Analysis of EDTA-soluble Cell Surface Components of Gram-positive Anaerobic Cocci

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The protein content of EDTA extracts from 76 strains of Gram-positive anaerobic cocci was examined using SDS-PAGE. Strains of *Peptostreptococcus anaerobius* produced almost identical profiles; greater heterogeneity was observed within the species *Peptococcus magnus*, *Peptococcus prevotii* and *Peptococcus asaccharolyticus*, but several strains within each biotype produced similar patterns. Serological investigation of these extracts by ELISA revealed numerous cross-reactions among the different biotypes. Immunoblot transfers from polyacrylamide gels demonstrated two common antigens within strains of the species *Ps. anaerobius*, but these were not species-specific.

INTRODUCTION

Gram-positive anaerobic cocci (GPAC) belonging to the genera *Peptococcus* and *Peptostreptococcus* are frequently recovered from a variety of human infections. These include pulmonary and cerebral abscesses (Gorbach & Bartlett, 1974; Ingham *et al.*, 1977), and intra-abdominal (Gorbach & Bartlett, 1974), female genital tract (Thadepalli *et al.*, 1973) and orofacial infections (Busch, 1984). In most cases GPAC are present with other anaerobic and facultative organisms; therefore the precise contribution of GPAC to the overall disease process is obscure. A considerable hindrance to studies of the pathogenicity of GPAC is the absence of a practical identification scheme for diagnostic laboratories (Watt *et al.*, 1984). Current schemes (Holdeman *et al.*, 1977; Dowell & Hawkins, 1974; Sutter *et al.*, 1975) involve numerous biochemical tests and many species are defined on the basis of negative results. Polyacrylamide gel electrophoresis (PAGE) of cellular proteins has been utilized in studies of several micro-organisms presenting taxonomic problems, for example mycoplasmas (Razim & Rottem, 1967), corynebacteria (Jackman, 1982) and clostridia (Cato *et al.*, 1982). Results obtained by this procedure have agreed with those from DNA-DNA homology studies. An alternative approach to the classification of micro-organisms is examination of their serological characteristics, including identification of cellular components which induce the formation of specific antibodies in laboratory animals. The detection of such antibodies in the sera of patients is of diagnostic and epidemiological importance.

The aims of the present study were to extract cell surface components from a variety of GPAC, and then to examine their protein profiles using SDS-PAGE and determine their antigenic content using the techniques of immunoblot transfer to nitrocellulose and ELISA.

METHODS

Bacteria. Seventy-six strains representing four species of GPAC (*Peptostreptococcus anaerobius*, *Peptococcus magnus*, *Peptococcus prevotii* and *Peptococcus asaccharolyticus*) were selected for study. These comprised one ATCC and five NCTC strains and 70 clinical isolates. All were obligate anaerobes as defined by Watt & Jack

Abbreviations: GPAC, Gram-positive anaerobic cocci; CMB, Robertson's cooked meat broth; TBS, Tris-buffered saline; TTBS, Tween 20-TBS; HRP, horseradish peroxidase; HSA, human serum albumin.

(1977) and were identified to species level according to VPI (Virginia Polytechnic Institute) criteria (Holdeman *et al.*, 1977). Strains of GPAC were stored as lyophilized preparations or in 10% (w/v) skimmed milk at -20°C .

Culture and harvest of bacteria. Organisms were reconstituted with pre-reduced modified Robertson's cooked meat broth (CMB; Watt, 1972) and incubated anaerobically at 37°C for 48 h, according to the method of Collee *et al.* (1972). CMB cultures were then used to inoculate 200 ml pre-reduced CMB (Deacon *et al.*, 1978). After anaerobic incubation for 48 h at 37°C , cells were harvested by centrifugation (20000 g, 10 min) washed twice in 40 ml phosphate-buffered saline (PBS: 50 mM-phosphate buffer, pH 7.5, with 0.15 mM-NaCl) and finally centrifuged as before.

EDTA extraction. Washed cells were resuspended in 2 ml PBS containing 10 mM-EDTA (pH 7.4) and transferred to a 45°C water bath for 60 min. This suspension was centrifuged (10000 g, 3 min) and the supernatant, which contained the extract, was transferred to acid-washed Bijou bottles. The protein content of individual EDTA extracts was estimated by the Lowry method, using bovine serum albumin (2 mg ml^{-1}) as a standard.

Antiserum preparation. Four clinical isolates of GPAC were selected for the production of antisera in rabbits, based on the method of Poxton (1979). The strains were *Ps. anaerobius* (GPAC 56393) from a case of otorrhoea, *Pc. magnus* (GPAC 149) and *Pc. prevotii* (GPAC 150), both from wound infections, and *Pc. asaccharolyticus* (STD 34) from the vagina of a female attending a sexually-transmitted disease clinic. Two New Zealand White rabbits per strain were inoculated intravenously with 10^9 bacteria in 1 ml PBS on three consecutive days each week for four weeks. Antibody titres were determined from test bleeds using an ELISA procedure (see below). The majority of animals were exsanguinated in the fifth week; the pair inoculated with *Pc. prevotii* received two further doses of 10^{10} and 10^{11} bacteria in weeks five and six before exsanguination.

SDS-PAGE. A modified version of the method of Laemmli (1970) was used, as described by Poxton & Brown (1979). Vertical slab gels ($170 \times 140 \times 1\text{ mm}$) consisted of a 10 mm, 4% (w/v) acrylamide stacking gel above a 10% (w/v) acrylamide separating gel. EDTA extracts containing 100 μg protein, suspended in 100 μl single strength sample buffer, were boiled for 3 min, and then added to wells in the stacking gel: 60 V were applied to the gel to move samples through the stacking gel (1–2 h), followed by 150 V (2–3 h) for separation. Gels were stained with Coomassie blue (Poxton & Sutherland, 1976).

Immunoblot transfer. The technique described by Cousland & Poxton (1983) was adopted. EDTA extracts adjusted to 10 μg protein in 50 μl sample buffer (boiled for 3 min) were run in duplicate SDS-polyacrylamide gels as described, but electrophoresis was halted when the gel front had travelled 7.5 cm into the separating gel. One gel was stained with silver (Oakley *et al.*, 1980) to demonstrate protein profiles. To detect antigens in EDTA extracts the duplicate gel was used in an immunoblot transfer procedure, with a BioRad Immunoblot assay kit, according to the method of Towbin *et al.* (1979). Material from the gel was transferred to a nitrocellulose membrane (0.2 μm pores) by electrophoresis in Tris/glycine/methanol buffer (pH 8.3) for 18 h at 12 V (40 mA). After a 10 min wash in Tris-buffered saline (TBS: 20 mM-Tris/HCl, 500 mM-NaCl, pH 7.5) and blocking for 45 min in 3% (w/v) gelatin-TBS, the nitrocellulose was placed in first antibody solution for 2.5 h (antisera to *Pc. magnus*, *Pc. prevotii* and *Pc. asaccharolyticus* were diluted 1:50 in 1% (w/v) gelatin-TBS, whereas *Ps. anaerobius* antiserum was diluted 1:250). Following two 10 min washes in 0.025% (v/v) Tween 20-TBS (TTBS), the nitrocellulose was incubated for 1 h in second antibody solution containing goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate diluted 1:3000 in 1% (w/v) gelatin-TBS. The nitrocellulose was then washed as before and transferred to HRP colour development solution containing 4-chloro-1-naphthol and hydrogen peroxide. Development was stopped by immersing the nitrocellulose in distilled water after 15–30 min.

ELISA. The method used was essentially that of Engvall & Perlmann (1972) adapted for microtitre plates. EDTA extracts were adjusted to 30 μg protein ml^{-1} and results were read in a Titretrek Multiskan (Organon Teknika, St Neots, UK). End-points of titrations were taken as the first dilution of antisera to give an A_{405} value of 1.0.

Treatment of EDTA-extracted antigens. An EDTA extract from *Ps. anaerobius* (GPAC 56393) was adjusted to 200 μg protein ml^{-1} and subjected to the following treatments, as described by Poxton & Byrne (1981). (i) Heat treatment (121°C for 15 min). (ii) Formalin (as 20%, w/v, formaldehyde) for 16 h at 20°C , followed by dialysis for 18 h at 4°C against PBS. This treatment denatures proteins. (iii) Sodium periodate (0.1 M) for 16 h at 20°C in the dark; excess periodate was removed with ethylene glycol; this was followed by dialysis for 18 h at 4°C against PBS. This treatment oxidizes carbohydrates.

Two controls were included: an untreated EDTA extract and, as the periodate control, an EDTA extract treated as described in (iii) above, but with distilled water instead of periodate. Control and treated extracts (75 μl) were mixed with equal volumes of sample buffer containing SDS, boiled for 3 min and run in duplicate polyacrylamide gels (10 μg protein per track). One gel was stained with silver for proteins; the other was used in an immunoblot transfer to nitrocellulose. Incubation of the latter with antiserum to GPAC 56393 revealed denaturation of antigens associated with each treatment, relative to untreated controls, which gave an indication of their chemical nature.

RESULTS

Antibody titres

Sera from each pair of rabbits were pooled and antibody titres determined by ELISA, using EDTA extracts as antigens, with the following results: *Ps. anaerobius* 1:8000; *Pc. asaccharolyticus* and *Pc. magnus* 1:1600; *Pc. prevotii* 1:200. Despite two booster injections, *Pc. prevotii* proved to be poorly immunogenic relative to the other strains.

SDS-PAGE

The protein profiles of five strains each of *Ps. anaerobius* and *Pc. magnus* stained with Coomassie blue are shown in Fig. 1. Striking similarities are apparent between strains of *Ps. anaerobius* (tracks 2–6), and the patterns are dominated by a single darkly-staining band corresponding in mobility to ovotransferrin (M_r 76000–78000; track 1); other regions in the patterns show qualitative and quantitative variations. These features were observed in all strains of *Ps. anaerobius* tested. The profiles of *Pc. magnus* (tracks 7–11) show greater heterogeneity, particularly track 10 which contains an extract from NCTC 9815.

Strains of *Pc. prevotii* also exhibited heterogeneous protein profiles (Fig. 2), but patterns in tracks 4 and 5 are very similar, and tracks 8 and 9 have many bands in common. Despite receiving the same amount of protein, bands in track 7 stained very faintly. Similar features were displayed by strains of *Pc. asaccharolyticus* (not shown), i.e. overall heterogeneity, but with several well-defined bands common to all extracts. Only *Ps. anaerobius* produced protein profiles which were visually almost identical. The observation of groups of strains with almost identical protein profiles within species suggested a potential method for classifying GPAC.

Immunoblot transfer

This technique was chosen to investigate the antigenic content of EDTA extracts from GPAC. Nitrocellulose membranes were probed with antisera homologous to the species run in the corresponding polyacrylamide gels. In the case of *Ps. anaerobius*, the high M_r band seen in earlier gels proved to be an antigen common to the 13 strains investigated, including ATCC 27337. A second common antigen of lower M_r (approximately equivalent to myoglobin, M_r 17200, in mobility) was also demonstrated. Some strains of *Ps. anaerobius* possessed antigens not shared with other isolates of this species (results not shown).

Similar procedures with EDTA extracts from seven strains each of *Pc. prevotii*, *Pc. magnus* and *Pc. asaccharolyticus* highlighted the following features: many strains within each species possessed common antigens and a small number of strains (two strains of *Pc. magnus* and one strain each of *Pc. prevotii* and *Pc. asaccharolyticus*) displayed additional unshared antigens; one strain of *Pc. prevotii*, three strains of *Pc. magnus* and one strain of *Pc. asaccharolyticus* did not react with homologous antisera which suggested misidentification of these strains or the vaccine strain by conventional means.

Cross-reactivity between GPAC

To determine the presence of cross-reacting antigens, EDTA extracts from 50 strains of GPAC were tested in an ELISA procedure against each of the four antisera. A single dilution of each antiserum was used as follows: *Ps. anaerobius* 1:1000; *Pc. magnus* and *Pc. asaccharolyticus* 1:500; *Pc. prevotii* 1:100. An A_{405} value of ≥ 1.0 indicated a significant positive reaction, but a value of 0.8 was included as a 'borderline' reaction. All 15 strains of *Ps. anaerobius* reacted strongly with the homologous antiserum; six strains also reacted with *Pc. magnus* antiserum and three of these also reacted with *Pc. prevotii* antiserum. Only six strains of *Pc. magnus* reacted strongly with the homologous antiserum and one of these was borderline; cross-reactions were observed between *Pc. magnus* and antisera to *Pc. prevotii*, *Ps. anaerobius* and *Pc. asaccharolyticus*. One strain of *Pc. asaccharolyticus* cross-reacted with antisera to *Ps. anaerobius* and *Pc. magnus*; a further strain reacted with antiserum to *Pc. prevotii*. Of the strains of *Pc. prevotii* tested only an extract from the vaccine strain (GPAC 150) reacted strongly with its antiserum. Two strains biotyped as *Pc. prevotii* were serologically identified as *Pc. magnus* and *Ps. asaccharolyticus* respectively, but the latter also had antigens in common with *Ps. anaerobius*.

Fig. 1. PAGE of EDTA extracts from five strains of *Ps. anaerobius* (tracks 2-6) and five strains of *Pc. magnus* (tracks 7-11). Track 1 contains the following M_r marker proteins (BDH) in order from the top of the gel downwards: ovotransferrin (M_r 76-78000); albumin (66250); ovalbumin (45000); chymotrypsinogen A (25700); myoglobin (17200); cytochrome *c* (12300). The gel was stained with Coomassie blue.

Fig. 2. PAGE of EDTA extracts from 10 strains of *Pc. prevotii* (tracks 2-11). Track 1 contains M_r markers (see Fig. 1). The gel was stained with Coomassie blue.

Fig. 3. Immunoblot transfer from a polyacrylamide gel of control and treated EDTA extracts from *Ps. anaerobius*. Track 1, untreated control with high (H) and low (L) M_r antigens common to this species; track 2, heat-treated extract; track 3, formalin-treated extract; track 4, periodate-treated extract; track 5, periodate control.

Treatment of EDTA-extracted antigens of Ps. anaerobius

The use of PAGE and immunoblot transfer to visualize the effects of various treatments on the two common antigens contained in an EDTA extract from *Ps. anaerobius* is illustrated in Fig. 3: track 1 contains the untreated extract and demonstrates the high and low M_r antigens common to all strains of *Ps. anaerobius* examined by this procedure; track 2 contains the heat-treated extract from which the low M_r antigen has disappeared; track 3 contains the formalin-treated extract from which both antigens have disappeared; track 4 contains the periodate-treated extract which lacks the low M_r antigen, similar to track 2; track 5 contains the periodate control, in which both antigens are still present. The reactions of the high M_r antigen after heat and periodate treatments (tracks 2 and 4) are reduced in intensity; this suggests some degree of denaturation, since all tracks received the same amount of protein.

DISCUSSION

In the present study, the protein content of EDTA extracts from a variety of GPAC was studied using SDS-PAGE. Incubation of Gram-positive cells with EDTA causes the solubilization of non-covalently bound cell surface polymers, including proteins and membrane-

associated lipocarbohydrates (Poxton & Byrne, 1981). This treatment, however, does not break covalent bonds; therefore secondary wall polymers (teichoic acids or their analogues) are not removed. Autolysis of GPAC cells was not observed during the 60 min incubation period.

The method of immunizing rabbits used produced antibody titres that compared favourably with previous reports (Stone, 1940; Markowitz & Lerner, 1977); the titre obtained for *Ps. anaerobius* confirms the report of Wong *et al.* (1980) that this species is strongly immunogenic. The technique of SDS-PAGE has been successfully used in taxonomic studies of several groups of micro-organisms. Cato *et al.* (1983) observed a 'general similarity' in the patterns of whole cell proteins extracted from strains of *Pc. magnus*, but greater variation within the species *Ps. micros*. In the present study, EDTA extracts from *Pc. magnus*, *Pc. prevotii* and *Pc. asaccharolyticus* produced heterogeneous profiles in gels stained with Coomassie blue that were not characteristic of their biotype. Within these species, however, a number of strains produced very similar patterns, suggesting that many GPAC could be assigned to distinct groups by this technique. Strains of *Ps. anaerobius* consistently produced almost identical protein patterns dominated by one high M_r band. Immunoblots of 13 strains revealed that this band and another of low M_r were antigens common to this species, which confirms previous reports (Wong *et al.*, 1980; Graham & Falkler, 1979). The high M_r antigen was denatured by formalin, but not by heat or periodate, which suggests that its determinant sites contain protein. Heat, periodate and formalin denatured the low M_r antigen, suggesting that it contains both carbohydrate and protein moieties. Sensitivity to proteolytic enzymes, but resistance to heat and periodate, are characteristics of a receptor for human serum albumin (HSA) on the surface of *Pc. magnus* recently described by Myhre (1984); however, no indication of its M_r was given. Serological cross-reactions between *Pc. magnus* and *Ps. anaerobius* were observed in this study and it would be of interest to determine whether or not these involve a molecule on the surface of *Ps. anaerobius* capable of binding HSA.

Numerous serological cross-reactions between different biotypes of GPAC were demonstrated by ELISA, indicating the presence of shared antigens. For three of the 50 strains tested, ELISA suggested identities contrary to those deduced from biochemical tests; a total of 19 strains could not be identified with the antisera used. Graham & Falkler (1979) and Wong *et al.* (1980) produced evidence that the antigens common to strains of *Ps. anaerobius* were not possessed by other peptostreptococci. The present study involves different antigen and antiserum preparations; therefore although results are not directly comparable, they indicate that the antigens contained in EDTA extracts of *Ps. anaerobius* are not unique to this species because these antigens were also detected by antisera to *Pc. magnus* and *Pc. prevotii*. In addition, antiserum to *Ps. anaerobius* recognized antigens in extracts from *Pc. magnus* and *Pc. asaccharolyticus*.

This study has shown that GPAC identified to species level by conventional biochemical tests possess a range of cell surface antigens, some of which are not species-specific. Extracts containing these antigens were easily prepared from GPAC, and the technique of immunoblotting offered a simple and sensitive means for their detection. We suggest that further investigation of the serological characteristics of such extracts following SDS-PAGE may aid the classification of GPAC.

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