A study of the surface and somatic antigens of *Bacteroides fragilis*

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SUMMARY

Antisera against live cultures of 20 strains of *Bacteroides fragilis* produced in rabbits agglutinated homologous whole-cell as well as O-antigen suspensions with several cross-reactions. Pure specific antisera were produced by absorbing cross-reacting antigens. O-antisera reacted against O-antigens but failed to do so against most whole-cell suspensions, suggesting the presence of surface structures which might have blocked the reactions. The titres of the whole-cell antisera were not significantly higher than those of O-antisera, but the reactions were more definite and easier to read.

This system may prove useful in serological studies of *B. fragilis*.

INTRODUCTION

The serology of *Bacteroides fragilis* was investigated by many workers and several serological classification schemes, based on the heat stable lipopolysaccharide (LPS), were proposed (Beerens, Wattre, Shinjo & Romond, 1971; Sharpe, 1971; Lambe & Moroz, 1976; Elhag, Bettelheim & Tabaqchali, 1977). However, the agglutination titres were always found to be low and cross-reactions of *B. fragilis* O-antigens were frequently reported. Hofstad (1975, 1977) suggested that the cross-reactions were due to the presence of several antigenic factors within the LPS, which are shared by some strains of *B. fragilis*.

Other antigens have also been explored. Kasper & Seiler (1975) and Kasper (1976) described an outer membrane of the *B. fragilis* cell-envelope and a true polysaccharide capsule in all strains of *B. fragilis fragilis*. He was able to produce antibodies to these surface structures by immunizing rabbits with live cultures of the *B. fragilis* strains. Furthermore, he suggested that this polysaccharide antigen may form a basis of a serogrouping system for *B. fragilis*.

Using simple serological techniques, we set out to produce antisera against live cultures of *B. fragilis* and to compare their reactivity and specificity with those of O-antisera previously prepared (Elhag *et al.* 1977).

MATERIALS AND METHOD

Bacterial strains

Twenty strains of *B. fragilis*, used in this study, were designated 1–20. Types 1–17 were the same as those used in a previous study (Elhag *et al.* 1977). Two strains of

B. fragilis subsp. *fragilis* were now used for serotypes 18 and 19. A new serotype 20 was a strain of *B. fragilis* subsp. *fragilis* originally isolated from the blood of a patient with septicaemia, which was not agglutinated by any of the antisera raised against the 19 strains.

Culture media

DST agar (Oxoid) 4 % (w/v), lysed horse blood 5% (v/v) incorporating gentamicin sulphate 0·01 % (w/w) was used to culture the *Bacteroides* strains.

Peptone yeast broth (Cato *et al*. 1970) was used for the production of *B. fragilis* antigens.

Identification of B. fragilis

Bacteroides strains were identified on the basis of their colonial appearance, cell morphology as well as the detection of the end products of glucose metabolism by gas liquid chromatography (Cato *et al*. 1970). They were further identified by API 20A anaerobe system (Holdeman & Moore, 1972; Starr, Thompson, Dowell & Balows, 1973).

Preparation of antigens

Whole-cell antigens. Cultures of *B. fragilis* strains were grown anaerobically for 48 h in 30 ml of peptone yeast broth to a density of approximately 5×10^8 bacteria/ ml. Formalin was then added giving a final concentration of 0·5 % (v/v). This preparation was kept at room temperature for 1 h and centrifuged. The deposit was then washed three times and resuspended in buffered physiological saline (BPS), pH 7·2, and adjusted to give a reading of No. 5 Wellcome Opacity Tube (Wellcome Reagent Ltd). The antigens were then stored at $-20\ ^\circ$C and tested within 7 days.

O-antigens. These were prepared as previously described (Elhag *et al*. 1977).

Preparation of antisera

The antisera were raised in New Zealand white rabbits, weighing approximately 2 kg. Before inoculation, 10 ml of blood were withdrawn from each rabbit to provide control sera.

Whole-cell antisera. Cultures of *B. fragilis* strains grown for 18 h in peptone yeast broth to a density of approximately 4×10^8 bacteria/ml were formalized as previously described and kept at room temperature for 1 h. 0·5 ml of this preparation was then injected intravenously into a rabbit. This was followed by 1·0 ml and 2·0 ml at 4 days interval. Five days after the third inoculation, the rabbit was injected with 2·0 ml of a similar live unformalized culture, followed 5 days later by a final injection of 4·0 ml of live culture.

Three days after the final injection, 40 ml were withdrawn from the marginal vein of the rabbit's ear. Subsequently 60 ml were withdrawn twice at 3–4 days interval. Four days after the last bleed, the rabbit was exsanguinated by cardiac puncture. The serum removed from the four samples was pooled and stored at $-20\ ^\circ$C.

O-antisera. These were prepared as described by Elhag *et al*. (1977).

Testing of antisera

Antisera were tested by the tube agglutination technique. Each whole-cell antiserum was tested against its homologous and heterologous whole-cell antigen suspensions and against O-antigens prepared from all the strains.

Each antiserum was diluted 1/10 (v/v) in BPS, pH 7·2. Merthiolate was added as a preservative, giving a final concentration of 1/10000 (w/v). To a series of 0·3 ml volumes of each diluted serum were added equal amounts of the appropriate antigen, giving a final dilution of 1/20. These were then incubated at 50 °C for 18 h. The length of further incubation at 4 °C depended on the antiserum tested, 4 h being required for whole-cell antisera whereas O-antisera were reincubated overnight.

Agglutination read visually was considered to have occurred when there was definite clumping at the bottom of the tube and the supernatant solution was completely clear and, on tapping, the agglutinated clumps were visible. Any suspensions giving agglutination reactions at 1/20 were further tested against doubling dilutions of the antiserum those giving no reactions at 1/20 were considered as negative.

Preparation of pure antisera

Pure antisera were obtained by absorption with any cross-reacting antigen. The growth from a heavily streaked DST agar (Oxoid) plate was suspended in the serum, diluted 1/10 (v/v) in 0·1 % mercuric iodide + 0·4% potassium iodide in buffered formal saline and incubated for 2 h at 50 °C (Kauffmann, 1944; Bettelheim, 1969). The bacteria were finally removed by centrifugation.

The absorbed sera were tested against all antigens with which the unabsorbed sera had reacted. If the absorbed serum reacted only with homologous antigen, then a pure specific antiserum was considered to have been produced. Final titres were obtained by testing each antigen against doubling dilutions of its homologous absorbed antiserum.

RESULTS

The pre-inoculation control sera showed no reactions when tested against all the antigen suspensions. The agglutination results of whole-cell antisera tested against whole-cell antigens are shown in Table 1. All the antisera except (13) reacted with their homologous antigens and only three agglutinated heterologous antigens as well. The titres ranged between 40–640 with the homologous being generally higher than the heterologous reactions. When these antisera were tested against O-antigens, reactions occurred with all the homologous strains, but there were more cross-reactions (Table 2). Fig. 1 shows a schematic representation of the cross-reactions between *B. fragilis* O-antigens.

The results of testing O-antigens against O-antisera were similar to those obtained in our previous study (Elhag *et al.* 1977). However, when whole-cell antigens were tested against these antisera, a number of reactions were lost and those remaining were present at low titres except serum 19 (Table 3).

Table 1. Homologous and heterologous agglutination reactions of B. fragilis whole-cell antigens against whole-cell antisera

Antigen		Antisera against:																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
B. fragilis fragilis NCTC 9343	1	320																			
B. fragilis fragilis NCTC 10584	2		320									40								80	
B. fragilis thetaiotaomicron NCTC 10582	3			80																	
B. fragilis vulgatus NCTC 10583	4				160																
B. fragilis fragilis NCTC 9344	5					320															
B. fragilis fragilis NCTC 8560	6						320										40				
B. fragilis fragilis NCTC 10581	7							320													
B. fragilis ovatus ATCC 8483	8								80												
B. fragilis distasonis ATCC 8503	9									160											
B. fragilis fragilis	10										40										
B. fragilis fragilis	11		40									80									
B. fragilis fragilis	12												320								
B. fragilis distasonis	13													<20							
B. fragilis distasonis	14														40						
B. fragilis distasonis	15															320					
B. fragilis fragilis	16					40											160				
B. fragilis fragilis	17																	80			
B. fragilis fragilis	18	80																	160		
B. fragilis fragilis	19																			640	
B. fragilis fragilis	20																				40

Table 2. *Homologous and heterologous agglutination reactions of B. fragilis O-antigens against B. fragilis whole-cell antisera*

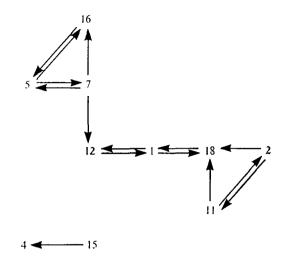| Antigen		Antisera against:																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
B. fragilis fragilis NCTC 9343	1	160											20						80		
B. fragilis fragilis NCTC 10584	2		160									80									
B. fragilis thetaiotaomicron NCTC 10582	3			80																	
B. fragilis vulgatus NCTC 10583	4				160											40					
B. fragilis fragilis NCTC 9344	5					640		160									160				
B. fragilis fragilis NCTC 8560	6						160														
B. fragilis fragilis NCTC 10581	7					40		320													
B. fragilis ovatus ATCC 8483	8								320												
B. fragilis distasonis ATCC 8503	9									160											
B. fragilis fragilis	10										80										
B. fragilis fragilis	11	80										160									
B. fragilis fragilis	12	40						80					160								
B. fragilis distasonis	13													40							
B. fragilis distasonis	14														80						
B. fragilis distasonis	15															160					
B. fragilis fragilis	16					40		80									320				
B. fragilis fragilis	17	80	40															160			
B. fragilis fragilis	18											20							160		
B. fragilis fragilis	19																			160	
B. fragilis fragilis	20																				160

Fig. 1. Antigenic relationships between various *Bacteroides fragilis* serotypes.
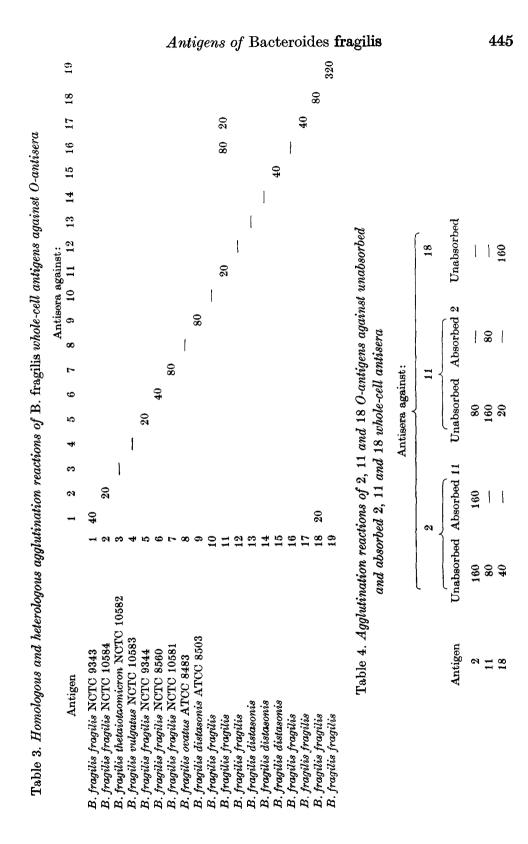
The absorption studies of whole-cell antisera are illustrated in Tables 4 and 5. The agglutination reactions of types 2, 11 and 18 are shown on Table 4. Absorption of antiserum 2 with antigen 11 resulted in the removal of agglutinins against both antigens 11 and 18. Similarly pure type 11 antiserum was produced by absorbing it only with antigen 2. Table 5 shows the reactions of types 5, 7, 12 and 16. Pure type 7 antiserum was produced only after absorption with all the three cross-reacting antigens. On the other hand, all the cross-reactions of type 5 antiserum were removed by absorbing it with type 16 only.

Pure whole-cell antisera were finally produced after absorption with the cross-reacting antigens. On testing, the antisera reacted only against the homologous whole-cell and O-antigens at titres ranging between 40–320 (Tables 6 and 7).
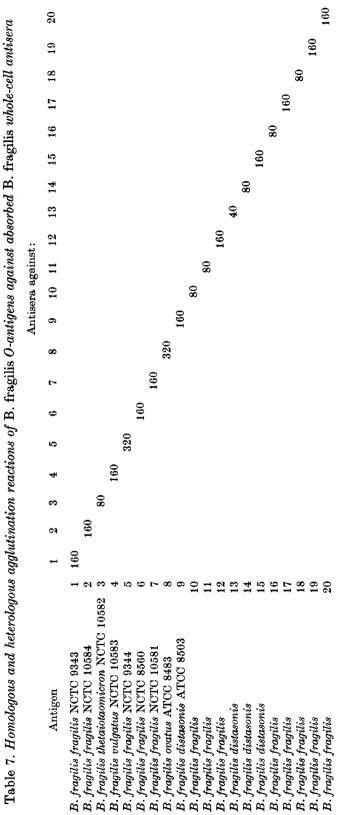
DISCUSSION

Previously we demonstrated that different serotypes exist within the *Bacteroides* spp. and that specific O-antisera can be produced against the heat stable O-antigens of these microorganisms (Elhag *et al.* 1977). The titres obtained however were low. In an attempt to obtain higher titres, whole-cell suspensions of live cultures of 20 strains of *B. fragilis* were used to immunize rabbits. Specific antisera against these antigens were produced but the titres were not significantly higher than those obtained by O-antisera. This may be because the immunization scheme consisted of 3 formalized and only 2 live cultures. Perhaps more intensive immunization with live cultures may provide higher titres. However, despite the low titres, the agglutination reactions produced by the whole-cell antisera needed shorter incubation periods, were more definite, easier to read and gave a distinct end-point.

The absence of many reactions between O-antisera and whole-cell antigen suspensions (Table 3) suggests the presence of a surface structure on these strains which masked the O-antigen and prevented agglutination. Normal reaction, however, occurred with type 19; perhaps this strain lacks the surface structures.

Table 3. *Homologous and heterologous agglutination reactions of B. fragilis whole-cell antigens against O-antisera*

Antigen		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
																			Antisera against:	
B. fragilis fragilis NCTC 9343	1	40																		
B. fragilis fragilis NCTC 10584	2		20																	
B. fragilis thetaiotaomicron NCTC 10582	3			—																
B. fragilis vulgatus NCTC 10583	4				—															
B. fragilis fragilis NCTC 9344	5					20														
B. fragilis fragilis NCTC 8560	6						40													
B. fragilis fragilis NCTC 10581	7							80												
B. fragilis ovatus ATCC 8483	8								—											
B. fragilis distasonis ATCC 8503	9									80										
B. fragilis fragilis	10										—									
B. fragilis fragilis	11											20					80	20		
B. fragilis fragilis	12												—							
B. fragilis distasonis	13													—						
B. fragilis distasonis	14														—					
B. fragilis distasonis	15															40				
B. fragilis fragilis	16																—			
B. fragilis fragilis	17																	40		
B. fragilis fragilis	18		20																80	
B. fragilis fragilis	19																			320

Table 4. *Agglutination reactions of 2, 11 and 18 O-antigens against unabsorbed and absorbed 2, 11 and 18 whole-cell antisera*

	Antisera against:					
	2		11		18	
Antigen	Unabsorbed	Absorbed 11	Unabsorbed	Absorbed 2	Absorbed 2	Unabsorbed
2	160	160	80	—	—	—
11	80	—	160	80	80	—
18	40	—	20	—	—	160

Table 5. *Agglutination reactions o 5, 7, 12 and 16 O-antigens against unabsorbed and absorbed 5, 7, 12 and 16 antisera*

Antigen	5		7						12	16	
	Unabsorbed	Absorbed with 16	Unabsorbed	5 & 12	12 & 16	5 & 16	5, 12 & 16	Unabsorbed	Unabsorbed	Absorbed with 5
				Absorbed with							
5	640	320	160	—	40	—	—	—	160	—
7	40	—	320	320	320	320	160	—	—	—
12	—	—	80	—	—	40	—	160	—	—
16	40	—	80	80	—	—	—	—	320	80

Table 6. *Homologous and heterologous agglutination reactions of B. fragilis whole-cell antigens against absorbed whole-cell antisera*

Antigen		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
												Antisera against:									
B. fragilis fragilis NCTC 9343	1	160																			
B. fragilis fragilis NCTC 10584	2		160																		
B. fragilis thetaiotaomicron NCTC 10582	3			80																	
B. fragilis vulgatus NCTC 10583	4				160																
B. fragilis fragilis NCTC 9344	5					160															
B. fragilis fragilis NCTC 8560	6						320														
B. fragilis fragilis NCTC 10581	7							32													
B. fragilis ovatus ATCC 8483	8								80												
B. fragilis distasonis ATCC 8503	9									160											
B. fragilis fragilis	10										40										
B. fragilis fragilis	11											40									
B. fragilis fragilis	12												320								
B. fragilis distasonis	13													<20							
B. fragilis distasonis	14														40						
B. fragilis distasonis	15															320					
B. fragilis fragilis	16																160				
B. fragilis fragilis	17																	80			
B. fragilis fragilis	18																		160		
B. fragilis fragilis	19																			640	
B. fragilis fragilis	20																				40

Table 7. *Homologous and heterologous agglutination reactions of B. fragilis O-antigens against absorbed B. fragilis whole-cell antisera*

Antisera against:

Antigen		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
B. fragilis fragilis NCTC 9343	1	160																			
B. fragilis fragilis NCTC 10584	2		160																		
B. fragilis thetaiotaomicron NCTC 10582	3			80																	
B. fragilis vulgatus NCTC 10583	4				160																
B. fragilis fragilis NCTC 9344	5					320															
B. fragilis fragilis NCTC 8560	6						160														
B. fragilis fragilis NCTC 10581	7							160													
B. fragilis ovatus ATCC 8483	8								320												
B. fragilis distasonis ATCC 8503	9									160											
B. fragilis fragilis	10										80										
B. fragilis fragilis	11											80									
B. fragilis fragilis	12												160								
B. fragilis distasonis	13													40							
B. fragilis distasonis	14														80						
B. fragilis distasonis	15															160					
B. fragilis fragilis	16																80				
B. fragilis fragilis	17																	160			
B. fragilis fragilis	18																		80		
B. fragilis fragilis	19																			160	
B. fragilis fragilis	20																				160

These surface structures were successfully removed by heating, after which reactions occurred. We have not explored the nature of these surface structures yet, but Kasper & Seiler (1975) and Kasper (1976) were able to demonstrate the presence of an outer membrane consisting of protein and polysaccharide in all strains of *Bacteroides* and showed that only strains of *B. fragilis* subsp. *fragilis* have a true polysaccharide capsule. In this study, however, absence of reactions was demonstrated with other *B. fragilis* subspecies also.

Whole-cell antisera presumably containing antibodies against both the heat labile surface antigens and the heat stable LPS, agglutinated whole-cell as well as O-antigens with the exception of type 13 whole-cell antigen (Tables 1 and 2). Perhaps the surface structures of this strain are poorly immunogenic. More cross-reactions occurred when these whole-cell antisera were tested against O-antigens. This may be due to the presence of common group antigens within the LPS in contrast to the surface antigens, which appear to be more type specific.

It is noteworthy that more cross-reactions occurred with O-antigens when tested against whole-cell antisera than against O-antisera. Whether these reactions were due to similarity between some surface and somatic antigens or were noticed because of the improved agglutination reactions produced by whole-cell antisera, cannot be assessed.

During absorption studies it was found that some of the multiple antigenic cross-reactions could be removed by fewer antigens than the number with which they originally cross-reacted. The relations between the O-antigens of some *B. fragilis* strains illustrated in Fig. 1 and the cross-absorption tests (Tables 4 and 5) confirm earlier findings by Hofstad (1975, 1977), who suggested that several antigenic determinants or factors are present in the LPS moiety and that some of these factors may be shared by some strains but not by others within the species. Finally pure specific antisera were produced to the 20 strains of *B. fragilis* used by absorbing all the cross-reacting antigens.

Surface structures of many bacteria have been shown to contain immunologically important antigens, most likely because of their exterior location and closer contact with the host defensive system. In this study the antisera raised against *B. fragilis* surface antigens by the use of live cultures gave better reactions than that produced against O-antigens. It is felt therefore that whole-cell antisera may prove more useful in the study of *B. fragilis* serology.

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