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Immunological Characteristics of the Glycolipid Antigen of *Leptospira interrogans* Serovar lai

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The protective antigen (PAg), a glycolipid substance, was extracted from *Leptospira interrogans* serovar lai strain 017 with a chloroform-methanol-water (1:2:0.8 [vol/vol/vol]) solution and partially purified by silica gel column chromatography. The PAg was not detected by Coomassie brilliant blue staining in sodium dodecyl sulfate-polyacrylamide gel electrophoresis but was observed as a smearlike band, which corresponded to a 24- to 30-kilodalton standard protein, by silver staining. The outer envelope (OE) fraction showed the same band, suggesting that the PAg was one of the chemical components of the OE. The immunogenicity and protective activity of the PAg were compared with those of the OE. The PAg as well as the OE and whole cells was able to induce agglutinating antibody against *L. interrogans*. Furthermore, the immune sera exhibited opsonic activity against *L. interrogans*, as observed by measurement of chemical luminescence derived from reactive oxygen. The PAg exhibited protective activity in hamsters challenged with lethal doses of *L. interrogans*. Therefore, the antigen may be useful as a component vaccine against leptospiral infection.

Leptospirosis is widespread in human and domestic animals. At present, vaccination is the best way to control leptospirosis, and chemically inactivated leptospires are used in many countries (8). For protection against leptospiral infection, an outer envelope (OE) is more effective than lyophilized whole cells (WC) (5, 7, 24). It has been reported that the lipopolysaccharide (LPS) or the glycolipid antigen may be the protective principle of leptospires (2, 17, 24). Agglutinating antibody towards leptospiral cells appears to be most important in protection against leptospiral infection (1, 2), and pathogenic leptospires are ingested by macrophages containing opsonic antibody against leptospires (11, 26).

To classify leptospires, we have established hybridoma clones producing monoclonal antibodies (MAbs) against *Leptospira interrogans* serovar lai strain 017, which was identified by cross-absorption with the serovars of the Icterohaemorragiae serogroup in China (10). These MAbs recognize the carbohydrate moiety of OE as an antigenic determinant of leptospiral cells (20). In this paper, we describe the isolation and partial purification of the agglutinogen recognized by the MAbs. The protective activity, agglutinating antibody-inducing activity, and opsonin-inducing activity of the extract were compared with those of OE and WC to examine the possibility of the development of a component vaccine against leptospiral infection.

MATERIALS AND METHODS

Strain and cultivation. *L. interrogans* serovar lai strain 017 was used as the virulent leptospire; it was isolated in China as one serovar of serogroup Icterohaemorrhagiae (10). The organisms were cultivated in Baseman-Cox medium (6) at 30°C for 7 days. Virulent organisms were maintained by passage in hamsters and were cultivated in Korthof medium (18) at 30°C.

Extraction and purification of glycolipid antigen. Agglutinogen was extracted from lyophilized cells with a chloroform-methanol-water (CMW; 1:2:0.8 [vol/vol/vol]) solution by the method of Bligh and Dyer (9). The CMW extract (ca. 430 mg), dissolved in a CMW (1:2:0.6 [vol/vol/vol]) mixture, was applied to a silica gel (silica gel 60; E. Merck AG, Darmstadt, Federal Republic of Germany) column (3 by 40 cm). The elution profile was determined by sodium dodecyl sulfate-poylacrylamide gel electrophoresis (SDS-PAGE), and two fractions, I and II, were obtained. Fraction I (Fr I; ca. 200 mg) and fraction II (Fr II; protective antigen [PAg]; ca. 140 mg) were eluted with three bed volumes of CMW (1:2:0.6) solution and with three bed volumes of CMW (1:2:0.8) solution, respectively. After evaporation of the solvent under reduced pressure below 40°C, each fraction was suspended in a small amount of distilled water and lyophilized. OE was prepared by SDS extraction by the method of Auran et al. (5).

SDS-PAGE and immunoblotting. SDS-PAGE was carried out by the method of Laemmli (19) with a vertical slab gel of 15% polyacrylamide and a stacking gel of 5% polyacrylamide. Each gel was stained with Coomassie brilliant blue or silver stain (14).

Antigens from another gel strip were electrophoretically transferred onto nitrocellulose membranes (Toyo-Roshi Co., Tokyo, Japan) by the method of Towbin et al. (25). The PAg band was immunologically stained with MAbs LW2 (20) and AG-1. The nitrocellulose membranes were incubated with the MAbs at 37°C for 2 h. Peroxidase-conjugated anti-mouse immunoglobulin G (IgG)-IgM-IgA (Organon Teknika, Malvern, Pa.) as the second antibody was added and incubated at 37°C for 1 h. Substrate solution prepared by the method of Hawkes et al. (13) was added, and specific antigen bands were visualized. Other nitrocellulose membranes were stained for detection of protein bands with India ink (12).

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Immunization of mice with leptospiral antigen and protective activity test. Four-week-old male ddY mice and golden hamsters were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan). The immunogenicity of the antigen was examined by immunization of mice. The antigens (Fr I, Fr II, and OE isolated from serovar lai and WC) were intraperitoneally (i.p.) injected into ddY mice six times at intervals of 3 days. Blood was taken from the retro-orbital venous sinus at intervals of 7 days, and the serum was separated.

The protective activity of the antigens was examined in golden hamsters. The animals were given the serovar lai preparations i.p. or subcutaneously on days 0 and 7 and were challenged i.p. with homologous leptospires on day 14. After observation for 14 days, survivors were sacrificed and the kidneys were incubated in Korthof medium at 30°C for 14 days to detect the presence of leptospires.

MAT. The agglutinating antibody titers in the immunized mice were estimated by the microscopic agglutination test (MAT). The MAT was carried out in accordance with World Health Organization guidelines (28). Culture medium (25 μl) containing leptospires and the same volume of a twofold serial dilution of each mouse serum were mixed and incubated at 30°C for 2 h. The MAT titer was the reciprocal of the highest dilution in which 50% of the leptospires were agglutinated.

ELISA. The antibody levels in immunized mice were measured by an enzyme-linked immunosorbent assay (ELISA) with antigen disrupted from WC by ultrasonication. The sonicated antigen of leptospiral cells was prepared by the method of Adler et al. (3), and the microplate (Falcon 3915) was coated with the antigen overnight at 4°C. Each well was washed with phosphate-buffered saline containing 0.02% Tween 20 (pH 7.4) and was blocked with 5% newborn calf serum–phosphate-buffered saline at room temperature for 1 h. Wells coated with the antigen were washed with 1% newborn calf serum–phosphate-buffered saline. Mouse sera, each diluted 1/100 with 0.02% Tween 20–phosphate-buffered saline, were added to wells, and the plates were stored overnight at 4°C.

The plates were washed with 0.02% Tween 20–phosphate-buffered saline, and peroxidase-conjugated anti-mouse IgG-IgM-IgA was added. After incubation at 37°C for 2 h, each well was washed with phosphate-buffered saline and the substrate solution (0.56 mg of 2,2′-azino-di-3-ethyl-benzthiazoline-sulfonate per ml, 0.07 μg of H_2O_2 per ml) was added. After incubation at 37°C for 15 min, the optical density was measured at 415 nm with an EIA reader (Corona Electric Co., Tokyo, Japan).

Assay of opsonic activity. The opsonic activity of each mouse serum against homologous leptospires was determined by measurement of chemical luminescence (CL) derived from reactive oxygen, which was produced by peritoneal exudate macrophages. The peritoneal exudate macrophages were induced by i.p. injection of 3% thioglycolate medium. After 3 to 4 days, the peritoneal exudate macrophages were harvested from the peritoneal cavity and washed with Hanks balanced salt solution (pH 7.2) without phenol red. The cells were suspended (10^6 cells per ml) in Eagle minimum essential medium (pH 7.4) without phenol red. The cell suspension (500 μl) and luminol (20 μl; Sigma Chemical Co., St. Louis, Mo.) solution (2 mg/ml) were added to a CL tube and incubated at 37°C for 30 min in a humidified CO_2 incubator. After incubation, 2 μl of mouse serum and leptospiral cells (6×10^7 cells) were added to the tube. The intensity of CL was measured for 15 min with a TD-4000 lumiphotometer (Laboscience Co., Tokyo, Japan). The opsonic activity was expressed as the peak intensity of CL over the 15-min period.

RESULTS

Preparation and SDS-PAGE profiles of the PAg. The leptospiral PAg was extracted with a CMW solution. First, the antigen was extracted with a CMW (1:2:0.8) solution from lyophilized cells and the CMW extract was separated into two layers, the chloroform fraction and the methanol-water fraction, by the addition of chloroform and water. There were no differences in SDS-PAGE profiles or in protective activities between the chloroform and methanol-water fractions (data not shown); both fractions were collected as the CMW extract. Second, the CMW extract was separated into two fractions, Fr I and Fr II, by silica gel column chromatography. Fr II was a glycolipid substance, and its protein content was very low.

The PAg was not detected by Coomassie brilliant blue staining in SDS-PAGE but was detected as a smearlike band (24 to 30 kilodaltons) by silver staining in Fr II (Fig. 1; black arrowheads). No bands were detected in Fr II by India ink staining of the blot on a nitrocellulose membrane, while many protein bands were found in WC and OE. Furthermore, the PAg band was also detected at 24 to 30 kilodaltons by immunostaining with MAb LW2 against WC and MAb AG-1 against the PAg. On the other hand, 24- to 30-kilodalton bands were also observed in OE and WC, but the intensity of staining was weak. Nonspecific binding of the second antibody to WC and OE antigens was observed on the immunoblots (Fig. 1, white arrows).

Antibody titers determined by MAT and ELISA. Agglutination titers of sera from mice injected with Fr II, OE, and WC increased from days 13 to 27 and decreased in order from WC to OE to Fr II (Table 1). Fr I could not induce agglutinating antibody against leptospires. Antibody titers against all leptospiral antigens as determined by the ELISA rose from days 13 to 20 and nearly plateaued on day 27 (Table 2). The optical density values decreased in order from WC to OE to Fr II to Fr I. From MAT and ELISA results, we concluded that Fr II as well as OE and WC induces antibody against leptospires but that Fr I does not induce any antibody.

Opsonic activity. Enhancement of CL by sera from mice immunized with OE and WC was found on day 13 and reached a plateau on days 20 to 27 (Table 3). Enhancement of CL by Fr II began on day 20 and was almost the same as that by OE and WC on day 27. On the other hand, sera from mice immunized with Fr I did not induce a CL response. These findings indicated that Fr II is able to induce opsonic antibody against leptospires.

Protective activity. Animals given 100 or 10 μg of each material were protected completely from challenge with homologous leptospires (Table 4, experiment 1). Three of five animals survived a dose of 0.1 μg of OE, and leptospiral growth was not observed in kidney cultures of survivors (experiment 2). However, the same dose of Fr II did not result in any activity. These results indicated that the protective activity of Fr II is weaker than that of OE. In experiment 2, 1 of five animals in the control group survived, and leptospiral growth was not observed in kidney cultures of survivor. Hamsters occasionally survived from leptospiral infection without the administration of vaccine. The reason why normal hamsters exhibited resistance to leptospiral infection is not clear.

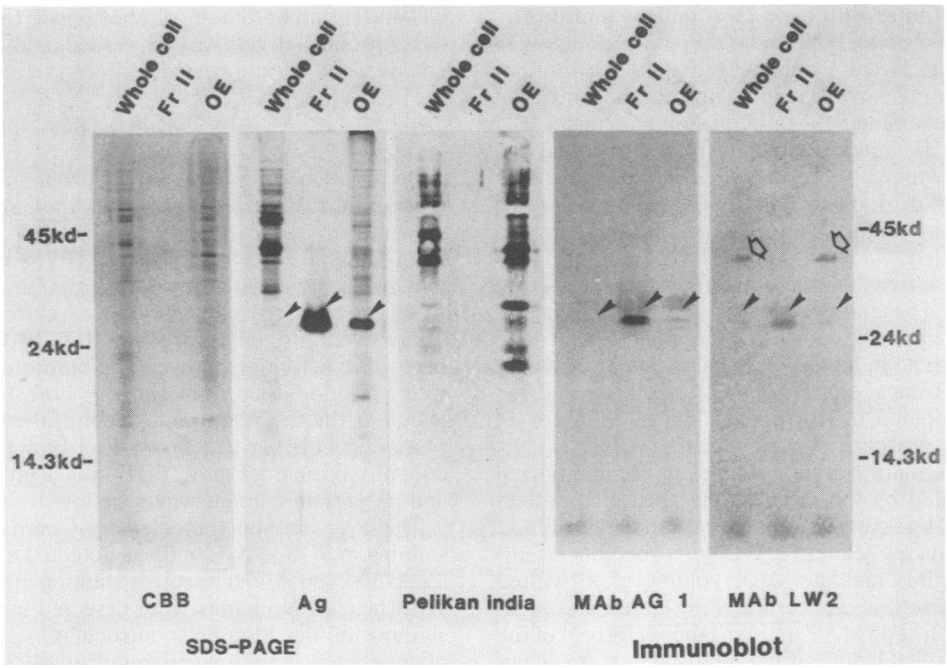

FIG. 1. SDS-PAGE and immunoblotting of serovar lai preparations. Each gel was stained with Coomassie brilliant blue stain (CBB) or silver stain (Ag). Antigens were transferred from the gel strip to nitrocellulose membranes and stained with MAbs LW2 and AG-1. Other nitrocellulose membranes were stained for the detection of protein bands with India ink. Numbers on the left indicate the molecular masses (in kilodaltons [kd]) of the standard proteins used. Black arrowheads and white arrows indicate the PAg bands and the bands showing nonspecific binding of the second antibody to WC and OE antigens, respectively.

DISCUSSION

It has been reported that leptospiral LPS, an important PAg, is capable of producing a protective antibody (2, 17). However, purified LPS, extracted with hot phenol water (27), was of a very low yield (15, 23). It seems that the hot phenol water method was not appropriate for extraction of the PAg from leptospiral cells. Ono et al. (22) extracted glycolipid antigen from serovar canicola with chloroform-methanol (2:1 [vol/vol]). However, glycolipid antigen was recovered as a small percentage of the starting material.

In this paper, the PAg, extracted from serovar lai with a CMW (1:2:0.8) solution, was eluted with the same solvent from a silica gel column, and the yield was about 20% of the lyophilized cells. Similar glycolipid antigens were efficiently extracted with a CMW (1:2:0.8) solution from lyophilized cells of other serovars (serovars canicola, copenhageni, australis, autumnalis, and hebdomadis). Therefore the CMW method appears to be an efficient way to extract glycolipid antigen from leptospires.

SDS-PAGE and immunoblotting showed that the PAg was one of the chemical components of OE. The SDS-PAGE profile of LPS (17) or LPS-like substance (23) was similar to that of the PAg. The PAg is thought to be a substance similar to LPS and LPS-like substance. Furthermore, the band reacted with the serogroup-specific MAbs LW2 and AG-1 against serovar lai in immunoblotting, showing that the PAg is a serogroup-specific antigen.

TABLE 1. MAT of sera from mice immunized with various preparations of *L. interrogans* serovar lai[a]

Sample	Dose (μg/mouse)	Mean agglutination titer ± SD[b] on day:			
		5	13	20	27
Fr I	100	<40	<40	<40	<40
	10	<40	<40	<40	<40
Fr II	100	40 ± 0	1,387 ± 1,124	4,693 ± 4,846	5,120 ± 4,434
	10	66 ± 23	427 ± 185	2,347 ± 2,423	3,413 ± 1,478
OE	100	213 ± 92	2,987 ± 1,955	10,240 ± 0	13,653 ± 5,912
	10	60 ± 28	240 ± 139	2,987 ± 1,955	4,693 ± 4,846
WC	100	173 ± 140	3,413 ± 1,478	13,653 ± 5,912	27,307 ± 11,824
	10	53 ± 23	853 ± 370	5,120 ± 4,434	5,120 ± 4,434

[a] Each preparation was given i.p. to ddY mice on days 0, 3, 6, 9, 12, and 15. Blood samples were collected from the retro-orbital venous sinus on days 5, 13, 20, and 27.
[b] Three mice per group.

TABLE 2. ELISA of antibody levels in sera from mice immunized with various preparations of *L. interrogans* serovar lai[a]

Sample	Dose (μg/mouse)	Mean optical density at 415 nm ± SD (10^2)[b] on day:			
		5	13	20	27
Fr I	100	30 ± 16	60 ± 12	126 ± 16	90 ± 10
	10	16 ± 16	80 ± 4	111 ± 52	69 ± 38
Fr II	100	23 ± 27	119 ± 63	211 ± 16	190 ± 55
	10	11 ± 13	96 ± 28	194 ± 41	214 ± 39
OE	100	46 ± 10	297 ± 19	427 ± 103	405 ± 114
	10	17 ± 13	92 ± 52	213 ± 37	205 ± 16
WC	100	112 ± 45	586 ± 35	637 ± 27	659 ± 49
	10	72 ± 58	312 ± 116	423 ± 54	471 ± 113

[a] Each preparation was given i.p. to ddY mice on days 0, 3, 6, 9, 12, and 15. Ultrasonically disrupted cells of *L. interrogans* serovar lai were used as antigens in the ELISA. Blood samples were collected from the retro-orbital venous sinus on days 5, 13, 20, and 27.
[b] Three mice per group.

TABLE 4. Protective effects of immunization of hamsters with preparations of *L. interrogans* serovar lai[a]

Expt	Sample	Dose/animal (μg)	Administration route	No. of survivors at 14 days/no. tested (%)	Result of kidney cultures of survivors[b]
1	Fr II	100	i.p.	5/5 (100)	ND
		10	i.p.	5/5 (100)	ND
		10	s.c.[c]	5/5 (100)	ND
	OE	100	i.p.	5/5 (100)	ND
		10	i.p.	4/5 (80)	ND
		10	s.c.	3/3 (100)	ND
	Control	0		0/4 (0)	NT
2	Fr II	10	i.p.	3/5 (60)	ND
		1	i.p.	4/5 (80)	ND
		0.1	i.p.	1/5 (20)	ND
	OE	10	i.p.	5/5 (100)	ND
		1	i.p.	5/5 (100)	ND
		0.1	i.p.	3/5 (60)	ND
	Control	0		1/5 (20)	ND

[a] Animals were injected with each sample on days 0 and 7. Animals were challenged i.p. with serovar lai strain 017 (2.5×10^8 cells per hamster) on day 14.
[b] ND, Leptospires not detected; NT, not tested.
[c] s.c., Subcutaneously.

Fr II, which was mainly composed of the PAg, induced agglutinating antibody efficiently, as did OE and WC. Agglutinating antibody against leptospiral cells appeared to be most important in protection against leptospirosis (1, 2).

It has been reported that polymorphonuclear leukocytes and macrophages play an important role in protection from leptospiral infection (11, 16, 26). A correlation between the CL response and phagocytosis of polymorphonuclear leukocytes was reported (4), and the CL response was applied to studies of the interaction of phagocytes with leptospires (17, 21). Fr II as well as the OE and WC induced opsonic antibody against leptospires in CL response studies. These results suggested that the PAg stimulates protective-antibody production and leads to protective activity against leptospiral infection.

In the experimental leptospiral infection, Fr II exhibited protective activity at doses of 100 to 1 μg per hamster, but OE showed more potent activity at a dose of 0.1 μg per hamster. The results correlated well with the results of tests of agglutinating antibody-inducing activity and opsonic antibody-inducing activity of Fr II and OE. It is well known that immunogenicity is usually correlated with the molecular weight of an antigen. Auran et al. (5) demonstrated that the OE fraction extracted with 0.02% SDS retained a membrane structure arranged in micelles, as observed by electron microscopy. It seemed likely that the immunogenicity of the PAg was reduced by the reduction in the molecular weight of the antigen resulting from extraction of the antigen. This result suggested that the maintenance of immunogenicity, for example, by incorporation of the PAg into a liposome membrane, is needed to include the PAg in a component vaccine.

On the basis of chemical nature and SDS-PAGE profiles, a type-specific main antigen (24), an F4 antigen (2), a glycolipid antigen (22), LPS (15, 17), and an LPS-like substance (23) prepared from various kinds of leptospiral serovars seems to be similar to the PAg. However, there are a few reports on the protective activities of a type-specific main antigen (24) and an F4 antigen (2) that suggest they are weak in comparison with WC and the PAg. The differences in the protective activities may result in methods and strains

TABLE 3. CL response of sera from mice immunized with various preparations of *L. interrogans* serovar lai[a]

Sample	Dose (μg/mouse)	Mean CL response ± SD (light level unit)[b] on day:			
		5	13	20	27
Fr I	100	0.41 ± 0.06	0.69 ± 0.09	0.42 ± 0.10	0.59 ± 0.09
	10	0.75 ± 0.05	0.90 ± 0.03	0.45 ± 0.17	0.52 ± 0.13
Fr II	100	0.66 ± 0.24	1.25 ± 0.73	7.18 ± 4.35	11.07 ± 1.43
	10	0.62 ± 0.18	1.16 ± 0.38	4.22 ± 0.80	5.91 ± 1.71
OE	100	0.84 ± 0.04	11.76 ± 1.02	9.98 ± 0.45	9.48 ± 2.77
	10	0.67 ± 0.16	0.81 ± 0.27	5.48 ± 2.37	5.52 ± 1.39
WC	100	0.88 ± 0.18	7.48 ± 2.93	10.28 ± 3.06	11.04 ± 6.56
	10	0.69 ± 0.19	3.60 ± 2.08	8.86 ± 2.28	6.05 ± 1.80

[a] Each preparation was given i.p. to ddY mice on days 0, 3, 6, 9, 12, and 15. Blood samples were collected from the retro-orbital venous sinus on days 5, 13, 20, and 27.
[b] Three mice per group.

used in antigen preparations. Determination of the similarities of these antigens is necessary for further comparative studies of antigens prepared from the same strains.

A chemical analysis of Fr II is currently in progress in our laboratory.

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