Serological and Chemical Interrelationship of Antigens from *Leptospira interrogans* serovar *canicola*

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Antigens of the outer envelope from *Leptospira interrogans* serovar *canicola* (Hond Utrecht IV) were extracted by 50% (v/v) ethanol or by sodium dodecyl sulphate and serological analysis suggested that they were identical. The 'fraction 4' extracted by alkali was found to contain glycoproteins of high (retentate) and low (filtrate) molecular weight; the latter behaved like a hapten in serology and in animal immunization experiments. Antibodies were raised in rabbits against this hapten by conjugating it to bovine albumin fraction V. The antiserum was found to react with both the low molecular weight and high molecular weight glycoproteins. This anti-hapten serum contained little or no whole-cell-agglutinating antibodies. The fraction 4 retentate behaved like a complete antigen in serological and immunization studies. Fraction 4 retentate and the outer envelope preparations were serologically related but they were not identical. Chemical studies revealed similarities between the carbohydrate component of the outer envelope obtained by ethanol extraction and fraction 4. The outer envelope extracted by ethanol, fraction 4 and its low and high molecular weight glycoproteins contained arabinose, rhamnose, fucose, xylose, mannose, galactose, glucose, glucosamine and glucuronic acid. Three unidentified peaks were observed in gas-liquid chromatographic analysis of the *O*-trimethylsilyl derivatives of methyl glycosides of all these samples and one of these peaks co-eluted with the *O*-trimethylsilyl derivative of 3-*O*-methylmannose.

INTRODUCTION

Hindle & White (1934) reported the isolation of a leptospiral antigen by an alkali extraction procedure. Faine *et al.* (1974*a*) modified this method of extraction and isolated a polysaccharide-containing, non-toxic material which they called fraction 4 (F₄). Faine *et al.* (1974*b*) demonstrated that F₄ could protect hopping mice against lethal or carrier infection by homologous virulent leptospires. Auran *et al.* (1972) isolated outer sheath by sodium dodecyl sulphate extraction from serovar *canicola* and demonstrated its immunogenic property in hamsters. Palit *et al.* (1974) extracted outer sheath by a 50% (v/v) ethanol extraction procedure and this extract was found to have a close antigenic relationship with the alkali extract F₄ in serovar *hardjo* (Palit & Harrison, 1977).

In our present study, we have investigated the serological relationship between F₄ (Faine *et al.*, 1974*a*) and outer envelope extracted by 50% (v/v) ethanol (Palit *et al.*, 1974) or by the sodium dodecyl sulphate procedure of Auran *et al.* (1972). Some of the chemical characteristics of the F₄ material from serovar *canicola* have also been elucidated.

METHODS

Leptospiral strain and growth. *Leptospira interrogans* serovar *canicola* (Hond Utrecht IV) was obtained through the courtesy of the WHO *Leptospira* Reference Laboratory (Department of Health, Brisbane, Queensland, Australia). Methods for maintenance and growth of the organisms have been described previously (Palit & Harrison, 1977).

Preparation of antigens. Fraction 4 was extracted as described by Faine *et al.* (1974*a*) and taken up in distilled water after 90% (v/v) ethanol precipitation. Any particulate material was then removed by filtration through a Millipore filter (1.2 μ m pore size). A sample of F₄ was diluted 10-fold in distilled water and filtered through an ultrafiltration cell (Amicon, Lexington, Mass., U.S.A.) equipped with a PM10 membrane that had been thoroughly pre-washed by passing distilled water through it under nitrogen pressure until the filtered water was free from glycerol as determined by the indole test (Kabat & Mayer, 1964*a*). After ultrafiltration of F₄, another 3 vol. distilled water was passed through the membrane. The filtered fraction 4 was called 'F₄ filtrate'; the material left on the PM10 membrane was washed off with distilled water and called 'F₄ retentate'. The F₄ filtrate and the F₄ retentate were concentrated separately by freeze-drying so that each was of the same volume as the original F₄.

The outer envelope was extracted by treating the washed leptospires with 50% (v/v) ethanol as described previously (Palit *et al.*, 1974; Palit & Harrison, 1977). The outer envelope was also extracted according to the method of Auran *et al.* (1972). Ultrafiltration of the sodium dodecyl sulphate-treated material was as described above. The ultrafiltered material was dialysed against a large volume of distilled water until the preparation failed to react (Palmer *et al.*, 1971) with healthy rabbit serum in the agar gel diffusion test described below.

Dry weights of all antigen preparations were determined by drying to a constant weight a measured volume of the sample in distilled water, or the freeze-dried antigen, in a vacuum desiccator containing P₂O₅ and NaOH pellets.

Preparation of antisera. Anti-whole culture serum was raised in rabbits according to the method described by Palit & Gulasekharan (1973).

Anti-retentate serum was prepared in a rabbit by injecting intravenously approximately 3 mg dry wt of the material in 1 ml phosphate buffered saline (pH 7.2). The rabbit was bled by cardiac puncture 20 d later.

Anti-filtrate serum was prepared in three ways. (i) Approximately 4 mg dry wt of filtrate in 2 ml of sterile distilled water was emulsified with an equal volume of Freund's complete adjuvant (Difco) and the emulsion was injected intradermally into the shaved back and flank of a rabbit at 10 to 15 sites. Blood was collected for serum by cardiac puncture 4 and 8 weeks after inoculation and the serum was stored at -20 °C in small volumes. (ii) The filtrate was conjugated with bovine albumin fraction V (BSA; Miles Laboratories, Kankakee, Ill., U.S.A.) by diazo-coupling according to the method of Fielder *et al.* (1970). 2,4,6-Trichlorotriazine was from Matheson Coleman & Bell, Norwood, Ohio, U.S.A., and redistilled dimethyl formamide (BDH) was used for conjugation. The dialysed conjugate was mixed with an equal volume of Freund's complete adjuvant and the emulsion, containing approximately 4 mg of filtrate, was injected intradermally into a rabbit as described above. After 4 weeks the rabbit was injected intravenously with approximately 3 mg of the conjugated filtrate in phosphate buffered saline (pH 7.2). Samples of blood were collected as above 4, 7 and 8 weeks after the first inoculation. The separated serum samples were stored at -20 °C. (iii) The filtrate was conjugated with BSA using toluene-2,4-diisocyanate ('pure'; Koch-Light) according to the method of Singer & Schick (1961) as described for ferritin by Likhite & Schon (1967), except that BSA was used instead of gamma-globulin solution. The purified, dialysed conjugate was emulsified with an equal volume of Freund's complete adjuvant. Emulsion containing approximately 3.5 mg dry wt of original antigen was injected into a rabbit as described in (ii). Samples of blood were taken by cardiac puncture 4 and 8 weeks after the first inoculation.

Serology. The warm complement fixation test was performed as described by Alton *et al.* (1975). Throughout the test procedure, 'the standard (macro) technique' was used where all reagents were in 0.25 ml volumes. Individual guinea pig sera from the guinea pig colony of the Commonwealth Serum Laboratories were tested for the presence of natural lytic antibodies against washed sheep red cell suspensions (1.5%, v/v); sera from five guinea pigs which did not show the presence of such natural antibodies at 1:10 dilution, were pooled and freeze-dried in 1 ml volumes. Glycerinated haemolysin marketed by the Commonwealth Serum Laboratories (Parkville, Victoria, Australia) was used. Necessary pro- and anti-complementary controls for antigen and antiserum were set up. The fixation of complement was allowed to proceed at 37 °C in a water bath for 1 h; the indicator system was then added and the test sera were incubated at 37 °C for 30 min. The highest dilution of the antiserum showing 50% lysis in the presence of a particular antigen dilution was considered to be the titre for the antiserum.

The methods for the passive haemagglutination and the microscopic agglutination tests have been described elsewhere (Palit & Gulasekharan, 1973). The agar gel precipitation test (Ouchterlony, 1949) was set

Fig. 1. Double diffusion precipitin reaction in agar gel: 1, anti-whole culture serum; 2, outer envelope extracted by sodium dodecyl sulphate; 3, outer envelope extracted by ethanol; 4, F_4 retentate.

Table 1. Serological reactivity of F_4 , F_4 filtrate and F_4 retentate with homologous immune serum

	Dry weight of antigen ($\mu\text{g ml}^{-1}$)/ reciprocal serum titre			
	Haem- agglutination test	Tannic acid- modified haem- agglutination test	Complement fixation test	Agar gel precipitation test
F_4	190/1024	200/64	8/128	One band
F_4 retentate	50/1024	60/64	4/128	One band
F_4 filtrate	920/0	920/0	63/0	No reaction

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

up as described by Palit & Harrison (1977). The tannic acid-modified haemagglutination test was as described by Palit & Gulasekharam (1973) except that freshly washed red cells were used for the tannic acid treatment. The passive haemagglutination serum absorption test was done by mixing a known dry weight of filtrate with a particular dilution of the anti-filtrate serum and incubating the mixture at 37 °C for 1 h. The reacted mixture was checked for the presence of antibodies by setting up a haemagglutination test with the retentate-sensitized red cells.

Analytical procedures. The thiobarbituric acid test (Weissbach & Hurwitz, 1959) was employed for the detection of 2-keto-3-deoxyoctonate (KDO), as described previously (Palit & Harrison, 1977). Carbohydrate was determined by the indole test (Kabat & Mayer, 1964*a*) with D-glucose as standard. Amino sugars were determined by the method of Elson & Morgan (Kabat & Mayer, 1964*b*). Chloroform-soluble lipid was estimated as described elsewhere (Palit *et al.*, 1974) and total nitrogen by the micro-Kjeldahl method (Lauber, 1976). Descending paper chromatography for the preliminary qualitative sugar analysis and quantitative carbohydrate analysis by gas-liquid chromatography (g.l.c.) have been described previously (Palit & Harrison, 1977).

RESULTS

Immunological studies

In the agar gel precipitation test, anti-whole culture serum developed identical precipitin bands with outer envelope extracted by 50% (v/v) ethanol or by sodium dodecyl sulphate, while F_4 retentate showed a reaction of partial identity (Fig. 1).

The F_4 filtrate did not react with the anti-whole culture serum in serological tests (Table 1). Amongst all these tests, the tannic acid-modified haemagglutination showed the lowest serum titres and required the largest amount (dry weight) of F_4 and F_4 retentate.

Table 2. *Chemical composition of F₄, F₄ filtrate and F₄ retentate*

All values are the average of three to five estimations.

Component	Composition (% w/w)		
	F ₄	F ₄ filtrate	F ₄ retentate
Protein*	43	18.5	29.2
Carbohydrate	35.5 (35.1)†	44 (53.2)†	18.5 (22.5)†
Lipid	13	Not done	34
Amino sugar (glucosamine.HCl)	5.2	4.7	5

* Calculated from total nitrogen by multiplying by 6.25 after correcting for amino sugar.

† Values in parentheses were determined by gas-liquid chromatography.

Antiserum prepared against the F₄ filtrate was tested by the passive haemagglutination test with F₄-coated red cells. Freund's adjuvant and filtrate mixture yielded very little (1:2) haemagglutinating antibody in the rabbit. The filtrate conjugated with BSA by 2,4,6-trichloro-s-triazine gave antiserum with a haemagglutinating titre of 1:16 or 1:32, but toluene-2,4-diisocyanate coupling resulted in the production of higher titred (1:1024) haemagglutinating antiserum. Filtrate-coated (up to 920 µg ml⁻¹) red cells failed to react with any of the anti-filtrate sera. F₄ retentate-coated red cells gave the same haemagglutinating titre as F₄-treated red cells.

The possibility that the filtrate behaved as a hapten was examined by the serum absorption experiments. In the absorption test, anti-filtrate serum raised in a rabbit by 2,4,6-trichloro-s-triazine conjugate required 36 µg of the filtrate to absorb haemagglutinating antibodies from 0.4 ml of 1:4 diluted serum. With toluene-2,4-diisocyanate-coupled filtrate, 840 µg of filtrate was required to neutralize haemagglutinating antibodies.

The anti-filtrate serum obtained by the two procedures demonstrated only a minimal amount (1:2) of reactivity in the leptospiral agglutination test. Adler & Faine (1978*a, b*) reported that F₄ antibodies in human and rabbit sera were distinct from the agglutinins.

The anti-retentate serum raised in a rabbit reacted up to a dilution of 1:512 in the passive haemagglutination test in which the retentate-sensitized (50 µg ml⁻¹) red cells were employed.

Chemical investigations

F₄, F₄ filtrate and F₄ retentate contained protein, carbohydrate, amino sugar and lipid (Table 2). The F₄ filtrate contained a higher ratio of carbohydrate to protein than did F₄ or the F₄ retentate. A large amount of lipid was found in the retentate.

From the results of quantitative carbohydrate analyses (Table 3), it is evident that there is a close resemblance between the carbohydrate composition of the ethanol extract of the outer envelope and the F₄ preparation, as previously reported for serotype *hardjo* (Palit & Harrison, 1977). F₄ and its fractions contained no detectable KDO.

In the g.l.c. analysis of the *O*-trimethylsilyl methyl glycosides, all samples gave three unidentified peaks. Of these, the first peak (0.35 retention time relative to *O*-trimethylsilyl mannitol) was found to co-elute (Table 3) with a derivatized authentic sample of 3-*O*-methylmannose which was kindly supplied by Dr R. W. Wheat (Department of Biochemistry, Duke University, Medical Center, Durham, N.C., U.S.A.). The other two peaks (0.50 and 0.67 retention time relative to *O*-trimethylsilyl mannitol) could not be identified.

In studies of serovar *hardjo*, the uronic acid found (Palit & Harrison, 1977) in both outer envelope extracted by 50% (v/v) ethanol and F₄ preparations was identified as galacturonic acid. However, during the present investigation it became apparent that this assignment was incorrect and that the uronic acid found previously and in the present serovar *canicola* preparations was glucuronic acid. Some substantial differences between the carbohydrate composition of the filtrate and the retentate were noted (Table 3).

Table 3. *Quantitative carbohydrate analysis of Leptospira interrogans serovar canicola extracts determined by gas-liquid chromatography*

Results are the average of three to five determinations using separate batches of extract. Values in parentheses are percentages (w/w) of the total carbohydrate in each extract.

Carbohydrate	Carbohydrate content (% of total weight)			
	Outer envelope extracted by ethanol	F ₄	F ₄ filtrate	F ₄ retentate
Arabinose	9.5 (28.4)	9.8 (27.9)	14.6 (27.4)	6.4 (28.4)
Rhamnose	8.1 (24.2)	8.2 (23.4)	10.0 (18.8)	6.7 (29.8)
Fucose	1.1 (3.3)	0.9 (2.6)	1.1 (2.1)	1.0 (4.4)
Xylose	3.7 (11.0)	2.5 (7.1)	3.2 (6.0)	1.8 (8.0)
Mannose	4.0 (12.0)	5.1 (14.5)	10.3 (19.4)	2.3 (10.2)
Galactose	3.0 (9.0)	4.9 (14.0)	8.8 (16.5)	2.4 (10.7)
Glucose	1.1 (3.3)	1.3 (3.7)	0.9 (1.7)	0.4 (1.8)
Glucuronic acid	1.1 (3.3)	1.0 (2.9)	2.0 (3.8)	0.4 (1.8)
Unknown 1 (3-O-methylmannose)	1.9 (5.7)	1.4 (4.0)	2.3 (4.3)	1.1 (4.9)
Total	33.5	35.1	53.2	22.5

The recovery, on a mass basis, of F₄ after ultrafiltration through a PM10 membrane was 70 to 86 %, of which 40 to 60 % was the filtrate and 26 to 31 % was the retentate.

DISCUSSION

In this investigation it was established that the precipitating antigen in the outer envelope extracted by 50 % (v/v) ethanol (Palit & Harrison, 1977; Palit *et al.*, 1974) is antigenically identical to the outer envelope preparation reported by Auran *et al.* (1972). The precipitating antigens in F₄ retentate and outer envelope extracted by 50 % (v/v) ethanol have antigenic similarities but were not identical. These findings are consistent with previous studies with serovar *hardjo* (Palit & Harrison, 1977), while Faine *et al.* (1974a) have suggested that F₄ is a constituent of the outer envelope.

It has been established that purified F₄ antigen when injected in soluble form into rabbits is not immunogenic but its intradermal injection in emulsified form with Freund's adjuvant elicits antibody production (Faine *et al.*, 1974a; Adler & Faine, 1978a).

Our preliminary analysis by high pressure liquid chromatography indicates that F₄ is a mixture of glycoproteins which, in the present work, have been fractionated by ultrafiltration on a PM10 membrane. The homogeneity of the fractionated material is currently under investigation, but the broad chemical and serological properties of the fractionated material have been elucidated in this work. One of the glycoproteins in F₄ (PM10 filtrate) has a molecular weight of less than 10000. This is supported by the fact that the filtrate behaved as a low molecular weight 'hapten' in all serological tests employed and failed to stimulate production of antibodies in rabbit immunization experiments. However, when the filtrate was conjugated to BSA, either by the carbohydrate (Fielder *et al.*, 1970) or protein (Singer & Schick, 1961) portion of the glycoprotein molecule, it produced specific antibodies in rabbits. The protein-protein conjugate produced with toluene-2,4-diisocyanate induced a better antibody production than the carbohydrate-protein conjugate formed with 2,4,6-trichloro-s-triazine; this could possibly be due to biological variation. On the other hand, the other glycoprotein in F₄ retentate appears to have a molecular weight in excess of 10000 and behaved as a complete antigen in all serological tests and immunization studies.

The anti-filtrate serum was highly specific for the filtrate as demonstrated by the serum absorption test. It also reacted with the retentate-sensitized red cells to a high titre in the

passive haemagglutination test. These findings suggest that the antigenic determinants in glycoprotein molecules of filtrate and retentate are the same. It is possible that these glycoproteins originated from a large molecule of glycoprotein cleaved during the alkali treatment (Lüderitz *et al.*, 1966).

The tannic acid-modified haemagglutination test with F₄ retentate showed lower titre and required more antigen (on a dry weight basis) as compared to the passive haemagglutination test, suggesting that the protein part (Neter, 1956) of the glycoprotein molecule sensitizes erythrocytes less efficiently than the carbohydrate part of the molecule.

Azuma *et al.* (1976) identified 3-*O*-methylmannose in the polysaccharide antigen of *Leptospira biflexa* (Urawa) and we found that one of the unidentified peaks in the *O*-trimethylsilyl methyl glycosides of outer envelope extracted by ethanol, F₄, filtrate and retentate co-eluted with the *O*-trimethylsilyl derivative of an authentic sample of 3-*O*-methylmannose.

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