Identification of leptospiral flagellar antigens by gel electrophoresis and immunoblotting

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Summary. Flagella extracted from five serovars, representative of the pathogenic and saprophytic species of the Leptospiraceae, were morphologically similar. Analysis of *Leptospira interrogans* flagellar preparations by polyacrylamide gel electrophoresis revealed three common major bands in the $(30-40) \times 10^3$ -mol. wt region, and serovar-specific bands in the lower region of the gels. Although some differences were observed, flagella extracted from *L. biflexa* serovar *patoc* and *Leptonema illini* revealed similar electrophoretic profiles to those seen in *L. interrogans* flagella. Immunoblot analysis showed that while flagellar components in the $(20-30) \times 10^3$ -mol. wt region were recognised only by homologous rabbit antisera, a major protein doublet of $(33-34) \times 10^3$ or $(35-36) \times 10^3$ -mol. wt, depending on the species, was also demonstrated by heterologous antisera. The serovar-specific bands in the $(20-30) \times 10^3$ -mol. wt region were composed of lipopolysaccharide (LPS). These results show that leptospiral flagella are immunogenic and contain antigens which are conserved among the different genera of the family Leptospiraceae.

Introduction

The genus *Leptospira* is divided into two species, *L. interrogans*, which includes pathogenic serovars and the saprophytic *L. biflexa*. A third species, originally classified as *Leptospira illini* (Faine, 1982), has now been reclassified as *Leptonema illini* on the basis of morphological and DNA differences (Hovind-Hougen, 1979).

Each leptospire possesses two flagella (axial filaments) inserted subterminally into the protoplasmic cylinder. The filament, sheath, hook and basal body of leptospiral flagella are similar to the corresponding structures of other bacterial flagella (Smibert, 1973). Chang and Faine (1970) showed that purified leptospiral flagella were immunogenic and on immunodiffusion formed a characteristic precipitation line. The pattern of cross-reactivity between serovars by immunodiffusion was different from that seen in the agglutination-absorption system used for classification (Chang *et al.*, 1974).

Nauman *et al.* (1969) identified six protein bands when acid-dissociated leptospiral flagella were subjected to polyacrylamide gel electrophoresis (PAGE). In contrast, other bacterial flagella usually possessed a single band. However, the antigenicity of these protein components was not studied, nor was their flagellar origin proved. There have been

no further studies on either the characteristics or antigenicity of leptospiral flagellar components.

In this present study, the composition of flagella prepared from serovars representative of each of the three species in the family Leptospiraceae was investigated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting to determine their immunogenicity and cross-reactivity.

Materials and methods

Organisms, antigens, antibodies and serological methods

The growth and culture of leptospires and the preparation of rabbit antisera against whole, live leptospires have been described previously (Chang and Faine, 1970; Adler and Faine, 1978).

Antiserum was prepared against SDS-PAGE separated flagella components by the method of Boulard and Lecroisey (1982). Coomassie blue stained bands were excised from the gel, macerated and emulsified with incomplete Freund's adjuvant (1:1.2 v:v). Female New Zealand White rabbits were given intradermal injections of this material at approximately five sites and bled 4 weeks later. The production and characterisation of monoclonal antibodies reacting with homologous LPS epitopes of serovars *hardjo* and *pomona* were performed as described previously (Adler and Faine, 1983; Farrelly *et al.*, 1987).

The microscopic agglutination test (MAT) was de-

scribed by Adler and Faine (1983). Flagella, purified by differential centrifugation followed by caesium chloride gradient centrifugation, were negatively stained (Chang and Faine, 1970; Adler and Faine, 1979) and examined with a Philips EM 300 electronmicroscope at an accelerating voltage of 80 kV. Flagella were stained with gold labelled anti-rabbit immunoglobulins (Janssen auroprobe EMGAR, 5 nm diameter) by the procedure described by Barbour *et al.* (1984) with the following modifications. Grids were incubated with antibody at 37°C in a humid atmosphere, Tween 20 (Sigma) 0.05% was added to the washing buffer and grids were washed with distilled water before negative staining.

Electrophoresis and immunoblotting

SDS-PAGE analysis of the purified flagellar preparations was performed with the discontinuous buffer system of Laemmli (1970) with resolving and stacking gels containing polyacrylamide 10% and 4% respectively. Flagellar protein 5 µg/lane was loaded on to the gels and electrophoresed at 30mA/gel, together with low mol. wt markers (Pharmacia Fine Chemicals, Sweden). After electrophoresis, the gels were either silver-stained (Morrisey, 1981) or the separated flagellar proteins were transferred to nitrocellulose (0.45 µm pore size; Schleicher and Schuell Co.) with a Biorad Transblot Cell (Biorad, USA) run at 60 V for 3 h. The transblot buffer described by Towbin *et al.* (1979) was diluted 1 in 2 to facilitate transfer of high mol. wt proteins. Transblotted mol. wt markers were stained with india ink (Hancock and Tsang, 1983) to check efficiency of transfer. After transfer, antigenic components were detected by immunostaining (Chapman *et al.*, 1987) with rabbit antisera or

mouse monoclonal antibodies, followed by either peroxidase conjugated goat anti-rabbit immunoglobulins (Biorad) or sheep anti-mouse immunoglobulins (Silenus, Australia) respectively. Bands were visualised with 4-chloro-1-naphthol (Hawkes *et al.*, 1982).

Results

Electronmicroscopy

Electronmicroscopic examination of purified flagellar preparations revealed only filamentous structures (fig. 1). The filaments had a striated appearance whereas thinner, apparently un-sheathed, filaments were seen occasionally. Flagellar preparations extracted from each of the five serovars tested were morphologically indistinguishable.

Electrophoresis and immunostaining

Flagellar preparations were electrophoretically separated on SDS-PAGE 10% gels and stained for protein. Except for minor variations in the mol. wts of the bands, similar profiles were obtained for flagella preparations from each serovar (fig. 2).

Flagellar preparations extracted from the *L. interrogans* serovars revealed an identical banding profile in the mol. wt region $(30-40) \times 10^3$, each showing major bands of approximate mol. wts $37(10^3)$ and $31(10^3)$, and a $(33-34) \times 10^3$ -mol. wt doublet.

Fig. 1. Electronmicrograph of flagella extracted from *L. interrogans* serovar *pomona* negatively stained with uranyl acetate 0.05%. Arrows indicate partially sheathed flagella. Bar = 0.2 µm.

Fig. 2. Silver-stained SDS-PAGE gel of leptospiral flagellar preparations. The positions of mol. wt markers are indicated (10^3). Lane A—*L. interrogans* serovar *pomona*; B—*L. interrogans* serovar *hardjo*; C—*L. interrogans* serovar *copenhageni*; D—*L. biflexa* serovar *patoc*; E—*L. illini*.

In the $(20-30) \times 10^3$ -mol. wt region of the gel, profiles unique to each serovar were observed. Flagellar preparations from serovars *pomona* and *hardjo* had two and three additional bands respectively (fig. 2), whereas flagella from serovar *copenhageni* revealed no bands below mol. wt 30×10^3 . Flagellar preparations from *L. biflexa* serovar *patoc* had a profile in the $(30-40) \times 10^3$ -mol. wt region similar to those seen in *L. interrogans* although mol. wts were slightly higher (fig. 2). In this case, the mol. wts (10^3) of the major bands were 38, 34 and the doublet 35–36. In contrast, flagella extracted from *L. illini* revealed two doublets of approximate mol. wts (10^3) 36–37 and 33–34 respectively. Additional bands of mol. wts (10^3) 31, 39 and 41 were also observed (fig. 2).

After transfer to nitrocellulose, the antigenicity of the flagellar proteins was examined by probing the separated proteins with rabbit antisera. These

Table. Serological cross-reactivity of different leptospiral serovars measured by MAT with rabbit antisera

Serovar of test strain	MAT titre with antiserum raised against serovar				
	<i>pomona</i>	<i>hardjo</i>	<i>copenhageni</i>	<i>patoc</i>	<i>illini</i>
<i>pomona</i>	512	8	0	2	0
<i>hardjo</i>	0	1024	2	0	0
<i>copenhageni</i>	4	0	512	0	0
<i>patoc</i>	0	0	0	256	8
<i>illini</i>	0	0	0	0	512

sera had high homologous agglutinating titres but only very low titres against heterologous serovars (table). All antisera produced against *L. interrogans* serovars recognised the three major flagellar components extracted from other serovars of *L. interrogans*, as exemplified by the *pomona* antiserum (fig. 3), although the band of mol. wt 31×10^3 was only faintly detectable. The sera also reacted with bands of mol. wts (10^3) 38 and 35–36 in flagellar preparations of *L. biflexa* serovar *patoc* and with the $(33-34) \times 10^3$ -mol. wt doublet band of *L. illini* flagella. The cross-reactivity pattern was identical regardless of the *L. interrogans* serovar used to produce the antisera. Rabbit antisera to serovar *patoc* reacted strongly with the $(33-34) \times 10^3$ -mol. wt doublet of both *L. interrogans* and *L. illini* flagella. There was also faint reactivity with the 37×10^3 -mol. wt band of *L. interrogans* and the $(36-37) \times 10^3$ -mol. wt doublet of *L. illini* (fig. 3). *L. illini* antiserum reacted with the doublet from all other serovars and with additional homologous components.

As well as recognising heterologous bands, all sera reacted with low-mol. wt serovar-specific bands, when they were present (fig. 3), which were identical to those seen in silver stained gels (fig. 2).

Identity of low-mol. wt bands

In preparations from serovars *hardjo* and *pomona*, bands in the $(20-30) \times 10^3$ -mol. wt region were immunostained with monoclonal antibodies specific for homologous LPS, producing identical profiles to those seen when purified LPS was similarly electrophoresed and stained (data not shown).

Investigation of the cross-reactive flagellar components

Rabbit antiserum prepared against the $(33-34) \times 10^3$ -mol. wt doublet of flagellar preparations from

Fig. 3. Immunoblot of leptospiral flagellar preparations stained with rabbit antisera against *L. interrogans* serovar *pomona* (lanes A-E) or *L. biflexa* serovar *patoc* (lanes F-J). The positions of mol. wt markers are indicated (10). Lanes A and F—*L. interrogans* serovar *pomona*; B and G—*L. interrogans* serovar *hardjo*; C and H—*L. interrogans* serovar *copenhageni*; D and I—*L. biflexa* serovar *patoc*; E and J—*L. illini*.

L. interrogans serovar *pomona* (anti-doublet serum) failed to agglutinate whole leptospire. Analysis by immunoblotting showed that the same antiserum reacted strongly with both the (33–34)- and 37-mol. wt (10^3) bands of flagellar preparations of the three *L. interrogans* serovars, as well as the (35–36)- and the (33–34)-mol. wt (10^3) doublets of *L. biflexa* serovar *patoc* and *L. illini* respectively (fig. 4). The anti-doublet serum failed to react with transblotted, purified LPS or other cellular components of *L. interrogans* serovar *pomona*.

To determine whether the antigens recognised by the anti-doublet serum were indeed of flagellar origin, this antiserum was allowed to react with serovar *pomona* flagella and immunogold stained. The gold particles preferentially labelled the entire length of the flagella (fig. 5). In contrast, flagella similarly allowed to react with normal rabbit serum (NRS) failed to bind the gold particles (fig. 5).

Discussion

Electronmicroscopic examination of all the flagellar preparations revealed the presence of thick and thin filamentous structures. The thin filaments were probably unsheathed thick filaments, some of which had their sheaths partially removed. Similar structures have been described for flagellar preparations extracted from other leptospire (Nauman *et al.*, 1969; Smibert, 1973) and treponemes (Bharier and Rittenberg, 1971; Sand Petersen *et al.*, 1981). The striated appearance of the filaments has also been noted in other leptospire and treponemes (Nauman *et al.*, 1969; Bharier *et al.*, 1971; Jackson and Black, 1971).

Analysis of the flagellar preparations by SDS-PAGE revealed similar banding profiles in the (30–40) $\times 10^3$ -mol. wt range, comprising a doublet and two satellite bands. Banding profiles identical to those reported here in the (30–40) $\times 10^3$ -mol. wt

Fig. 4. Immunoblot of leptospiral flagellar preparations stained with anti-doublet serum. The positions of mol. wt markers are indicated (10^3). Lane A—*L. interrogans* serovar *pomona*; B—*L. interrogans* serovar *hardjo*; C—*L. interrogans* serovar *copenhageni*; D—*L. biflexa* serovar *patoc*; E—*L. illini*.

range have been described for flagellar preparations extracted from treponemes (Bharier and Allis, 1974; Sand Petersen *et al.*, 1981; Penn *et al.*, 1985) although the mol. wt of the components differed slightly. The major protein components of *Borrelia* spp. flagella also appear as a doublet of either 39 or 41 mol. wt (10^3) depending on the species (Barbour *et al.*, 1986).

However, unique, low-mol. wt bands— $(20-30) \times 10^3$ -mol. wt range—were also observed in flagella extracted from *L. interrogans* serovars *pomona* and *hardjo* and *L. biflexa* serovar *patoc*. These bands were shown to be LPS antigens by their reactivity with LPS specific monoclonal antibodies. The association of LPS components with the flagellar structure is unclear because flagellar preparations lacking low-mol. wt components (as seen with serovar *copenhageni*) appeared morphologically identical to those with them. Contamination of flagellar preparations with LPS during extraction

may be a possible explanation as this is commonly encountered when attempting to purify antigens from gram-negative bacteria (Gregory, 1986).

The anti-doublet serum reacted with both the $(33-34) \times 10^3$ -mol. wt doublet of serovar *pomona*, against which it was raised, and the band of mol. wt 37×10^3 , suggesting either shared antigenic determinants or contamination with the 37×10^3 -mol. wt band during excision from PAGE gels. Immuno-electronmicroscopy of *pomona* flagella showed that this serum reacted specifically with native flagella, demonstrating that the $(33-34) \times 10^3$ -mol. wt doublet or the 37×10^3 -mol. wt band, or both, of serovar *pomona* are flagellar components. In similar experiments, polyclonal rabbit antisera against denatured treponemal flagellar components failed to react with native flagella (Limberger and Charon, 1986). This may reflect differences in the extraction of the denatured proteins or the immunisation procedure from those used here. Immuno-electronmicroscopy with monoclonal antibodies has previously identified the major doublet of both treponemal and borrelial flagellar preparations to be of flagellar origin (Barbour *et al.*, 1986; Limberger and Charon, 1986).

The extent of cross-reactivity of the major flagellar proteins, especially that of the doublet, suggests that flagella from both genera of Leptospiraceae share several antigenic determinants indicating that flagella may be a conserved structure. The cross-reactivity of leptospiral or leptoneal flagella with those from other spirochaetes was not examined. The possibility that leptospiral flagella may also cross-react serologically with other, less closely related, spirochaetes cannot therefore be excluded. However, the major flagellar protein of *Borrelia* spp., which was also found to cross-react within its genus, did not react with other spirochaetes (Barbour *et al.*, 1986).

In a previous study of leptospiral flagella (Chang *et al.*, 1974), the overall cross-reactivities between serovars and species which we have reported were not observed. In view of the relatively insensitive immunodiffusion technique used by Chang *et al.* (1974) compared with immunoblotting, it is possible that some cross-reactions were not detected. For example, in their study, neither serovar *patoc* flagella nor serovar *patoc* antisera reacted with serovars *pomona*, *hardjo* or *copenhageni*. Nor did they observe any cross-reactions between these serovars of *L. interrogans*, while we found strong reciprocal cross-reactions.

A second possibility is that because we used SDS-dissociated flagella, we detected antigens or epitopes not accessible on native flagella. Thus, the

Fig. 5. Electronmicrograph of flagella extracted from *L. interrogans* serovar *pomona* stained with (A) normal rabbit serum, or (B) anti-doublet serum, followed by colloidal gold-conjugated anti-rabbit immunoglobulins. Negative staining was performed with ammonium molybdate 2%. Bar = 0.5 μ m.

doublet may represent internal flagellar antigens which are conserved throughout the family Leptospiraceae. Similar internal antigens have been described for the flagella of *Vibrio* spp. (Shinoda *et al.*, 1980) and treponemes (Limberger and Charon, 1986). This second possibility is unlikely because we have shown by immuno-electronmicroscopy that the major flagellar components seen in denaturing gels are recognised on native flagella.

The lack of cross-reactivity between serovars by MAT and the high level of cross-reactivity shown by flagellar antigens in immunoblotting indicate that it is unlikely that flagella are involved in the agglutination of leptospires by immune rabbit serum. Previous reports have shown that anti-flagella sera agglutinate whole leptospires (Chang and Faine, 1973). This may well have been due to contamination of flagellar preparations with LPS

which is immunogenic and can elicit the production of agglutinating antibodies (Adler and Faine, 1983; Farrelly *et al.*, 1987). Leptospiral flagella are strongly immunogenic, being recognised by antibodies induced by natural and experimental leptospirosis in humans and rabbits (Chang and Faine, 1973; Adler and Faine, 1979) and carry antigenic determinants common to all three species. Since flagella of other gram-negative organisms are involved in antibody-mediated immunity (Holder *et al.*, 1982; Tamura *et al.*, 1984), further study of the role of anti-flagella antibodies in immunity to leptospirosis is warranted.

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