Antibodies to a Common Outer Envelope Antigen of *Treponema hyodysenteriae* with Antibacterial Activity

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Outer envelopes of *Treponema hyodysenteriae* strains P18A and VS1 were prepared and characterized by SDS-PAGE. In Western blot analysis of eleven strains of *T. hyodysenteriae* and two intestinal non-pathogenic spirochaetes, polyclonal antiserum raised to the outer envelopes of strain P18A contained antibodies primarily to two polypeptides. A 45 kDa polypeptide was present in only two strains of *T. hyodysenteriae*, P18A and MC52/80, whereas another antigen of 16 kDa was common to all eleven strains of *T. hyodysenteriae* but was not present in the two non-pathogens. Immunogold labelling of whole organisms suggested that the 16 kDa antigen was present on the surface of the spirochaetes. In *in vitro* tests the serum agglutinated and inhibited growth of only the *T. hyodysenteriae* strains, suggesting that antibodies to the 16 kDa antigen were responsible for these activities. Serum from a gnotobiotic pig infected with *T. hyodysenteriae* strain P18A had antibodies to the 16 kDa antigen alone and also possessed agglutinating and growth-inhibitory activities.

INTRODUCTION

The aetiological agent of swine dysentery is *Treponema hyodysenteriae* (Taylor & Alexander, 1971; Harris et al., 1972). It is an intestinal spirochaete which colonizes the mucosa of the large intestine of the growing pig and together with other bacterial species (e.g. *Fusobacterium* and *Bacteroides* spp.) gives rise to mucohaemorrhagic diarrhoea (Harris et al., 1978; Lysons et al., 1978; Joens et al., 1981) leading to dehydration and occasionally death of the infected animal. Pigs that have been infected with virulent strains of *T. hyodysenteriae* and recover from the disease have been shown to be immune to further infection (Olson, 1974; Joens et al., 1979; Rees et al., 1989).

In a comparison of the antigens of a virulent strain of *T. hyodysenteriae* and a non-pathogenic intestinal spirochaete (*Treponema innocens*) numerous cross-reacting antigens were detected by Western blotting with sera from pigs with clinical disease and also with convalescent porcine sera (Joens & Marquez, 1986). One antigen (16 kDa) was specific to *T. hyodysenteriae*. In a study by Chatfield *et al.* (1988) three antigens unique to virulent strains of *T. hyodysenteriae* were identified using hyperimmune rabbit sera raised against *T. hyodysenteriae* and absorbed with the non-pathogenic *T. innocens*; but the cellular location of these antigens was not described. More recent investigations (Kent *et al.*, 1989) have demonstrated an immune response to the axial filament polypeptides of *T. hyodysenteriae* using immunoblotting techniques with serum from a pig recovered from swine dysentery. However, antibacterial activity of hyperimmune pig serum raised against axial filaments could not be demonstrated.

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Surface-orientated antigens of the outer envelope of the spirochaetes are probably encountered by the host early in infection and an immune response directed against these components may, in part, be responsible for immunity of pigs to swine dysentery. Although there is no direct evidence to support this hypothesis in swine dysentery, investigations of the immune response in humans to *Treponema pallidum* have revealed the presence of antibodies to highly antigenic polypeptides (Hanff *et al.*, 1982; Baker-Zander *et al.*, 1985) and one of 47 kDa has been shown to reside in the outer envelope (Jones *et al.*, 1984; Marchitto *et al.*, 1986). An investigation with monoclonal antibodies to this surface-exposed antigen revealed that the antibodies were effective both in *in vitro* complement-dependent immobilization and in neutralization tests (Jones *et al.*, 1984; Marchitto *et al.*, 1986). Bailey *et al.* (1987) also demonstrated immobilization of *T. pallidum* with a monoclonal antibody to a surface-associated 44 kDa polypeptide.

In studies of the spirochaete *Borrelia burgdorferi*, antibodies to outer envelope antigens have been detected in the sera and synovial fluid of patients with Lyme disease (Barbour *et al.*, 1983a, 1985; Craft *et al.*, 1986), and monoclonal antibodies have been raised to an outer membrane polypeptide of 31 kDa (Barbour *et al.*, 1983b) and also against polypeptides described as the 34 kDa range of polypeptides (Barbour *et al.*, 1984). Likewise, patients infected with *Leptospira interrogans* serovar *hardjo* had antibodies to surface-exposed antigens (Chapman *et al.*, 1988). However, little information exists of the antibacterial activity attributable to these antibodies.

This study is concerned with identification of polypeptide antigens present in the outer envelope of *T. hyodysenteriae* which (a) are specific to *T. hyodysenteriae*, (b) are exposed on the surface of the spirochaete and (c) may stimulate the production of antibodies that have antibacterial activity.

**METHODS**

*Bacterial strains.* Eight strains of *T. hyodysenteriae* representing the LPS serotypes described by Kent *et al.* (1989), two additional strains from the USA, B204 (serotype 2) and B234 (serotype 1), and JWPM (serotype 2; isolated in the Netherlands) were used in this study. Two non-pathogenic, intestinal spirochaetes, PWS/A and M1, were isolated from healthy pigs (Hudson *et al.*, 1976; Lemcke & Burrows, 1979). The spirochaetes were cultivated in trypticase soya broth supplemented with 5% (v/v) rabbit serum (Lemcke *et al.*, 1979) and grown in large volumes as described by Kent *et al.* (1988).

*Preparation of outer envelopes.* The outer envelopes of *T. hyodysenteriae* strains P18A and VS1 were prepared by a modification of the method of Johnson *et al.* (1973). A one-litre, 24 h culture of strain P18A was centrifuged at 6000 *g* at 20 °C and the bacterial pellet was washed once with phosphate-buffered saline (PBS; 0.17 m-NaCl, 3.35 mM-KCl, 0.01 M-Na2HPO4, 1.84 mM-KH2PO4, pH 7.2) and resuspended in 40 ml of PBS. Sodium dodecyl sulphate (SDS) solution (10%, w/v) was added to give a final concentration of 0.005% (w/v) and the suspension was incubated for 15 min at room temperature with occasional mixing. The suspension was then centrifuged at 20000 *g* for 30 min and the clear supernatant fluid was filtered through a 0.45 μm pore size membrane filter (Millipore). The outer envelopes were reaggregated by dialysis against several changes of distilled water for 7 d at 4 °C and were collected by centrifugation at 90000 *g* for 1 h at 4 °C. The pellets were resuspended in distilled water and stored at −20 °C.

*SDS-PAGE and Western blotting.* The method of Laemmli (1970) was used to separate polypeptides on 12.5% (w/v) polyacrylamide gels using a 3.5% (w/v) stacking gel. Samples were dissolved in sample buffer consisting of 62.5 mM-Tris/HCl, 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 1% (v/v) glycerol at 100 °C for 5 min. Gels were run for 18 h at a constant voltage of 35 V and were either stained with PAGE Blue 83 (BDH) or electrophoretically transferred at 70 V for 4 h to nitrocellulose (Schleicher and Schuell) by the method of Towbin *et al.* (1979).

The method used for probing nitrocellulose filters with serum antibody was that described by Kent *et al.* (1989). The filter was treated with 20% (v/v) foetal calf serum (FCS) in 0.05 M-Tris-buffered saline, pH 8.0 (TBS) for 1 h with gentle agitation. The FCS was replaced with hyperimmune antisera diluted 1/100 or antisera from infected gnotobiotic pigs diluted 1/10 in TBS containing 20% FCS for 1 h. The antiserum was removed and the filter was successively washed (15 min each step) in TBS, TBS containing 0.1% Nonidet-P40, and finally TBS. The filter was then placed in TBS/FCS containing 0.5 μCi (18.5 kBq) 125I-labelled Protein A (Amersham) for 1 h, washed as above, dried and exposed with RX type X-ray film (Fuji) using enhancing screens at −70 °C for 18–72 h.

*Antisera.* Gnotobiotic pig antisera were raised against outer envelopes (serum B50) and axial filaments (serum C11) from *T. hyodysenteriae* strain P18A as described by Kent *et al.* (1989). Convalescent porcine serum (CPS) was obtained from a pig that had persistent swine dysentery.
Two gnotobiotic pigs (67A and 67B) were orally dosed on two consecutive days with 50 ml of broth cultures (5×10⁸ live organisms ml⁻¹) of T. hyodysenteriae strain P18A and sera were collected 21 d after infection.

**Slide agglutination test.** The slide agglutination test was that described by Burrows & Lemcke (1981).

**Growth inhibition test.** Inhibition of the growth of T. hyodysenteriae by antisera was tested by the method of Lemcke & Burrows (1979).

**Electron microscopy.** Preparations of the outer envelope was negatively stained with 2% (w/v) ammonium molybdate on Formvar-coated copper grids. For ultra-thin sectioning, membrane pellets were fixed in 3% (w/v) glutaraldehyde in 0.1 M-phosphate buffer and post-fixed in 1% (w/v) osmium tetroxide. Washed pellets were further fixed in 1% (w/v) uranyl acetate, dehydrated, transferred to propylene oxide and embedded in Araldite. Immunogold labelling was performed by first allowing a drop of the washed suspension of spirochaetes to dry on a Formvar-coated nickel grid. The grids were treated with dilutions of hyperimmune gnotobiotic pig antiserum for 30 min. The grids were rinsed with PBS and treated with 10 nm Protein A-gold particles (Janssen) for 15 min. The grids were rinsed and negatively stained with ammonium molybdate. All grids were viewed in a Philips EM300 electron microscope.

### RESULTS

#### Outer envelope preparation

SDS treatment of cultures of T. hyodysenteriae produced low yields of outer envelopes that were free of contaminating axial filaments. An electron micrograph of negatively stained outer envelopes of strain P18A is shown in Fig. 1 (a). Dialysis of the SDS-soluble fraction against distilled water gave preparations of outer envelope that were predominantly lipid bilayers. Transmission electron microscopy of ultrathin sections (Fig. 1 b) confirmed the trilaminar structure of these outer envelopes. These preparations were used, without further purification, for the production of antiserum.

#### SDS-PAGE and Western blotting

SDS-PAGE analysis of the outer envelope preparations showed the presence of many bands (Fig. 2). Similar bands were observed in both P18A and VS1 except for a band at 16 kDa that was present in the outer envelope preparation from strain VS1 (lane 2) but was apparently absent in the outer envelope of strain P18A (lane 1). Fig. 3 shows the Western blot analysis of all strains of porcine intestinal spirochaetes probed with outer envelope serum B50. The serum reacted strongly with two antigens of approximately 45 kDa and 16 kDa of the homologous strain P18A (lane 7), and strain MC52/80 reacted similarly (lane 4). The antiserum reacted only with the 16 kDa antigen in all other strains of T. hyodysenteriae. Although similar amounts of each strain were applied to the gel the intensity of the reaction to the 16 kDa antigen was variable. Strain VS1 (lane 5) reacted more strongly than any other strain, whereas P35/2 (lane 3), B169 (lane 8) and B234 (lane 13) reacted only weakly. The antiserum did not react with any polypeptides of the non-pathogenic spirochaetes (lanes 1 and 2). Very weak reactions were also observed with all thirteen strains to antigens in the range 30 to 45 kDa, which corresponded to the axial filament polypeptides. When axial filament polypeptides of eight strains of T. hyodysenteriae (Kent et al., 1989) were separated by electrophoresis and transferred to nitrocellulose the B50 serum detected these polypeptides only after extended exposure of the autoradiograph (data not shown).

Fig. 4 shows an immunoblot of strain P18A probed with CPS, B50, C11, 67A and 67B sera. The 16 kDa antigen was recognized by all sera except the axial filament serum C11 (lane 3). The serum from 67B (lane 2) also reacted with the axial filament polypeptides AF2, 3 and 4 but the serum from 67A only detected the 16 kDa antigen (lane 1). When these pigs were dosed with P18A, shedding of the organism from pig 67A was not observed for all 21 d and the animal was clinically normal. In contrast, pig 67B excreted the organism for 10 d commencing 2 d after infection and had abundant clear mucus in the faeces during this period.

The 45 kDa polypeptide common to strains P18A and MC52/80 that was detected by the B50 antiserum (lane 4) was of similar size to the largest polypeptide (AF1) of axial filaments of P18A detected by the C11 serum (lane 3).
Fig. 1. Electron micrographs of outer envelopes of *T. hyodysenteriae* strain P18A. (a) Outer envelopes negatively stained with ammonium molybdate; (b) ultrathin sections of outer envelopes stained with uranyl acetate. Bars 0.1 μm.

**Immunogold labelling**

Surface labelling of the spirochaetes was apparent when strains P18A (Fig. 5a) and VS1 (Fig. 5b) were treated with B50 antiserum and Protein A-gold. The gold label appeared attached only to the outer envelope of both P18A and VS1; there was no apparent labelling of the axial filaments of either strain. Furthermore, there was also no labelling of the non-pathogenic strains M1 and PWS/A (Fig. 5c).

**Slide agglutination and growth inhibition tests**

Hyperimmune serum B50 agglutinated all *T. hyodysenteriae* strains and also the non-pathogen M1. However, when B50 was absorbed with both M1 and PWS/A, only strains of *T. hyodysenteriae* were agglutinated. The degree of agglutination observed was variable and several strains (P35/2, MC52/80 and B169) agglutinated only weakly. Antiserum 67A also agglutinated the *T. hyodysenteriae* strains but not the non-pathogens.
Fig. 2. Outer envelopes of *T. hyodysenteriae* strains P18A (lane 1) and VSI (lane 2) separated by SDS-PAGE and stained with PAGE Blue 83.

Fig. 3. Western blots of whole cells from 11 strains of *T. hyodysenteriae* (lanes 3–13) and two non-pathogenic intestinal spirochaetes (lanes 1 and 2). Polypeptides were separated on a 12.5% SDS-PAGE gel, transferred to nitrocellulose and probed with hyperimmune gnotobiotic pig serum (B50). Lane 1, M1; lane 2, PWS/A; lane 3, P35/2; lane 4, MC52/80; lane 5, VSI; lane 6, KF9; lane 7, P18A; lane 8, B169; lane 9, JWPM; lane 10, S75/1; lane 11, B204; lane 12, B78; lane 13, B234.

Table 1. Growth inhibition tests on strains of *T. hyodysenteriae* using (a) hyperimmune gnotobiotic pig serum (B50) raised against outer envelopes of *T. hyodysenteriae* strain P18A and (b) serum (67A) from a gnotobiotic pig orally dosed with P18A.

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<td>B169</td>
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<td>3.3</td>
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<td>P18A</td>
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NT, Not tested.
*Inhibition of growth is the distance (mm) from the disc to the edge of the clear zone (mean of two results).

Table 1 shows the results of growth inhibition tests using B50 and 67A sera. There was no inhibition of growth of the non-pathogens M1 and PWS/A but there was a marked inhibition of growth of all the *T. hyodysenteriae* strains tested except B169, which was not inhibited by 67A. Strain P35/2 did not grow on the medium used in the assay and was not tested.
DISCUSSION

Preparation of outer envelopes using a low concentration of SDS and dialysis against distilled water was used in order that trilaminar membranes could be seen by electron microscopy since dialysis against $\text{Mg}^{2+}$ at concentrations greater than 5 mM produces aggregates which are devoid of trilaminar structures (Johnson et al., 1973). Mild treatment with 0.005% SDS produced outer envelopes with minimal contamination from axial filaments as judged by electron microscopy. Immunoblots of purified axial filaments versus the hyperimmune gnotobiotic porcine serum (B50) demonstrated that the preparation of outer envelopes was not completely devoid of axial filaments. However, contamination of outer envelopes with axial filaments can easily be monitored with polyclonal antibodies that are specific to axial filaments (Kent et al., 1989).

Studies of the polypeptides of outer envelopes of spirochaetes have in general been difficult, particularly with the non-cultivable spirochaete *T. pallidum*. Outer envelope polypeptides have been the subject of many investigations but such studies have, in addition, often identified axial filament and other periplasmic polypeptides (Stamm et al., 1987). However, the most conclusive evidence for the presence of surface-orientated polypeptides has come from the use of monoclonal antibodies to *T. pallidum* (Jones et al., 1984; Marchitto et al., 1984, 1986; Bailey et al., 1987). Similar studies have also been made with *Leptospira interrogans* (Jost et al., 1988) and *Borrelia burgdorferi* (Barbour et al., 1983b, 1984). *T. hyodysenteriae* is relatively easy to culture and prepare the outer envelope.
Fig. 5. Immunogold labelling of whole organisms of *T. hyodysenteriae* strains P18A (a) and VS1 (b), and of the non-pathogenic intestinal spirochaete PWS/A (c), using hyperimmune gnotobiotic pig serum B50. Bars, 0.1 μm.
The outer envelope preparations contained many polypeptides as identified by PAGE Blue 83 staining and it was surprising to discover that only two of these were strongly antigenic. Although the 16 kDa antigen was apparently in low abundance (as determined by the poor sensitivity of staining with PAGE Blue 83) in outer envelope preparations of strain P18A, high titre antiserum was obtained, suggesting that it is highly antigenic. The 45 kDa antigen was common to two strains only (P18A and MCS2/80) of different LPS serotypes and therefore did not show strain or serotype specificity. The 16 kDa antigen was present in all strains of T. hyodysenteriae but not the non-pathogens and is therefore probably species specific. The variation in the concentration of this antigen in each strain could not be explained by the small differences in the amount of material applied to the gel. Either the 16 kDa antigen was present in different amounts in the outer envelope of each strain or the serum (raised against outer envelopes of P18A) reacted weakly in particular with strain B169, suggesting only partial conservation of the epitopes in the strains tested. Also the 16 kDa antigen could not be detected in all preparations of outer envelopes from P18A by immunoblotting with the B50 antiserum. Although a standard method for cultivation of the strains was adopted, variation either in expression of this antigen or in preparation of the outer envelopes may have contributed to these observations. Changes in the cell surface properties of other spirochaetes have also been observed; for example in vitro incubation of T. pallidum for more than 8 h was required before antibodies in rabbit syphilitic sera recognized three protein antigens (Stamm et al., 1987).

Immunogold labelling of the surface of strains P18A and VS1 suggests that the 16 kDa polypeptide is a surface antigen because the 45 kDa antigen, also detected by B50 serum in P18A, was not present in VS1. This conclusion is supported by the agglutination of all the strains by B50 serum and also 67A, a serum which had antibodies only to the 16 kDa polypeptide. Although the 45 kDa polypeptide migrated in SDS-PAGE close to the largest axial filament polypeptide it is probably an outer envelope polypeptide because of its presence in only two of the strains. Additional support for this conclusion is provided by the investigations of Kent et al. (1989), which demonstrated that the axial filament polypeptides were conserved among all strains of T. hyodysenteriae.

Inhibition of growth of strains of T. hyodysenteriae by B50 and 67A sera also suggests that antibodies to the 16 kDa polypeptide are responsible for this activity. These results demonstrate that antibodies to the 16 kDa polypeptide have in vitro antibacterial activity and may contribute to an antibacterial mechanism in vivo which could lead to protection of the pig from swine dysentery. Joens & Marquez (1986) also identified a 16 kDa polypeptide in sera and in colonic secretions from convalescent pigs and suggested that it might be important in stimulating a protective immune response. In the investigations presented here a conventional pig with swine dysentery and two gnotobiotic pigs challenged with strain P18A responded strongly to the 16 kDa polypeptide, which indicates that it is also expressed in vivo.

Protection against swine dysentery may therefore be achieved, in part, by antibodies to the polypeptide described above. However, greater knowledge of the surface antigens of T. hyodysenteriae is necessary before the mechanisms of immunity can be understood.

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REFERENCES


