Analysis of the Axial Filaments of *Treponema hyodysenteriae* by SDS-PAGE and Immunoblotting

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Purified axial filaments from eight serotypes of *Treponema hyodysenteriae* and two non-pathogenic intestinal spirochaetes were characterized by SDS-PAGE and Western blotting. Axial filaments of all ten strains had similar SDS-PAGE profiles; five major axial filament polypeptides were identified, with molecular masses of 43·8, 38, 34·8, 32·8 and 29·4 kDa. Hyperimmune gnotobiotic pig serum raised against purified axial filaments of strain P18A (serotype 4) cross-reacted with all other serotypes and with the non-pathogens, and convalescent serum taken from a pig with persistent swine dysentery also showed a strong response to the axial filament polypeptides. Hyperimmune gnotobiotic pig serum raised against axial filaments failed to agglutinate viable organisms and did not inhibit growth *in vitro*. Hence, the axial filaments of *T. hyodysenteriae* have been identified as major immunodominant antigens, although the role that antibodies to these antigens play in protection has yet to be established.

INTRODUCTION

Swine dysentery is a severe mucohaemorrhagic diarrhoea which affects the colon of the growing pig (Harris & Glock, 1986). *Treponema hyodysenteriae* has been identified as the causative agent (Taylor & Alexander, 1971; Harris *et al.*, 1972) and, together with other bacterial species such as *Fusobacterium* and *Bacteroides* spp., gives rise to the severe clinical disease seen in conventional pigs (Harris *et al.*, 1978; Lysons *et al.*, 1978; Joens *et al.*, 1981). Only a mild form of the disease is seen when gnotobiotic pigs are infected with *T. hyodysenteriae* alone (Whipp *et al.*, 1982).

Humoral (Joens *et al.*, 1979; Egan *et al.*, 1983; Burrows *et al.*, 1984) and mucosal (Joens *et al.*, 1984) immune responses to infection have been observed although little is known of the immune response to specific antigens of *T. hyodysenteriae*. Joens & Marquez (1986) compared antigens of a virulent strain of *T. hyodysenteriae* with those of a non-pathogenic intestinal spirochaete. They showed that an immune response to numerous antigens could be detected by immunoblotting with sera taken from pigs during clinical disease and convalescence although the cellular location of these antigens was not established. A single antigen unique to *T. hyodysenteriae* was also observed. Chatfield *et al.* (1988) observed antigens unique to a virulent strain of *T. hyodysenteriae* which were thought to be outer envelope polypeptides when hyperimmune rabbit sera and post-vaccination porcine sera were used in immunoblotting experiments.

In studies of *Leptospira* spp. (Chapman *et al.*, 1988), *Borrelia* spp. (Craft *et al.*, 1986; Barbour *et al.*, 1983) and *Treponema* spp. (Hanff *et al.*, 1982; Baker-Zander *et al.*, 1985) antibodies to axial filament polypeptides have been demonstrated in patients with disease. Moreover,

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treponemicidal activity of normal human serum against *Treponema pallidum* has been correlated with immunoglobulin G directed against endoflagellar polypeptides (Blanco *et al.*, 1986). Therefore, antibodies to the axial filaments of *T. hyodysenteriae* may play an important role in protection of pigs from swine dysentery.

This investigation was concerned with the characterization of the axial filaments of ten different spirochaetes. Eight strains of *T. hyodysenteriae* representative of all known lipopolysaccharide (LPS) serotypes and two strains of a non-pathogenic, intestinal spirochaete were examined by SDS-PAGE and immunoblotting using convalescent and polyclonal hyperimmune porcine sera.

METHODS

Bacterial strains. Eight strains of *Treponema hyodysenteriae* were used throughout this investigation, together with two porcine isolates that could not be identified as *T. hyodysenteriae* and were not pathogenic for pigs (Table 1). B78, S75/1, B169, P18A, KF9, VS1 and MC52/80 were used as representative of the seven LPS serotypes of *T. hyodysenteriae* identified by Lemcke & Bew (1984). P35/2 was a new field isolate which is serologically distinct from other serotypes of *T. hyodysenteriae* and hence represents a new serotype (unpublished observation). The non-pathogens PWS/A and M1 were isolated from healthy pigs (Hudson *et al.*, 1976; Lemcke & Burrows, 1979).

All strains were cultivated in trypticase soya broth supplemented with 5% (v/v) rabbit serum as described by Kent *et al.* (1988).

Preparation and purification of axial filaments. Axial filaments were prepared from all strains by a modification of the method described by Hardy *et al.* (1975). One-litre cultures were harvested by centrifugation at 15000 *g* for 30 min at 4 °C and washed once with phosphate-buffered saline (0.17 M-NaCl, 3.35 mM-KCl, 0.01 M-Na₂HPO₄, 1.84 mM-KH₂PO₄, pH 7.2) containing 0.01 M-MgCl₂ and 0.01 M-CaCl₂ (complete PBS) and pellets were suspended in the same buffer. Outer envelopes were removed by the addition of SDS to a final concentration of 0.01%. The suspension was incubated for 15 min at room temperature with occasional agitation and the organisms then collected by centrifugation at 25000 *g* for 30 min at 4 °C. The pellets were resuspended in 10 ml complete PBS and blended for 2 min using a Universal blender (MSE). Filaments were recovered in the supernatant by pelleting the remaining organisms at 30000 *g* for 30 min at 4 °C. The blending was repeated up to 10 times for each strain, and filaments from each step were checked by electron microscopy for contaminating structures, e.g. membranes. The filament preparations were pooled and sodium lauroyl sarkosinate (Sarkosyl) was added to 0.2% (w/v) final concentration. Filaments were sedimented by centrifugation at 94000 *g* for 60 min at 4 °C and were purified by isopycnic gradient centrifugation in caesium chloride (0.359 g CsCl + 1 ml double-distilled water, $\rho = 1.25$) at 100000 *g* for 48–65 h at 10 °C. A band at approximately 1.3 ρ was collected in double-distilled water and then centrifuged at 94000 *g* for 60 min at 4 °C. Purified filaments were resuspended in double-distilled water, checked by electron microscopy and SDS-PAGE and stored at -20 °C.

SDS-PAGE and Western blotting. SDS-PAGE was carried out using the discontinuous buffer system of Laemmli (1970). Vertical slab gels were prepared with a 12% (w/v) acrylamide running gel and 3.75% stacking gel. Samples were dissociated by boiling in 62.5 mM-Tris/HCl pH 6.8, 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 1% (v/v) glycerol. Low-molecular-mass markers were obtained from Pharmacia. Gels were run at a constant voltage of 150 V for 3–5 h and were either stained with PAGE Blue 83 (BDH) or Western blots were performed using the method described by Towbin *et al.* (1979). Polypeptides were transferred to nitrocellulose (Schleicher & Schuell) overnight at 35 V. After transfer, the nitrocellulose was blocked with 20% (v/v) foetal calf serum (FCS) in 0.05 M-Tris-buffered saline, pH 8.0 (TBS) for 1 h on a rocking platform. The nitrocellulose was then incubated with dilutions of antisera (1/100) in TBS containing 20% FCS for 1 h. After incubation, the antiserum was removed and the nitrocellulose washed for 15 min each with TBS, TBS + Nonidet-P40 (0.1%, v/v) and TBS before addition of 0.1 μ Ci (37 kBq) ¹²⁵I-Protein A per ml TBS (containing 20% FCS) for 1 h. The washing steps were repeated and the nitrocellulose allowed to dry. Autoradiographs using RX-type X-ray film (Fuji) were exposed with enhancing screens for 4–24 h at -70 °C.

Preparation of antisera. Antiserum to purified axial filaments of *T. hyodysenteriae* strain P18A was raised in a 14-d-old gnotobiotic pig. Protein (1.5 mg) was emulsified in Freund's incomplete adjuvant (Gibco) in a total volume of 2 ml and administered subcutaneously. The immunization was repeated after 2 weeks at a second subcutaneous site. The animal was test bled 14 d later and the serum collected at exsanguination was designated C11.

Rabbit antisera against intact bacteria were raised by subcutaneous and intravenous inoculation as described by Lemcke & Burrows (1979).

Convalescent serum (CPS) was obtained from a conventionally reared pig. The animal was infected at 6 weeks of age and had persistent swine dysentery for several weeks. Serum was collected at post-mortem 12 weeks post-infection.

Table 1. *Strains used*

Strain	Origin	Serotype	Reference
B78	USA	1	Baum & Joens (1979)
S75/1	GB	2	Baum & Joens (1979); Lemcke & Bew (1984)
B169	Canada	3	Baum & Joens (1979)
P18A	GB	4	Baum & Joens (1979); Lemcke & Bew (1984)
KF9	GB	5	Lemcke & Bew (1984)
VS1	GB	6	Lemcke & Bew (1984); Lysons <i>et al.</i> (1982)
MC52/80	GB	7	Lemcke & Bew (1984)
P35/2	GB	New	(K. A. Kent, unpublished observation)
PWS/A	GB	Non-pathogen	Hudson <i>et al.</i> (1976)
M1	GB	Non-pathogen	Lemcke & Burrows (1979)

Slide agglutination test. This was done according to the method described by Burrows & Lemcke (1981).

Growth inhibition test. The ability of sera to inhibit growth of *T. hyodysenteriae* was determined by the method of Lemcke & Burrows (1979). Briefly, suspensions of broth-grown *T. hyodysenteriae* strains were inoculated onto rabbit serum agar and excess inoculum was removed. Plates were allowed to dry and then antibiotic assay discs (6 mm diameter) impregnated with undiluted hyperimmune rabbit serum were applied. Plates were incubated under anaerobic conditions at ambient temperature for 18 h followed by 3–5 d at 37 °C. After incubation, the sizes of the zones of growth inhibition were measured from the outer edge of the disc to the edge of the inhibition zone.

Electron microscopy. The axial filament preparations and suspensions of spirochaetes were negatively stained with 2% (w/v) ammonium molybdate on Formvar coated copper grids. Immunogold labelling was performed as follows. Axial filaments or spirochaete suspensions were placed on Formvar-coated nickel grids and allowed to dry. The grids were treated with hyperimmune porcine serum (C11) for 30 min. After rinsing in distilled water the grids were treated with 10 nm Protein A–gold particles (Janssen) for 15 min. After further rinsing the suspensions were negatively stained with ammonium molybdate and viewed in a Philips EM300 electron microscope.

RESULTS

Purification and SDS-PAGE analysis of axial filaments

An electron micrograph of a CsCl-purified preparation of axial filaments from *T. hyodysenteriae* strain P18A is shown in Fig. 1. Two distinct bands were sometimes observed after gradient centrifugation but both bands appeared similar by electron microscopy and SDS-PAGE. Preparations from all the strains investigated were of similar appearance. Filaments that possessed a hook were occasionally observed and the filaments generally had a striated appearance. 'Thick' and 'thin' filaments were frequently observed. SDS-PAGE analysis of axial filaments from eight strains of *T. hyodysenteriae* and two non-pathogenic strains revealed the presence of several major polypeptides (Fig. 2), which ranged in size from approximately 29 kDa to 46 kDa. The axial filaments of *T. hyodysenteriae* strain P18A (lane 4), the representative serotype of British isolates, gave five major polypeptide bands: AF1 43.8 kDa; AF2, 38.0 kDa; AF3, 34.8 kDa; AF4, 32.8 kDa; and AF5, 29.4 kDa. Two of these bands (AF1 and AF3) appeared to contain more than one component. Filaments of other strains gave bands of similar size but with one or two obvious differences. The 43.8 kDa (AF1) polypeptide of all other strains of *T. hyodysenteriae* did not resolve clearly as a doublet. AF1 of strains P35/2 (lane 8) and PWS/A (lane 9) had a lower molecular mass (42.0 kDa) than that of other strains, whereas the non-pathogen M1 (lane 10) had a higher molecular mass of 46.0 kDa. AF2 of all strains appeared similar. The 34.8 kDa polypeptide (AF3) appeared in all *T. hyodysenteriae* strains as a triplet, except in strains B78 (lane 1) and MC52/80 (lane 7), where it was a doublet. In the two non-pathogenic spirochaetes (lanes 9 and 10) this was a single band. AF4 and AF5 were similar in all strains, with AF4 resolving as a single band and AF5 resolving as a doublet.

Immunogold labelling

Fig. 3 shows the protein A–gold labelling of *T. hyodysenteriae* strain P18A labelled with hyperimmune gnotobiotic pig serum (C11) raised against purified axial filaments. The gold particles labelled the surface of the filaments that had been released from the organism during

Fig. 1. Electron micrograph of caesium chloride purified axial filaments of *T. hyodysenteriae* strain P18A negatively stained with ammonium molybdate. Bar, 0.1 μ m.

Fig. 2. Axial filament polypeptides of eight strains of *T. hyodysenteriae* and two non-pathogenic intestinal spirochaetes separated by SDS-PAGE and stained with PAGE Blue 83. Lane 1, B78; lane 2, S75/1; lane 3, B169; lane 4, P18A; lane 5, KF9; lane 6, VS1; lane 7, MC52/80; lane 8, P 35/2; lane 9, PWS/A; lane 10, M1. Molecular mass markers are indicated by arrows.

preparation of the sample on the copper grid. Gold particles were not observed attached to the outer envelope of the spirochaete.

Slide agglutination and growth inhibition tests

There was no agglutination of any strain of *T. hyodysenteriae* or of the non-pathogenic spirochaetes by C11 serum, whereas hyperimmune rabbit serum raised against whole P18A agglutinated all eight strains of *T. hyodysenteriae* but not the non-pathogenic spirochaetes M1 and PWS-A.

In the growth inhibition test, C11 serum gave no zones of inhibition with any of the strains of spirochaete tested. The hyperimmune rabbit serum raised against whole P18A gave 4–8 mm zones of inhibition of growth with all strains of *T. hyodysenteriae* but no inhibition with the non-pathogenic spirochaetes.

Western blotting

In Western blot analysis, convalescent porcine serum (CPS) reacted with the five polypeptides of the axial filaments of all ten strains, including the non-pathogens (Fig. 4a).

Fig. 3. Immunogold labelling of whole organisms of *T. hyodysenteriae* strain P18A using hyperimmune gnotobiotic pig serum (C11). Bar, 0.5 μ m.

Fig. 4. Western blots of the axial filament polypeptides from eight strains of *T. hyodysenteriae* and two non-pathogenic intestinal spirochaetes. Polypeptides were separated on a 12% acrylamide SDS-PAGE gel, blotted onto nitrocellulose and probed with (a) convalescent porcine serum (CPS) and (b) hyperimmune gnotobiotic pig serum (C11). Lane 1, B78; lane 2, S75/1; lane 3, B169; lane 4, P18A; lane 5, KF9; lane 6, VS1; lane 7, MC52/80; lane 8, P35/2; lane 9, PWS/A; lane 10, M1. Molecular masses of the major polypeptides are shown.

When the C11 serum was used to probe the transfer blots of all the strains, only the four higher molecular mass bands AF1 to AF4 reacted strongly, and all four bands reacted with similar intensity (Fig. 4*b*). The band of lowest molecular mass (AF5) reacted very weakly and was only observed after extended exposure of the autoradiograph.

DISCUSSION

The axial filament preparations from all strains of intestinal spirochaetes were morphologically similar when examined by electron microscopy. In axial filament preparations from *T. hyodysenteriae* and the non-pathogenic spirochaetes 'thick' and 'thin' filaments were observed, a finding similar to that reported for *T. pallidum* (Sand-Petersen *et al.*, 1981; Cockayne *et al.*, 1987) and *Leptospira* spp. (Nauman *et al.*, 1969; Kelson *et al.*, 1988), where 'thick' and 'thin' filaments are known to be sheathed and unsheathed filaments respectively. In contrast, the axial filaments of *Borrelia* spp. are reported to be of uniform thickness along their length (Hovind-Hougen, 1974; Barbour & Hayes, 1986). The hook structure reported to be present in most spirochaetes was occasionally observed in preparations from the intestinal spirochaetes.

The SDS-PAGE profiles of axial filaments of all strains examined were also similar and only minor variations were observed. The five polypeptides of 43.8, 38, 34.8, 32.8 and 29.4 kDa are similar to those described for *T. pallidum* and *T. phagedenis* biotype Reiter (Blanco *et al.*, 1986), in which five polypeptides of 35, 33 (doublet), 30 and 27 kDa have been described. Axial filaments from *Leptospira* spp. have six polypeptides (Nauman *et al.*, 1969) whereas Hansen *et al.* (1988) report the presence of only one polypeptide in *Borrelia burgdorferi* axial filaments. Occasionally when axial filaments were purified on CsCl two bands were observed close together. Both bands contained morphologically identical axial filaments which had the same SDS-PAGE profile. In the purification of *T. phagedenis*, *T. pallidum* and *B. burgdorferi* axial filaments, Blanco *et al.* (1986) and Hansen *et al.* (1988) have also observed this phenomenon. These bands may have arisen due to the association of non-protein material with some of the axial filaments, thus affecting their buoyant density.

The cross-reaction of the hyperimmune gnotobiotic porcine serum (C11) and the convalescent serum (CPS) with the axial filament bands of all the strains of intestinal spirochaetes demonstrates the presence of shared epitopes. In a study of *T. hyodysenteriae* and non-pathogenic intestinal spirochaetes (*T. innocens*), Joens & Marquez (1986) demonstrated that sera taken from pigs with acute clinical swine dysentery cross-reacted with polypeptides from both species. Numerous common antigens were identified as well as some that were specific for *T. hyodysenteriae*. However, the nature of these antigens was not determined. Cross-reaction between axial filament polypeptides of *T. pallidum* and *T. phagedenis* biotype Reiter with hyperimmune rabbit (Blanco *et al.*, 1988), normal human (Blanco *et al.*, 1986) and human syphilitic sera (Hardy *et al.*, 1975) has been reported. Baker-Zander & Lukehart (1984) and Bailey *et al.* (1987) have also reported the presence of common epitopes of *T. pallidum* and *T. hyodysenteriae* axial filaments. Similarly, Chapman *et al.* (1988) observed cross-reaction of axial filaments among the *Leptospira* spp. using hyperimmune rabbit serum, and Barbour *et al.* (1986) reported cross-reaction of a monoclonal antibody with axial filaments of several species of *Borrelia*. These observations suggest that the axial filament polypeptides of each genus are highly conserved.

Polypeptide AF5 was detected by SDS-PAGE in all serotypes and antibody to this antigen was present in porcine convalescent serum. The observation that antibody binding to this polypeptide was very weak with hyperimmune gnotobiotic pig serum (C11) cannot yet be explained. Similar observations were made by Blanco *et al.* (1986) with hyperimmune rabbit serum against *T. pallidum*, where the polypeptide of lowest molecular mass could not be detected. The reason for these findings is uncertain although it is possible that the colonic mucosa of pigs recognized axial filament epitopes of whole, intact organisms which may have been modified during the preparation of purified axial filaments for parenteral immunization.

This investigation has identified the axial filaments of *T. hyodysenteriae* as major immunodominant antigens. Similarly, Western blot analyses of sera from patients infected with

T. pallidum (Moskophidis & Muller, 1984; Hanff *et al.*, 1982; Baker-Zander *et al.*, 1985), *L. interrogans* serovar *hardjo* (Chapman *et al.*, 1988) and *B. burgdorferi* (Hansen *et al.*, 1988) have also identified the axial filaments as immunodominant antigens.

In a recent study by Chatfield *et al.* (1988), pigs protected by vaccination using an intramuscular inoculation of formalin-killed *T. hyodysenteriae* showed a predominant antibody response to five *T. hyodysenteriae* polypeptides with molecular masses in the range 29–45 kDa. Although these antigens were described as outer envelope polypeptides, the SDS-PAGE profiles are similar to those presented in this investigation as axial filament polypeptides. It is possible that the outer envelope preparations described by Chatfield *et al.* (1988) contained axial filaments.

It has been suggested that antibodies to axial filaments may be bactericidal (Blanco *et al.*, 1986) but the growth inhibition tests described here suggest that antibodies to axial filaments alone are not bactericidal. It is likely that hyperimmune serum was unable to agglutinate intact viable organisms as the axial filaments are located within the outer envelope and therefore inaccessible to antibody. However, antibodies to axial filament antigens may operate in conjunction with antibodies to other components of the spirochaete, e.g. the outer envelope, thus producing an effective anti-bacterial mechanism.

Further investigations of the immune response to specific antigens of *T. hyodysenteriae* should lead to a better understanding of the mechanisms of immunity and possibly to the formulation of improved vaccines.

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