Serological Study of Trichloroacetic Acid Extracts of *Bacteroides fragilis*

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Immunodiffusion techniques were used on trichloroacetic acid extracts from 10 strains of *Bacteroides fragilis* in detecting precipitating antibodies against this species in immune rabbit sera. Species and even strain specificities were observed in these precipitin reactions. Multiple antigens were detected in the extracts from some strains, whereas only one precipitin band per extract developed during agar-gel diffusion tests of others. The antigen extracts were found to be both heat stable and resistant to hydrolysis by alpha-chymotrypsin. Four serological patterns were demonstrated in homologous and heterologous reactions with the *B. fragilis* antigen-antibody systems used. The results showed that some strains were serologically distinct from others, indicating that the strains tested are of more than one serotype.

The clinical importance of *Bacteroides fragilis* (formerly classified as *B. fragilis* subspecies *fragilis*; see ref. 5) in various human infections has been well documented (11, 12, 14, 22, 30, 31).

Results of serological studies with agglutination (2, 7, 20, 21, 25, 26), fluorescent-antibody (FA) (1, 8, 20, 28; G. L. Jones, Dr. P.H. thesis, University of North Carolina, Chapel Hill, 1974; G. L. Lombard, Dr. P.H. thesis, University of North Carolina, Chapel Hill, 1972), and immunodiffusion techniques (7, 20, 23, 24; J. D. Quick, Ph.D. thesis, University of Missouri, Columbia, 1972) show that the species has several different serotypes. Also, chemical analyses of extracted lipopolysaccharides from cells of *B. fragilis* show that the cell wall antigens of certain strains are multispecific (16, 17). This study is a sequel to one in which these same strains of *B. fragilis* were studied with agglutination and FA tests (1). This paper describes results of precipitin tests performed with a double diffusion in agar procedure on trichloroacetic acid extracts of ten strains of *B. fragilis*.

MATERIALS AND METHODS

Strains. The strains of *Bacteroides* and *Fusobacterium* are shown in Table 1. These organisms were obtained from the Anaerobe Section, Center for Disease Control (CDC), Atlanta, Ga. The cultures were all subjected to a battery of tests and identified according to the criteria established by Dowell and Hawkins (10), Holdeman and Moore (15), and Cato and Johnson (5).

Preparation of antisera. Antisera against seven

strains of *B. fragilis* (11710, 12103, 12330, 12336, 12959, 13712, and 14787) were prepared as previously described (1). Other antisera to strains 5462, 9053, and 14462, prepared as reported earlier (Jones, Dr. P.H. thesis), were furnished by G. L. Jones of the CDC.

Preparation of antigens. Cells to be used as antigens in precipitin tests were grown on Schaedler agar (Baltimore Biological Laboratory, Cockeysville, Md.) supplemented with 0.001% each of menadione and hemin. Bacterial cultures were incubated anaerobically in a glove box at 37°C for 48 to 72 h and harvested in saline (0.85% NaCl) containing 0.5% Formalin. Approximately 0.5 g (wet weight) of packed cells were suspended in 3 ml of 10% trichloroacetic acid and heated in a water bath at 56°C for 30 min with occasional shaking. Trichloroacetic acid-treated cells were removed from the water bath, allowed to cool, and centrifuged (3,000 × g, 30 min). The supernatant fluid was then decanted. Acetone (4 ml) and 5% sodium acetate (1 ml) were added to each supernatant, and the mixture was refrigerated for 24 to 48 h at 4°C. Precipitates were recovered by centrifugation, dissolved in 1 ml of deionized water, and neutralized with N/20 KOH to the end point of phenol red. Cummins reported using similar methods with *Propionibacterium acnes* (6).

Absorption of antisera. Test sera which cross-reacted were absorbed and used to determine antigenic relationships. Formalin-killed (0.5% Formalin in 0.85% saline, vol/vol) and washed whole cells were collected from a suspension (adjusted to 20% transmission at 540 nm) and mixed with 1 ml of undiluted antiserum. The serum-cell suspension was incubated in a water bath at 37°C for 1 h and refrigerated overnight at 4°C. The absorbed antiserum was then cleared by centrifugation.

Treatment of extracts with enzyme. Crystalline

TABLE 1. *Strains of Bacteroides and Fusobacterium included in the study*

Strain	CDC no.	Source or other designation ^a
<i>B. fragilis</i>	11710	Abdominal incision, NY
<i>B. fragilis</i>	12103	VPI 7058
<i>B. fragilis</i>	12330	Bowel incision, NY
<i>B. fragilis</i>	12336	Blood, NY
<i>B. fragilis</i>	12959	Incision, NY
<i>B. fragilis</i>	13712	Bowel incision, NC
<i>B. fragilis</i>	14787	Pasteur Institute E504
<i>B. fragilis</i>	5462	Chest fluid, NJ
<i>B. fragilis</i>	9053	VPI 2553
<i>B. fragilis</i>	14462	ATCC 23745
<i>B. ovatus</i>	11296	VPI 4244
<i>B. distasonis</i>	11298	VPI 4243
<i>B. distasonis</i>	15988	Pelvic infection, AK
<i>B. vulgatus</i>	14464	ATCC 8482
<i>B. vulgatus</i>	14363	Blood, TX
<i>B. thetaiotaomicron</i>	14463	ATCC 8492
<i>B. thetaiotaomicron</i>	12660	VPI C11-24
<i>B. thetaiotaomicron</i>	14389	Blood, NC
<i>B. melaninogenicus</i>	19003	Gingival swab, GA
<i>B. oralis</i>	17624	Lesion on thumb, AZ
<i>F. necrophorum</i>	5164	Exudate-knee, MA
<i>F. necrophorum</i>	16298	Abscess, CO
<i>F. mortiferum</i>	8178	ATCC 25557
<i>F. nucleatum</i>	9052	VPI 4355
<i>F. varium</i>	16043	Skin lesion, WY
<i>F. russii</i>	9054	VPI 0307

^a VPI, Virginia Polytechnic Institute, Blacksburg; PI, Pasteur Institute, Paris, France; ATCC, American Type Culture Collection, Rockville, Md; NY, New York; NC, North Carolina; NJ, New Jersey; AK, Alaska; TX, Texas; GA, Georgia; AZ, Arizona; MA, Massachusetts; CO, Colorado; WY, Wyoming.

alpha-chymotrypsin (Zolyse, Alcon Laboratories, Inc., Ft. Worth, Tex.) was reconstituted with its balanced salt diluent (pH 6.9) as instructed by the manufacturer. Equal parts of each trichloroacetic acid extract and the enzyme solution were mixed, incubated at 37°C for 2 h, and placed in an agar well located amid wells containing serial dilutions of homologous antiserum to determine the effect of proteolytic enzyme on the extracted factor(s) (Fig. 6). Treated and untreated extracts were tested for the presence of protein by the Bio-Rad protein assay method (3).

Titration of antigen-antibody systems. Autologous precipitin titrations of nine antisera varied from 1:2 to 1:16 (14787 did not react). When antigens were diluted greater than 1:2, precipitation results were affected. As a result of these low titers in preliminary tests, antisera and antigenic preparations were not diluted in later tests.

Ouchterlony serology. Formation of antigen-antibody complexes was determined with agar-gel diffusion methods in petri plates (15 by 100 mm) prepared with 1% Ionagar no. 2 (Colab Laboratories, Inc., Chicago Heights, Ill.) in 0.85% saline. Aqueous merthiolate was added to a final concentration of 0.01%. Two

designs of well arrangements were used: (i) a satellite pattern (Fig. 1-3) and (ii) a horizontal tandem pattern (Fig. 4). All reservoirs were 4 mm deep, 8 mm in diameter, and spaced 13 mm apart (well center to well center). Wells were charged with 0.25 ml of undiluted antiserum or trichloroacetic acid extract. Negative controls consisted of normal rabbit sera which did not produce detectable precipitin bands. Test plates were stored at room temperature in a moist environment for 48 to 72 h, then refrigerated at 4°C for 10 to 14 days. Results of daily checks for the development of immunoprecipitin lines were recorded. Bands of fusion, partial fusion, or lack of identity were interpreted as described by Kabat and Mayer (18).

RESULTS

Immunodiffusion test results are shown in Table 2. From one to four precipitin bands were formed in homologous reactions. Antiserum 12103 formed four bands with its antiserum (Fig. 1); anti-*B. fragilis* sera 11710 and 13712 (Fig. 2) each produced three immunoprecipitin lines; and 12330, 12336, and 129595 (Fig. 3) antisera formed two precipitates with their trichloroacetic acid extracts. Sera against 5462, 9053, and 14462 each yielded only one line of precipitation. Antigen-antibody components of strain 14787 did not react in homologous tests.

Eight of the test antisera contained precipitating antibody to at least one other extract, and extracts from nine of the *B. fragilis* strains formed precipitates with at least one heterologous antiserum. Some of the antisera formed two precipitin bands in cross-reactions (Fig. 4). Antiserum 12330 only formed a precipitate with homologous antigenic factors, and anti-1478 serum did not form a precipitate with any of the precipitinogen preparations tested. Furthermore, extracts from the latter strains did not form precipitin bands with any of the other nine *B. fragilis* antisera.

Lines of partial fusion (spurs) developed in some of the cross-reactions (i.e., serum 11710 with 12103 or 12336 extract and antiserum 12103 with 11710 or 13712 extract). Comparable serological relationships were noted in seven heterologous reactions. In three cross-reactions, precipitates intersected with the autologous precipitin lines instead of coalescing in arcs; hence, lines of nonidentity formed in some tests (e.g., antisera 12103, 13712, and 5462 with extracts 12336, 12103, and 13712, respectively). Faint precipitin lines formed in seven heterologous reactions, but it could not be determined whether they were identical.

Results obtained with absorbed antisera are shown in Table 3. Only a few cross-absorptions were done because there were limited quantities of some of the antisera and because the fusion

FIG. 1. Homologous precipitin reaction of *B. fragilis* 12103. X, Saline control; 03, 12103 antiserum. Outer wells contain extract from strain 12103.

FIG. 2. Precipitin reaction of *B. fragilis* 13712 extract and antiserum. X, Saline control; 12, 13712 antiserum. Outer wells contain 13712-trichloroacetic acid extract.

FIG. 3. Precipitin bands observed in homologous reaction using *B. fragilis* 12959 antiserum and extract. X, Saline control; 59, 12959 antiserum. Outer wells contain 12959 extract.

FIG. 4. Cross precipitin reactions. 54, 5462 antiserum; 54, trichloroacetic acid extract from strain 5462; 12, extract from *B. fragilis* 13712; 14, extract from 14787.

FIG. 5. Pooled antiserum from ten strains of *B. fragilis* (see text). P, pooled antiserum. Circumferential wells contain extract from *B. fragilis* 12103 (03).

FIG. 6. Reactions of extract from *B. fragilis* 12959, treated and untreated with alpha-chymotrypsin. E, Untreated extract; EC, extract treated with enzyme; U, undiluted antiserum. 2, 4, 8, 16, 32, Serial dilutions of anti-12959 serum placed in agar wells.

of precipitates in some preliminary cross-reactions suggested reactions of identity. From these results, we postulated that strains 11710, 12103, 12959, 13712, and 5462 share a common antigen,

that other strains (5462, 9053, and 14462) contain an antigenic factor which differs from the first one, and that at least one strain (12330) contains a precipitating antigen unique to it.

TABLE 2. *Precipitin bands formed in agar-gel diffusion tests by reacting antisera and trichloroacetic acid extracts of B. fragilis*

Trichloroacetic acid extract	No. of bands formed with the following antisera: ^a									
	11710	12103	12330	12336	12959	13712	14787	5464	9053	14462
11710	3	2 (1 common, 1 partial)	0	2 (partial)	1 (common)	1 (common)	0	1 (common)	0	0
12103	2 (1 common, 1 partial)	4	0	0	1 (weak)	1 (nonidentical)	0	1 (common)	0	1 (common)
12330	0	0	2	0	1 (weak)	0	0	1 (weak)	1 (weak)	0
12336	2 (partial)	1 (nonidentical)	0	2	0	1 (common)	0	0	1 (weak)	0
12959	1 (common)	1 (common)	0	0	2	1 (common)	0	0	1 (common)	0
13712	1 (common)	2 (1 common, 1 partial)	0	2 (1 common, 1 partial)	2 (1 common, 1 partial)	3	0	2 (1 common, 1 nonidentical)	0	0
14787	0	0	0	0	0	0	0	0	0	0
5462	1 (common)	2 (common)	0	0	0	1 (common)	0	1	1 (common)	1 (common)
9053	0	0	0	0	1 (weak)	0	0	1 (common)	1	0
14462	0	1 (weak)	0	0	0	0	0	1 (common)	0	1

^a Common, bands fused; partial, bands formed spurs; nonidentical, bands intersected; weak, faint or diffused band.TABLE 3. *Precipitin test results using absorbed B. fragilis antisera and trichloroacetic extracts*

Antiserum	No. of bands observed after absorption of trichloroacetic acid antigenic extracts						
	11710	12103	12336	12959	13712	5462	9053
Anti-11710	3	2	2	1	1	1	0
Anti-11710/12103 ^a	1	0	2	0	0	0	ND ^b
Anti-11710/12336	3	2	1	1	1	1	ND
Anti-12103	2	4	1	1	2	2	0
Anti-12103/11710	1	2	1	0	0	1	ND
Anti-12103/12336	2	3	1	1	1	1	ND
Anti-12103/13712	0	2	0	0	0	0	ND
Anti-12103/5462	0	1	1	0	1	0	ND
Anti-12336	2	0	2	0	2	0	0
Anti-12336/11710	2	ND	2	ND	1	ND	ND
Anti-12336/13712	1	ND	0	ND	1	ND	ND
Anti-12959	1	1	0	2	2	0	1
Anti-12959/13712	0	0	ND	0	0	ND	1
Anti-5462	1	1	0	0	2	1	1
Anti-5462/13712	0	0	ND	ND	1	1	1
Anti-5462/9053	1	1	ND	ND	2	0	0

^a Denominator denotes absorbing antigen.^b ND, Not done.

A pooled antiserum (containing equal quantities of the ten antisera) was reacted with the individual extracts. Both multiple and single bands were formed with each of the extracts except with those of strains 14787 and 9053. The pooled reagent formed from one band (12330) to as many as three bands (12103 [Fig. 5]) with eight of the extracts. In concurrent tests of the extracts, lines of identity, partial identity, and

nonidentity were formed. Some of the precipitates were distinct, whereas others were blurred and difficult to interpret.

Cellular extracts from strains of *B. oralis*, *B. melaninogenicus*, *B. distasonis*, *B. thetaio-taomicron*, and *B. ovatus* did not form precipitates with any of the antisera. Negative reactions were also observed when extracts from five species of *Fusobacterium* were tested. A weak band

was visible between antisera 11710 and 12959 and the extract from *B. vulgatus* 14363. In neither case did the bands coalesce with homologous precipitate; i.e., 11710-*B. vulgatus* complex did not meet and fuse with the homologous band, and 12959-*B. vulgatus* precipitate intersected the autologous line.

No precipitins were detected in rabbit antisera to various other gram-negative bacilli, namely, *Escherichia coli* ATCC 11303, *Enterobacter aerogenes* ATCC 9621, *Klebsiella pneumoniae* ATCC 10031, *Yersinia enterocolitica* (sewage isolate), *Moraxella lacunata* ATCC 11748, and *Pseudomonas aeruginosa* ATCC 15442.

Gram stains of cells extracted with trichloroacetic acid showed that most were intact but stained only faintly. In other studies, when cells were heated in a water bath at 100°C for 1 h before they were extracted, the extracts formed comparable precipitin lines to those formed by unheated cells extracts. Additionally, titers and specificities of the antisera were not affected by exposing the trichloroacetic acid extracts to alpha-chymotrypsin enzyme (Fig. 6).

Ouchterlony test results correlated well with those obtained in an earlier study involving immunofluorescence (1). Generally, antisera that produced higher FA titers and reacted with more heterologous strains of *B. fragilis* also formed a greater number of and more intense precipitin bands. These antisera (11710, 12103, 12959, and 13712) also had a wider range of precipitin activity.

Precipitin test results were corroborative with the FA results obtained by Jones (Dr. P.H. thesis). Antiserum 5462 reacted in precipitin tests with extracts from 9053 and 14462. Antisera to the latter two strains reacted with extract 5462, but there were no cross-reactions between extract 9053 and antiserum 14462, and vice versa. Strain 5462 was more widely reactive than 9053 or 14462.

DISCUSSION

Precipitating antibodies to *B. fragilis* in immune rabbit sera were detected with immunodiffusion techniques. The absence of precipitin lines in sera obtained from these same animals before they were immunized indicated an active immunological response.

The development of fused immunoprecipitin lines with multiple antigens indicated antigenic similarities, whereas the formation of faint lines or the absence of bands suggested immunological individuality. Different FA titers (1) and the formation of nonidentical precipitin lines also suggested that type-specific determinants were

present in the trichloroacetic acid extracts. In all, four serological patterns were observed in this study: (i) bands formed with only homologous systems, (ii) identical immunoprecipitin lines formed with one or more of the remaining test strains, (iii) bands of partial fusion (spurs formed), and (iv) bands intersected rather than fused with autologous precipitate. Serogrouping and serotyping results for this species have been reported by others (7, 8, 20, 21, 25, 26). Apparently, the ten strains of *B. fragilis* tested are of more than one serotype.

The fact that bands did not form with extracts from the other species and members of heterologous genera implies that the *B. fragilis* antigen-antibody systems we tested are group specific. We saw no cross-reactions between our antisera and extracts from *B. distasonis*, *B. thetaiotaomicron*, or *B. ovatus*; however, such serological reactions with these groups have been reported (2, 17, 23-26; Quick, Ph.D. thesis). Two of the antisera to *B. fragilis* cross-reacted with a strain of *B. vulgatus*, but the antigens were shown to be dissimilar in both cases. Hofstad (17) also observed cross-reactivity between these two groups. Various growth conditions or methods of extraction may have influenced these differences.

Results of staining, heating, and treating with alpha-chymotrypsin indicate: (i) that only cell wall material was removed by the trichloroacetic acid extraction method; (ii) that the antigenic components were heat stable; and (iii) that antigenic factors lacked protein moieties, that if such moieties were present, they were not hydrolyzed by the enzyme alpha-chymotrypsin, or that protein molecules were not involved in the serological activities of the determinants. Although we did not detect proteins in our extracts, their presence in cell wall preparations of *B. fragilis* has been established (16, 17, 19). The thermostability of these antigens agrees with earlier reports (1, 21) but not with the findings of de la Cruz and Cuadra (9). This problem should be studied further.

Somewhat surprising is the fact that strain 14787 was nonreactive. This antiserum was shown earlier (1) to have a rather high agglutination titer (1:4,096), but its FA titer was quite low (1:128) when compared to those of the other test antisera. It is possible that insufficient antibody was present in the antiserum, or that only nonprecipitating antibody was present. Kasper (19) has shown that there are differences in the amounts of antigen present on the bacterial surface, which could have been the case with strain 14787. Also, zones of equivalence may never have formed between 14787 antigens or antibod-

ies and those of other strains. This problem needs further study.

We agree with Lambe and Moroz (21) that a schema for serological classification of *B. fragilis* and related organisms is needed. Such a system would have substantial etiological and epidemiological significance. The development of polyvalent pools, which would allow screening of isolates, would make it possible to recognize additional serotypes and would complement progress already made in this area.

Ultrasonic (7, 10, 24) and phenolic extracts (13, 16, 17, 27, 29) have been used in studying the antigenic components of *B. fragilis*. Cold trichloroacetic acid has been used to extract lipopolysaccharide from *B. melaninogenicus* (4), but we know of no studies of *B. fragilis* in which heated trichloroacetic acid has been used. This method of extracting precipitinogens proved useful in our study.

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