Immunochemistry of Extracts from *Leptospira interrogans* Serotype *hardjo*

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SUMMARY

Antigens from *Leptospira interrogans* serotype *hardjo* grown in modified Korthof's medium were obtained by ethanol and alkaline extraction procedures and their chemical and serological properties were compared. The protein to polysaccharide ratio in the ethanol extract was 1:1.8 and in the alkali extract was 1.7:1. The lipid content of the latter was twice that of the former (8.5%, w/w). There was an inverse relationship of the protein and carbohydrate contents of the two preparations, the total reducing sugar being higher in the ethanol extract whereas protein was higher in the alkali extract. Both preparations contained arabinose, rhamnose, fucose, xylose, mannose, galactose, glucose and galacturonic acid but in different amounts. No muramic acid or 2-keto-3-deoxyoctonate was detected.

Both extracts contained erythrocyte sensitizing substances which, in the passive haemagglutination absorption test, appeared to be closely related antigenically but not identical. In the electron microscope, thin sections of the ethanol extract showed trilaminar outer envelope-like material.

INTRODUCTION

Extraction of various leptospiral antigens and their application in sero-diagnosis of leptospirosis have been extensively reported (Hindle & White, 1934; Chang & McComb, 1954; Schneider 1954; Rothstein & Hiatt, 1956; Schricker & Hanson, 1963; Palit & Sharma, 1971). Isolation and identification of structural components of leptospirae have also been described recently (Ritchie & Ellinghausen, 1965; Nauman, Holt & Cox, 1969; Yanagihara & Mifuchi, 1968; Palit, Hamilton & Gulasekharam, 1974). Zeigler & Vaneseltine (1975) described the gross chemical composition of the outer envelope in serotype *pomona*. Immunochemical properties of a type-specific antigen extracted by the phenol-water method from the Kyto strain of the *hebdomadis* group have been described by Shinagawa & Yanagawa (1972). Subsequently, Kasai & Yanagawa (1974) partially characterized the type-specific antigen in serotype *canicola* (Hond Utrecht IV). Serological, morphological and chemical properties of a genus-specific, ethanol-extractable outer envelope preparation from serotype *biflexa*, strain Patoc I, have been reported by Palit *et al.* (1974). The ability of the outer envelope to protect hamsters against homologous challenge was shown by Auran, Johnson & Ritzi (1973) and subsequently confirmed by Glosser *et al.* (1973) and Bey, Auran & Johnson (1974). A polysaccharide material designated Fraction 4 (Faine, Adler & Palit, 1974a) protected hopping mice against lethal or carrier infection (Faine, Adler & Ruta, 1974b).

This paper deals with the serological and chemical interrelationship between Fraction 4 and outer envelope antigens of *Leptospira interrogans* serotype *hardjo*.

METHODS

Leptospiral strains. *Leptospira interrogans* serotype *hardjo* was obtained from Dr T. M. Alexander (Department of Agriculture, Launceston, Tasmania) and was originally isolated from dairy cattle in 1969. It was maintained in a modified Korthof's medium (Alston & Broom, 1958) supplemented with sheep serum derivative (Faine, 1969). Serotypes *tarassovi* (Mitis-Johnson), *icterohaemorrhagiae* (RGA), *pomona*, *canicola* (Hond Utrecht IV) and *biflexa* Patoc I were obtained from the WHO *Leptospira* Reference Laboratory (Department of Health, Brisbane, Queensland, Australia). *Leptospira biflexa* Waz was obtained through the courtesy of Professor S. Faine (Monash Medical School, Prahran, Victoria, Australia). These strains were maintained in Korthof's medium containing 10% (v/v) rabbit serum.

Extraction of antigens. The procedure for growth and harvesting of organisms and the ethanol extraction method for isolating antigen were those described by Palit & Gulasekharan (1973) but with the following minor modifications. After the 50% (v/v) ethanol extraction, the mixture was centrifuged at 27000 g for 35 min in a Sorvall RC2-B refrigerated centrifuge at 4 °C. The ethanol concentration in the supernatant fluid was then increased to 90% (v/v), and the precipitate was recovered by centrifuging at 12000 g for 20 min, taken up in a small volume of distilled water and dialysed against 4 l sterile distilled water. The dialysate was re-precipitated with 90% (v/v) ethanol.

The alkali extract (Fraction 4, F4) was isolated as described by Faine *et al.* (1974a). For analyses, F4 was dissolved and the ethanol extract was suspended in sterile distilled water. Otherwise they were taken up in phosphate-buffered saline, pH 7.2 (Palit & Gulasekharan, 1973).

Serology. Anti-whole culture serum prepared against each serotype used in this study was obtained as described by Palit & Gulasekharan (1973). The microscopic agglutination (MAT) and passive haemagglutination (HA) tests were done as described previously (Palit & Gulasekharan, 1973).

For the serum absorption test, washed sheep red cells were sensitized with the optimum dilution of F4 or ethanol extract. After washing, the sensitized, packed cells were added to a 1:2 dilution of antiserum. The mixture was kept at 37 °C for 1.5 h, centrifuged at 1500 g for 10 min and the supernatant serum was assayed for antibodies by the HA test. The absorption process was repeated until the specific antibodies were completely removed from the supernatant serum. The pre- and post-absorption HA tests were done with the same optimum antigen dilution used for antiserum absorption. After complete absorption of the antibodies, the supernatant serum was further reacted with the antigen-coated red cells at least three or four times.

Ouchterlony test. To prepare antiserum, the ethanol extract was mixed with an equal volume of Freund's complete adjuvant (Difco) and a total of 0.9 mg antigen in 1 ml was injected intradermally into 8 to 10 sites on the shaved back of a rabbit. Blood was taken at weekly intervals after inoculation.

The agar gel precipitation test (Ouchterlony, 1949) was set up with 1% (w/v) agarose gel in veronal buffer (pH 7.5) on glass microscope slides. The wells were 6 mm diam. and 4 mm apart. The gel was incubated for 1 to 3 days at room temperature in a humid, air-tight chamber.

Analytical procedures. Total carbohydrate was determined by the indole test as described by Kabat & Mayer (1964a) with D-glucose as standard. Total nitrogen was determined by the micro-Kjeldahl method (Kabat & Mayer, 1964b) and protein by the micro-modification of the Folin-Ciocalteu test (Kabat & Mayer, 1964c) with bovine serum albumin as

standard. The chloroform-soluble lipid was estimated as described by Palit *et al.* (1974). Amino sugars were determined by the method of Elson & Morgan as described by Kabat & Mayer (1964*d*).

Descending paper chromatography, for the preliminary qualitative analysis of the sugars, was conducted in two different solvent systems: (i) butanol/pyridine/acetic acid/water (60:40:30:3, by vol.) and (ii) ethyl acetate/pyridine/water (8:2:1, by vol.). Hydrolysis conditions and other details were as described by Palit *et al.* (1974).

The thiobarbituric acid test (Weissbach & Hurwitz, 1959) was used for detecting 2-keto-3-deoxyoctonate (KDO). *Salmonella adelaide* purified lipopolysaccharide (Kabat & Mayer, 1964*e*) was used as a positive control. To release KDO, the test samples and *S. adelaide* lipopolysaccharide were boiled in a water bath in sealed ampoules containing 0.2 M-HCl for different times (15 to 120 min). Another set of samples was hydrolysed with 0.5 M-HCl. These hydrolysates and also unhydrolysed samples were used in the test.

Muramic acid was tested for in a Beckman liquid chromatograph (Munich multi-chrome model B type 4255 fitted with the ultramicro accessory, giving a detection limit of 0.01 μ mol). Samples were first hydrolysed in 6 M-HCl for 24 h in sealed ampoules and dried over P₂O₅ and NaOH pellets.

For quantitative sugar analysis by gas-liquid chromatography (g.l.c.) methyl esters were prepared as follows. Each sample, together with 0.1 mg mannitol as internal standard, was dried in a vacuum desiccator over P₂O₅ and 2 ml anhydrous 1 M-HCl in dry methanol was then added. The tube was sealed under N₂ and heated at 85 °C for 24 h; the excess HCl was then neutralized with Ag₂CO₃, and 50 μ l acetic anhydride was added. This mixture was stored at room temperature for 6 h. The supernatant fluid was kept and the precipitate was washed several times with dry methanol. The supernatant fluid and washings were evaporated under reduced pressure at 35 °C and finally dried over P₂O₅. Trimethylsilyl derivatives were prepared from samples in a sealed tube by adding 0.1 ml dry pyridine (analytical grade), 0.09 ml hexamethyldisilazane (Pierce Chemical Co.) and 0.01 ml trifluoroacetic acid. The mixture was kept at room temperature for 30 min with occasional shaking. The clear solution obtained could easily be injected into the gas chromatograph. The g.l.c. analysis was done in a Hewlett Packard 7620A research chromatograph with a flame ionization detector. Separations were effected in a 12 ft $\times$ 1/16th in glass column packed with 3% OVI (Pierce Chemical Co.) on 100 mesh Chromosorb W (Pierce Chemical Co.). The column temperature was maintained at 150 °C for 2 min after injection and then increased to 200 °C at 1 °C per min. Peaks were identified by comparison with known standards. Peak areas relative to internal standards were calculated with a HP 3352 B Laboratory Data System.

Electron microscopy. Thin sections were prepared and stained as described by Palit *et al.* (1974).

RESULTS

Serological investigations

Sheep red cells sensitized with 333 μ g ethanol extract antigen ml⁻¹ from serotype *hardjo* reacted in the haemagglutination test with the homologous anti-whole culture serum. Similarly F4, from the same organism, was required at 166 μ g ml⁻¹ to sensitize red cells which reacted with the same anti-whole culture serum. The F4-sensitized red cells had a higher haemagglutination titre (1:2048) than cells treated with the ethanol extract antigen (1:128).

Haemagglutination absorption studies. Repeated absorption of the diluted antiserum (1:2) with F4-sensitized red cells led to complete removal of the specific antibodies. At this

Fig. 1. Double diffusion precipitin reaction in agar gel. (1) 90 % ethanol extract outer envelope (OE); (2, 3) anti-OE serum from rabbit 1; (4, 5) anti-OE serum from rabbit 2.

Table 1. *Haemagglutination reactivity of homologous (hardjo) and heterologous immune serum with F4- and ethanol extract antigen-sensitized red cells*

Serum from <i>L. interrogans</i> serotype	Reciprocal HA titres with:		MAT titre (live, whole <i>hardjo</i>)
	F4-sensitized red cells	Ethanol extract antigen-sensitized red cells	
<i>hardjo</i>	2048	128	1024
<i>tarassovi</i> (Mitis-Johnson)	16	0	4
<i>icterohaemorrhagiae</i> (RGA)	2	0	0
<i>pomona</i>	4	0	2
<i>canicola</i>	32	0	2
<i>L. biflexa</i> Patoc I	16	0	2
<i>L. biflexa</i> Waz	2	0	0

0 indicates no HA titre at 1:2 dilution of the antiserum.

stage the reciprocal titre of the absorbed serum with ethanol extract antigen-coated red cells was 32 compared to its pre-absorption titre of 128. After complete absorption of the F4 antibodies, the antiserum was further treated three or four times with F4-sensitized red cells. No further reduction in the HA titre with the ethanol extract antigen-coated red cells was observed. The reciprocal MAT titres of the antiserum before and after absorption with F4-absorbed and unabsorbed anti-whole culture serum were 512 and 64 respectively.

Double diffusion agar gel precipitation test. Two antisera prepared against ethanol extract outer envelope showed one precipitin line (Fig. 1).

HA cross-reactivity. The red cells coated with ethanol extract antigen showed very little cross-reaction with antiserum from heterologous serotypes of *Leptospira interrogans* or *L. biflexa* groups (Table 1). The F4-sensitized red cells reacted with antiserum against serotypes *tarassovi*, *canicola* and *biflexa* Patoc I, to low titres, but with other antisera it showed very little reaction. In microscopic agglutination tests, serotype *hardjo* showed very little reaction with antisera to heterologous strains.

Table 2. *Quantitative sugar analysis by g.l.c. of F4 and ethanol extract*

Results given are the average of three to five determinations using individual batches of antigens. Numbers in parentheses are the values relative to rhamnose.

Component	Percentage (w/w) of total sugar	
	F4	Ethanol extract
Arabinose	6.06 (0.87)	9.82 (0.84)
Rhamnose	6.91 (1.00)	11.74 (1.00)
Fucose	1.66 (0.24)	2.87 (0.24)
Xylose	1.9 (0.27)	2.87 (0.24)
Mannose	4.38 (0.63)	7.37 (0.63)
Galactose	3.87 (0.56)	5.67 (0.48)
Glucose	2.36 (0.34)	3.72 (0.32)
Galacturonic acid	5.42 (0.78)	7.20 (0.61)

Fig. 2. Electron micrograph of a section of 90 % ethanol-extracted material of serotype *hardjo* showing rows of triple-layered outer envelope, with each row in close proximity to another. Bar marker represents 10 μ m.

Chemical and ultrastructural investigations

Paper and gas-liquid chromatographic analyses revealed the same sugars in both F4 and the ethanol extract but there were quantitative differences (Table 2). Rhamnose was the most abundant and fucose the least abundant sugar in both preparations.

The ethanol extract contained 44.6 % (w/w) total reducing sugar and 25 % (w/w) protein; F4 contained 45 % (w/w) protein and 26 % (w/w) total reducing sugar. The values for the protein contents were confirmed from their total nitrogen contents by multiplying the latter

values by 6.25: F4 contained 6.6% N (41% protein) while the ethanol extract contained 4.0% N (25% protein). The lipid content of F4 (18%, w/w) was more than double that in the ethanol extract (8.5%, w/w). F4 had only half the amount of amino sugar (4%, w/w) found in the ethanol extract. Appreciable amounts of galacturonic acid were found in both preparations (Table 2).

Neither preparation from serotype *hardjo* contained detectable KDO. Tests were done on both hydrolysed and unhydrolysed preparations in parallel with lipopolysaccharide from *Salmonella adelaide* as a positive control. With the latter the hydrolysed samples gave stronger reactions than the unhydrolysed ones. Muramic acid was not detected (detection limit of 0.01 μ mol) in either preparation.

Thin sections of the ethanol extract showed rows of triple-layered envelope material in close proximity to each other (Fig. 2).

DISCUSSION

The alkali extract F4 from *L. interrogans* serotype *hardjo* contained protein and carbohydrate in the ratio of 1.7:1. A similar protein to carbohydrate ratio has been reported in F4 from other serotypes of *Leptospira* grown in similar conditions (Faine *et al.*, 1974a). The lipid content of F4 from serotype *hardjo* is higher than that from serotypes *icterohaemorrhagiae*, *pomona* and some strains of non-pathogenic *Leptospira* (Faine *et al.*, 1974a). In the ethanol extract from serotype *hardjo*, the protein to polysaccharide ratio was 1:1.8. The inverse relationship of the protein and carbohydrate content between F4 and ethanol extract has not previously been reported. Zeigler & Vaneseltine (1975) obtained outer envelope from serotype *pomona* grown in a chemically defined, modified minimal medium by treating cells with 0.01 M-phosphate buffer. Here the protein to carbohydrate ratio was approximately 1.8:1. Thus the difference in protein to carbohydrate ratio between *hardjo* and *pomona* outer envelope preparations may be due to the different growth media used.

The presence of uronic acid in type-specific antigen extracted from serotype *canicola* has been reported by Kasai & Yanagawa (1974), but the kind of uronic acid was not identified. In this study, galacturonic acid was present in both F4 and ethanol extract, the amount being considerably higher in the latter.

The ethanol extract from serotype *hardjo* contained more fucose than did F4. This 6-deoxyhexose has been found in some strains of *Escherichia coli* and in the *Salmonella* group of organisms, but its presence in *Leptospira interrogans* does not seem to have been reported. F4 and ethanol extract contained the same monosaccharides but in different amounts. However, in both preparations, the concentrations of sugars relative to each other was virtually the same, which may indicate a similar chemical origin.

KDO was not detected either in F4 or ethanol extract hydrolysates or in unhydrolysed materials. Many Gram-negative bacteria contain KDO as one of the basic constituents of the O-specific polysaccharides (Lüderitz, Staub & Westphal, 1966). Recently, Zeigler & Vaneseltine (1975) failed to detect KDO in serotype *pomona* outer envelopes. Osborn (1963) suggested that KDO in the Enterobacteriaceae may link the polysaccharide and lipid A in lipopolysaccharide.

In this study, electron microscopy (Fig. 2) of the ethanol extract from serotype *hardjo* showed characteristically layered outer envelope material in rows similar to the outer envelope material extracted by 50% ethanol treatment of *L. biflexa* strain Patoc I (Palit *et al.*, 1974). The absence of muramic acid in the outer envelope and F4 preparations suggested that they were not contaminated with mucopeptide components.

The passive haemagglutination and chemical tests suggest that there is a close relationship between the two preparations but they are not identical. Perhaps the F4 material is situated close to the outer envelope in the leptospiral cell, or during the alkali extraction the same antigenic complex which is present in ethanol extract is being isolated in some chemically and serologically modified form. Definitive evidence for these possibilities may be obtained by immunolabelling procedures which may indicate the site of origin of F4 in the leptospiral cells.

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