DETECTION OF SPECIES-SPECIFIC AND CROSS-REACTIVE CELL-SURFACE ANTIGENS OF *BACTEROIDES* SPECIES BY AN INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY

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SUMMARY. EDTA-released outer-membrane antigen complexes were prepared from 172 laboratory and reference strains and 43 fresh clinical or faecal isolates representing 20 species or subspecies of *Bacteroides*, together with 13 species of other genera. These antigens were titrated against antisera to whole, live cells of 21 species or subspecies of Bacteroidaceae in an indirect enzyme-linked immunosorbent assay (ELISA). The presence of species-specific antigens was investigated and cross reactions between species were noted. Results showed that a high proportion of the species possess species-specific antigens with little significant cross reactivity.

We believe that the ELISA system described here detects antigens that represent the whole cell surface of bacteroides organisms. We have exploited the sensitivity of the system and its quantitative potential to define approaches for those wishing to use serological approaches for either the identification of *Bacteroides* species or for the titration of serum antibodies in patients with a possible bacteroides infection.

INTRODUCTION

In the laboratory investigation of infections involving anaerobes at mucosal surfaces, estimation of the numbers of the various organisms present, and a distinction between commensals and potential pathogens, could influence clinical assessment and management. In the development of this expertise, it would be helpful if diagnostic laboratories could identify bacteroides organisms to specific or subspecific level as a routine, but this is still a complex and time-consuming exercise. Although the direct observation of fluorescence of a specimen in ultraviolet light and the direct examination of pus by gas chromatography may be useful adjuncts to direct microscopy and may expedite the detection of anaerobes and the determination of the genera involved, more detailed identification still generally rests upon an extended series of biochemical and other tests (see Duerden *et al.*, 1980).

Serological approaches to detailed identification of *Bacteroides* species and subspecies have been explored by several groups of workers (Beerens *et al.*, 1971;

Lambe and Jerri, 1976; Abshire, Lombard and Dowell, 1977; Elhag and Tabaqchali, 1978). Commercial kits have been developed (Holland, Stauffer and Altemeier, 1979; Labbé *et al.*, 1980) and an immunoperoxidase method has been described (Hsu *et al.*, 1979). Other workers have explored the possibility of detecting specific antibodies in the serum of patients with bacteroides infections (Hofstad, 1979; Sonnenwirth, 1979).

Our lack of knowledge of the significant surface antigens of these organisms in relation to existing and evolving taxonomic groupings limits the development of serological approaches to the characterisation of gram-negative anaerobes.

Outer membrane (OM) complexes released by EDTA from several species of *Bacteroides* have been shown to contain species-specific antigens that can be demonstrated by enzyme-linked immunosorbent assay (Poxton, 1979). The present study extends this investigation and examines cross reactions that may occur between species.

MATERIALS AND METHODS

The test bacteria included 172 strains representing 20 species or subspecies of *Bacteroides*, five strains of *Capnocytophaga ochracea*, six strains representing four species of *Fusobacterium*, and eight strains representing eight genera of aerobic or facultative gram-negative bacteria.

The following strains were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London NW9 5HT: *Bacteroides fragilis* NCTC nos. 9343, 9344, 8560, 10581 and 10584; *B. thetaiotaomicron* NCTC10582; *B. vulgatus* NCTC10583; *B. eggerthi* NCTC11155; *B. splanchnicus* NCTC nos. 10825 and 10826; *B. melaninogenicus* ss. *intermedius* NCTC nos. 9336 and 9338; *B. asaccharolyticus* NCTC9337; *B. corrodens* NCTC10939; *Fusobacterium necrophorum* NCTC nos. 10576 and 10577; *F. necrogenes* NCTC10723; *F. polymorphum* NCTC10562; *F. varium* NCTC10560; *Proteus mirabilis* NCTC6197; *Salmonella abortus equi* NCTC5727; *Shigella sonnei* NCTC8220; *Escherichia coli* NCTC10418; *Pseudomonas aeruginosa* NCTC10662; *Enterobacter aerogenes* NCTC8172; *Branhamella catarrhalis* NCTC3622 and *Haemophilus influenzae* NCTC4560.

B. melaninogenicus ss. *melaninogenicus* ATCC15930 was obtained from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md 20852. *B. distasonis* ATCC8503, *B. ovatus* ATCC8483 and *B. uniformis* ATCC8492 were from Dr Ella M. Barnes, Agricultural Research Council Food Research Institute, Colney Lane, Norwich. *B. variabilis* VPI11368, *B. bivius* VPI6318 and VPI6822, *B. disiens* VPI8057, *B. melaninogenicus* ss. *melaninogenicus* VPI4196 and *B. melaninogenicus* ss. *levi* VPI3300 were obtained from the Virginia Polytechnic Institute and State University (VPI), Blacksburg, Va. 24060. *B. oralis* strain 1210 was obtained from Dr S. Socransky, Forsythe Dental Center, Boston, Mass, and *B. ruminicola* NP333 was obtained from Dr G. H. Bowden, London Hospital Medical College. *B. ureolyticus* WPH58 was obtained from Dr A. L. James, Newcastle Polytechnic, Newcastle upon Tyne. *Capnocytophaga ochracea* (*Ristella ochraceus*) strain 1956c was obtained from Dr M. Sebald, Institut Pasteur, 25 Rue du Docteur Roux, Paris.

Other strains were received in connection with collaborative studies initiated by the ICSB, Taxonomic Sub-committee on Gram-negative Anaerobic Rods (see Holbrook, Duerden and Deacon, 1977; Deacon, Duerden and Holbrook, 1978), or were isolated in this laboratory from clinical material; 43 strains of *Bacteroides* spp. were isolated for use in the fresh isolate study (see below). All strains were identified by the methods of Duerden *et al.* (1976, 1980).

Growth of organisms and preparation of OM antigens was modified from the method of Poxton (1979). Cultures in 50 ml of PPY medium were incubated for up to 48 h to yield good growth. The suspended, washed cells were incubated with 10mM EDTA at 45°C for 2.5 h; after removal of cells by centrifugation, the undialysed supernate was used as the OM antigen.

Antisera were prepared as described by Poxton (1979).

Absorption of antisera. Cross-reacting antibodies were removed by incubating 1 ml of serum with bacteria harvested from 50 ml of an 18-h PPY culture and washed with phosphate-buffered saline. The suspension was shaken during the absorption period of 30 min at 37°C on a

blood-cell suspension mixer. Cells were removed by centrifugation at 20 000 *g* for 30 min at 4 °C. The absorption procedure was repeated once.

Micro indirect enzyme-linked immunosorbent assay (ELISA) was modified from that of Poxton (1979). Considerable loss of protein can occur during dialysis and, because dialysed and undialysed OM antigens behaved similarly in the ELISA assay, undialysed preparations were used. No difference in homologous titre was generally found when the OM antigens were used at 30 µg protein/ml or 60 µg protein/ml, so the lower concentration was used. However, for comparison with results already published by Poxton (1979) the 60-µg concentration was used in cross-reactivity studies. The results were read on a Titertek Multiscan Spectrophotometer (Organon Teknika, St Neots, Cambs); the highest dilution of serum that gave an E_{405} value > 1.0 was recorded as the titre.

Study of fresh isolates. Primary isolation plates from clinical specimens that contained anaerobic gram-negative rods, were kindly supplied from the Bacteriology Laboratory, Royal Infirmary of Edinburgh, and the Central Microbiology Laboratories, Western General Hospital, Edinburgh. When possible, a single colony was subcultured into 50 ml of PPY medium and incubated for 18–48 h. This culture was checked for purity, then 5 ml were lyophilised and the remaining 45 ml were used for the preparation of OM antigen. If it was not possible to pick a single colony pure from the primary isolation plate, a subculture was made on BM-based lysed-blood agar containing kanamycin 75 µg/ml and vancomycin 2.5 µg/ml (Holbrook, Ogston and Ross, 1978). After incubation for 48 h, a single colony was subcultured to PPY medium and processed as above.

OM antigens prepared from the fresh isolates were tested initially in a screening assay with selected pools of rabbit antisera before they were titrated against individual sera within any pool. A pool of antisera was prepared by mixing different volumes of several sera so that, when the pool was diluted and tested against its homologous OM antigens, they all reacted to the same titre. The pools were: 1, *B. fragilis* group (BFG) comprising 11 sera; 2a, *B. melaninogenicus* group (BMG, pigmented) comprising four sera; 2b, *B. melaninogenicus* group (BMG, non-pigmented) comprising six sera and 3, asaccharolytic group (AG) comprising three sera.

OM antigens that reacted significantly in screening tests with the BFG pool were further screened against two other pools of BFG sera. They were: 1a, four sera representing three indole-negative *Bacteroides* species and 1b, seven sera representing six indole-positive *Bacteroides* species.

RESULTS

Studies with OM antigens and homologous sera

Outer-membrane antigen preparations from 28 strains representing 23 species or subspecies of non-sporing anaerobes and one strain of *Capno. ochracea* were included in the indirect ELISA with antisera raised against the same strains, except for three of the *Fusobacterium* species, in rabbits and referred to here as species reference sera. OM antigen preparations from the eight control aerobic or facultative species were included in the study. The results are summarised in table I and the figure. Titres in the range 3200–51 200 were recorded for strictly homologous interactions, *i.e.*, when an OM preparation was tested against a reference serum raised specifically against the same strain. Minor heterologous cross reactions were observed in some cases and are indicated as fractions of the homologous titres. When OM antigens from a range of strains within the same species were tested against the species reference antiserum, the results indicated the antigenic homogeneity or heterogeneity of the species (table I). Results obtained with reference and laboratory stock strains are analysed separately from those obtained with freshly isolated strains.

Results with reference and laboratory strains. OM antigen preparations from all *B. fragilis*, *B. vulgatus*, *B. eggerthi*, *B. uniformis*, *B. splanchnicus*, *B. melaninogenicus* ss.

TABLE I
Reactions of *OM* antigens from reference, laboratory and freshly isolated strains with species reference antisera by indirect ELISA

Species reference antiserum raised against (species and strain no.)	Homologous titre (T)	Number of reference and laboratory strains reacting to a titre of					Number of freshly isolated strains reacting to a titre of					Total number of strains tested	Number of strains reacting to a titre of <T/8
		T/2 T/4 T/8 <T/8					T T/2 T/4 T/8 <T/8						
		T	T/2	T/4	T/8	<T/8	T	T/2	T/4	T/8	<T/8		
<i>B. fragilis</i> NCTC9344	12800	8	1	1	...	...	3	17	3	...	1	34	1
<i>B. vulgatus</i> NCTC10583	25600	2	6	2	...	...	...	1	...	...	1*	11	0
<i>B. distasonis</i> ATCC8503	12800	2	...	...	...	5	...	...	...	...	1}	8	2
<i>B. distasonis</i> GNAB26	12800	3	...	1	...	3	...	...	...	...	1}	...	...
<i>B. thetaiotaomicron</i> NCTC10582	25600	1	...	1	2	3	...	...	...	2	1}	10	2
<i>B. thetaiotaomicron</i> GNAB11	3200	2	2	...	2	1	...	1	1	...	1}	...	...
<i>B. ovatus</i> ATCC8483	6400	1	...	...	...	1	...	...	1	...	...	3	1
<i>B. eggerthi</i> NCTC11155	3200	1	4	...	...	...	...	...	...	...	...	5	0
<i>B. uniformis</i> ATCC8492	3200	1	1	1	...	...	...	...	...	...	...	3	0
<i>B. variabilis</i> VPI11368	6400	1	...	...	...	1	...	...	...	...	...	2	1
<i>B. splanchnicus</i> NCTC10826	12800	1	...	1	...	...	...	...	...	...	...	2	0
<i>B. mel. ss. int.</i> NCTC9338	12800	5	3	3	...	...	...	...	...	...	...	11	0
<i>B. mel. ss. levii</i> VPI3300	3200	1	...	...	...	...	...	...	...	...	...	1	0
<i>B. mel. ss. mel.</i> ATCC15930	12800	1	...	...	...	10	...	...	...	...	...	11	5
<i>B. mel. ss. mel.</i> VPI4196	25600	1	2	1	1	6	...	...	...	...	...	17	12
<i>B. oralis</i> 1210	51200	3	1	1	...	12	...	...	...	...	...	14	8
<i>B. ruminicola</i> NP333	25600	2	...	1	2	9	...	...	...	...	...	15	4
<i>B. ruminicola</i> GA33	6400	1	...	...	...	13	...	...	...	...	...	3	1
<i>B. hiius</i> VPI6822	51200	1	...	2	...	3	...	3	2	4	...	27	3†
<i>B. hiius</i> VPI6318	6400	1	2	...	1	2	2	2	2	1	2	1	0
<i>B. disiens</i> VPI8057	25600	1	1	...	...	...	...	...	...	...	1	3	1
<i>B. asaccharolyticus</i> NCTC9337	25600	1	1	10	11	3†	...	...	...	...	...	27	3†
<i>B. corrodens</i> NCTC10939	3200	1	...	...	...	...	...	...	...	...	...	1	0
<i>B. ureolyticus</i> WPH58	25600	2	...	...	2	...	...	...	...	...	...	4	0
<i>Capno. ochracea</i> 1956C	3200	1	...	...	3	1	...	...	...	...	...	5	1
<i>F. necrophorum</i> NCTC10577	12800	1	...	...	...	1	...	...	...	...	...	2	1

* Results combined when two species reference sera were used.

† Two of these strains are now known to be *B. gingivalis*.

intermedius, and *B. disiens* strains reacted strongly with their respective species reference antisera. Two strains of *B. ureolyticus* reacted less strongly, but the reference serum did not cross react with any other species. Two strains labelled *B. asaccharolyticus* and later shown to be *B. gingivalis* did not react with the *B. asaccharolyticus* antiserum.

When it became apparent that some strains did not react with an antiserum prepared against the same species, sera were raised against other strains in an attempt to find a more representative strain or to determine whether more than one serogroup existed within the species. For example, strains of *B. thetaiotaomicron* reacted more uniformly and more strongly with GNAB11 serum than with NCTC10582 serum, but one strain failed to react with either serum. Two strains of *B. distasonis* reacted with ATCC8503 serum, and four other strains reacted with GNAB26 serum, but one strain failed to react with either serum. These findings indicate that there are two or more serogroups in each of these species. For some species or subspecies the small number of available test strains limited our study.

OM antigens from a significant proportion of the strains of *B. melaninogenicus* ss. *melaninogenicus*, *B. oralis* and *B. ruminicola* failed to react with their species reference antisera. Six of 11 strains of *B. melaninogenicus* ss. *melaninogenicus*, 12 of 17 strains of *B. oralis* and eight of 14 strains of *B. ruminicola* failed to react with their species reference antisera.

Results with freshly isolated strains. After the OM antigens prepared from fresh isolates had been screened against pooled sera (see *Methods*) the following results were obtained with appropriate species reference sera (table I): 23 out of 24 strains of *B. fragilis*, one strain of *B. vulgatus*, two of three strains of *B. thetaiotaomicron*, one strain of *B. ovatus* and seven out of nine strains of *B. bivius* reacted to a titre one-eighth or greater of the respective homologous titres. However, one strain each of *B. fragilis*, *B. distasonis*, *B. thetaiotaomicron* and *B. disiens*, and two strains of *B. bivius* failed to react with their species reference sera. Three strains that failed to react with any group of pooled antisera were later identified as *B. capillosus* for which we do not have an antiserum. The OM antigen prepared from *B. bivius* GNAB136 reacted significantly with more than one serum; it reacted to half of the homologous titre with the species reference serum (VPI6318) and to one-eighth of the homologous titres with the *B. disiens* VPI8057 serum and the *B. oralis* 1210 serum.

Cross reactions between species

B. fragilis group (BFG) OM antigens. When OM antigens from 11 strains representing the nine species comprising the BFG were titrated with rabbit antisera raised against washed whole bacteria, homologous titres were significantly greater and usually at least 16-fold greater than any heterologous cross reactions (figure). However, there were four reactions between BFG OM antigens and BFG sera, in which the difference was 16-fold or less; there were significant cross reactions between a *B. ovatus* antigen and the two *B. thetaiotaomicron* antisera, a *B. uniformis* antigen and a *B. fragilis* serum and a *B. variabilis* antigen and a *B. eggerthi* serum. In addition, three BFG OM antigens cross reacted with *B. melaninogenicus*/*B. oralis*/*B. ruminicola* group antisera to one-sixteenth of the homologous titre. Serum raised against *Capno. ochracea* cross reacted with the OM antigen from *B. distasonis* GNAB26.

FIG.—Specificity of the interaction of OM antigens and antisera prepared from bacteroides and other organisms in an indirect ELISA system. BFG = *B. fragilis* group; BMG = *B. melaninogenicus/oralis/ruminicola* group; AG = asaccharolytic group.

B. melaninogenicus/oralis/ruminicola group (BMG) OM antigens. The OM antigens of this group did not cross react with antisera raised against species in the BFG or the asaccharolytic group. When these OM antigens were titrated against antisera raised against the BMG species there was usually at least a 16-fold difference between the homologous and cross-reacting titres, but there were 12 cross reactions where the difference was 16-fold or less (figure).

Asaccharolytic group (AG) OM antigens. OM antigens of this group did not cross react with antisera raised against species in the BFG or AG series, but there were some minor cross reactions with antisera raised against the BMG species (figure).

OM antigens of aerobic and facultative gram-negative bacilli. OM antigens from these organisms usually failed to react with the bacteroides antisera or reacted to less than one-sixteenth of the homologous titre. The exception was the OM antigen from *Esch. coli* NCTC10418 which reacted with some of the BFG and AG antisera and the *Capno. ochracea* antiserum to a titre one-sixteenth or greater of the homologous titre, but did not react with the BMG antisera.

Cross reactions with Fusobacterium necrophorum antiserum. Serum raised against *F. necrophorum* cross reacted with many OM antigens prepared from *Bacteroides* spp. but not with the OM antigens of other *Fusobacterium* spp.

Reactions with absorbed antisera. After absorption of cross-reacting antibodies (see *Methods*) the heterologous titres of 10 out of 13 cross-reacting sera were reduced to < 400 with no more than a twofold decrease in the homologous titres (table II).

TABLE II

Absorption of cross-reacting antibodies by treatment of antiserum with whole cells of cross-reacting strains

Antiserum raised against strain	Cross-reacting antigen from strain	Titre when homologous antigen was reacted with:		Titre when cross-reacting antigen was reacted with:	
		unabsorbed serum	serum absorbed with whole cells of cross-reacting strain	unabsorbed serum	serum absorbed with whole cells of cross-reacting strain
NCTC9344	ATCC8492	6400	3200	400	< 400
NCTC10582	ATCC8483	25600	25600	1600	< 400
GNAB11	ATCC8483	3200	3200	400	< 400
NCTC11155	VPI11368	6400	3200	400	< 400
ATCC8492	NCTC10418	12800	12800	3200	< 400
NCTC9338	VPI4196	25600	25600	6400	< 400
VPI4196	NCTC9338	51200	51200	3200	< 400
VPI4196	ATCC8503	25600	12800	1600	800
VPI4196	GNAB26	25600	12800	1600	800
VPI4196	VPI3300	25600	25600	1600	800
VPI4196	1210	25600	12800	1600	< 400
VPI4196	VPI8057	25600	12800	800	< 400
1210	VPI8057	25600	12800	1600	< 400
VPI6318	VPI4196	12800	6400	1600	< 400
VPI8057	NCTC9338	12800	12800	400	< 400

DISCUSSION

We have shown here, and previously (Poxton, 1979), that *Bacteroides* species generally have cell-surface antigens that are species specific. The cross reactions that do occur are mainly between similar species; cross-reactive antibodies can be absorbed

with whole cells of the cross-reactive species. Our results suggest that it will be possible to define species-specific surface antigens for many species of *Bacteroides* especially within the *B. fragilis* group. This has already been done for *B. vulgatus* where the species-specific antigen is the major outer-membrane protein (Poxton and Ip, 1981) and for *B. fragilis* where the "capsular" polysaccharide, which appears to be membrane bound and is thus amenable to investigation by ELISA, is the species-specific antigen (Kasper *et al.*, 1977). More strains within the species *B. bivius*, *B. melaninogenicus* ss. *intermedius*, *B. asaccharolyticus* and *B. ureolyticus* also appear to possess species-specific or subspecies-specific antigens. Some species such as *B. melaninogenicus* ss. *melaninogenicus*, *B. oralis* and *B. ruminicola*, are as yet ill defined and there were significant cross reactions within the group; more taxonomic studies must be made before this group can be investigated serologically. For other species, we have not been able to obtain sufficient strains to make a full serological investigation.

In any identification scheme based on serological techniques, the antigens that are to be detected must be clearly defined; their predominance within a species, subspecies or serogroup should be known, and their cross reactions with related and unrelated species must be determined. Moreover, the stability of these antigens and their expression in laboratory-passaged strains must be ascertained. Only one of 24 fresh isolates of *B. fragilis* failed to react with the *B. fragilis* species reference serum. More work is needed to evaluate the significance of the few non-reactors when small numbers of fresh isolates of other species were tested against apparently appropriate species reference sera. A cautious approach to the identification of wild strains by sera raised against stock strains is clearly advisable on principle, but the results with *B. fragilis* strains are particularly encouraging.

The indirect enzyme-linked immunosorbent assay that we used has provided useful results in our investigation of material released from bacteria by mild heat, EDTA and sonication. Our system appears to provide a valid representation of all of the antigens present at the surface of whole cells. The antigens detected could include protein, lipopolysaccharide (LPS) and capsular material; because extremely high dilutions of serum are used, the system will detect the more immunogenic molecules, which have the greatest affinity for the antibodies that we raised against whole cells. Less immunogenic molecules which may be more cross reactive, such as LPS (Elhag and Tabaqchali, 1978), will not be detected at such high dilutions of serum. The extreme sensitivity of the system, its specificity and its quantitative potential will be of use in defining approaches that might exploit immunofluorescence methods for the direct examination of clinical specimens, or might allow the titration of serum antibodies in patients with a possible bacteroides infection.

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