

## The relationship between the serogroup antigen and lipopolysaccharide of *Legionella pneumophila*

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(Received 8 July 1985; accepted 30 August 1985)

### SUMMARY

Serogroup-specific antigen was extracted from a number of *Legionella pneumophila* strains and compared with phenol-water extracted lipopolysaccharide on the basis of gel filtration, chemical analysis, SDS-PAGE and reaction with serogroup-specific antibody in immunoblots. Serogroup specificity is apparently borne by the O side-chains of the lipopolysaccharide, which, although smooth in type, partitions in the phenol phase. For four serogroup 1 strains tested, there was no qualitative correlation between O side-chain length and pulmonary virulence for guinea-pigs.

### INTRODUCTION

The specificity of a major heat-stable, pronase-resistant antigen provides a means of serogrouping strains of *Legionella pneumophila*. Antibodies to the serogroup antigen are therefore of diagnostic importance (Harrison & Taylor, 1982; Stanek *et al.* 1983). However, the nature of the serogroup antigen is uncertain, although results from studies of its chemical composition (Johnson *et al.* 1979; Wong *et al.* 1979; Flesher *et al.* 1982), its role in endotoxicity (Wong *et al.* 1982) and its behaviour in crossed immunoelectrophoresis (Joly & Kenny, 1982; Collins *et al.* 1983), suggest that it is lipopolysaccharide (LPS).

Many of the specificities utilized in serotyping Gram-negative organisms are expressed on the O-specific polysaccharide of their LPS. This has been most studied in the salmonellae and *Escherichia coli*. The LPS from these organisms has been shown to display heterogeneity with respect to O-specific chain length (Palva & Makela, 1980; Goldman & Leive, 1980). In both cases there is also good correlation between possession of O side-chain and virulence. Other Gram-negative organisms, e.g. *Neisseria gonorrhoeae* (Connelly & Allen, 1983) and *Bordetella pertussis* (Peppler, 1984), have been shown to possess LPS with O-specific polysaccharide of only short chain length and with little heterogeneity.

In this study we have examined the serogroup antigens from one virulent serogroup 3 strain of *L. pneumophila* and from four strains of serogroup 1 which differ from each other in virulence as determined by a guinea-pig model of infection (Fitzgeorge *et al.* 1983; Fitzgeorge, 1985).

The purposes of this study were to determine the relationship between the serogroup antigen of *L. pneumophila* and its LPS, the extent of the heterogeneity

in the LPS O side-chain length and whether possession of O side-chain was a prerequisite for virulence.

#### MATERIALS AND METHODS

*L. pneumophila strains.* Serogroup 1 strains were Corby (a human isolate kindly provided by Dr R. A. Swann, John Radcliffe Hospital, Oxford.), Corby avirulent (prepared from the above strain by multiple passage on charcoal yeast extract agar), W74/81 (isolated in this laboratory from a naturally contaminated water supply) and Philadelphia 1 (NCTC 11192), also passaged repeatedly since its initial isolation. Serogroup 3 strain W166/81, isolated in this laboratory, was also from a water supply. Of the serogroup 1 strains, Corby is the most virulent for guinea-pigs by the aerosol route (Fitzgeorge, 1985), W74/81 is of intermediate virulence whilst NCTC 11192 and Corby avirulent fail to cause respiratory infection (Fitzgeorge *et al.* 1983; R. B. Fitzgeorge, personal communication). The Philadelphia 1 strain (NCTC 11192) was cultured once on charcoal yeast extract (CYE) agar (Edelstein, 1981) in this laboratory. The other strains, with the exception of Corby avirulent, had been subcultured only three times on CYE agar prior to use in this study.

*Growth conditions.* Starter cultures of 100 ml yeast extract broth (YEB) (Ristroph, Hedlund & Allen, 1980) in 500 ml conical flasks were inoculated with organisms grown on CYE agar and incubated aerobically at 37 °C for 24 h on an orbital shaker. Purity was checked by Gram's stain and by the ability to grow on CYE agar but not on blood agar. Starter cultures were used to inoculate fresh YEB (5 ml into 500 ml in 2 l flasks, 12 flasks per strain) which was incubated as above for 16 h. Organisms were harvested by centrifugation at 1500 g for 1 h and washed once in sterile water.

For each strain the cell pellet was divided into two parts (*ca.* 5 g wet weight each). Half was extracted for serogroup antigen and half for LPS.

*Preparation of serogroup antigen.* Cells were extracted in 1 ml 0.9% (w/v) saline per 100 mg wet weight, heated to 100 °C for 1 h with constant stirring, cooled, centrifuged at 17000 g for 15 min and the supernatant dialysed overnight against phosphate-buffered saline (8.0 g NaCl, 0.2 g KCl, 1.15 g Na<sub>2</sub>HPO<sub>4</sub>, 0.2 g KH<sub>2</sub>PO<sub>4</sub>/l). Extracts were treated sequentially for 2 h at 37 °C with ribonuclease 1, deoxyribonuclease 1 (in the presence of 0.1 M-MgCl<sub>2</sub>) and pronase (all enzymes from BDH) each at a final concentration of 0.01 mg/ml. At the end of this treatment residual enzyme activity was destroyed (100 °C for 5 min) and the extracts dialysed against Tris-HCl buffer (0.1 M, pH 8.2). Twenty-millilitre samples of each extract were gel filtered on a 2.6 × 90 cm column of Sepharose 4B at a flow rate of 10.0 ml/h. The eluate was monitored for absorbance at 280 nm and assayed for serogroup antigen by ELISA.

*Preparation of LPS.* Cells were extracted by the phenol-water procedure (Westphal & Jann, 1965). For each strain, pooled aqueous phases and the phenol phase were dialysed separately for 3 days against running tap water and then against PBS. Phenol phase preparations were subjected to the same enzyme treatments and gel filtration used for serogroup antigen.

*Chemical analyses.* 2-Keto-3-deoxyoctonate (KDO) was assayed by the method of Karkhanis *et al.* (1978), carbohydrate by the method of Dubois *et al.* (1956) and protein using the Lowry assay system.

*Antisera and conjugates.* Antisera to serogroup antigens 1 and 3 were obtained by immunizing rabbits with saline extracts of strains W74/81 and W166/81, respectively. After a preliminary blood sample had been taken each rabbit was injected with 300  $\mu$ l of antigen (15  $\mu$ g carbohydrate) in incomplete Freund's adjuvant (Difco) distributed between two intramuscular and two dorsal subcutaneous sites. A boost was given with the same dose after 2 weeks and blood was taken at weekly intervals for antibody assay. Sera were titrated by an indirect ELISA (see below). Two weeks after a further boost (about 12 weeks after starting the injections), the rabbits were exsanguinated under barbital anaesthesia. High-titre sera were pooled and the IgG fraction obtained by protein A affinity chromatography; IgG was conjugated to horseradish peroxidase (HRP) according to the methods of Nakane & Kawoosi (1974).

*ELISA for antibody.* Sera were titrated by an indirect ELISA in 96-well plates (Dynatech M 129A) at room temperature. Wells were coated overnight with homologous saline extract (10  $\mu$ g/ml carbohydrate) in 0.05 M carbonate buffer (pH 9.5), then washed. Wash solution was 0.1% Tween 20 in PBS, which was also used for serial dilution of the test sera in a second plate. Volumes of 100  $\mu$ l were transferred from each well to the corresponding well on the washed coated plate, which was incubated for 2 h with shaking. The plate was washed and sheep anti-rabbit HRP conjugate added (100  $\mu$ l/well at 10  $\mu$ g/ml) and incubation continued for 2 h. After washing again, substrate was added and colour read at 450 nm. Substrate was 5-aminosalicylic acid (5-ASA) in water (1 mg/ml) adjusted to pH 6.0, with H<sub>2</sub>O<sub>2</sub> added to 0.007%. Reaction with heterologous antigen was < 0.4%.

*SDS-PAGE.* Samples were run on slab gels using the Laemmli (1970) buffer system. The separating gel was 15% (w/v) acrylamide, 0.5% bis-acrylamide containing 4.0 M urea. Samples containing 0.5 mg/ml LPS were diluted 1:1 with double-strength sample buffer and 50  $\mu$ l applied to each track of the gel (total 12.5  $\mu$ g LPS/track). Gels were run at 125 V until the bromophenol blue tracking dye had migrated to within 1.0 cm from the end of the gel. The gel was stained for LPS using a silver stain (Tsai & Frasch, 1982). A Transblot apparatus (Biorad) was used for immunoblotting the gels, and blots were visualized (Irons, Ashworth & Wilton-Smith, 1983) using an HRP conjugate of antibody to serogroup antigen.

*ELISA for antigen.* The wells of 96-well plates (Nunc Immunoplate 1) were coated overnight with 100  $\mu$ l antibody to serogroup antigen at 10  $\mu$ g IgG/ml in carbonate buffer (pH 9.5), then washed as in the ELISA for antibody above. Serial twofold dilutions of test materials were made in a second plate using wash solution as diluent. Volumes of 100  $\mu$ l were transferred from each well to the corresponding well on the washed, coated plate, which was incubated for 2 h with shaking. The plate was washed, HRP conjugate of antibody to serogroup antigen added (100  $\mu$ l/well at 10  $\mu$ g IgG/ml), and incubation continued for 2 h. After washing again, substrate (5-ASA) was added and colour read at 450 nm. The activity of serogroup 1 antigen in the serogroup 3 ELISA was < 1%, and vice versa.

Table 1. Results of chemical analysis on purified saline extracted (SE) and phenol phase (P) antigen of *Legionella pneumophila*

Sample	Chemical analysis of Sepharose 4B column fractions*			
	Carbohydrate (% dry wt.)	Protein (% dry wt.)	KDO (% dry wt.)	Carbohydrate:KDO
Corby (SE) pool A	5.5	16.0	1.15	4.8:1
Corby (SE) pool B	6.7	7.6	1.65	4.1:1
Corby AV† (SE) pool A	4.0	16.2	0.80	5.0:1
Corby AV (SE) pool B	4.6	7.4	1.25	3.7:1
NCTC 11192 (SE) pool A	2.5	10.0	0.25	10.0:1
NCTC 11192 (SE) pool B	6.2	9.5	1.20	5.2:1
W74/81 (SE) pool A+B	5.2	12.0	1.40	3.7:1
W166/81 (SE) pool A+B	8.5	12.0	1.55	5.5:1
Corby (P) pool A	7.2	6.2	1.90	3.8:1
Corby AV (P) pool A	6.4	6.0	1.85	3.5:1
<i>S. typhimurium</i> LPS (Sigma)	53.5	6.2	7.15	7.5:1

\* See fraction 1 for fraction description.

† AV, Avirulent.

## RESULTS

Corby-strain saline extract eluted from a Sepharose 4B column as two peaks of 280 nm absorbance and two ELISA peaks of serogroup 1 antigen (Fig. 1a). The elution profiles were similar for strains other than Corby. The peaks monitored by the two techniques (Fig. 1a) were not fully coincident, indicating that much of the protein present after heating and pronase treatments was a contaminant of the serogroup antigen. The asymmetric first antigen peak (approximately 85% of eluted antigen) is broad, suggesting either heterogeneity of a high-molecular-weight material or considerable aggregation. The second ELISA peak might represent unaggregated antigen. The elution profile of phenol phase LPS from the same column is shown in Fig. 1b. In this case both the peak of 280 nm absorbance and the ELISA peak coincide; there is no second peak.

Fractions were pooled (A, B, C) as indicated (Fig. 1a), lyophilized and assayed for carbohydrate, protein and 2-keto-3-deoxyoctonate (KDO). The data from these analyses are presented in Table 1. The first ELISA peak maximum (pools B, Fig. 1a) contained 4.6–6.7% (w/w) carbohydrate, 1.2–1.65% (w/w) KDO and 7.6–9.5% (w/w) protein. Whilst the first peak of 280 nm absorbance (pools A, Fig. 1a) contained consistently higher amounts of protein, 10–16% (w/w), the ratios of carbohydrate to KDO in pools A and B were similar to each other and to phenol phase antigen. The second antigen peak (pool C) contained insufficient material to allow detection of carbohydrate or KDO.

In contrast, aqueous phases contained little or no antigen detectable by ELISA. They might have contained lipid-A core material as KDO was present in significant amounts (data not shown).

For saline extracts and for phenol-phase LPS, silver staining of SDS-PAGE gels (Fig. 2) revealed ladder-like patterns typical of smooth LPS (Tsai & Frasch, 1982), although over a slightly more restricted range compared to the LPS from

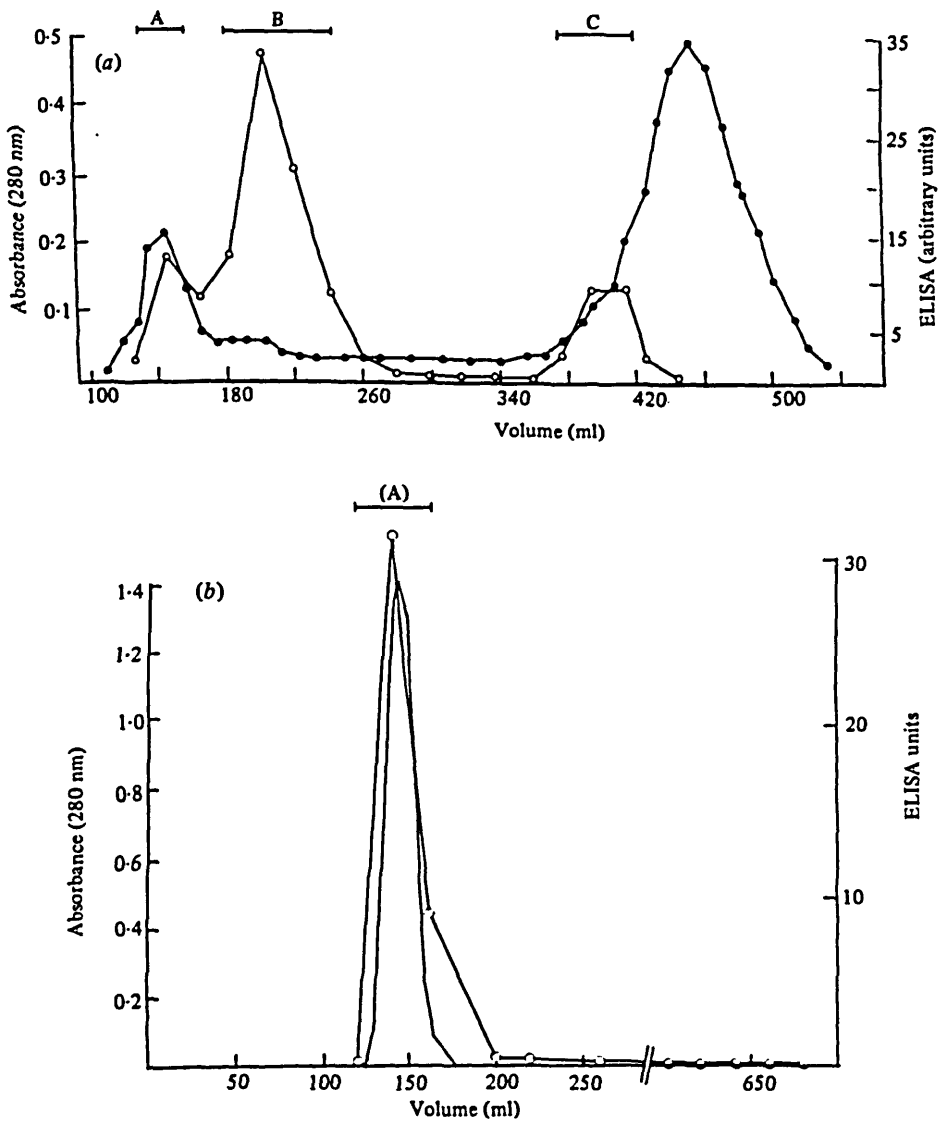


Fig. 1. (a) Elution profile of strain Corby saline extract from a Sepharose 4B column. ●, Absorbance at 280 nm; ○, serogroup antigen by ELISA (arbitrary units). (b) Elution profile of strain Corby phenol phase LPS: continuous line, absorbance at 280 nm; ○, ELISA.

*Salmonella typhimurium*. There was a marked difference in the band spacing between the latter and the *L. pneumophila* patterns.

Saline extracts of four serogroup 1 strains (e.g. Fig. 2, lane 3) and of a serogroup 3 strain (lane 2) resolved into at least 35 bands including the two more intense staining bands (B). Phenol phase LPS (Fig. 2, lanes 6 and 7) lacked the bands but otherwise resolved similarly to saline extracts. The saline extracts and phenol phase LPS from both virulent and avirulent *L. pneumophila* gave identical



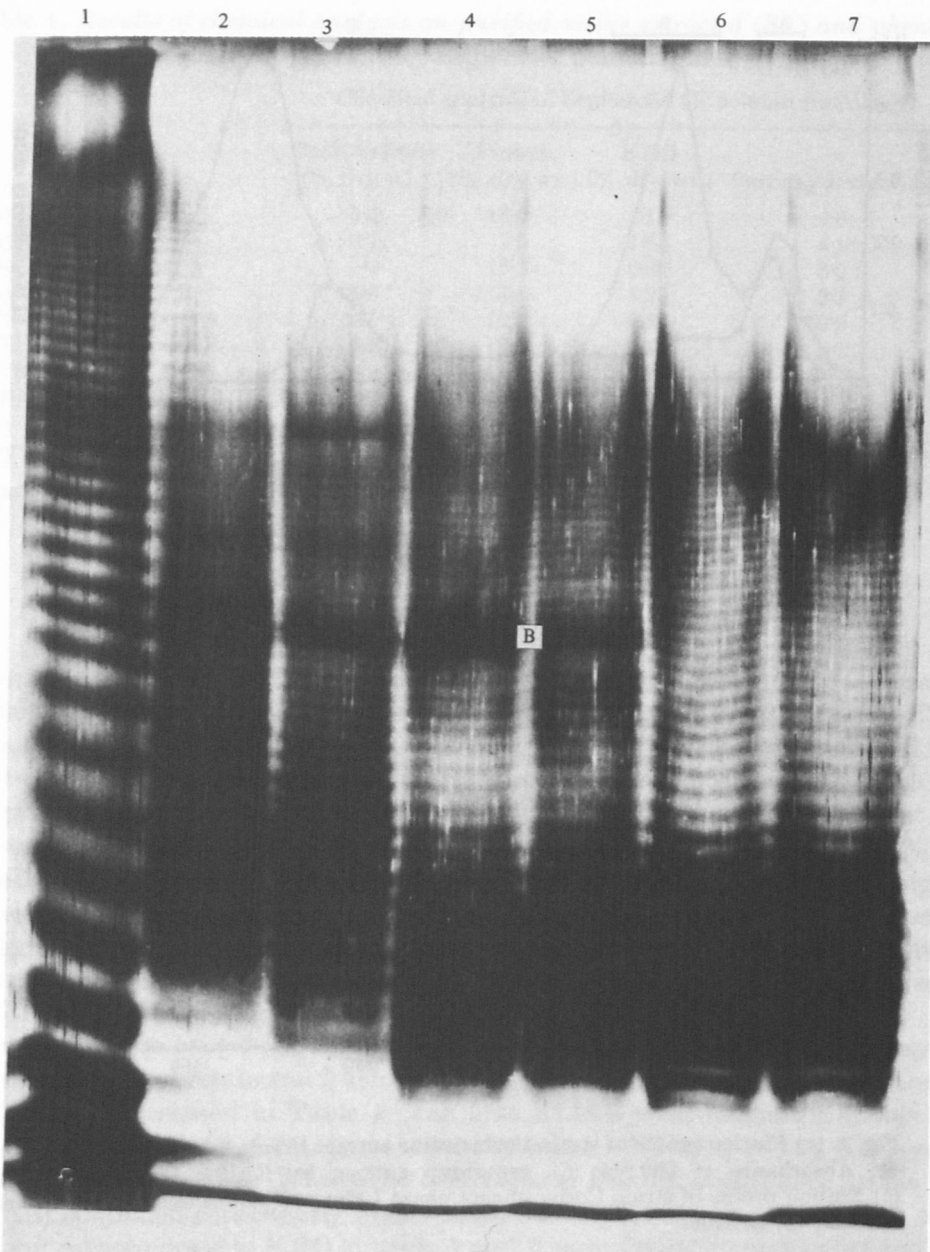
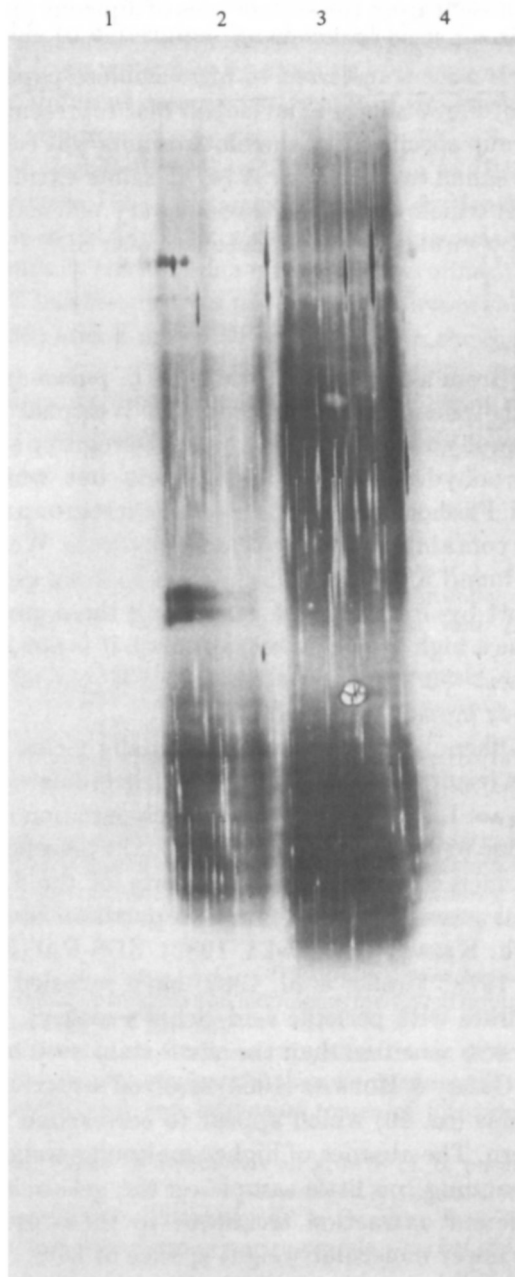


Fig. 2. SDS-PAGE analysis of serogroup antigen. Lanes were loaded with 12.5  $\mu\text{g}$  LPS. Lane 1, *S. typhimurium* LPS (Sigma); lane 2, W166/81 (SE); lane 3 W74/81 (SE); lane 4, Corby (SE); lane 5 NCTC 11192 (SE); lane 6, Corby (P); lane 7, Corby AV (P). SE, Saline extract; P, phenol phase; AV, avirulent.



**Fig. 3.** Immunoblot analysis of serogroup antigen. Lane 1, W166/81 (SE); lane 2, W74/81 (SE); lane 3, Corby (P); lane 4, W74/81 aqueous phase.

patterns which were absent from the stained gels of aqueous phase material (result not shown).

Samples run on gels were transferred to nitrocellulose paper and visualized as described in methods; Fig. 3 shows a developed blot representative of the results obtained. The serogroup specificity of the blot staining was confirmed by the lack of reaction of W166 saline extract. The W74/81 saline extract immunoblot had two major bands (M) which appear to transfer very efficiently and which were absent from the Corby virulent phenol phase (see also SDS-PAGE above).

#### DISCUSSION

Serogroup antigen from a number of strains of *L. pneumophila* was extracted with saline and by the phenol/water procedure of Westphal & Jann (1965). Gel exclusion chromatography on these extracts yielded serogroup antigen preparations which contained carbohydrate, KDO and protein but which otherwise were chemically undefined. Flesher *et al.* (1982) reported a serogroup antigen preparation from *L. pneumophila* containing 10% (w/w) carbohydrate. Wong *et al.* (1982) gave a figure of 13% and found KDO associated with serogroup antigen, in agreement with a previous report by Johnson *et al.* (1979). All these groups found that the serogroup antigen has a high lipid content, although it is not known whether this could account for the > 80% (saline extract pool B, phenol phase pool A) still uncharacterized in our preparations.

Application of the phenol/water procedure normally yields LPS in the aqueous phase. However, the results of SDS-PAGE analysis indicate that the serogroup antigen preparations are LPS of smooth type which partition in the phenol phase. This could either be due to a high lipid content or to the presence of dideoxy-sugars in the O side-chain increasing the hydrophobicity of the LPS. The legionella serogroup antigen has previously been found to partition in this manner (Wong *et al.* 1982; Schramek, Kazar & Bazovska, 1982). SDS-PAGE analyses by other groups (Wong *et al.* 1979; Flesher *et al.* 1982) have revealed only a single band staining as carbohydrate with periodic acid-Schiff's reagent. This procedure has been shown to be far less sensitive than the silver stain used here (Tsai & Frasch, 1982). More recently Gabay & Horwitz (1985) resolved serogroup 1 *L. pneumophila* LPS into several bands (*ca.* 10) which appear to correspond to the lower region of our banding pattern. The absence of higher-molecular-weight bands may have been due either to running too little sample on the gels or to the use by these researchers of a different extraction technique to those used here, one which possibly selects only lower-molecular-weight species of LPS.

The band spacing in *L. pneumophila* LPS (Fig. 2) differs markedly from that of *S. typhimurium*. This may be due to the LPS of *L. pneumophila* having a much higher lipid-A content than that of *S. typhimurium* so that addition of O side-chain makes a proportionately smaller difference to the molecular weight. Alternatively the difference in band spacing could be due to a smaller O side-chain repeat unit in *L. pneumophila*.

The staining of the immunoblots demonstrates that the serogroup specificity of *L. pneumophila* is in fact the O specificity of its LPS. The absence of stained bands of high molecular weight from the blots may be due either to inefficient transfer



or to a lack of sensitivity because of low antigen concentration in this region. It should now be possible to determine the chemical basis for the specificity of the serogroup antigens of *L. pneumophila* by analysis of the sugar compositions of the LPS O side-chains of different serogroups. Study of the exceptionally large lipid moiety of the LPS is also needed.

For the salmonellae and *Escherichia coli*, possession of O side-chain correlates with virulence (Roantree, 1967; Medearis, Camitta & Heath, 1968). From the similarity of SDS-PAGE findings for virulent and avirulent strains of the same serogroup, it seems unlikely that LPS is a virulence determinant for *L. pneumophila*. Nevertheless the LPS has been shown to elicit an unfavourable immune response (Baskerville *et al.* 1983) and it may also act directly in the disease process.

This work was supported by an M.R.C. project grant. We thank Drs A. Baskerville, R. B. Fitzgeorge and A. Robinson for helpful advice and discussion.

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