Vaccination of mice with lipopolysaccharide (LPS) and LPS-derived immuno-conjugates from *Leptospira interrogans*

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Summary. Mice were vaccinated with lipopolysaccharide (LPS) from *Leptospira interrogans* serovar *pomona* or *hardjo*, or with the polysaccharide (PS) fraction of the LPS, or with an immunoconjugate of PS and diphtheria toxoid (DT). Maximum agglutinin titres were found 6–10 weeks after vaccination with LPS or PS-DT conjugate; the latter elicited antibody titres at least 10 times higher than those produced in response to LPS. Animals failed to react significantly to PS. Titres elicited by antigens of serovar *pomona* were higher than those elicited by serovar *hardjo*.

Introduction

Leptospirosis is an acute febrile illness caused by serovars of *Leptospira interrogans*. The two most commonly associated with disease in Australia and New Zealand are serovars *pomona* and *hardjo*.^{1,2} Immunity to infection by *L. interrogans* depends on the production of serum agglutinins^{3,4} against leptospiral antigens. Many antigen preparations have been studied,⁵ the most significant being lipopolysaccharide (LPS). Leptospiral LPS has chemical and biological properties different from those of the classical LPS of gram-negative organisms such as *Escherichia coli*,^{6–8} and appears to be neither toxic nor pyrogenic. Monoclonal antibody (MAb) against a polysaccharide epitope of leptospiral LPS protected guinea-pigs passively against leptospirosis,⁹ and MAbs against LPS determinants are opsonic.¹⁰ Furthermore, leptospiral LPS is a major antigen recognised by human antibodies produced in response to infection with *L. interrogans*.¹¹ Immunisation with leptospiral LPS or the polysaccharide (PS) fraction of LPS from serovar *copenhageni* protected hamsters from lethal challenge with virulent organisms of the same serovar.¹² This suggests that LPS antigens could be used as immunising agents against leptospirosis. The LPS and PS of leptospires appear to be T-cell-independent antigens which elicit mainly IgM responses.^{12,13} The covalent binding of carbohy-

drate antigens to antigenic proteins, first described in 1929 by Avery and Goebel,¹⁴ confers T-cell-dependent properties on several polysaccharide antigens,^{15–18} offering an attractive method for increasing the immunogenicity of such antigens and for stimulating a longer-lived IgG response. The aim of this study was to investigate the serological responses of mice to LPS and PS antigen preparations of serovars *pomona* and *hardjo* and to prepare and assess the potential of PS-protein conjugate preparations as immunising agents against leptospirosis.

Materials and methods

Bacteria, antigens and serological methods

Leptospira interrogans serovar *pomona* strain L10 was isolated from a human case of leptospirosis.¹⁹ Serovar *hardjo* strain L13 was the Hardjoprajitno reference strain²⁰ obtained in 1979 from the National Health Institute, Wellington, New Zealand. Cultivation of leptospires and the microscopic agglutination test (MAT) were performed as described previously.²¹ Briefly, serial two-fold dilutions of sera were incubated with viable leptospires at 28°C for 1 h and agglutination determined by examining a loopful on a glass slide by dark-field microscopy. LPS and PS were prepared from both serovars as described previously.⁹ Antiserum against whole leptospires was produced by giving mice 2×10^8 live leptospires by intraperitoneal injection on two occasions with a 2-week interval; mice were bled 4 weeks after the second injection. MAb that was used has been described previously.²²

Preparation of polysaccharide and diphtheria toxoid (PS-DT) conjugates

This was based on the method of Schneerson *et al.*²³ The PS fraction of the LPS of serovar *pomona* or serovar *hardjo* was dissolved in 0.2 M NaHCO₃ (pH 9.5) to give a 1 mg/ml solution which was stirred over ice while cyanogen bromide 1 mg/mg of polysaccharide was added. After 6 min, adipic acid dihydrazide (Sigma) dissolved in 0.5 M NaHCO₃ was added to a final concentration of 0.3 M. The pH was adjusted to 8.5 with 0.2 M HCl and the reaction mixture was stirred overnight at 4°C. The solution was dialysed against phosphate-buffered saline, pH 7.2 (PBS) for 72 h at 4°C, and lyophilised; the carbohydrate content was assayed by the phenol and sulphuric acid method of Dubois *et al.*²⁴ This activated polysaccharide was stored at -20°C.

Diphtheria toxoid (DT) (Commonwealth Serum Laboratories, Melbourne, Australia) 100 Lf/ml was purified by passing 5-ml samples through a 70-cm column of Sephacryl S-300 Superfine (Pharmacia, LKB Biotechnology AB, Uppsala, Sweden) equilibrated with PBS. Fractions containing the 70-Kda toxoid peak²⁵ were pooled, concentrated with an Amicon PM-10 ultrafiltration membrane (Amicon Div., WR Grace & Co, Danvers, MA, USA) and chromatographed again on Sephacryl S-300. Fractions were pooled and concentrated again and the protein concentration was measured by the micro-method of Bradford.²⁶ Purified toxoid was then passed through a sterile 0.45-µm filter and stored at 4°C.

The reaction mixture containing c. 0.1 mg of carbohydrate and 0.1 mg of protein per ml and 0.1 M 1-ethyl-3-dimethylaminopropyl carbodiimide (Sigma) was stirred at a constant pH 5.0 over ice for 3 h, and then dialysed against PBS overnight and passed through a column of Sephacryl S-300. The first peak, containing both protein and carbohydrate was concentrated by Amicon PM10 ultrafiltration and filtered through a sterile 0.45-µm filter. The carbohydrate and protein content of the conjugated polysaccharide was assayed and the conjugate was stored at 4°C.

Electrophoresis, transblotting and staining

Purified DT (100 µg of protein/lane for Coomassie Blue staining or 25 µg of protein/lane for Western blotting), PS-DT conjugate (15 µg or 6 µg of carbohydrate/lane) and PS (25.5 µg or 64 µg of carbohydrate/lane) were subjected to electrophoresis on 1.0-mm thick, SDS-polyacrylamide 10% gels (SDS-PAGE) as described by Laemmli²⁷ in a Protean I gel apparatus (Bio-Rad, Richmond, CA, USA). Gels were silver-stained for LPS by the method of Hitchcock and Brown,²⁸ or stained with Coomassie Blue 0.25%, or the resolved proteins and carbohydrates were transblotted²⁹ on to nitrocellulose (Schleicher and Schuell, 0.45 µm) in a Bio-Rad transblot cell and immunostained with anti-LPS MAb, or with mouse antisera against whole leptospires, peroxidase-conjugated rabbit anti-mouse serum (Silenus Laboratories, Melbourne, Australia) and 4-chloro-naphthol.³⁰

Vaccination of mice

Groups of inbred BALB/c mice (Monash University Animal House, Melbourne, Australia) aged 6-8 weeks were given LPS, PS or PS-DT conjugate by subcutaneous injection in 0.1 ml of PBS on days 0 and 14 and were bled at intervals from the orbital plexus.

Results

Antigenicity of LPS

In mice vaccinated with LPS, the highest MAT titres were reached after c. 10 weeks. All mice receiving 25 µg of serovar *pomona* LPS produced detectable antibody by week 10 and this persisted until the end of the study at week 42 (table I). Mice receiving 5-10 µg of LPS did not all react; those receiving 2.5 µg or less produced low or undetectable levels of agglutinin.

Mice vaccinated with similar doses of serovar *hardjo* LPS also showed peak MAT titres after c. 10 weeks (table II). However, no group showed lasting 100% sero-conversion, and the average titres were significantly lower than those elicited by *pomona* LPS. The differences between groups receiving

Table I. Serum agglutinin titres of groups of six mice vaccinated at 0 and 2 weeks with LPS of serovar *pomona* strain L10

Weeks after vaccination	Geometric mean titre (range) of agglutinin after vaccination with LPS doses (µg) of						
	0.25	0.5	1.0	2.5	5.0	10.0	25.0
2	1.2 (0-2)	0	0	0	0	0	0
3	0	0	0	0	1.3 (0-2)	1.3 (0-2)	1.6 (0-2)
4	0	0	0	0	1.8 (0-4)	4.0 (0-32)	2.6 (0-32)
5	0	0	0	0	5.3 (0-32)	5.7 (0-64)	4.0 (0-32)
6	0	0	0	0	3.4 (0-16)	5.0 (0-32)	5.7 (0-32)
10	0	0	0	0	13 (0-128)	4.8 (0-32)	42 (4-256)
16	1.4 (0-2)	0	0	0	7.1 (0-64)	6.1 (0-128)	23 (2-128)
24	0	0	0	0	4.5 (0-16)	3.0 (0-64)	14 (2-128)
35	1.6 (0-4)	0	0	2.6 (0-32)	11 (0-256)	5.7 (0-128)	26 (2-256)
42	0	0	2.0 (0-4)	1.6 (0-4)	7.1 (0-256)	6.4 (2-32)	14 (2-128)

0, titre <2.

Table II. Serum agglutinin titres of groups of six mice vaccinated at 0 and 2 weeks with LPS of serovar *hardjo* strain L13

Weeks after vaccination	Geometric mean titre (range)* of agglutinin after vaccination with LPS doses (μ g) of							
	0.25	0.5	1.0	2.5	5.0	10.0	25.0	
2	0	0	0	2	2	2	4	
3	0	1.3	0	2.8	1.5	1.2	0	
		(0-2)		(0-8)	(0-8)	(0-2)		
4	1.3	0	0	1.5	1.1	0	0	
	(0-2)			(0-4)	(0-2)			
5	1.2	1.3	1.4	1.7	0	1.2	0	
	(0-2)	(0-2)	(0-4)	(0-16)	(0-2)			
6	2.3	2.4	2.2	2.5	2.2	1.6	1.8	
	(2-4)	(2-4)	(0-4)	(0-4)	(0-8)	(0-8)	(0-16)	
10	0	0	1.3	2.0	2.6	2.2	3.2	
			(0-2)		(2-4)	(2-4)	(2-8)	
14	0	0	1.2	1.4	0	0	1.3	
			(0-2)	(0-4)			(0-4)	
18	1.2	1.3	1.3	1.7	3.2	2.3	3.2	
	(0-2)	(0-2)	(0-2)	(0-4)	(0-8)	(0-4)	(2-8)	
31	0	1.6	1.3	1.5	2.0	2.0	2.5	
		(0-2)	(0-2)	(0-2)		(0-4)	(0-8)	
39	ND	ND	ND	1.6	1.2	1.2	1.8	
				(0-2)	(0-2)	(0-2)	(0-2)	

* Where no range is given, all mice had the same titre. 0, titre < 2. ND, not done.

different doses of *hardjo* LPS were not as marked as those with *pomona* LPS.

Mice vaccinated with a mixture containing equal amounts of LPS from serovars *pomona* and *hardjo* reacted to each component similarly to those receiving the individual components. The response to *pomona* LPS was considerably stronger (mean titre 20.2 at week 6) than to *hardjo* LPS (mean titre 1.4). There was neither a synergic nor an antagonistic effect.

Antigenicity of PS

Mice were inoculated with doses of PS from 0.5 μ g to 100 μ g. The minimum antigenic dose of PS from serovar *pomona* was 50 μ g, which produced a seroconversion rate of 20% with a mean titre of 1.1 at week 10. Mice inoculated with PS from serovar *hardjo* did not produce detectable antibody.

Examination of PS-DT conjugate

The carbohydrate and protein content of the conjugated polysaccharides were 240 μ g/ml and 262 μ g/ml respectively for serovar *pomona* and

240 μ g/ml and 228 μ g/ml respectively for serovar *hardjo*. PS alone contained less than 5% protein. The PS-DT conjugates were examined by PAGE and Western blotting (serovar *pomona* shown in the figure). The PS-DT conjugate was seen as a single discrete band with an apparent mol. wt of 67 Kda detected by Coomassie Blue (lane B) and as an unresolved high mol. wt smear when Western blotted with a MAb against serovar *pomona* LPS (lane E). The conjugate was distinct from the purified DT (lanes A and F) and from the PS (lanes C and D). The DT appeared as an unresolved band of 45-66 Kda when stained with Coomassie Blue (lane A) and could not be detected at all when Western blotted with a MAb against a PS epitope of *pomona* LPS (lane F). In contrast *pomona* PS did not stain with Coomassie Blue (lane C) but appeared as a single arched band of 30 Kda when Western blotted with the MAb (lane D).

Figure. (a) SDS-PAGE stained with Coomassie Blue; (b) Western blot immunostained with an anti-LPS mouse MAb. Lanes A and F: purified DT; B and E: *pomona* PS-DT conjugate; C and D: *pomona* PS.

Vaccination with PS-DT conjugate

Mice were inoculated with PS-DT containing 2.4, 12.0 or 48 µg of carbohydrate from serovar *pomona* (table III). All mice seroconverted by week 4 and the mean MAT titres at week 10 were 15–100 times those elicited by the equivalent amount of LPS.

Therefore, mice were inoculated with serovar *hardjo* PS-DT conjugate containing 2.4 µg of carbohydrate-equivalent alone (table III). All mice seroconverted, producing at week 10 a mean MAT titre of 128, 40 times the maximum mean titre elicited by LPS.

Discussion

Previous studies^{9,10} showed that antibodies produced against LPS determinants of *Leptospira* are protective and that the LPS and PS of some serovars are highly antigenic and protective.¹² The range of doses tested in this study was equivalent to c. (2.5×10^7) – (2.5×10^9) organisms or 0.25–25 µg of LPS.⁶ However not all animals produced agglutinin. The LPS of serovar *pomona* stimulated a dose-dependent antibody response in most of the animals, although only amongst those receiving 25 µg of LPS did all the animals seroconvert. These titres declined only slowly during the following 30 weeks, indicating a lasting immune response. In contrast, the LPS of serovar *hardjo* produced low, apparently dose-independent titres. The initial high response with rapid fall-off was probably due to a

short-lived IgM response. Similar differential responses to *pomona* and *hardjo* components of whole-cell vaccines have been reported previously^{31,32} (see below). Titres achieved with a bivalent preparation containing LPS from both serovars were similar to those from each serovar alone and indicated neither synergic nor antagonistic effects.

Neither the *pomona* nor *hardjo* PS induced significant levels of agglutinin. These findings contrast with previous results with serovar *copenhageni*¹² in which the PS, although less immunogenic than LPS, could still elicit significant levels of agglutinin and protect from infection. These differences are probably due to intrinsic differences in the chemical composition and structure of the LPS,^{6,33} reflecting significant biological differences between the serovars.

In an effort to improve antigenicity, the PS of the two serovars was linked to DT to produce immunoconjugate vaccines. By gel electrophoresis and Western blotting, the PS-DT conjugate appeared quite distinct from both the purified DT and PS. Although the apparent mol. wt of the PS-DT conjugate was 67 Kda (lane B), somewhat higher than that of the purified DT (lane A),²⁵ the apparent mol. wt of the PS (30 Kda) is only an estimate, as charge differences and branching of the polysaccharide chain can lead to a higher apparent mol. wt. Thus the PS-DT conjugate seen in the Coomassie-stained gel probably has a low number of low mol. wt PS sub-units attached while the more sensitive Western blot shows PS-DT conjugate with a range of numbers of PS sub-units attached. Serovar *pomona* PS-DT conjugate containing 2.4 µg of carbohydrate-equivalent had approximately the same amount of carbohydrate as 5 µg of LPS but the PS-DT conjugate stimulated antibody titres which were 15–100 times higher than those elicited by the equivalent amount of LPS. The *hardjo* conjugate had the same amount of carbohydrate as the *pomona* conjugate, 2.4 µg of carbohydrate-equivalent being approximately equal to 6.7 µg of *hardjo* LPS. The difference was due to the composition of the LPS; *pomona* has a higher carbohydrate content than *hardjo* (data not shown). This PS-DT conjugate also stimulated 40 times the antibody titres produced by the LPS. Conjugation to antigenic carrier proteins has been used to confer both greater immunogenicity and T-cell-dependent properties on carbohydrate antigens from various different bacteria^{15–18} and we have shown this approach to be feasible for *Leptospira*.

As previously noted, the response of mice to *pomona* PS-DT conjugate was much higher than the response to *hardjo* conjugate, despite the use of

Table III. Serum agglutinin titres of groups of six mice vaccinated at 0 and 2 weeks with PS-DT conjugate from serovar *pomona* strain L10 or serovar *hardjo* strain L13

Weeks after vaccination	Geometric mean titre (range) of agglutinin after vaccination with conjugate in carbohydrate-doses (µg) of			
	serovar <i>pomona</i>			serovar <i>hardjo</i>
	2.4	12.0	48.0	2.4
2	0	0	0	1.6 (0–4)
4	108 (32–512)	446 (256–1024)	724 (256–2048)	10 (4–16)
6	57 (4–512)	338 (8–2048)	1218 (512–2048)	40 (32–64)
10	338 (128–1024)	1024 (512–4096)	676 (256–2048)	128 (32–512)

0, titre < 2.

equivalent amounts of LPS or equal amounts of carbohydrate as PS linked to DT. Future detailed structural and chemical analysis of reactive epitopes of PS may provide a better understanding of this difference in response.

Experimental infection has been prevented in animals with agglutinin titres as low as 2^{21} ,³⁴ which suggests that the LPS of serovar *pomona* could possibly be used on its own as a protective vaccine; but this is not the case for serovar *hardjo*. Although leptospiral LPS does not possess endo-

toxic properties,³⁵ potential adverse effects which may preclude its use as a vaccine have been reported.⁸ These effects might be obviated by the use of PS alone, and we have found that the poor immunogenicity of PS can be overcome by conjugation to DT. Further investigations into the side-effects of these vaccines and their ability to protect against disease are thus warranted.

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