Antigens recognised by the human immune response to infection with *Leptospira interrogans* serovar *hardjo*

A. J. CHAPMAN, B. ADLER and S. FAINE

Department of Microbiology, Monash University, Clayton, 3168, Victoria, Australia

Summary. Serum samples from patients infected with *Leptospira interrogans* serovar *hardjo* were tested by the microscopic agglutination test (MAT), enzyme immunoassay (EIA) and immunoblotting. There was no apparent correlation between MAT titre and EIA optical density (OD) for individual serum samples, but sequential serum samples produced similar profiles in both tests during the course of an infection. Immunoblotting of *hardjo* sonicate with patients' sera revealed reactions with a number of bands, in the mol. wt (10^3) range 14·4–95. However, all serum samples reacted with the major 28×10^3 -mol. wt sub-unit of *hardjo* lipopolysaccharide (LPS) and most reacted with a $(34\cdot5\text{--}35) \times 10^3$ -mol. wt flagella doublet. Examination of sequential serum samples obtained over a period of about 3 months after infection revealed little change in the antigens detected after the second to third week of infection. Absorption of patients' sera with whole viable leptospires revealed that antibodies to several exposed antigens, including LPS, were produced. Sera which reacted with *hardjo* flagella also reacted with bands of similar mol. wts in preparations from other serovars.

Introduction

Leptospirosis is an acute febrile illness, the severity of which varies from mild to rapidly fatal. The causative bacterium *Leptospira interrogans* is classified serologically into more than 180 serovars (Faine, 1982). In Australia and New Zealand, leptospirosis is most frequently caused by serovar *hardjo* or serovar *pomona* (Adler and Faine, 1980; Blackmore and Schollum, 1982). Infections with serovar *hardjo* are acquired mainly from cattle and are usually observed in dairy farmers, whereas serovar *pomona* infections occur in other occupational groups associated with animals, such as slaughtermen and meat inspectors (Mackintosh *et al.*, 1980; Blackmore and Schollum, 1982; Faine, 1982; Swart *et al.*, 1983).

Leptospirosis may remain undiagnosed because the symptoms are non-specific (Kaufmann, 1976) or may be confused with other illnesses, especially in the early stages of infection (Faine, 1982). Therefore, laboratory tests are frequently necessary to confirm the diagnosis. The microscopic agglutination test (MAT) for the detection of specific antileptospiral antibodies is currently the most commonly used diagnostic test (Faine, 1982), despite the limitations imposed by the need to

maintain cultures of several leptospiral serovars and the subjectivity involved in reading and interpreting the results.

Enzyme-linked immunosorbent assays (ELISA or EIA) were developed in an attempt to circumvent these problems. Adler *et al.* (1980) used ultrasonicated leptospires as the antigen, and found that the predominant class of reacting antibody in human sera was IgM. Terpstra *et al.* (1980) used a heated culture supernate as the antigen and found that their test was genus-specific, but they did not classify the reacting antibody. Milner *et al.* (1985) found that an IgM ELISA test with a sonicated antigen preparation was sensitive and reproducible for the detection of human anti-*hardjo* antibodies. They agreed with Adler *et al.* (1980) that the ELISA test was capable of detecting antigens in addition to those detected by the MAT. However, the nature of the antigens detected by these methods is unknown. Indeed, little is known about the identity of the antigens involved in the response to leptospiral infections.

Nunes-Edwards *et al.* (1985) investigated the protein antigens recognised by IgG antibodies in hyperimmune rabbit anti-*hardjo* sera. Most antigens detected by these sera had mol. wts between 30×10^3 and $66\cdot5 \times 10^3$ and were present in the outer envelope. It is difficult to relate this study to natural human infections because the reactivities

of IgG antibodies were investigated, whereas IgM is the predominant antibody class in man (Adler *et al.*, 1980). Moreover, only protein antigens were investigated and lipid and carbohydrate components of the outer envelope (Auran *et al.*, 1975; Zeigler and VanEseltine, 1975) may also be important antigens.

More information about the identity and nature of the antigens recognised during naturally acquired infections is needed to elucidate those antigens with significance for immunity and diagnosis. We have investigated by immunoblotting the antigens involved in the human antibody response to natural infections with *L. interrogans* serovar *hardjo*, and the time-course of the IgM and IgG responses to various antigens during different stages of infection.

Materials and methods

Leptospire

Three isolates of *L. interrogans* serovar *hardjo* were used. The reference strain Hardjoprajitno (designated L13) was isolated in 1938 from a Sumatran rubber-plantation worker (Wolff, 1953). Strain L171 was a bovine isolate obtained from R. Marshall, New Zealand, and strain L187 was isolated from a human placenta (Faine *et al.*, 1984) in Australia. Other serovars were obtained from previously described sources (Adler and Faine, 1983a). Leptospire were grown at 30°C in Tween-albumin EMJH medium with added pyruvate (Johnson *et al.*, 1973) and washed three times with phosphate buffered saline, pH 7.2 (PBS), to remove adherent bovine serum albumin (BSA).

Human serum samples

Sequential serum samples obtained from patients with culturally proven leptospirosis caused by serovar *hardjo* (patients 1-3) were provided by the National Health Institute, Wellington, New Zealand. Further individual serum samples from Australian patients (patients A-E), reactive with serovar *hardjo* by MAT, were selected from specimens submitted to this laboratory for leptospirosis serology. Human sera were absorbed with whole, viable leptospire as previously described (Adler and Faine, 1978).

Serological tests

The microscopic agglutination test (MAT) was performed as described by Turner (1968), with Cooke microtitration equipment (Cooke Engineering Co., Alexandria, VA, USA) used to prepare serum dilutions. Enzyme immunoassay (EIA) with ultrasonicated leptospiral antigens was based on the procedure described by Adler *et al.* (1980) with peroxidase-conjugated rabbit anti-human μ -chain (IgM) and γ -chain (IgG) specific immunoglobulins (DAKO Immunoglobulins a/s, Copen-

hagen). All sonicated leptospire were suspended in PBS. The optical density in each well was measured at 488 nm by a MicroELISA reader (Dynatech Laboratories, Alexandria, VA, USA).

Monoclonal antibodies

Monoclonal antibodies MUM/F1-1 (IgG1) and MUM/F1-4 (IgM) were produced from BALB/c mice immunised with *L. interrogans* serovar *hardjo* (L13) as previously described (Farrelly *et al.*, 1987).

Preparation of leptospiral antigens

Sonicated leptospiral antigen preparations were produced as previously described (Adler *et al.*, 1980), except that all sonicates were suspended in PBS. The protein content of leptospiral suspensions was determined by the method of Bradford (1976) with BSA as the standard.

Lipopolysaccharide (LPS) was extracted from *L. interrogans* serovar *hardjo* (L187) by the hot phenol-water method of Westphal and Jann (1965) as previously described (Vinh *et al.*, 1986a).

Flagella were prepared according to the method of Chang and Faine (1970), followed by further purification by caesium chloride density gradient centrifugation, in which flagella were concentrated at a buoyant density of 1.27 (Adler and Faine, 1979).

Gel electrophoresis and immunoblotting

Samples standardised for protein content were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 1.5 mm, 10% gels, with the discontinuous buffer system of Laemmli (1970). After electrophoresis, gels were either stained with Coomassie Brilliant Blue (BDH Chemicals, Kilsyth, Australia) 0.25% in water: isopropanol: acetic acid at a ratio of 68: 25: 7 (v/v), or transblotted on to nitrocellulose (pore size 0.45 μ m; Schleicher and Schuell, Dassel, W. Germany) in a Bio-Rad Trans-Blot cell at 60 volts for 3 h with the buffer of Towbin *et al.* (1979) diluted 1 in 2. The nitrocellulose was then stained with Indian ink (Hancock and Tsang, 1983), or immunostained with human serum. Blots to be immunostained were blocked with skimmed milk 5% (Johnson *et al.*, 1984) and Tween 20 0.05% (Sigma, St Louis, USA) (Batteiger *et al.*, 1982) in Tris-buffered saline, pH 7.4, for 40 min. Human serum and peroxidase-conjugated anti-human immunoglobulins (DAKO) were diluted, 1 in 100 and 1 in 200 respectively, in the blocking buffer and consecutively incubated with the blots for 2 h at 37°C. Blots were washed with four 10-min changes of blocking buffer after each incubation and developed with 4-chloro-1-naphthol (Merck, Darmstadt, W. Germany) (Hawkes *et al.*, 1982).

Molecular weights were determined by comparison with mol.-wt markers obtained from either BIO-RAD (Richmond, CA, USA) or Pharmacia AB (Uppsala, Sweden).

Results

SDS-PAGE profiles of three isolates of serovar *hardjo*

A complex pattern of protein bands with mol. wts ranging from $<20.1 \times 10^3$ to $>94 \times 10^3$ was observed for each of three serovar *hardjo* isolates (fig. 1). Many proteins were shared, but there were some differences between the local isolates L171 and L187, and the type strain Hardjoprajitno (L13).

Antibodies in sera, measured by MAT, EIA and immunoblotting

Individual serum samples that reacted with serovar *hardjo* by MAT were also tested against serovar *hardjo* by EIA (table) and by immunoblotting (fig. 2). There was no apparent relationship between MAT titre and EIA optical density (OD) for either IgM or IgG antibodies. EIA revealed

Table. The presence in human sera of antibodies reacting with *L. interrogans* serovar *hardjo* (strain L171) as judged by MAT and EIA

Patient	MAT titre	EIA OD (488 nm)*	
		IgM	IgG
A	2560	0.55	0.10
B	20 480	0.38	0.13
C	2560	0.49	0.25
D	2560	1.74	0.13
E	40 960	0.71	0.39

* Adjusted for background.

specific IgM and IgG in all samples although the OD levels for specific IgM were always higher. Individual serum samples from patients A–E reacted with bands in the mol.-wt (10^3) range 14.4–94 when immunoblotted with sonicated *hardjo* (strain L171) and IgM and IgG specific secondary antibodies (fig. 2). All sera contained IgM antibodies that reacted with a curved band of mol. wt $c. 28 \times 10^3$ to which most samples also contained IgG antibodies. Some samples also contained IgM and IgG antibodies or both, which reacted with a doublet of mol. wt $c. (34.5\text{--}35) \times 10^3$. However, a band of mol. wt $c. 65 \times 10^3$ reacted only with IgG antibodies.

Identification of some major antigens

Murine monoclonal antibodies reacting with serovar *hardjo* lipopolysaccharide (LPS), designated MUM/F1-1 and MUM/F1-4 (Farrelly *et al.*, 1987), were tested by immunoblotting against *hardjo* sonicate (fig. 3) and found to react with a curved band of mol. wt 28×10^3 . Immunoblotting of a purified *hardjo* LPS preparation with these anti-LPS monoclonal antibodies and human anti-*hardjo* serum (fig. 3) revealed that there were three major bands, of mol. wts (10^3) $c. 15, 23$ and 28 , in the LPS preparations. One epitope was present on all three major components, whereas another epitope was present on the mol. wt 28×10^3 component alone. Immunoblotting of a *hardjo* flagella preparation with human anti-*hardjo* sera (fig. 3) showed that both IgM and IgG antibodies reacted with a doublet of mol. wt $(34.5\text{--}35) \times 10^3$. Occasional reactions were also noted with bands of mol. wts (10^3) $c. 15$ and 30 .

Analysis of antibodies at different stages of infection

Serial sets of sera, from three patients whose leptospiral infection with serovar *hardjo* were

Fig. 1. SDS-PAGE profiles of three strains of *L. interrogans* serovar *hardjo* stained with Coomassie blue. Lane A strain Hardjoprajitno (L13); lane B, strain L171; lane C, strain L187. Numbers on the left indicate mol. wts (10^3) of the standard proteins used.

Fig. 2. Immunoblots of *L. interrogans* serovar *hardjo* sonicate with human sera positive, by MAT, for antibodies against serovar *hardjo*. "M" and "G" denote the detection of IgM and IgG antibodies respectively. Lanes 1 and 2, Normal human serum; lanes 3 and 4, patient A serum; lanes 5 and 6, patient B serum; lanes 7 and 8, patient C serum; lanes 9 and 10, patient D serum; lanes 11 and 12, patient E serum. Numbers on the left indicate mol. wts (10^3) of the standard proteins used.

Fig. 3. Immunoblots of *L. interrogans* serovar *hardjo* sonicate (lanes 1 and 2), lipopolysaccharide (lanes 3–6) and flagella (lanes 7–14) with anti-LPS monoclonal antibodies and human sera. “M” and “G” denote the detection of human IgM and IgG antibodies respectively. Lanes 1 and 3 monoclonal antibody MUM/F1-1; lanes 2 and 4 monoclonal antibody MUM/F1-4; lanes 5 and 6, patient B serum; lanes 7 and 8, patient A serum; lanes 9 and 10, patient B serum; lanes 11 and 12, patient C serum; lanes 13 and 14, patient D serum. Numbers on the left indicate mol. wts (10^3) of the standard proteins used.

Fig. 4. Time course of appearance of antibodies against *L. interrogans* serovar *hardjo* (L171) produced by patients 1-3, as measured by MAT and EIA. Serum samples were numbered according to the number of days after a positive blood culture was obtained. EIA optical densities were subtracted for background. MAT: ■—■; EIA IgM: ▲—▲; EIA IgG: ●—●.

proved by blood culture, were tested by MAT, EIA and immunoblotting. Fig. 4 shows the time-course of antibody appearance in patients 1-3 as measured by MAT and EIA. Peak antibody levels were recorded within 15-30 days of a positive blood culture, by both MAT and EIA. MAT titres gradually declined after the peak, although levels were still relatively high at the end of the three-month sampling period. The peak concentration of IgM was higher than that of IgG in the serum of patient 1 but this was not observed in sera from the other two patients.

In immunoblotting tests with serovar *hardjo* sonicate, sera from patient 1 (fig. 5) reacted strongly, by IgM, with three bands of mol. wts (10^3) c. 15, 23 and 28 from day 7 onwards. Weak reactivity was also observed with a doublet of mol. wt ($34.5-35$) $\times 10^3$. The major band detected by IgG was the 28×10^3 -mol. wt band observed to react with IgM antibodies; weak reactions with other bands of mol. wts (10^3) c. 15, 30, 34.5, 35, 44 and 62 were also observed. Similar results were also obtained from sera collected on days 25, 38, 44 and 77 (results not shown). Sera from patient 2 (fig. 5) differed in

their specific immunoglobulin content; only a small IgM response was observed, and this did not appear until day 26. It was directed mainly against a curved band with a mol. wt c. 28×10^3 and decreased dramatically in later samples but did not disappear. The IgG response was much greater than that of IgM; there was a strong reaction with a band of mol. wt c. 52×10^3 on day 1, at which time there was no demonstrable reactivity with IgM. Later samples reacted with bands of mol. wts (10^3) c. 36, 44, 47, 52, 60, 64, 72 and 95. Only a slight reaction with the curved 28×10^3 -mol. wt band was observed with these samples. Sera collected on days 63 and 90 produced a similar immunoblot profile to the day 26 serum (results not shown).

In sera from patient 3 (fig. 5) the IgM response was mainly directed against a curved band of mol. wt c. 28×10^3 . Other antigens were also recognised at day 15, but these antibodies had disappeared by day 48 (results not shown). In contrast, a strong, IgG response was directed against bands of mol. wts (10^3) c. 22, 32, 34.5, 35, 52, 62 and 95 as well as a diffuse band of mol. wt ($15-20$) $\times 10^3$. No IgG response to the curved 28×10^3 -mol. wt band was detected until day 83.

In an attempt to determine whether all the antigens detected by immunoblotting sera from patient 2, with *hardjo* sonicate, were present on the leptospiral surface, the day 26 serum was repeatedly absorbed with whole, viable leptospires until no MAT titre remained. Immunoblotting of *hardjo* sonicate with this absorbed serum revealed that all reactive IgM and IgG antibodies were removed, except for IgG antibodies reacting with a 52×10^3 -mol. wt band that also reacted with the day 1 sample from patient 2. A similar removal of reactivity was observed following absorption of sera from other patients tested (results not shown).

Cross-reactivity between serovars

IgG antibodies that reacted with a ($34.5-35$) $\times 10^3$ -mol. wt doublet in the homologous *hardjo* sonicate also appeared to react with a similar doublet in a sonicate prepared from serovar *pomona*, and with bands of mol. wts (10^3) 34 and 35 present in sonicates of *L. biflexa* serovar *patoc* and *Leptonema illini*, when serum obtained from patient 3 on day 48 was immunoblotted with homologous and heterologous sonicates (fig. 6). A reaction was also observed with a band of mol. wt c. 42×10^3 and a diffuse band of mol. wt ($14.4-23$) $\times 10^3$ that were present only in serovars *pomona* and *hardjo*. Use of a *hardjo* flagella preparation indicated that the

Fig. 5. Representative immunoblots of *L. interrogans* serovar *hardjo* sonicate with sequential serum samples from patients 1, 2 and 3. "M" and "G" denote the detection of IgM and IgG antibodies respectively. "Neg." denotes lanes immunostained with human serum negative by EIA and MAT as a control. Serum samples were numbered according to the number of days after a positive blood culture was obtained. Numbers on the left indicate the mol. wts (10^3) of the standard proteins used.

$(34.5-35) \times 10^3$ cross-reactive doublet was a flagellar component.

Discussion

SDS-PAGE protein profiles of three strains of *L. interrogans* serovar *hardjo* revealed minor differences between the isolates from Australia and New Zealand, and the type strain Hardjoprajitno. Others have found that *hardjo* isolates from Australia and New Zealand were indistinguishable by DNA restriction endonuclease analysis, but were markedly different from strain Hardjoprajitno (Robinson *et al.*, 1982), and that there were qualitative and quantitative differences between the protein antigens of recently isolated *hardjo* strains and those of Hardjoprajitno (Nunes-Edwards *et al.*, 1985). Since *hardjo* isolates obtained from Australia and New Zealand were very similar, the New Zealand strain

(L171) was selected for use in testing human sera obtained from Australia and New Zealand, because of the higher cell density obtainable from this isolate.

The lack of correlation between MAT titre and EIA OD, in tests of human sera for the presence of leptospiral antibodies, confirms previous observations (Adler *et al.*, 1980; Terpstra *et al.*, 1980; Milner *et al.*, 1985).

Immunoblotting revealed that IgM antibodies from each of the five patients A-E reacted with a curved band of mol. wt 28×10^3 . Some sera also contained IgG antibodies that reacted with this band. This band was identified as a component of LPS because anti-LPS monoclonal antibodies also reacted with it in sonicated preparations, although the shape of the band differed depending on whether sonicate or purified LPS was used as the antigen.

Immunoblotting purified *hardjo* LPS with anti-*hardjo* LPS monoclonal antibodies and serum from patient B showed that LPS was composed of three sub-units, and that the 28×10^3 -mol. wt sub-unit was the major component. Although the human sera and the MUM/F1-1 monoclonal antibody reacted with all three sub-units, MUM/F1-4 appeared to react only with the 28×10^3 -mol. wt sub-unit, thus indicating that LPS components may not share all epitopes.

Sera from patients A-D also reacted with a $(34.5-35) \times 10^3$ -mol. wt doublet which was shown by immunoblotting to consist of flagellar components. Antibodies against flagella (axial filaments) have been demonstrated previously in sera from patients with leptospirosis (Adler and Faine, 1979).

The identity and location of the antigen associated with the 65×10^3 -mol. wt band which reacted only with IgG are unknown. The reaction may indicate a secondary response to a previous subclinical or undiagnosed infection; alternatively, the antibodies to this antigen may appear later than those against LPS or flagella. However, as the stage of infection in these patients was not known, the significance of the 65×10^3 -mol. wt band and other minor antigens cannot be determined. Moreover, the identity of the infecting serovar was not proven by culture in these patients, and the highest MAT titre does not prove the identity of the infecting serovar (Turner, 1968).

To investigate these problems, serum samples were obtained from patients in whom infection with serovar *hardjo* had been proven by blood culture, a procedure that is usually successful only within the first week of illness (Turner, 1968). Although there was little correlation between MAT titre and EIA OD for individual serum samples,

Fig. 6. SDS-PAGE of *hardjo* flagella stained with Coomassie blue (lane 1), and immunoblots of *hardjo* flagella (lane 2) and leptospiral sonicates (lanes 3-6) with serum obtained from patient 3 on day 48. IgG antibodies detected only. Lanes 1 and 2, *L. interrogans* serovar *hardjo* flagella preparation; lane 3, *L. illini* sonicate; lane 4, *L. biflexa* serovar *patoc* sonicate; lane 5, *L. interrogans* serovar *pomona* sonicate; lane 6, *L. interrogans* serovar *hardjo* sonicate. Numbers on the left indicate the mol. wts (10^3) of the standard proteins used.

the times of appearance of antibodies as measured by MAT and EIA were similar and exhibited similar profiles during the course of infection. Differences observed between the IgM and IgG EIA profiles (fig. 4) may indicate a secondary response in the two patients in whom the IgM and IgG peaks occurred simultaneously. The finding that specific IgM levels detected by EIA declined after a strong initial response while specific IgG levels remained high suggests that EIA for the detection of specific IgM may be useful in discriminating between current and previous infections.

Immunoblotting *hardjo* sonicate with sera from patients 1–3 revealed that there was little change in the antigens detected after the second to third week of infection. However, all patients produced IgM antibodies that reacted with the 28×10^3 -mol. wt component of LPS, although reactions to other LPS components differed. It is possible that patients producing only IgM LPS-reactive antibodies were experiencing a primary infection, whereas those producing both IgM and IgG antibodies reacting with LPS may have had a previous infection with serovar *hardjo*. However, as past histories of these patients were not known, no firm conclusions can be drawn.

There was more variability in the IgG than in the IgM reactivities of these sera. The IgG response of patient 1 was directed almost exclusively against LPS, whereas IgG from patients 2 and 3 reacted with bands ranging in mol. wt (10^3) from 22 to 95. However, it is possible that this variable IgG response was due to previous leptospiral infections, because immunoblotting detected specific IgG antibodies in the first serum samples taken, but IgM antibodies were not detected until later in the infection.

Immunoblotting of absorbed serum obtained from one patient 26 days after the positive blood culture showed that antibodies are produced against several leptospiral antigens, other than LPS, which are apparently exposed on the surface of the organism.

Human sera cross-react widely when tested by MAT (Alston and Broom, 1958; Adler and Faine, 1978). Sera react even more widely by EIA, which also detects sub-surface, non-agglutinating antigens, and may, therefore, be used as a genus-specific test (Terpstra *et al.*, 1980). Such genus-specific antigen preparations have been used in the serodiagnosis of human leptospirosis (Chang *et al.*, 1957; Cox, 1957; Cox *et al.*, 1957; Palit and Gulasekharan, 1973). Results obtained by immunoblotting serum, collected on day 48 from patient 3, with homologous and heterologous sonicates (fig. 6) are consistent with cross-reactivity patterns we have previously observed with hyperimmune rabbit antisera (Chapman *et al.*, in press). As genus-specific tests have been found to be positive earlier than the MAT (Turner, 1968) and genus reactivity is due, at least in part, to flagellar components, the use of purified flagella may be useful in the early diagnosis of leptospirosis.

The observation that IgG antibodies in the serum obtained on day 26 from patient 2 also reacted with low-mol. wt material present only in the pathogenic serovars tested, may indicate that species-specific as well as genus-specific antigens were detected.

Our results show that LPS is a major antigen involved in the human antibody response to infection with *L. interrogans* serovar *hardjo*. The response to other antigens, including flagella, appears to be variable. As LPS elicits protective antibodies in mice (Faine *et al.*, 1974; Adler and Faine, 1983b; Farrelly *et al.*, 1987; Jost *et al.*, 1986) and lacks endotoxic activity (Vinh *et al.*, 1986b), LPS components may be useful as immunising agents. Studies are currently being undertaken to determine the antigens involved in the immune response to infection with other leptospiral serovars.

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