Purification and characterization of a protein antigen from *Leptospira interrogans* serovar *hardjo*, common to a wide range of bacteria

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A protein with a molecular mass of 64 kDa (P64) from *Leptospira interrogans* serovar *hardjo* was partially purified by using successively, phase partitioning with Triton X-114, ion-exchange chromatography and sucrose gradient centrifugation. Purification to homogeneity was obtained by electroelution of P64 from SDS-polyacrylamide gels. Monospecific rabbit antiserum (R α P64) was prepared using the purified protein preparation. P64 had a native molecular mass of > 670 kDa and was recognized by R α P64 as well as by human antisera. Western blotting of leptospiral serovars and 18 other bacterial species with R α P64 showed that P64 was cross-reactive with an equivalent antigen in a wide range of bacteria, indicating that it belongs to a family of antigens previously designated 'common antigen'. This putative common antigen from *Leptospira* appears to have a sub-surface location, but its function is not yet known.

Introduction

Leptospirosis is an acute febrile disease caused by members of the species *Leptospira interrogans*. In Australia and New Zealand, infections are most frequently caused by serovars *hardjo* and *pomona* acquired mainly from cattle and pigs, respectively. Information about the nature and identity of antigens recognized by antibodies produced during naturally acquired leptospirosis is important to elucidate those antigens with significance for pathogenesis, immunity or diagnosis. Immunity to leptospirosis is humorally mediated and antibodies involved in opsonization and agglutination of leptospires are of prime importance (McGrath *et al.*, 1984; Vinh *et al.*, 1982). To date, lipopolysaccharide (LPS) is the only agglutinating and opsonic leptospiral antigen to be identified (Adler & Faine, 1983; Farrelly *et al.*, 1987; Jost *et al.*, 1986) and recent studies indicate that immunization with LPS can confer protection from infection (Jost *et al.*, 1989).

The significance of protein antigens however is not clear. Nunes-Edwards *et al.* (1985) studied the protein antigens of serovar *hardjo* using radioimmuno-precipitation with immune rabbit sera. They identified a number of proteins with molecular masses between 30 and 67 kDa, most of which were present in the outer envelope and apparently exposed on the leptospiral cell

surface. However, nothing is known of the identity of these proteins nor of their roles in pathogenesis and immunity to infection. In other studies, protein antigens of 33, 34 and 37 kDa, recognized by human sera, were identified as flagellar components (Kelson *et al.*, 1988; Chapman *et al.*, 1988). Jost *et al.* (1988) identified a 35 kDa species-specific protein antigen in the outer envelope of the leptospire which is also recognized by human antisera. These antigens are neither opsonic nor do they elicit the production of agglutinating antibodies. Studies of a leptospiral genus-specific antigen, partially purified from serovar *kremastos*, was identified as a 62 kDa non-agglutinating sub-surface protein (Sakamoto *et al.*, 1985a). Whilst the significance of this protein in the leptospire is unknown it has been proposed as a useful screening antigen for the diagnosis of human leptospirosis (Sakamoto *et al.*, 1985b).

In recent years much attention has been paid to a family of conserved bacterial proteins referred to as common antigen(s). This antigen was originally identified as an immunoreactive protein with a molecular mass of 62 kDa in *Pseudomonas aeruginosa* (Hoiby, 1975; Sompolinsky *et al.*, 1980) and *Escherichia coli* (Kaijser, 1975). Since those observations, common antigen has been demonstrated in more than 60 different bacteria including Gram-negative and Gram-positive bacteria, spirochaetes and archaeobacteria (Hansen *et al.*, 1988). These proteins, which display common structural and

Abbreviation: LPS, lipopolysaccharide.

immunological features, are major immunogens and antigens during infections with corresponding bacteria.

A study of the human antibody response to infection with *L. interrogans* serovar *hardjo* by Chapman *et al.* (1988) identified a 64 kDa protein antigen (P64) recognized exclusively by IgG class antibodies. Antibodies against this protein were also produced by some humans following vaccination with a formalin-killed bivalent vaccine consisting of serovars *hardjo* and *pomona* grown in protein-free medium (A. J. Chapman, personal communication). This paper reports the purification of P64 and its characterization as the putative common antigen of *Leptospira*. As the mycobacterial common antigen has been identified as a heat-shock protein as well as being implicated in the pathogenesis of experimental autoimmune adjuvant arthritis (Van Eden *et al.*, 1988), knowledge of a leptospiral common antigen may have significance in the further understanding of leptospiral pathogenicity.

Methods

Bacterial strains. *Leptospira interrogans* serovar *hardjo* strain 171 was a bovine isolate obtained from R. B. Marshall, Massey University, New Zealand. All other leptospiral strains used were provided by the WHO Leptospira Reference Laboratory, Brisbane, Australia. Leptospires were grown in Tween 80/BSA medium with added pyruvate (Johnson *et al.*, 1973) and counted in a Petroff-Hauser bacterial counting chamber. *Treponema pallidum* (Nichols) sonicate was provided by D. Dickeson, Westmead Hospital, Sydney, Australia. *Borrelia burgdorferi* sonicate was provided by R. Munro, Liverpool Hospital, Sydney, Australia. *Mycobacterium bovis* BCG was provided by A. Basten, University of Sydney, Australia. Other Gram-negative and Gram-positive bacteria were obtained from the culture collection of the Department of Microbiology, Monash University, Melbourne, Australia.

Purification of P64. All purification procedures were done at 4 °C unless otherwise stated. Leptospires from 1 litre of an exponential phase culture were pelleted by centrifugation at 16300 g, washed three times with 50 mM-Tris/HCl, pH 7.4, containing 0.1 M-NaCl (buffer A), and disrupted by sonication (Adler *et al.*, 1980). Triton X-114 was added to a final concentration of 2% (v/v) and incubated overnight. Insoluble material was removed by centrifugation at 12100 g for 30 min, the supernatant was collected and subjected to phase partitioning (Radolf *et al.*, 1988). The aqueous phase was retained and concentrated in an Amicon stirred cell concentrator using an XM50 membrane prior to loading onto a DEAE-Sepharose CL-6B column (1.5 × 30 cm) (Pharmacia) equilibrated with buffer A. The column was washed with buffer A and 2 ml fractions collected and tested for P64 by SDS-PAGE and Western blotting with human serum. Fractions containing P64 were pooled and concentrated as described earlier, before loading onto a 12 ml 10–40% (w/v) sucrose/buffer A gradient and centrifuged at 284100 g in an SW 40 rotor for 40 h in a Beckman L2-65B ultracentrifuge. Sucrose fractions containing P64, determined by SDS-PAGE and Western blotting, were pooled and dialysed against buffer A and stored at 4 °C.

Antisera and serological methods. Antiserum was produced against SDS-PAGE-separated P64 by the method of Boulard & Lecroisey

(1987). Coomassie-blue-stained P64 was excised from the gel, macerated and emulsified with incomplete Freund's adjuvant (1:1.5, v/v). Female New Zealand White rabbits were given one intramuscular injection of this material containing approximately 20 µg protein. Rabbits were injected 3 weeks later, intradermally in 10 sites, with 20 µg P64 electro-eluted from SDS-polyacrylamide gels by the method of Jacobs & Clad (1986) and emulsified in adjuvant as before. Human sera from Australian patients reacting with P64 were selected from specimens submitted to this laboratory for leptospirosis diagnostic serology. Human anti-*T. pallidum* serum was provided by D. Dickeson, and rabbit anti-*B. burgdorferi* serum was provided by R. Munro. The anti-serovar *hardjo* LPS monoclonal antibody was described previously (Farrelly *et al.*, 1987) as were the methods for the microscopic agglutination test (Adler & Faine, 1978). Opsonization of leptospores for phagocytosis by mouse peritoneal macrophages and visualization by indirect immunofluorescence staining were done as described previously (Farrelly *et al.*, 1987). A fluorescein isothiocyanate-conjugated sheep anti-rabbit serum (Silenus Laboratories, Melbourne, Australia) was used as the second antibody. Indirect immunofluorescence was also done on acetone-fixed leptospores and visualized using a Zeiss IM35 inverted epifluorescence microscope (Vinh *et al.*, 1984).

Preparation of leptospiral antigens. Leptospiral outer envelope and protoplasmic cylinders were prepared from serovar *hardjo* as described by Auran *et al.* (1972). Cytoplasmic and membrane fractions were prepared as described by Ito *et al.* (1977). Flagella were prepared according to the method of Chang & Faine (1970) and leptospiral sonicates were prepared as described by Adler *et al.* (1980). The protein content of these leptospiral antigens, P64 and other bacterial strains were determined by the method of Bradford (1976) using bovine γ-globulin (Becton Dickinson) as the standard.

PAGE and Western blotting. Antigens were electrophoresed on discontinuous SDS-polyacrylamide 7% or 10% (w/v) gels in a Protean gel apparatus (Bio-Rad) and stained for protein using Coomassie brilliant blue (Chapman *et al.*, 1987) or silver-stained by the method of Morrissey (1981). Gels were also transblotted onto nitrocellulose (0.45 µm, Schleicher and Schuell) in a Bio-Rad transblot cell and immunostained with sera followed by peroxidase-conjugated goat anti-rabbit immunoglobulins (Bio-Rad), sheep anti-mouse immunoglobulins (Silenus Laboratories) or sheep anti-human immunoglobulins (Silenus Laboratories) as described by Chapman *et al.* (1987). Bands were visualized with 4-chloro-1-naphthol as described by Hawkes *et al.* (1982) and their molecular masses determined by comparison with standard proteins (Pharmacia). Blotted antigens digested with proteinase K (100 µg ml⁻¹; Protease-type XI, Sigma) in buffer A were incubated at 37 °C for 24 h. Control blots were incubated in buffer A only.

Native PAGE was done as for discontinuous SDS-PAGE with the exception that SDS was omitted from all reagents used. Samples were prepared in the absence of dithiothreitol and SDS without boiling and transblotting was done in the absence of methanol.

Two-dimensional electrophoresis was done as described by Milner *et al.* (1987) using 7% (v/v) acrylamide slab gels in the second dimension.

Elution of antibodies from nitrocellulose following Western blotting was done as described by Beall & Mitchell (1986) with the following modifications. Nitrocellulose strips were washed with 0.1 M-Tris/HCl, pH 7.4, containing 0.5 M-NaCl (TBS) prior to elution of antibodies. Eluted antibodies were diluted 1:2 with 5% (w/v) skim milk in TBS containing 0.05% (v/v) Tween 20 and used immediately.

Enzyme activity assays. Protease activity of purified P64 was assayed using Azocoll (10 mg ml⁻¹; <50 mesh, Calbiochem) as a substrate according to the assay procedure recommended by the manufacturer. Proteinase K (1 to 20 µg ml⁻¹) was used as a positive control. Soluble products produced as a result of enzymic degradation of Azocoll were measured by spectrophotometry at 520 nm.

Haemolysis of sheep erythrocytes by serovar *hardjo* [10^9 cells ml^{-1} in phosphate buffered saline (PBS, 0.15 M-sodium chloride, 0.15 M-sodium phosphate, pH 7.2)] and P64 ($10 \mu\text{g ml}^{-1}$ PBS) was measured photometrically at 540 nm. Erythrocytes were washed and resuspended in PBS to a concentration of 4% (v/v); 0.5 ml of washed leptospires or P64 were mixed with 0.25 ml erythrocytes and 0.25 ml PBS and then incubated at 37 °C for 18 h. The optical density of supernatants measured was converted to percentage lysis of erythrocytes by reference to a standard curve of optical densities of different dilutions of erythrocytes completely haemolysed by distilled water. Haemolysis inhibition studies were done by replacing 0.25 ml PBS with R α P64 or normal rabbit serum in the assay procedure.

Cytotoxicity assays on L929 fibroblasts were done as described by Vinh *et al.* (1986) by measuring release of lactate dehydrogenase (LDH) from damaged L cells (Mitchell *et al.*, 1980).

Results

Purification of P64

Leptospires from a 1 litre culture were washed, sonicated and subjected to phase partitioning by Triton X-114. P64 was found exclusively in the water phase. This fraction was applied to a DEAE-Sephadex CL-6B column and eluted in a single peak using buffer A. Fractions containing P64 were pooled and concentrated prior to loading onto sucrose gradients. After centrifugation, P64 was recovered from fraction 1 and dialysed against

buffer A; approximately 500 μg of protein was obtained from 10^{12} leptospires. The purification procedure was monitored by SDS-PAGE and Western blotting with human serum and an anti-LPS monoclonal antibody. P64 appeared as a single band when stained by Coomassie brilliant blue and Western blotting with human serum; in addition, contaminating LPS could not be detected in this preparation when immunostained using an anti-LPS monoclonal antibody (Fig. 1). However, protein silver staining of P64 in SDS-PAGE gels revealed the presence of contaminating material. Subsequently P64 was further purified by SDS-PAGE and electro-elution and was designated pP64; pP64 appeared as a single band of protein by gel electrophoresis when silver-stained. The protein nature of P64 was confirmed when Western blots were treated with proteinase K and stained with human serum. Proteinase K treatment completely abolished the reaction of human serum with P64 whereas the control blot was unaffected (not illustrated).

Production and characterization of antiserum to pP64

Rabbit antiserum was produced by immunization with pP64 and tested for reactivity by Western blotting of serovar *hardjo* sonicate and P64, electrophoretically resolved on 7% (w/v) SDS-polyacrylamide gels. Rabbit

Fig. 1. Evaluation of the P64 purification procedure by SDS-PAGE. Gels were either stained with Coomassie blue (a), silver-stained for protein (d), or immunoblotted with human antiserum (b) or an anti-LPS monoclonal antibody (c). Lanes: A, serovar *hardjo* sonicate; B, pooled ion-exchange fractions containing P64; C, sucrose gradient fraction containing P64; D, P64 after electroelution from SDS-polyacrylamide gels. The left-hand lane in (a) contains standard proteins.

Table 1. Cross-reactivity of RαP64 with leptospiral sonicates

RαP64 cross-reacted with sonicates of each of the serovars listed.

Species	Serogroup	Serovar
<i>L. interrogans</i>	Australis	<i>australis</i>
	Autumnalis	<i>autumnalis</i>
	Ballum	<i>ballum</i>
	Bataviae	<i>bataviae</i>
	Canicola	<i>canicola</i>
	Celledoni	<i>celledoni</i>
	Cynopteri	<i>cynopteri</i>
	Djasiman	<i>djasiman</i>
	Grippotyphosa	<i>grippotyphosa</i>
	Hebdomadis	<i>hebdomadis</i>
	Icterohaemorrhagiae	<i>copenhageni</i>
	Javanica	<i>javanica</i>
	Mini	<i>mini</i>
	Panama	<i>panama</i>
	Pomona	<i>pomona</i>
	Pyrogenes	<i>zanoni</i>
	Sarmin	<i>sarmin</i>
	Shermani	<i>shermani</i>
	Tarassovi	<i>tarassovi</i>
<i>L. biflex</i>	Semarang	<i>patoc</i>

antiserum to pP64 (RαP64) recognized a 60 to 64 kDa band in both sonicate and the P64 preparation; no reactivity was observed with normal rabbit serum. However, this band was occasionally observed to resolve into two distinct bands of 60 kDa and 64 kDa (Fig. 2); some bands of 120 kDa were also observed in P64, which were not seen in serovar *hardjo* sonicate. Boiling of samples and the presence or absence of dithiothreitol prior to SDS-PAGE had no effect on this banding profile. In an attempt to further resolve the doublet profile of P60-64, serovar *hardjo* sonicate was subjected to two-dimensional electrophoresis followed by Western blotting with RαP64. P60-64 migrated as a single spot in the pH region of 5 to 7 (not illustrated).

Because RαP64 was prepared using SDS-PAGE-purified (denatured) protein it was tested against non-denatured serovar *hardjo* sonicate and P64 using a native gel system. Serovar *hardjo* sonicate and P64 were electrophoretically separated on 5, 7 and 10% polyacrylamide gels in the absence of SDS and dithiothreitol; gels were Coomassie-blue-stained for protein or transblotted and immunostained with RαP64 (Fig. 3). In all cases only one protein of >670 kDa was observed in the P64 preparation by Coomassie blue staining. RαP64, as well as human antisera (not illustrated), reacted with this band in both sonicate and P64 by Western blotting,

Table 2. Cross-reactivity of RαP64 with sonicates of various bacterial species

Bacterial species	Reactivity with RαP64
<i>Leptonema illini</i>	+
<i>Treponema pallidum</i>	+
<i>Borrelia burgdorferi</i>	+
<i>Clostridium perfringens</i>	+
<i>Pseudomonas aeruginosa</i>	+
<i>Escherichia coli</i>	+
<i>Shigella flexneri</i>	+
<i>Neisseria gonorrhoeae</i>	+*
<i>Neisseria pharyngis</i>	+
<i>Proteus mirabilis</i>	+*
<i>Campylobacter jejuni</i>	-
<i>Bacteroides fragilis</i>	-
<i>Streptococcus pyogenes</i>	+
<i>Corynebacterium murium</i>	+*
<i>Pasteurella multocida</i>	+
<i>Staphylococcus epidermidis</i>	+
<i>Mycobacterium bovis</i> (BCG)	+
<i>Bacillus subtilis</i>	-

* Reactivity of RαP64 with a protein of 42 kDa.

indicating that P64 exists as a homomultimeric protein in the native state.

RαP64 did not agglutinate homologous leptospires nor did it opsonize them for phagocytosis by murine macrophages as determined by indirect immunofluorescence.

Examination of cross-reactivity of RαP64

The cross-reactivity of RαP64 with representative serovars from serogroups of *L. interrogans* (Table 1) was examined by Western blotting. Immunoblots showed identical profiles to those obtained with serovar *hardjo* (not illustrated). However, RαP64 also reacted with a 60 kDa band in sonicate from serovar *patoc* (Fig. 4), a member of the saprophytic species *L. biflexa*; this reactivity was considerably weaker than that obtained with serovar *hardjo* and the other *L. interrogans* serovars. The cross-reactivity of RαP64 against other spirochaetes and various Gram-negative and Gram-positive organisms was further investigated (Table 2); some of these are illustrated in Fig. 4. RαP64 reacted with a component in these and other bacteria in the 60-67 kDa region, with the exception of *Neisseria gonorrhoeae*, *Proteus mirabilis* and *Corynebacterium murium* where the cross-reactivity was directed against a 42 kDa component. In addition, human sera from a syphilis patient reacted with P64, as did rabbit anti-*Borrelia burgdorferi* antiserum. Both of

Fig. 2. Serovar *hardjo* sonicate (A) and P64 (B) separated by SDS-PAGE and immunoblotted with R α P64 antiserum.

these sera reacted with P64 in an identical manner to R α P64 and no cross-reactivity was observed with normal rabbit serum or normal human serum in the regions of interest.

P64 was electrophoresed on a 7% (w/v) SDS-polyacrylamide gel, transblotted onto nitrocellulose and immunostained with R α P64 antibodies that had been eluted from the 62 kDa band of *Pseudomonas aeruginosa*. These eluted antibodies reacted exclusively with the 64 kDa band of P64. In the reverse experiment, R α P64 antibodies eluted from the 64 kDa band of serovar *hardjo* sonicate reacted exclusively with the 62 kDa band of *P. aeruginosa* sonicate.

Localization of P64 in the leptospire

In order to localize P60-64, leptospires were separated into total membrane and cytoplasmic fractions by

Fig. 4. Immunoblotting of sonicates of bacterial species with R α P64. Lanes: A, serovar *hardjo*; B, *Leptospira biflexa* serovar *patoc*; C, *Leptonema illini*; D, *Clostridium perfringens*; E, *Pseudomonas aeruginosa*; F, *Escherichia coli*.

Fig. 3. Native PAGE of serovar *hardjo* sonicate (A) and P64 (B) on a 7% polyacrylamide gel, immunoblotted with R α P64 (a) or stained with Coomassie blue (b). The right-hand lane in (b) contains standard proteins.

Fig. 5. Localization of P64 by cell fractionation. Evaluation using SDS-PAGE and immunoblotting with R α P64. Lanes: A, cytoplasmic fraction; B, membrane fraction; C, leptospiral flagella; D, protoplasmic cylinders; E, outer envelope.

adapting a method used for *E. coli*. The outer envelope, protoplasmic cylinders and flagella of leptospires were purified using methods specifically devised for *Leptospira*. These five fractions were examined for the presence of P60-64 by Western blotting using R α P64 (Fig. 5). P60-64 was observed in both the membrane fraction and cytoplasmic fractions in equal amounts; proteins of >94 kDa were also observed in the cytoplasmic fraction. In contrast, P60-64 was found predominantly in the protoplasmic cylinders of leptospires compared with the outer envelope fraction. R α P64 did not react with a flagella preparation. This apparent sub-surface location was consistent with the inability of R α P64 to react with whole leptospires when tested by immunofluorescence (not illustrated).

Biological properties of P64

P64, purified from serovar *hardjo* in the absence of protease inhibitors, was examined for protease activity, ability to haemolyse sheep erythrocytes and cytotoxic

effects on a fibroblastic cell line. P64 had no detectable protease activity at concentrations of up to 100 μ g ml⁻¹ when tested using Azocoll as the substrate and compared with proteinase K standards. P64 also failed to haemolyse sheep erythrocytes, and R α P64 could not inhibit the haemolysis of sheep erythrocytes by serovar *hardjo*. Finally, P64 had no effect on L cells as measured by LDH leakage.

Discussion

Antigens shared by a broad spectrum of micro-organisms, referred to as common antigen(s), display similar structural and immunological features. In particular they can be isolated as homomultimers of 250–900 kDa comprising 60 kDa subunits (Shinnick *et al.*, 1988) and all contain species-specific and genus-specific epitopes as well as cross-reactive epitopes (Gillis *et al.*, 1985). Perhaps more importantly they are major immunogens and antigens during infection with the corresponding bacteria.

In this study, we purified a major protein with a molecular mass of 64 kDa (P64) from *Leptospira interrogans* serovar *hardjo*, recognized by humans during naturally acquired leptospirosis. Immunological characterization of P64 has led to the preliminary identification of this protein as the common antigen of *Leptospira*. The native molecular mass of P64 was calculated, using non-denaturing PAGE, to be greater than 670 kDa occurring as a homomultimeric protein composed of 64 kDa subunits. When tested by Western blotting, P64 reacted with human antiserum, rabbit antiserum produced against other spirochaetes, namely *B. burgdorferi* and *T. pallidum*, as well as with monospecific rabbit antiserum raised against P64 (R α P64). To investigate this immunological cross-reactivity further, R α P64 was used to screen a variety of other micro-organisms as well as representative serovars from the genus *Leptospira*. On immunoblots, this antiserum reacted with a 64 kDa band in all leptospiral serovars tested (Table 1) as well as with a 60–64 kDa region in 12 out of 18 Gram-negative and Gram-positive organisms tested; three organisms exhibited a cross-reactive protein of 42 kDa and the remaining three organisms gave no reaction (Table 2). The common antigen of *P. aeruginosa* consists of 62 kDa subunits (Sompolinsky *et al.*, 1980) and it is to this standard that the findings for other bacteria have been compared, the subunit molecular mass of common antigens falling in the narrow range of 59 to 65 kDa. However the Tr-c antigen of *T. phagedenis* has been characterized as a homomultimeric protein composed of 48 kDa subunits which cross-reacts immunologically with the common antigen of *P. aeruginosa* (Hindersson *et al.*, 1984).

The level of cross-reactivity of RαP64 outside the genus *Leptospira* was considerably weaker than that within the genus, indicating the probable existence of at least genus-specific epitopes, as well as cross-reactive epitopes. To confirm the existence of cross-reactive epitopes, RαP64 was reacted with serovar *hardjo*, and P64-specific antibodies were eluted from the 64 kDa band and tested against *P. aeruginosa* by Western blotting. In the reverse experiment, antibodies were eluted from the cross-reactive 62 kDa protein in *P. aeruginosa* immunoblots and tested against P64. In both cases eluted antibodies reacted exclusively with the homologous protein, confirming that the cross-reactivity observed with RαP64 serum was a function of anti-P64 antibodies and not due to non-specific cross-reactivity of the rabbit antisera used.

The reactivity of RαP64 with the purified protein and serovar *hardjo* sonicate occasionally manifested as a doublet, leading to initial concerns that this antiserum was not monospecific. Bands observed with a molecular mass of 120 kDa could be explained as dimers of P64 resulting from incomplete degradation of the homomultimeric form. Two-dimensional electrophoresis was done in an attempt to resolve this doublet profile further; however when immunoblotted with RαP64 only one reactive spot was observed which had a slightly acidic isoelectric point characteristic of other common antigens (Sompolinsky *et al.*, 1980). The common antigen in other bacteria was also partially degraded to low-molecular-mass products or observed as doublets (Gillis *et al.*, 1985; Thole *et al.*, 1988; Shinnick *et al.*, 1988). The source of this doublet profile is unknown and could be the result of enzymic cleavage or degradation of the P64 subunit. Alternatively, these proteins may result from a normal precursor/product relationship such as that observed with proteins requiring signal peptides to traverse the bacterial membrane.

Initial attempts to localize P64 in the leptospire, using immunogold-labelling of ultra-thin sections of whole leptospires, were unsuccessful. However, in cell fractionation experiments, P64 appears to have a sub-surface location, occurring predominantly in the cytoplasm with some membrane involvement. Whilst P64 was found in the outer envelope of the leptospire, it is unlikely that it is surface-exposed as intact leptospires did not react with RαP64 when tested by immunofluorescence. Gillis *et al.* (1985) found that the common antigen of *Mycobacterium leprae* was associated with the cell wall, whereas Thole *et al.* (1988) showed a cytoplasmic location for the common antigen of *Mycobacterium bovis* using immunogold-labelling. In other studies, the common antigen of *B. burgdorferi* and *P. aeruginosa* had a sub-surface location (Hansen *et al.*, 1988).

Humans produce antibodies to P64 during naturally

acquired leptospirosis but it is unlikely that P64 plays a role in immunity to infection as it does not give rise to agglutinating antibodies, nor did RαP64 antibodies opsonize whole leptospires for phagocytosis by murine macrophages. This is not surprising considering the apparent sub-surface location of P64.

The common antigen of *M. bovis* has been implicated in the pathogenesis of experimental adjuvant arthritis. Using T cell clones, capable of inducing arthritis in rats, along with truncated peptides of cloned *M. bovis* common antigen, the arthritogenic epitope was localized (Van Eden *et al.*, 1988). The presence of this or a cross-reactive epitope in the common antigen of different bacteria, including *Leptospira*, remains to be elucidated, but may explain why only some of the many bacteria expressing common antigen are associated with arthritis. Although the role of T cell immunity during infection with *Leptospira* is unclear, the fact that arthritis is not a common clinical feature of leptospirosis seems to indicate that the leptospiral common antigen may differ from that of *M. bovis* in this aspect. In an attempt to identify a cytotoxic function for P64 the purified protein was tested for protease activity, ability to haemolyse sheep erythrocytes and L cell cytotoxicity. P64 was inactive in all experiments. Although the possibility of some cytotoxic function is not precluded it seems unlikely that P64 would play such a role in the pathogenesis of leptospirosis.

The phylogenetic stability of common antigen is remarkable and suggests a vital but elusive physiological function. Recently the common antigen of *M. tuberculosis* has been identified as a heat-shock protein produced in response to environmental stress (Young *et al.*, 1988). Subsequently, the common antigen of *Coxiella burnetii* has been implicated in the heat-shock response of this organism (Vodkin & Williams, 1988). Both of these common antigens corresponded to a similar antigen in *E. coli* (the GroEL protein) which is also expressed as a heat-shock protein. The mechanism of virulence in *Leptospira* is obscure, but it has been shown that virulent leptospires survive and multiply in host tissues, whereas avirulent leptospires do not (Faine, 1957). Faine (1959) showed that avirulent strains were susceptible to physiological concentrations of NaCl at 37 °C but not at 30 °C, whereas virulent leptospires grew normally. In addition, virulent strains grew in rabbit serum medium at 40 °C and avirulent strains did not. It is interesting to speculate whether or not P64 may play a role in the survival of leptospires during heat stress in a similar manner to the heat-shock protein/common antigen of other bacteria. This and other studies will be the subject of further investigation.

Addendum. Two mouse monoclonal antibodies,

mc5205 and mc0445, against the 65 kDa common antigen/heat-shock protein of Mycobacteria, were obtained from the UNPD/World Bank/WHO Special Programme for Research and Training in Tropical Diseases [Engers *et al.* (1985). Letter. *Infection and Immunity* **48**, 603–605]. These antibodies were tested for reactivity against P64 and serovar *hardjo* sonicate by Western blotting. Both antibodies reacted with a single band of 64 kDa in these antigen preparations, confirming the identification of P64 as the common antigen of *Leptospira*.

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