with viable B. fragilis UC was as effective in promoting

abscess formation as live B. fragilis C alone (Table 6). Heat-killed B. fragilis alone had the capacity to promote abscesses in 13 of 20 (65%) recipients. Cultures obtained from exudate in this group were sterile; gram-stain of exudate showed no important differences in these abscesses compared to recipients of viable microorganisms except for the presence of bacteria in the latter group. These results indicate that B. fragilis C contains a heat stable factor which is able to potentiate abscess formation even when viable bacteria are not present.

Implantation of B. fragilis C cell components

Since it was observed that heat-killed encapsulated B. fragilis alone could cause abscess, an effort was directed toward defining whether an outer membrane component was responsible for this effect. Four cell wall fractions were obtained from B. fragilis C including the entire outer membrane, the capsular polysaccharide protein complex of outer membrane, the lipopolysaccharide fraction of outer membrane and the purified capsular polysaccharide. Analysis of these components showed that each contains either capsular polysaccharide or carbohydrates which are similar to those of the capsular polysaccharide or carbohydrates which are similar to those of the capsular polysaccharide. Each fraction was implanted in combination with B. fragilis UC (ss. distasonis ATCC 8503) at two doses. Initial doses were calculated to correspond to the approximate concentration of the cell component recovered from a culture containing 10^8 CFU/ml. The results of these implants (Table 7) indicate that all four cell components were effective in abscess formation in combination with B. fragilis UC and that this was a dose related phenomenon, the common demoninator being capsular polysaccharide.

Table 7: Results of implanting B. fragilis UC with cell wall components of B. fragilis C

Group	Cell wall component*	% Capsular polysaccharide	Amount (ug)	No. tested	No. with abscesses	Mean concentration antipolysaccharide ug/ Days after implant 0	7
8	Outer membrane	25	400	10	0	2.0	87.4
			1000	8	4(50%)	-	-
9	Protein polysaccharide	50	200	10	0	2.5	30.5
			500	8	4(50%)	-	-
10	Lipopolysaccharide	0**	90	9	0	2.0	8.4
			250	8	6(75%)	-	-
11	Capsular polysaccharide	99	100	10	5(50%)	2.0	8.9
			200	8	8(100%)	-	-

*All implants contain 1 x 10^8 CFU/ml B. fragilis UC

** 30-40% dry wt of lipopolysaccharide are carbohydrates found in capsular polysaccharide

Outer membrane, which contains the least amount of capsular polysaccharide on a dry weight basis, required 1000 ug of material to get abscesses in 50% of recipients; the capsular polysaccharide-protein, on the other hand, caused abscess formation in 50% of animals implanted with 500 ug. The lipopolysaccharide induced abscesses in 75% of the animals at 250 ug and the capsular polysaccharide caused all animals to develop abscesses at a 200 ug dose. Thus, there is a correlation between the concentration of B. fragilis capsular polysaccharide (or similar carbohydrates) and the total amount of cell wall component required to produce abscesses. By contrast,

implantation of 250 ug of E. coli 07:K1(L):NM capsular polysaccharide in combination with B. fragilis UC produced abscesses in just 3 of 10 animals. Similarly, heat-killed S. pneumoniae with B. fragilis UC caused abscesses in only 1 of 10 animals. These results implicate the capsular polysaccharide of B. fragilis as one of the factors responsible for promoting abscess formation in this animal model.

Implants of purified capsular polysaccharide and controls

A final series of animals were implanted with 200 ug of B. fragilis capsular polysaccharide without viable bacteria. All ten recipients had abscesses on autopsy examination seven days after challenge (Table 8).

Table 8: Results of implanting cell wall components without viable bacteria

Group	Implant	No. tested	No. with abscesses	Mean concentration of anti-polysaccharide antibody ug/ml Days after implant 0	7
14	B. fragilis capsular polysaccharide (200 ug)	10	10(100%)	2.0	10.4
15	E. coli 07:K1(L):NN capsular polysaccharide	5	0	2.0	2.0
16	Heat-killed S. pneumoniae	8	0	2.0	2.0

Similar experiments using capsular polysaccharide of E. coli 07:K1(L):NM and heat-killed S. pneumoniae failed to produce any abscesses.

Control animals implanted with barium sulfate, sterile cecal contents, barium sulfate plus cecal contents failed to produce abscesses in any animals. While sterile cecal contents were included in all inocula, our data indicates that cecal contents from germ free rats can be used in place of sterile cecal contents in this model. This suggests that bacterial products potentially present in the sterile cecal contents from conventional animals are not a necessary component. Implantation of barium sulfate alone results in a transient peritoneal fluid accumulation, but no abscesses.

Several recent publications have documented the importance of obligate anaerobes in clinical infections (29,2,30). Isolation rates of these microbes with certain types of infection may approach 100% in cases where the large intestine has been violated. Among the various anaerobic species, B. fragilis has consistently been cited as the most common isolate in these infections. One traditional view of the involvement of anaerobes in infection has been that these microbes are merely commensals and play no important role in the septic process. This view, however, is not supported by experimental models in which the pathogenicity of obligate anaerobes has been tested. The polymicrobic nature of anaerobic species injected together into guinea pigs was studied by Altemeier (14). He observed that single species of anaerobes rarely caused lesions when injected into animals. On the other hand, two relatively avirulent strains combined prior to injection resulted in peritonitis. Similar studies by Hite et al, (31) and Onderdonk et al, (26,27) have shown that abscess formation in rats implanted with colonic bacteria was contigent on the presence of anaerobes as part of the inoculum. Antimicrobials effective against anaerobes administered concommitantly with a challenge of complex fecal flora prevented abscess formation, while antimicrobials effective against facultative species failed to prevent this effect (32). Additional studies have demonstrated the synergy between facultative species and B. fragilis in abscess formation. Other investigators have reported abscess lesions with injection of pure culture of anaerobes into animals. Hill et al, (33) and Abe et al, (34) have

both reported liver abscess models using only Fusobacterium as an inoculum. Reports by Hackmen (35) have documented subcutaneous abscess using B. fragilis injected into mice.

Our studies were designed to determine the relative ability of encapsulated and unencapsulated B. fragilis to promote abscess formation in an experimental rat model of intraabdominal sepsis. Initial experiments tested the frequency of abscess formation when either encapsulated or unencapsulated strains of B. fragilis were combined with enterococci. Abscesses resulted in a majority of animals in both groups. These results are similar to previously reported studies in which it was shown that B. fragilis usually required the presence of a facultative strains to produce abscesses in this animal model. In retrospect, the bacteroides fragilis strain utilized in that study was unencapsulated according to electron microscopic and IFA examination. In the present experiment it was found that encapsulated B. fragilis produced abscesses without the addition of a second organism to the inoculum. Of particular interest was the observation that similar results were obtained utilizing heat-killed encapsulated B. fragilis. This suggested the presence of a heat stable abscess promoting factor which did not require viable bacteria. Subsequent experiments focused attention on the cell wall component of the encapsulated strains which was responsible for this effect. The results of these studies showed a correlation between the incidence of abscesses and the amount of B. fragilis capsular polysaccharide in the inoculum.

The implication of a capsular polysaccharide as a factor associated with virulence is not a unique observation. With S. pneumoniae, for example, it has been noted that smooth strains are more virulent than rough strains. With this organism the capsular material inhibits phagocytosis but does not appear to have any well defined toxic properties of its own. On the other hand, lipopolysaccharide of gram negative bacteria can contribute to virulence but only by inhibition of phagocytosis but also by the direct toxic effects presumably associated with endotoxin (27). One interesting difference between the lipopolysaccharide of coliforms and that of B. fragilis is the relative absence of biologic toxicity in the endotoxin of the latter organism. The unique feature of the B. fragilis capsule appears to be its propensity to promote abscesses. Capsular polysaccharide of E. coli and heat-killed S. pneumoniae failed to induce abscesses in this animal model whereas capsular polysaccharide of B. fragilis produced abscesses in 100% of recipients.

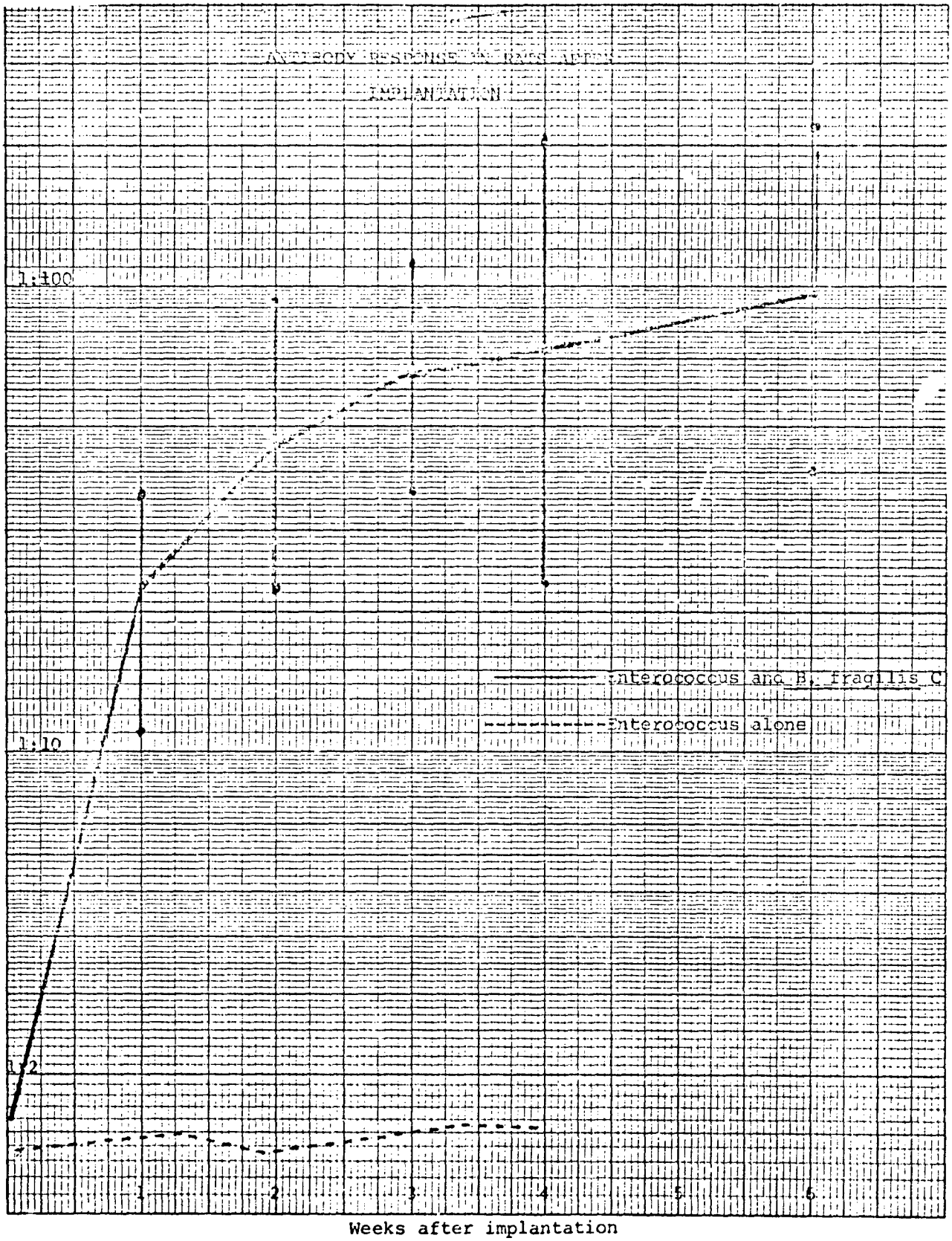
The "abscessogenic" qualities of the capsular polysaccharide of B. fragilis ss. fragilis may provide an explanation for the frequency of this organism in clinical infections, especially those associated with abscess formation. In addition, the identification of a specific factor associated with the virulence of this anaerobe should provide a clear outline for future studies of virulence factors associated with obligate anaerobes.

Antibody response in the rat model

Pre and post implantation sera were taken from the animals shown in Tables 6,7 and 8. Serum antibody concentrations are expressed as ug Ab/ml. The assay used was the RABA, described above, with 200 nanograms of antigen/test. The percent binding was converted to quantitative precipitin experiments using immune rat sera, according to the method outlined below (clinical studies).

All animals receiving encapsulated B. fragilis, heat-killed on live, cell wall components containing capsule, all developed high levels of serum anticapsular antibody. These concentrations continued to rise for 42 days. A typical antibody response curve is shown in Fig. 3. The mean and standard deviation is plotted for each time interval where serum was sampled.

Figure 3


1:100
1:10
1:2
Enterococcus and B. fragilis C
Enterococcus alone
1
2
3
4
5
6
Weeks after implantation

D. Clinical data in human pelvic inflammatory disease

Bacteroides fragilis have been reported to be frequent isolates from various infections of the female genital tract (16, 17). The role which these organisms play, particularly in pelvic inflammatory disease has been unclear. Eschenbach, et al (13), have described 2 clinically distinct types of pelvic inflammatory disease each with separate bacteriologic findings, gonococcal and non-gonococcal. Of the cases of non-gonococcal PID, the most common species recovered was B. fragilis. Of the 5 subspecies of B. fragilis, organisms of ss. fragilis were most frequently isolated. Isolation of these organisms may merely indicate isolation of normal vaginal flora from an area where anaerobic bacteria are common, however, they may be important in the infectious process. Evidence of an antibody response to these bacteria would elucidate the role of these organisms as pathogens.

In this study, antibody directed toward the common capsular polysaccharide of B. fragilis ss. fragilis is studied in paired sera of women with gonococcal and non-gonococcal PID using a quantitative radioactive antigen binding assay (RABA).

Selection of patients

Paired serums were obtained on 27 patients with acute PID from Drs.K.K. Holmes and D. Eschenbach. These sera were selected from patients with various bacterial species isolated from cul-de-sac specimens.

Antigen preparation

Intrinsically labelled (H^3 Na acetate) capsular polysaccharide of B. fragilis fragilis was isolated as described above. The specific activity of this antigen was 1000 counts/ug and 200 nanograms are used in the assay.

Quantitative precipitation analysis

Quantitative precipitin analysis of the sera of 3 normal individuals were performed by the methhod of Gotschlich, et al (36).

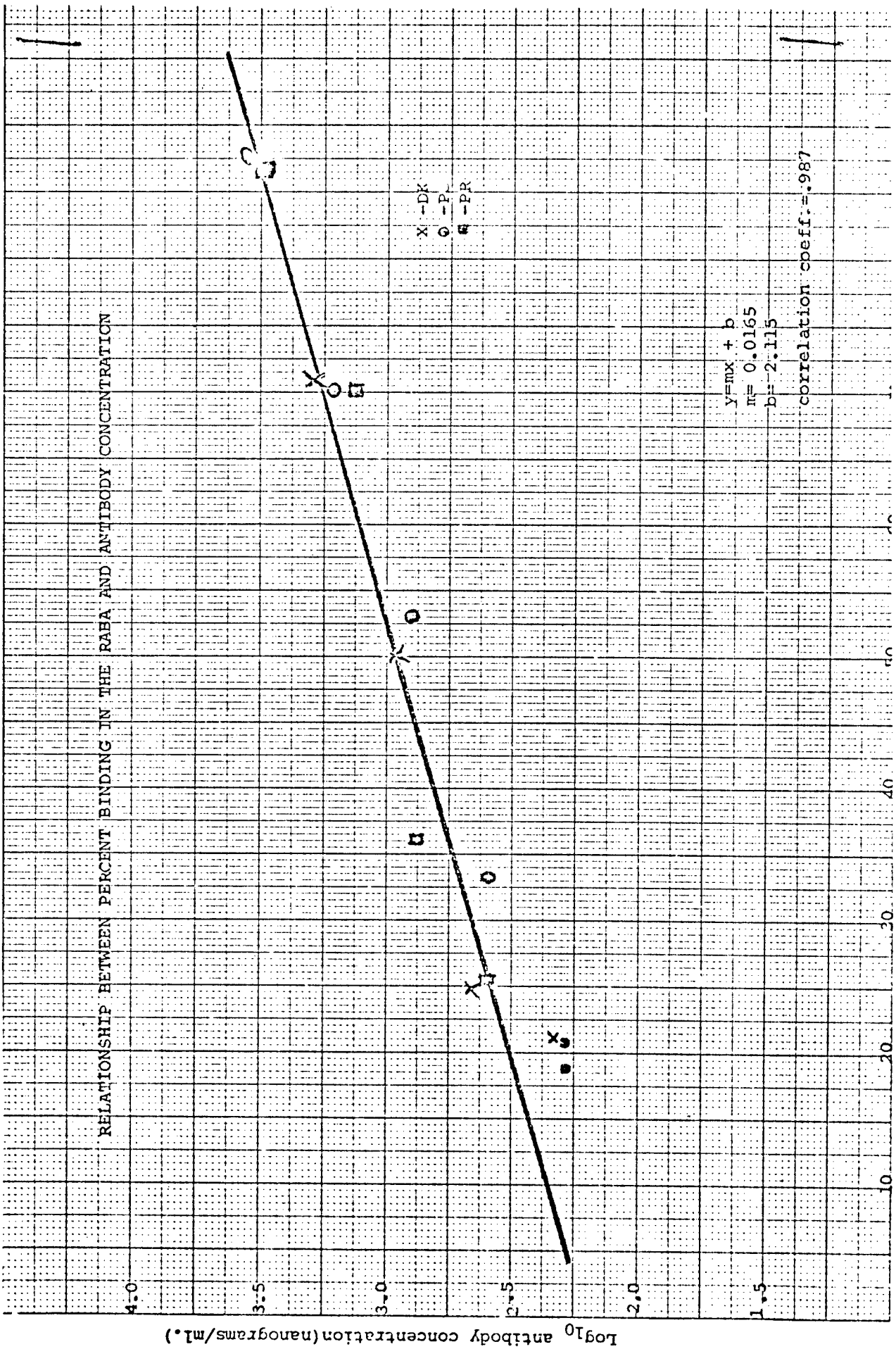
RABA

The RABA was done as described by Farr (37) performed as outlined above. The dilution of serum which bound 50% of the antigen was calculated from a series of 2 fold dilutions. Mean antibody concentrations were recorded from this 50% binding point. In as much as the percent binding was linearly related to the $\log_{10}$ of the antibody concentration as determined by the method of least squares, the concentration of antibody could be determined.

Quantitative determination of antibody by precipitation and by radioactive antigen binding capacity.

To relate antigen binding capacity to antibody concentration, the radioactive antigen binding capacity of three human sera with known content of precipitating antibody was determined. The concentration of antibody in these sera was determined by quantitative precipitation and were 38 ug/ml, 34.8 ug/ml and 32.3 ug/ml respectively. A highly significant linear relationship ($R = .989$, $r^2 = .976$) was observed between the percent of antigen bound and the log of the antibody concentration (Fig. 4), slope = .0165, intercept = 2.115.

Figure 4.


RELATIONSHIP BETWEEN PERCENT BINDING IN THE RABA AND ANTIBODY CONCENTRATION
X -DK
O -PL
■ -PR
y=mx + b
m= 0.0165
b= 2.115
correlation coeff.=.987
Log10 antibody concentration(nanograms/ml.)
4.0
3.5
3.0
2.5
2.0
1.5
10
20
30
40

Changes in antibody levels in pelvic inflammatory disease.

Paired sera from 27 women with acute pelvic inflammatory disease were studied for changes in level of antibody to the capsular polysaccharide of B. fragilis ss. fragilis. These sera were studied without knowledge of the culture results. Table 9 lists the acute and convalescent serum antibody levels of these women and the time interval between collection of the 2 serum samples.

Table 9: Culture results and serum antibody concentrations to B. fragilis ss. fragilis capsule in 27 selected cases of PID.

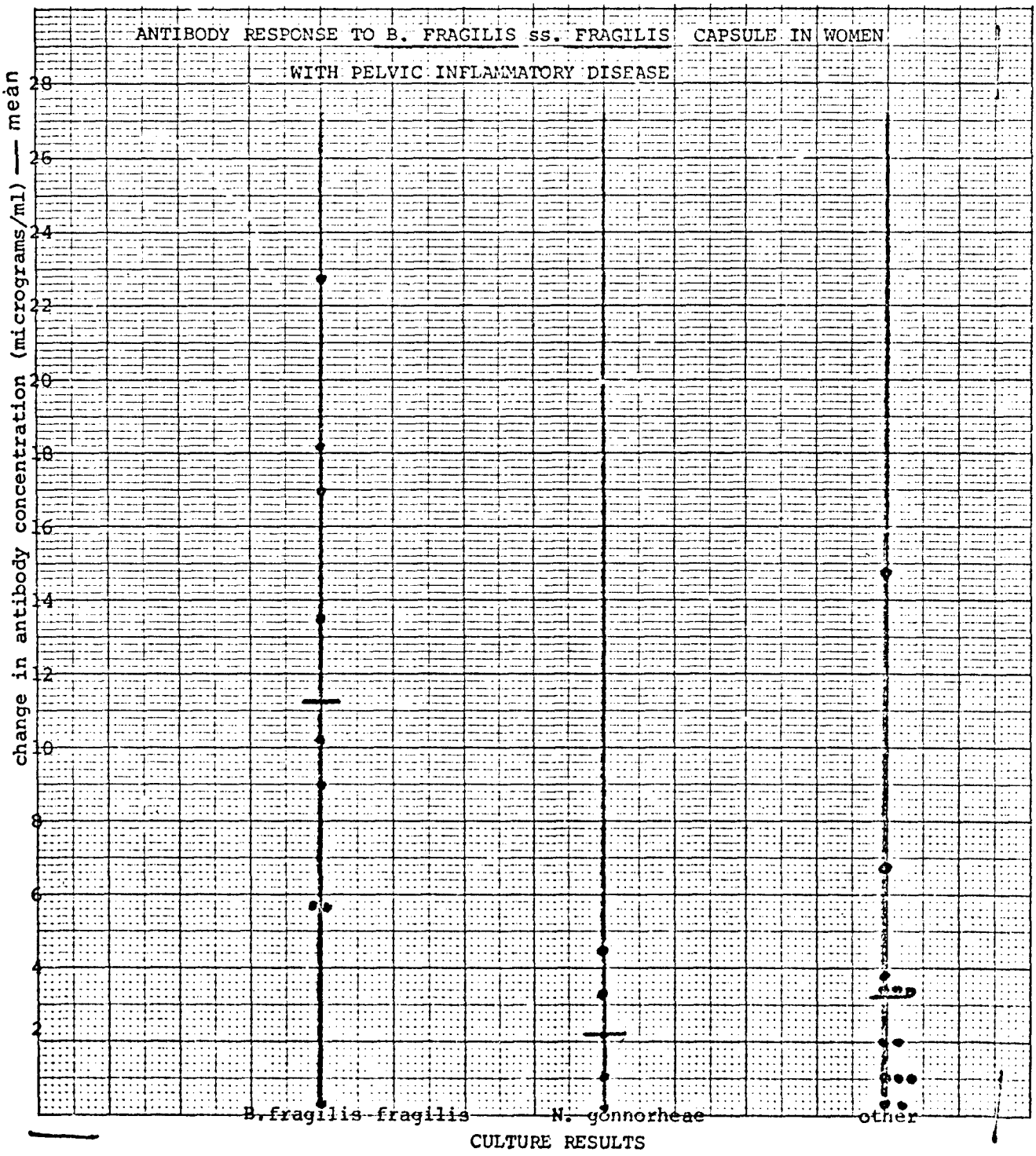
Patient	Organism	Acute ug/ml	Convalescent ug/ml ( )+	Change in antibody concentration (ug/ml
1	B. fragilis fragilis B. fragilis ss. thetaiotaomicron	9.06	14.72 (7)	5.66
2	B. fragilis fragilis	41.90	19.25 (30)	22.65
3	B. fragilis fragilis	11.30	5.66 (14)	5.64
4	B. fragilis fragilis B. fragilis ss. distasonis Peptostreptococcus intermedius B. melaninogenicus	11.30	11.30 (34)	0
5	B. fragilis fragilis	15.85	5.66 (7)	10.19
6	B. fragilis fragilis	26.04	7.93 (14)	18.11
7	B. fragilis fragilis Pseudomonas anaerobius H. vaginalis Lactobaccilus Fusobacterium sp.	11.30	20.38 (20)	9.08
8	B. fragilis	19.25	36.23 (23)	16.98
9	B. fragilis	14.72	28.31 (7)	13.59
10	N. gonorrhoeae	14.72	15.85 (30)	1.13
11	N. gonorrhoeae	16.98	12.46 (60)	4.52
12	N. gonorrhoeae	23.78	23.78 (64)	0
13	N. gonorrhoeae	6.79	10.19 (18)	3.40
14	N. gonorrhoeae Diptheroides Strepviridans	9.06	6.79 (34)	2.27
15	B. fragilis ss. distasonis	24.91	27.18 (20)	2.27
16	B. fragilis ss. distasonis	16.98	13.59 (46)	3.39
17	B. fragilis ss. distasonis B. fragilis ss. vulgatus B. fragilis ss. thetaiotaomicron	11.30	11.30 (21)	0
18	B. corrodens Pseudomonas anaerobius C. acnes	26.00	19.25 (21)	6.75
19	B. capalosis B. melaninogenicus	7.93	6.79 (10)	1.14
20	?	15.80	12.46 (14)	3.34
21	no isolate	19.25	19.25 (10)	0
22	Hemophilus influenzae Strepviridans	4.53	5.66 (33)	1.13
23	Diptheroides	23.09	26.72 (34)	3.63
24	Peptococcus provodii Diptheroides	7.93	22.65 (28)	14.72
25	Peptococcus asacharolyticus Fusobacterium necrophirum	5.66	6.73 (14)	1.07
26	Staphococcus epidermitis	21.51	24.90 (21)	3.40
27	H. vaginales	11.30	13.59 (18)	2.29

+ Days between specimens

The culture results from the cul-de-sac of these patients are also reported. The mean difference in serum antibody concentration between acute and convalescent specimens were 11.32 ug/ml, 2.26 ug/ml, and 3.32 ug/ml for women whom B. fragilis fragilis, gonococcus and neither organism respectively, were isolated.

These differences were highly significant ($p < .01$, analysis of variants) and indicate a much greater change in serum levels of antibody to the capsule of B. fragilis fragilis in association with isolation of this organism from the cul-de-sac. This data is depicted in Fig. 6.

Figure 5.



The role of various bacteria in the etiology of pelvic inflammatory disease has been somewhat controversial. Eschenbach, et al (13) in a study of 204 women with pelvic inflammatory disease recovered N. gonorrhoeae from the endocervical culture of 90 of these women. Cultures were done on peritoneal exudates from 54 of these women. N. gonorrhoeae was found in peritoneal exudates from 8 of 21 patients with, and none of 33 without cervical gonococcal infection. Among patients with severe disease, other bacteria were recovered from peritoneal exudates of 5 of 16 with and 19 of 22 without, cervical gonococcal infection. Mixed anaerobic and aerobic bacterial peritoneal infection was common in non-gonococcal pelvic disease. The most common species recovered were B. fragilis, peptostreptococcus and peptococci. In contrast, Chow, et al (38) using different techniques reported poor correltaion between the isolation of the gonococcus from the cul de sac and the endocervix. They reported a preponderance of aerobic bacterial isolates from the cul de sac and recovered B. fragilis only from one patient. They also indicated poor correlation between endocervical and cul de sac cultures of N. gonorrhoeae. Recently Moniff (39) has suggested that gonococci may be responsible for PID early in infection, followed by later superinfections with anaerobes. This would suggest that the non-gonococcal and gonococcal forms of the disease are not distinct, as suggested by Eschenbach, but rather progressive.

In this study, we have examined paired sera from women with acute pelvic inflammatory disease for the presence of antibodies to the capsular polysaccharide of B. fragilis ss. fragilis. Because these sera were frequently obtained at varying periods of time during the course of the illness, we have used a rise or fall in antibody concentrations as indicative of infection. Antibody was measured using a sensitive radioactive antigen binding assay. A highly significant difference in antibody concentrations was found between groups of women from whom B. fragilis ss. fragilis was isolated when compared to groups from whom either gonococcus or any other bacteria were isolated. This indicates that B. fragilis is playing a significant role in acute pelvic inflammatory disease, and this organism is involved early in the infectious process, and supports Eschenbach's belief that non-gonococcal PID is at least in part due to B. fragilis.

The RABA developed by Farr is a useful method for the quantitative determination of low concentrations of antibody. The quantity of antigen bound by a serum is a direct function of the concentration of antibody in that serum and the affinity of this antibody for antigen. In this investigation the RABA was standardized by reference to antisera with known quantitites of precipitating antibody. The degree of antigen binding was converted to antibody concentration with full recognition of the possible errors resulting from this conversion (36). The method of least squares provides a valid description of the relationship between percent binding and $\log_{10}$ antibody concentration ($r^2 = .975$).

In view of this data, and the resistance of B. fragilis to penicillin, some question is raised as to the use of penicillin in all cases of pelvic inflammatory disease. McCormack, et al (40) in a randomized therpeutic study of gonococcal and non-gonococcal PID using aqueous procaine penicillin G and Spectinomycin HCl demonstrated a significantly higher incidence of clinical failures in women with non-gonococcal PID than gonococcal PID. Both antibiotics were equally effective in gonococcal infection and neither was highly effective in non-gonococcal PID. This is interesting in view of the demonstrated in vitro sensitivity of B.fragilis to Spectinomycin. Perhaps the failures in this group are due to other anaerobes or aerobes. Future therapeutic studies regarding the treatment of gonococcal and non-gonococcal pelvic inflammatory disease are indicated.

2. Bacteroides melaninogenicus

Bacteroides melaninogenicus, has been implicated as one of the microbes involved in periodontal disease (41). It has further been shown to play an essential role in at least one model of mixed anaerobic infection (42). B. melaninogenicus is the predominant Bacteroides species isolated from pleuro-pulmonary infections (43). It has also been isolated from anaerobic infection of the soft tissues, pelvic, abdomen and other sites (29).

Studies of the immune response to Bacteroides melaninogenicus are few; those that do exist are almost solely limited to investigation of the immune response to a lipopolysaccharide (LPS) fraction of the organism isolated by the phenol-water technque (44,45,46). Other investigators have used whole organisms as antigens (47,48). Immunologic heterogeneity has been demonstrated within the species using whole cell agglutination (49), by ring test precipitation, complement fixation, indirect hemagglutination, and agar gel diffusion (46), and by slide cell agglutination of whole organisms with antiserum to a K-antigen preparation (45). No distinct serological classification has been made.

The study of the human immune response to infection with B. melaninogenicus is currently limited to the finding of low titers of antibody to the lipopolysaccharide fraction in normal subjects, using the indirect hemagglutination test (45). There are no reports of antibody response to B. melaninogenicusin patients with clinical infection.

Recent work in this laboratory in the isolation of outer membrane antigens of Bacteroides fragilis has provided an opportunity to systematically approach the study of B. melaninogenicus.

Inasmuch as well over 50% of the adult population is affected to some extent by periodontal disease, a detailed investigation of its pathogenesis bears serious attention. This fact, along with the many other clinical infections which involve B. melaninogenicus, has stimulated our current investigation into the immunochemistry of this organism.

Bacterial strains

Reference strain 382 (Bacteroides melaninogenicus subspecies asaccharolyticus) and strain 376 (Bacteroides melaninogenicus subspecies asaccharolyticus) were obtained through the courtesty of Dr. A. Crawford (Forsyth Dental Laboratory, Boston, Mass). Strain 536 was obtained from Dr. S. Finegold (Wadsworth V.A. Hospital, Lost Angeles, CA). Subspeciation was confirmed by Dr. A. Ondersonk (Boston V.A. Hospital, Boston, MA) according to the criteria outlined in the Anaerobic Laboratory Manual (9).

Media and Growth Conditions

The strains were stored initially in a lyophilized state. The organisms were suspended in 0.15 M NaCl and transferred to blood agar plates supplemented with menadione (0.5 ug/ml). Incubation was carried out in an anaerobic jar (GasPak, Baltimore Biological Laboratories, Baltimore, MD) at 37 C for 48 hours. One loopful of organisms was then suspended in 1 ml of 0.15 M NaCl and inoculated into a 100 ml anaerobic starter flask containing trypticase yeast basal extract medium (50) supplemented with hemin (5 ug/ml) and menadione (0.5 ug.ml)and incubated overnight. Ten ml of suspended organisms ($\sim 5 \times 10^8$ organisms/ml) were then transferred to each of four 1.5 liter Erlenmeyer flasks and grown overnight in the same trypticase yeast basal extract medium under anaerobic conditions. In one experiment 25 mCi H^3 sodium acetate (New England Nuclear, Boston, Mass) were added to each 1.5 liter flask. After incubation for 24 hours, the organisms were centrifuged at 8,000 g at 4 C and washed three times with 0.15 M NaCl. All cultures were examined by gram stain and subcultured aerobically and anaerobically on blood agar to check for contamination.

Purification of the Outer Membrane

Pelleted organisms were suspended in approximately 200 ml of a buffer containing 0.05 M sodium phsophate, 0.15 M NaCl, and 0.01 M EDTA adjusted to pH 7.4. The suspension was then incubated in a 60 C water bath for 30 minutes followed by shearing through a 25 guage needle with manual pressure, and mixing in a Waring blender for 10 seconds. The suspension of organisms was then centrifuged at 12,000 g for 20 minutes (Sorvall RC-5, Sorvall, Inc., Norwalk, CT) and the pellet was discarded. The supernatant was centrifuged at 80,000 g for 2 hours, (Beckman L2-65B, Beckman Instruments, Inc., Fullerton, CA) and the pellet, a yellowish-lucent gel was suspended in water and lyophilized.

Sucrose Density Gradient

A discontinuous sucrose density gradient was made by sequentially layering 0.5 ml solutions of sucrose in 5% steps, from 60% to 20% (wt/wt) into cellulose nitrate centrifuge tubes. 500 ug of outer membrane, intrinsically labeled with H^3 sodium acetate were suspended in 0.5 ml of 20% sucrose and layered on top of the

density gradient. The gradient was centrifuged at 250,000 g (Beckman L2-65B) for 18 hrs. 0.2 ml fractions were collected through a needle puncture in the bottom of the centrifuge tube and density was calculated from a table based on the percentage of sucrose read directly from a refractometer (Bausch and Lomb, Rochester, N.Y.). Radioactivity was determined on 50 ul aliquots of each fraction, suspended in 10 ml of a scintillation fluid containing 60% toluene, 40% ethylene glycol monomethyl ether (v/v), 0.4 g/100 ml Omnifluor (New England Nuclear, Boston, MA) in a Beckman L-230 scintillation counter (Beckman Instruments, Fullerton, CA).

Fractionation of the Outer Membrane

Lyophilized outer membrane 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 9.0 with NaOH (45). The pH of the suspension was raised to 11.0 with 1 N NaOH, where the suspension clarified, and was then lowered to pH 9.0 with 1 N HCl, where it remained clear. The sample was then immediately chromatographed on a 1.6 x 82 column of Sephadex G-100 (Pharmacia, Uppsala, Sweden) equilibrated with 0.5% NaD buffer and 2 ml fractions were collected.

The void volume material (pool 1, 10^5 daltons) was collected and concentrated to a volume of 5 ml with use of an ultra-filtration cell (Amicon Corp., Lexington, MA) with a PM-30 membrane. This fraction was separated from the NaD buffer by precipitation with 0.2 M NaCl and 80% ethanol. The suspension was centrifuged at 10,000 g for 15 min. The alcohol precipitation was repeated, and, after centrifugation the pellet was dissolved in 2 ml of water and lyophilized. The remainder of the column eluant (Pool 2) was treated similarly, except that a UM-2 membrane was used to concentrate this fraction because of its smaller size. The small molecular size ($\sim$ 12,000 daltons) was attributed to the lipopolysaccharide (LPS) disaggregating properties of NaD buffer.

For some studies Pool 1 was further purfied. The lyophilized Pool 1 material was suspended in 0.05 M Tris buffer, pH 7.3, at a concentration of 2 mg/ml. This suspension was the incubated with Pronase (Calbiochem, San Diego, CA) in a ratio of 1 mg Pronase/2 mg Pool 1, overnight at 36 C. A similar amount of Pronase was added the following morning, and the mixture was incubated for another 4 hours. The undigested large molecular size component of Pool 1 was then separated from the digested material and Pronase by gel chromatography on a Sepharose 4B column, 2.6 x 80 cm (Pharmacia, Uppsala, Sweden), equilibrated with Tris buffer, pH 7.3. The void volume of the column was estimated with blue dextran. Molecular size of the undigested Pool 1 material was estimated using a PM-30 membrane, alcohol precipitated with 80% ethanol, lyophilized, and stored at -20 C until used.

Polyacrylamide Gel Electrophoresis

Discontinuous, neutral polyacrylamide gel electrophoresis (PAGE) was performed as described previously, using 7.5% acrylamide and 0.2% sodium dodecylsulfate (SDS). The gels were stained for protein with Coomasie Brilliant Blue.

Electron Microscopy

Whole bacteria and outer membranes were studied by electron microscopy as described previously and similarly stained for capsular polysaccharide with Ruthenium Red.

Analytical Methods

Protein was determined by the method of Lowry (51) using bovine serum albumin as a standard. Total sugars were measured by the phenol-sulfuric acid method (52) using glucose as a standards. Hexoses were determined by the anthrone reaction (53) with glucose standards. Hexosamines were determined by the Elson-Morgan procedure (54) using glucosamine standards. Methyl pentose was determined by the Dische and Shettles method (55) using fucose standards. 2-keto-3-deoxyoctonate (KDO) was determined by the method of Weissbach and Hurwitz (56) as modified by Osborn (57), using KDO as a standard. Total heptose was determined by the ratio of ultraviolet light absorption at 280 nm compared to that at 260 nm (58). Total lipid was determined on whole outer membrane, Pool 1, and Pool 2 by modification of the procedure described by Bligh and Dyer (59), as follows: eight milligram samples were extracted with chloroform:methanol:H_2O (2:2:0:8) and allowed to settle overnight. The lower (chloroform) phase was then dried under a stream of nitrogen (N-Evap, Oraganomation. Associates.

Inc., Shrewsbury, MA). The extraction was repeated and the chloroform phase again dried. The dried material was then suspended in 2 ml of chloroform:methanol (2:1) where it was completely soluble. The solution was again dried under a stream of nitrogen and the remaining material weighed. Protein and carbohydrate determinations were made on the lipid phase to assure purity.

Gas-liquid chromatography

Gas-liquid chromatographic analysis of the membrane fractions (Pool 1 and Pool 2) was performed after trimethylsilyl (TMS) derivatives of standard sugars were prepared by a modification of the method described by Davis (60,61). Briefly, the large molecular size material from Pool 1, which had been digested with Pronase and purified on a Sepharose 4B column, was methanolyzed overnight in 2 ml of 0.1 M methanolic-HCl at 80 C. Each methanolyzed sample was extracted three times with two volumes of hexane and the hexane phase was removed and discarded. The methanolic-HCl was neutralized with NH_4OH to pH 7.0. The sample was then dried in a rotary evaporator (Roty-S, Brinkmann Instruments, Westbury, NY) and the dried sample was washed from the sample by drying under a stream of nitrogen. Trimethylsilyl derivatives were then prepared by adding 0.5 ml of a freshly prepared solution of pyridine:hexamethyldisilazane: trimethylchlorosilane (10:4:1) and stored overnight in a refrigerator. The mixture was then centrifuged at slow speed to remove precipitated NH_4Cl. After centrifugation the pellet was discarded and the supernatant dried under a stream of nitrogen and dissolved in 0.2 ml of hexane. The positive identification of all sugars was proved by co-chromatography with similarly prepared standard sugars or endotoxin from Escherichia coli 0:111. The 0:111 endotoxin and Pool 2 were methanolyzed with 0.5 M methanolic-HCl, and otherwise treated the same as Pool 1. The identification of all sugars in the 0:111, including heptose, has been proved previously by mass spectroscopy (62).

Samples were studied on a Packard 421 gas chromatograph (Packard Instruments, Inc., Downers, IL), fitted with a six foot U-shaped glass column, packed with 3% SE-30 on 80/100 Gas Chrom Q (Supelco, Inc., Bellefonte, PA). Samples were applied with an oven temperature of 140 C for 1 minute to elute the solvent peak. The temperature was then raised from 140 to 180 C at a rate of 5^{o}/min.

Serologic methods

The double diffusion in agar method of OUchterlony was used (63). Antisera were prepared by three intravenous injections per week into New Zealand white rabbits for three weeks. A booster injection was given in the fourth week. Phlebotomy was performed in the fifth week. Each injection contained approximately 5×10^9 live bacteria suspended in 1 ml of 0.15 M NaCl. Antisera were stored at -20 C.

Neufeld (Quellung) (19) reactions were performed on anaerobically grown broth and blood agar cultures using homologous and heterologous antisera.

Electron Microscopy

Three strains of Bacteroides melaninogenicus subspecies asaccharolyticus were studied by electron microscopy, staining for capsular polysaccharide with Ruthenium Red. Micrographs of whole organisms demonstrated trilaminar inner and outer cell membranes separated by a thin peptidoglycan layer typical of gram-negative bacteria (figures 6,7,8).

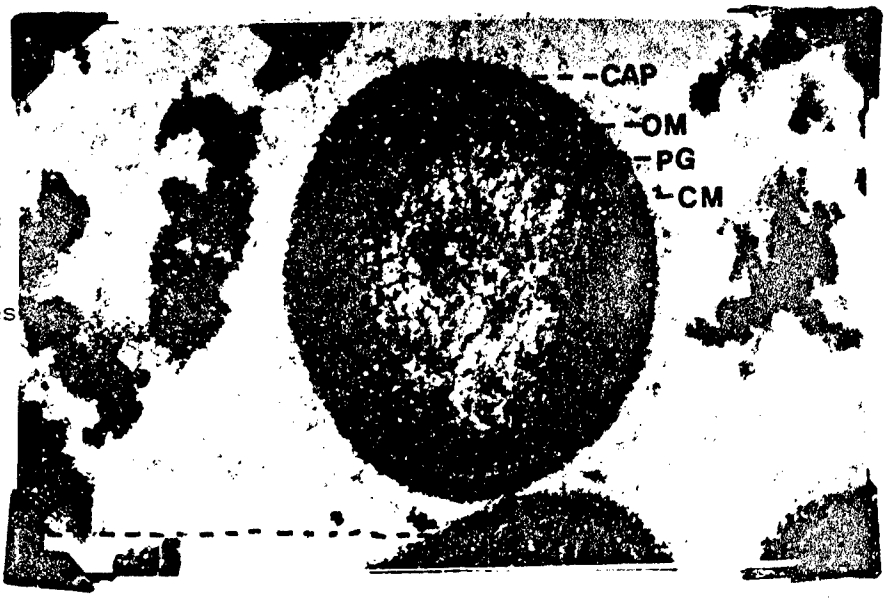


Fig. 6: Electron micrograph of thin sections of a 24 hr culture of Bacteroides melaninogenicus subspecies asaccharolyticus, strain 382, with Ruthenium Red in order to visualize the capsule (cap). OM = outer membrane; PG = peptidoglycan; CM = cytoplasmic membrane. The dashed line denotes 1 um (x 60,000).

Fig. 7: Electron micrograph of thin sections of a 24 hr culture of Bacteroides melaninogenicus subspecies asaccharolyticus, strain 376, stained with Ruthenium Red. Cap = capsule; OM = outer membrane; PG = peptidoglycan; CM = cytoplasmic membrane. The dashed line denotes 1 um (x 60,000).

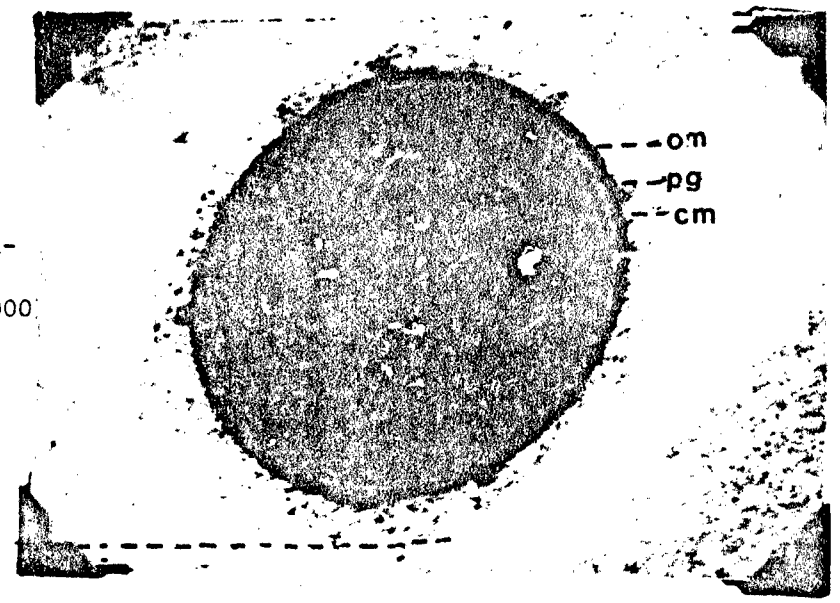
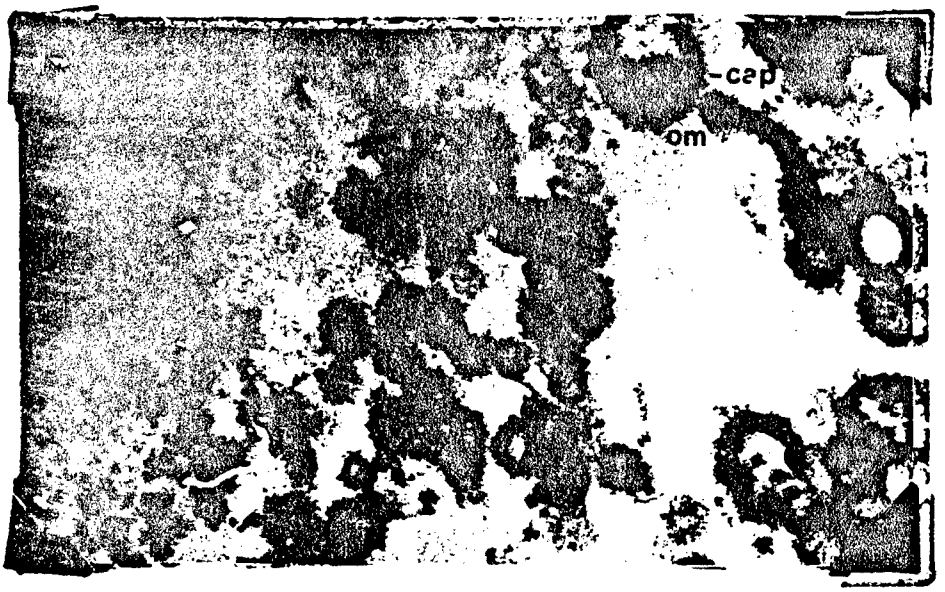


Fig. 8: Electron micrograph of thin sections of a 24 hr culture of Bacteroides melaninogenicus subspecies asaccharolyticus, strain 536, stained with Ruthenium Red. No capsule is present external to the outer membrane (OM). PG = peptidoglycan; CM = cytoplasmic membrane. The dashed line denotes 1 um (x 60,000)

The presence of a capsular material external to but associated with the outer membrane was noted in strains 382 and 376, but not in strain 536. Outer membrane preparations were also stained similarly (Figure 9).

Fig. 9: Electron micrograph of thin sections of a 24 culture of Bacteroides melaninogenicus subspecies asaccharolyticus, strain 382, stained with Ruthenium Red. Cap. = capsule; OM = outer membrane (x 60,000).

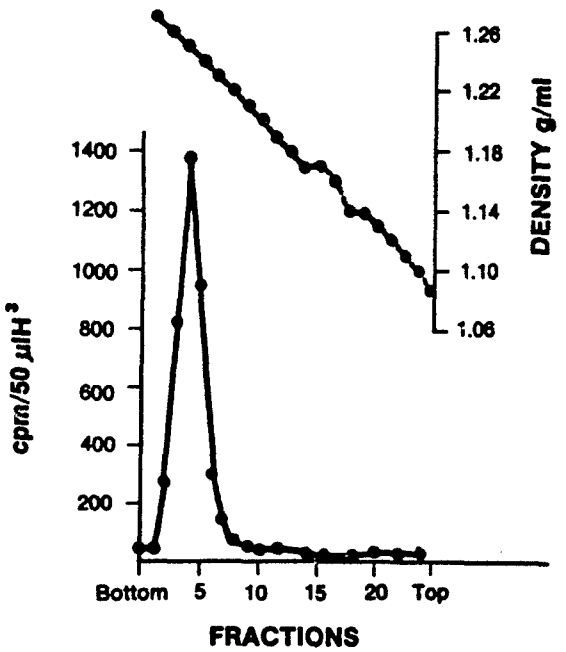


Enclosed structures, some with blebs extruding, and fragments were seen, all displaying a typical trilaminar membrane, without other cell wall or cytoplasmic structures evident. Capsular material was found to be present external to 95% of the membranes.

Purity of outer membrane in sucrose density gradients

The relative purity of the outer membrane was assessed by banding on a sucrose density gradient. When the H^3-sodium acetate labeled outer membrane preparation was placed on a sucrose density gradient (Figure 10), a single, sharp band resulted at a density of 1.24 g/ml.

Fig. 10: Discontinuous sucrose density gradient, ranging from 60% sucrose (bottom) to 20% sucrose (top). Concentrations of sucrose were converted to density (scale on right). Outer membrane of strain 382 was labeled with H^3-sodium acetate and 0.2 ml fractions were assayed for radioactivity (scale on left).



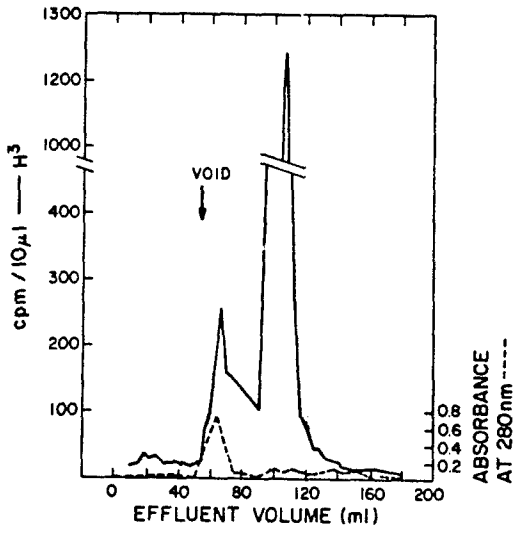
This finding confirms the relative purity of the outer membrane preparation, and indicates little contamination with peptidoglycan or cytoplasmic membrane. Furthermore, this density is similar to that reported for theouter membrane of Neisseria gonorrhoeae (64) and for B. fragilis, where the outer membrane density was shown to be clearly distinct from the density of the peptidoglycan and cytoplasmic membrane fractions.

Separation of the outer membrane components

The outer membrane preparation was found to be comprised of protein (23%), carbohydrate (13%, excluding amino sugars) and lipid (55%).

Outer membranes were extracted from organisms grown in six liter quantities labeled intrinsically with H^3-sodium acetate. The labeled outer membrane complex was then chromatographed on a Sephadex G-100 column equilibrated with 0.5% NaD buffer. The elution profile (figure 11) showed two distinct peaks of radioactivity.

Fig. 11: Elution profile of an outer membrane preparation of Bacteroides melaninogenicus subspecies asaccharolyticus chromatographed on a column of Sephadex G-100 Solid line indicates H^3 counts per minute (cpm) and broken line indicates protein content of fraction as determined by ultraviolet absorption at 280 nm.

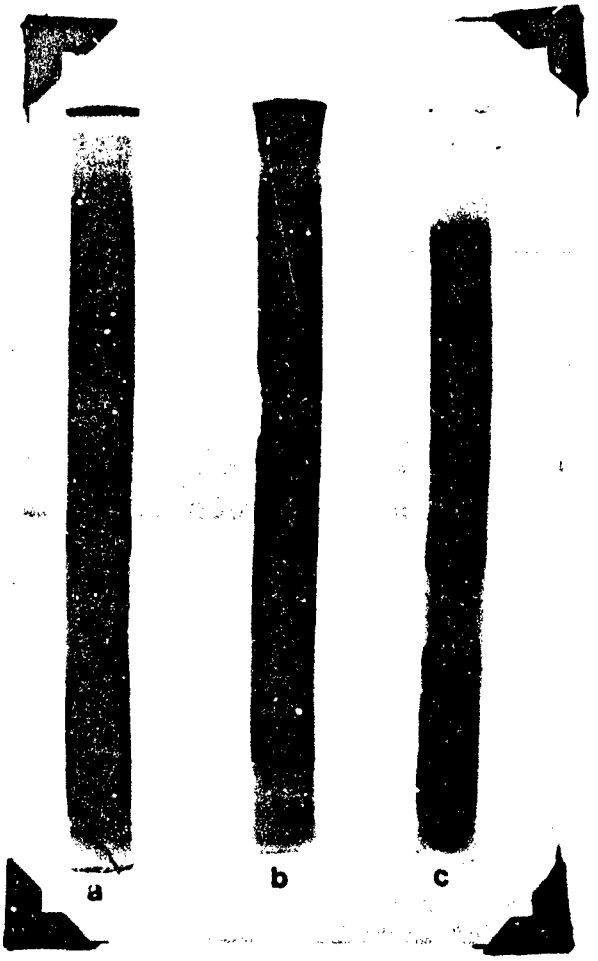


The void volume peak (Pool 1, 10^7 daltons) contained the majority of the membrane protein, and a second peak (Pool 2), eluted at a point where its estimated molecular size was 12,000 daltons, the size of the disaggregated lipopolysaccharide (LPS) monomer. This estimation was made by comparing elution profiles for proteins of known molecular size.

Measurement of loosely-bound lipid revealed that 55% of the whole outer membrane was lipid, while 26% of Pool 1 and 62% of Pool 2 were loosely-bound lipid.

Pool 1 (66% of the outer membrane) contained a large amount of protein (60% of Pool 1), while Pool 2 (33% of the outer membrane) had only small amounts (5% of Pool 2). The protein components of three strains (382, 376, 536) were further characterized by SDS-PAGE (figure 12).

Fig. 12: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of outer membrane preparations of three strains of Bacteroides melaninogenicus subspecies asaccharolyticus, stained for protein with Coomassie Brilliant Blue. A = Pool 1 (Sephadex G-100 column), strain 536, B = Pool 1, strain 376; C = Pool 1, strain 382. No bands were seen with any Pool 2 preparations.



When stained with Coomassie Brilliant Blue, unique peptide bands were noted for Pool 1 of all three strain. The Pool 1 patterns corresponded to those of the whole outer membrane preparations. There were no peptide bands noted in any of the Pool 2 fractions.

Molecular size of the peptide bands of each strain was determined by comparison with standards of known molecular size (cytochrome C, ovalbumin, transferrin). The major bands of strain 382 (figure 12, C) were estimated to be 90,000 and 30,000 daltons; those of strain 376 (figure 12, C)were 80,000 and 60,000 daltons; those of strain 536 (figure 12, A) were 80,000 and 52,000 daltons.

The carbohydrates of strain 382 were studied using spectrophotometric tests and gas-liquid chromatography. Spectrophotometric determinations showed that 58% of Pool 1 was measured as total carbohydrate, while 32% of Pool 2 was recovered as shuch. Each fraction was found to contain hexoses, hexosamines, methyl pentoses, in a ratio of 5:1:2 for Pool 1 and 9:3:2 for Pool 2. Neither Pool 1 nore Pool 2 had

Table 10: Gas-liquid chromatographic analysis of antigenic fractions of Bacteroides melaninogenicus subspecies asaccharolyticus.

Sugar	Pool 1	Pool 2
Rhamnose	N.D.	4
Mannose	N.D.	9
Glucose	18	32
Galactose	41	7
Glucosamine	13	11
Galactosamine	N.D.	N.D.
Heptose	N.D.	N.D.

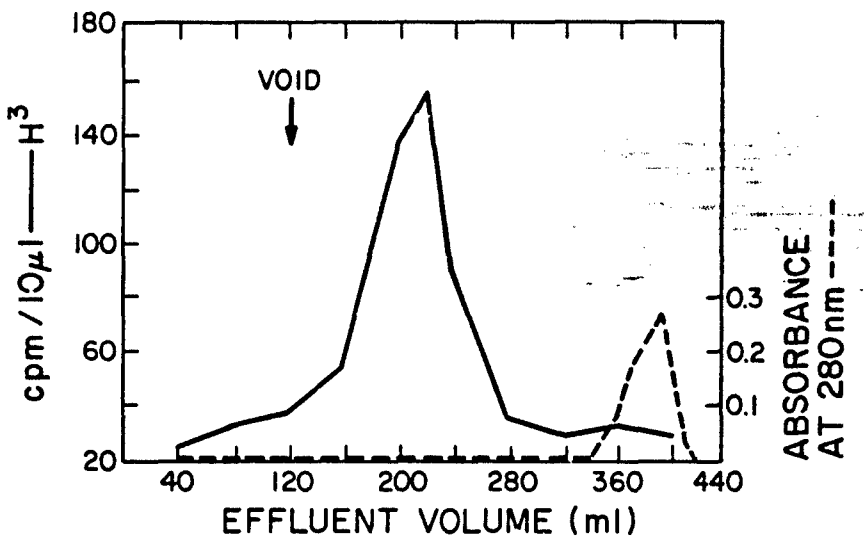
Note: Composition of sugars is given in percentages.

N.D. = not detectable in quantities 2%

detectable amounts of KDO, heptose or nucleic acid.

To isolate a large molecular weight polysaccharide from Pool 1, the Pool was digested with Pronase and the undigested material was then purified by gel chromatography on Sepharose 4B (figure 13).

Figure 13: Elution profile of a Pool 1 preparation (void volume of a Sephadex G-100 column) from strain 382. This material, after Pronase digestion, was chromatographed on a Sepharose 4B column. Solid line indicates H^3 counts per minute (cpm), and broken line indicates the protein content as determined by measuring ultraviolet absorption at 280 nm.



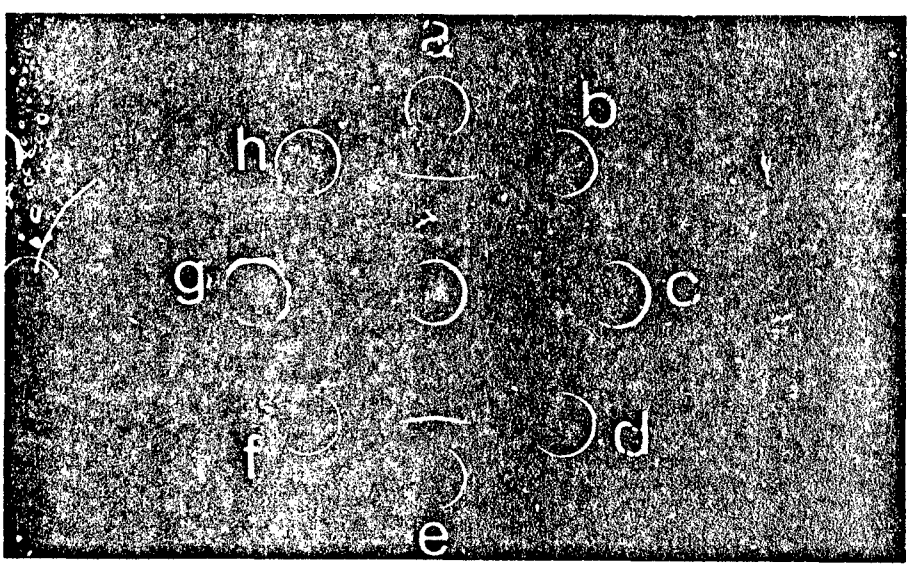
The purified material had less than 5% protein remaining. Its molecular size was estimated to be 7.2×10^5 daltons.

Gas-liquid chromatographic analysis of TMS derivatives of the methanolyzed Pool 1 polysaccharide (Table 10) demonstrated the following sugars: galactose (71%), glucose (18%), and glucosamine (13%). Similar analysis of Pool 2 revealed rhamnose (4%), mannose (9%), galactose (7%), glucose (32%), and glucosamine (11%). Neither of the fractions had detectable KDO or heptose by this method. Pool 2 contained two sugars, rhamnose and mannose (13% of Pool 2 carbohydrates), not present in Pool 1. The percentage composition of mutual sugars differed significantly between the two Pools.

Immunochemical characterization of the Outer Membrane

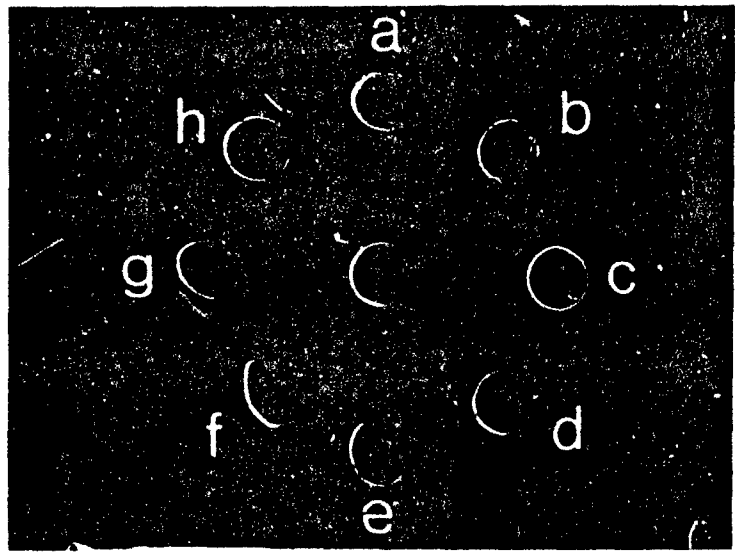
Immunodiffusion in agar was performed with the three strains of Bacteroides melaninogenicus subspecies asaccharolyticus, using the whole outer membrane, Pool 1 and Pool 2 with homologous and heterologous antisera. The whole outer membrane and Pool 1 preparations of strain 536 showed precipitin lines when tested with homologous antiserum (Figure 14).

Figure 14: Immunodiffusion in agar of antigenic components of three strains of Bacteroides melaninogenicus subspecies asaccharolyticus (382, 376, 536) against rabbit antiserum to strain 536 (center well). Outer wells contain the following components: A = outer membrane, strain 536; B = Pool 1, Sephadex G-100 column, strain 536; C = Pool 1, strain 382; D = Pool 1, strain 376; E = outer membrane, strain 536; F = Pool 2, strain 376; G = Pool 2, strain 382; H = Pool 2, strain 536. Concentration of antigens was adjusted to 3 mg/ml.



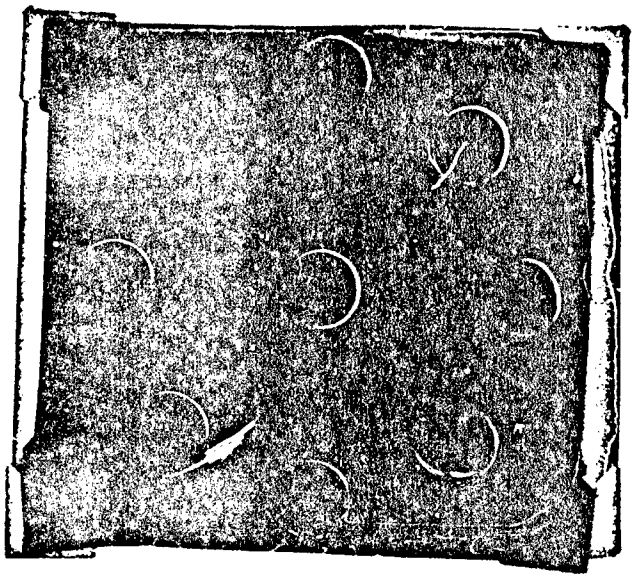
There were no precipitin lines seen when Pool 1 preparations from two other strains (382, 376) were tested with antiserum to strain 536, nor were there precipitin lines seen when Pool 2 preparations from either the homologous (536) or the two heterologous (382, 376) strains were tested with antiserum to strain 536. Strain 536, then demonstrated strain specificity in serologic testing using agar gel diffusion. When antigens from the three strains were tested with antiserum to strains 392 (figure 15), a different pattern was noted.

Figure 15: Immunodiffusion in agar of antigenic components of three strains of Bacteroides melaninogenicus subspecies asaccharolyticus (382, 376, 536) against rabbit antiserum to strains 382 (center well). Outer wells contain the following components: A = outer well, strain 382; B = Pool 1 (Sephadex G-100 column), strain 382; C = Pool 1, strain 376; D = outer membrane, strain 536; E = outer membrane, strain 536; F = Pool 2, strain 536; G = Pool 2, strain 376; H = Pool 2, strain 382. Concentration of antigens is 3 mg/ml.



One diffusion precipitin lines was seen with the homologous (382) outer membrane preparations. The vagueness of the line probably resulted from poor diffusion through the agar. Pool 1 of the homologous (382) formed two precipitin lines with its antiserum; Pool 1 of strain 376 also gave a precipitin line with antiserum to strain 382. Pool 1 of strain 536 did not react with antiserum to strain 382. Thus, immunologic cross-reactivity was demonstrable between strains 382 and 376 with antiserum to strain 382, but no cross-reactivity was seen with strain 536. When the antigens were tested with antiserum to strain 376 (figure 16) the homologous outer membrane preparation (376) shoed a diffuse precipitin line.

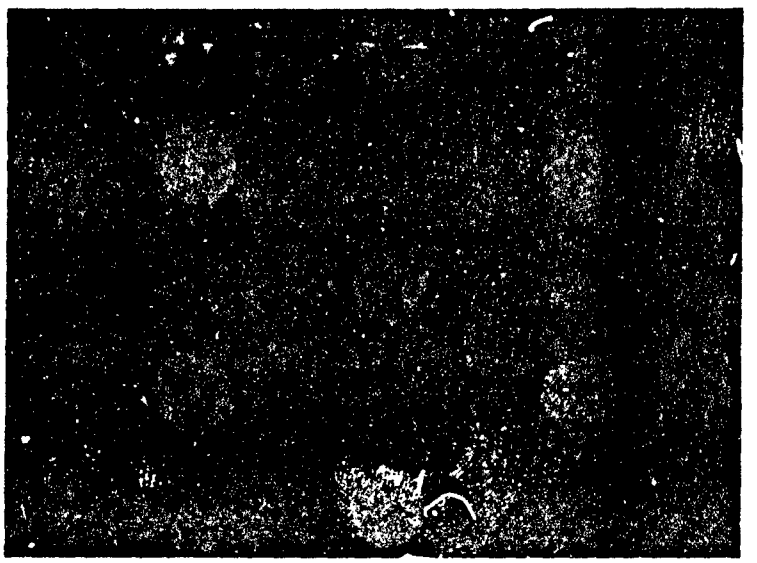
Fig. 16: Immunodiffusion in agar of antigenic components of three strains of Bacteroides melaninogenicus subspecies asaccharolyticus (382, 376, 536) against rabbit antiserum to strain 376 (center well). Outer wells contain the following components: A = outer membrane, strain 376; B = Pool 1, (Sephadex G-100 column), strain 376; C = Pool 1, strain 382; D = outer membrane, strain 536; E = Pool 1, strain 536; F = Pool 2, strain 382; H = Pool 2, strain 376. Concentration of antigens was adjusted to 3 mg/ml.



Pool 1 from the homologous strain formed two precipitin lines with its antiserum, Pool 1 from strain 382 demonstrated a single precipitin line with antiserum to strain 376. Pool 1 from strain 536 formed no precipitin line. None of the Pool 2 preparations gave precipitin reactions with antiserum to strain 376. Serologic cross-reactivity was thus confirmed between strain 382 and 376 with their respective antisera. Strain 536 formed precipitin lines with its antiserum only. None of the Pool 2 preparations demonstrated precipitin reactions with either homologous or heterologous antiserum.

After Pool 1 preparations from strains 382 and 376 were treated with Pronase the purified large molecular weight polysaccharide was again tested by agar gel diffusion. Whereas, prior to Pronase digestion, Pool 1 from strain 382 had formed two precipitin lines with its homologous antiserum, following Pronase digestion only one line remained (figure 17).

Figure 17: Immunodiffusion in agar of capsular antigens of strains 382 and 376 against rabbit antiserum to strain 382 (center well). The outer wells contain: A = Pool 1, strain 536 (no precipitin line); B = capsule (Pronase digested Pool 1 eluted from Sepharose 4B column), strain 382; C = capsule, strain 376. Concentration of antigens was adjusted to 3 mg/ml.



The cross-reacting line from strain 376 remained even after Pronase digestion. Again there was no reaction when strain 536 was tested with either antiserum to strain 382 or to strain 376. Similar results were noted when the Pronase digested Pool 1 preparations were tested with antiserum to strain 376.

Neufeld (quellung) reactions showed capsular swelling in all three strains under study when tested with homologous antiserum. Strains 382 and 376 showed capsular swelling when tested with each other's antiserum. Neither demonstrated swelling with antiserum to strain 536; nor did strain 536 show capsular swelling when tested with the other two heterologous antisera.

REFERENCES

1. Martin, W.J., Mayo Clin. Proc. 29:300, 1974.

2. Levison, M.E., Kaye, D. Drug Therapy. 6:117, 1976.

3. Wilson, W.R., Martin, J.M., Wilkowske, C.J. and Washington, J.A. Mayo Clin. Proc. 47:639, 1972.

4. Sutter, V.L., Attebery, H.R., Rosenblatt, J.E., Bricknell, K., Finegold, S.M. Anaerobic Bacteriology Manual. University of California Extension Division, Lost Angeles, 1972, 89 p.

5. Finegold, S.M., Attebery, H.R., Sutter, B.L. Am. J. Clin. Nutr. 27:1456,1974.

6. Moore, W.E.C., Holdeman, L.V. Am. J. Clin. Nutr.25:1306, 1972.

7. Jones, R.N., Fuchs, P.C., Antimic. Ag. Chemoth. 9:719, 1976.

8. Polk, B.F., Kasper, D.L.. Submitted for publication.

9. Holdeman, L.V., Moore, W.E.C. (ed.). Anaerobe Laboratory manual. Virginia Polytechnic Institute and State University Press, Blacksburg, Va., 1972, p. 132.

10. Chow, A.W., Guse, L.B., Medicine 53:93, 1974.

11. Felner, J.M., Dowell, V.R., Am. J. Med. 50:787, 1971.

12. Gorbach, S.L., Thadepalli, H., Norsen, J. in Anaerobic Bacteria: Role in Disease, Charles C. Thomas, Springfield, Ill. 1974, p. 399.

13. Eschenbach, D.A., Buchanan, T.M. et al N. Engl. J. Med. 293:166, 1975.

14. Stone, H.H. J. Ped. Surg. 11:37, 1976.

15. Stone, H.H., Martin, J.D., Ann. Surg. 175:702, 1972.

16. Thadepalli, H., Gorbach, S.L., Keith, L., Am J. Obstet. Gynecol. 117:1034, 1973.

17. Swenson, R.M., Michaelson, T.C., Daly, M.J. et al Obstet. Gynecol. 42:538, 1973.

18. Duguid, J.P. in R. Cruickshank (ed.) Medical Microbiology, Williams and Wilkins Co., Baltimore, 1965 p. 659.

19. Neufeld, F. Z. Hyg. Infektionskr, 40:54, 1902.

20. Cato, E.P. and Johnson, J.L. Intl. J. Syst. Bact. 26:230, 1976.

21. Rowley, D. J. Infect. Dis. 123:317, 1971.

22. MacLeod, C.M., Krauss, M.R. J. Exp. Med. 92:1, 1950

23. Casciato, D.A., Rosenblatt, J.E., Goldberg, L.S., Bluestone, R. Infect. Immun. 11:337, 1975.

24. Muschel, L.H. In G.E.W. Wolstenholme and J. Knight (ed.). Ciba Foundation Symposium on Complement. Little, Brown, Boston, 1965, p. 155.

25. Weinstein, W.M., Onderdonk, A.B., Bartlett, J.G. et al- Infect. Immun., 10 1250, 1974.

26. Onderdonk, A.B., Weinstein, W.M., Sullivan, N.M., Bartlett, J.G., Gorbach, S.L.Infect Immun.10:1256, 1974.

27. Onderdonk, A.B., Bartlett, J.G., Louie, T.J., Sullivan-Seigler, N., Gorbach, S.C. Infect. Immune. 13:22, 1976.

28. Kasper, D.L., Winkelhalke, J.L., Zollinger, W.D., et al. J. Immunol. 110: 262, 1973.

29. Gorbach, S.L., Bartlett, J.G., N. Engl. J. Med. 290:1177, 1237, 1289, 1974.

30. Levison, M.E., Kaye, D. Drug Therapy 76:65, 1976.

31. Hite, K.E., Locke, M., Hesseltine, H.C. J. Infect. Dis. 84:1, 1949.

32. Weinstein, W.M., Onderdonk, A.B., Bartlett, J.G., Louie, T.J. and Gorbach, S.L. J. Infect. Dis. 132:282, 1975.

33. Hill, G.B., Osterhout, S. and Pratt, C. Infect. Immun. 9:599, 1974.

34. Abe, P.M. Lennard, E.S. and Holland J.W. Infect. Immun. 13:1473, 1976.

35. Hackman, A.S. and Wilkins, R.D. Bacteriol. Proceedings A-12, 1975.

36. Gotschlich, E.C., Rey, M., Triau, R., Sparks, K.J. J. Clin. Invest. 51:89, 1972.

37. Farr, R.S. J. Infect. Dis. 103:239, 1958.

38. Chow, A.W. Malkasian, K.L., Marshall, J.R., et al. Amer. J. Obs. Gyn. 122:876, 1975.

39. Moniff, GRG et al, Amer. J. Obs. Gyn. in press.

40. McCormack, W.M., Lee, Y.H., Zowroozi, K. Ronken, J.J. Int. Sci. Cenf. Anti. Microb. Chemother. Ab. 1976.

41. Quayle, A.A. J. Oral Surg. 32:91, 1974.

42. Socransky, S.S., Gibbons, R.J. J. Infect. Dis. 115:247, 1965.

43. Bartlett, J.G., Finegold, S.M. Medicine (Balt.) 51:413, 1972.

44. Danielsson, D., Lambe, D.W., Jr., Persson, S., In . Balows, R.M. DeHaan, V.R. Dowell, Jr., and L.C. Guze (ed.). Anerobic Bacteria: role in disease. Charles C. Thomas, Springfield, Ill., 1974, p. 173.

45. Hofstad, T. In A. Balows, R.M. DeHaan, V.R. Dowell, Jr., and L.C. Guze (ed.). Anaerobic Bacteria: role in disease. Charles C. Thomas, Springfield, Ill., 1974, p. 295.

46. Hofstad, R., J. Bact. 97:1078, 1969.

47. Courant, P.R., Gibbons, R.J. Arch. Oral Biol. 7:685, 1962.

48. Okuda, K., Takazoe, I. Bull. Tokyo Dent. Coll. 15:43, 1974

49. Martin, W.J., Appl. Microbiol. 22:1168, 1971.

50. Sawyer, S.J., MacDonald, J.B., Gibbons, R.J. Arch. Oral Biol. 7:685, 1962.

51. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. J. Biol. Chem. 193:265, 1951.

52. Dubois, M., Gilles, K.A., Hamilton, J.K., Smith, F. Anal. Chem. 28:350, 1956.

53. Roe, J.H. J. Biol. Chem. 212:335, 1955.

54. Kabat, E.A., Mayer, M.M. Experimental Immunochemistry, 2nd ed. Charles C. Thomas, Springfield, Ill., 1961, p. 505-507.

55. Kabat, E.A, Mayer, M.M. Experimental Immunochemistry, 2nd ed. Charles C. Thomas, Springfield, Ill. 1961, p. 538.

56. Weissbach, A., Hurwitz, J.B. J.Biol. Chem. 234:705, 1959.

57. Osborne, M.J. Proc. Natl. Acad. Sci. U.S.A. 50:499, 1963.

58. Layne, E. In S.P. Colowick and N.O. Kaplan (ed.). Methods in Enzymology, Vol. 3. Academic Press, New York, 1957, p. 453.

59. Bligh, E.G., Dyer, W.J. Can. J. Biochem. Physiol. 37:911, 1959.

60. Davis, C.E., Arnold, K. J. Exp. Med. 140:159, 1974.

61. Baker, C.J., Kasper, D.L., Davis, C.E. J. Exp. Med. 143:258, 1976.

62. Davis, C.E., Freedman, S.D., Douglas, H., Braude, A.I. Anal. Biochem. 28:245, 1969.

63. Kabat, E.A., Mayer, M.M. Experimental Immunochemistry. 2nd. ed. Charles C. Thomas, Springfield, Ill., 1961, p. 82.

64. Johnston, K.H., Gotschlich, E.C. J. Bacteriol. 119:250, 1974.