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Comparative Analysis of Lipopolysaccharide and Lipid Antigens of *Leptospira interrogans* Serovars

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Lipopolysaccharide (LPS) or glycolipid antigens of Leptospira interrogans *have been candidates as sergroup or serotype specific antigen. In this study, therefore, we prepared the LPS and lipid antigens from* L. interrogans *serovars* lai, icterohaemorrhagiae, copenhageni, canicola, pomona, grippotyphosa, *and a Korean isolate 30R, The LPS antigens were analyzed by a polyacrylamide gel electrophoresis and lipid antigens by thin-layer chromatography, respectively. The seroreactivity of the antigens were also examined with homologous or heterologous antisera using an enzyme-linked immunosorbent assay. The LPS antigens from serovar* lai *and the strain 30R were closely related but different from serovar* icterohaemorrhagiae. *Particularly, the LPS angiens from serovars* icterohaemorrhagiae *and* grippotyphosa *were reactive only with the homologous antisera, thus indicating serovar specifictiy. However, the LPS antignes of the other serovars were reactive to the heterologous antisera. The lipid antigen of serovar* icterohaemorrhagiae *reacted only with the homologous antisera. In contrast, lipids of other serovars reacted broadly with heterologous antisera, particularly among serovars* lai, copenhageni, canicola, pomona, *and the strain 30R. The results thus indicated that the LPS and lipid antigens of* L. interrogans *may contain serovar-specific as well as cross-reactive epitopes.*

Key Words: *Leptospira interrogans,* lipopolysaccharide, lipid, glycolipid, antigen, antibody

Leptospirosis is an important zoonotic disease caused by *Leptospira interrogans*. The laboratory diagnosis of leptospirosis has been made by isolation of *L. interrogans* from patients and animals; however, due to frequent failure in growing the organisms and a long-term cultivation, the serological tests have been widely used (Farrar, 1990). The current standard method for the serodiagnosis is the microscopic agglutination (MA) test which has an excellent sensitivity in the detection of leptospiral infections in humans and animals (Alexander, 1985). Since the MA test uses the whole live organisms, besides the danger in handling the live patheogen, the method has some limitations such as low specificity, i.e., high level of cross-reactivity (Dikken and Kmety, 1978).

With the existence of more than 180 serovars in *L. interrogans* (Johnson and Faine, 1984), therefore, there should be a laborious and complicated cross-absorption experiments for serotyping of any unkonwn isolate by the MA test. The results were not definitive. For an example, since the first major outbreaks of leptospirosis in 1984 (Kim, 1984), there were numerous isolates of *L. interrogans* in Korea (Cho *et al.* 1984; Lee *et al.* 1984). Despite the extensive studies by the cross agglutinin absorption test, there had been some confusion in serotyping of the Korean isolates whether the majority of the isolates belong to servar *mwogolo* or *lai*, or an unidentified new serovars within the serogroup Icterohaemorrhagiae (Chang *et al.* 1989; Cho *et al.* 1989; Kim *et al.* 1987: Kim, 1987; Oh *et al.* 1986 & 1991).

As alternate tools, other serological tests have been developed such as the macroscopic slide agglutination test (Alexander, 1985), the indirect

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hemagglutination test (Alexander, 1985), the enzyme-linked immunosorbent assay (ELISA) (Terpstra *et al.* 1985), and the radioimmunoassay (Kawaoka *et al.* 1979), Because of using the whole cells or disrupted antigens, however, most of these tests could not still overcome the cross-reactivity among serovars of *L. interrogans*. Therefore, the identification of serogroup or serovar specific antigens of *L. interrogans* would be of great importance for epidemiological studies as well as for the clinical implications.

One of the candidates as specific antigen of *L. interrogans* has been lipopolysaccharides (LPS) (Shinagawa and Yanagawa, 1972) as used widely in serogrouping of the gram-negative bacteria such as *Salmonella* (LeMinor, 1984). Recently, there was a report of serovar specific glycolipid antigen of serovar *canicola* (Ono *et al.* 1987); particularly, the presence of specific glycolipid looked promising considering the importance of glycolipid antigens in serotyping of *Mycobacterium avium* (Brennan *et al.* 1978). In this study, therefore, we compared the LPS and lipid antigens of serovars *icterohaemorrhagiae, copenhageni, lai, canicola, grippotyphosa, pomona,* and a Korean isolate 30R to explore the possibility of identifying serovar specific antigen and of determining serovar of the Korean isolate.

MATERIALS AND METHODS

Organisms

Six serovars from four serogroups of *L. interrogans* and one Korean siolate were obtained from the National Institute of Health, Seoul, Korea and from the WHO/FAO Collaborating Center for Reference and Research on Leptospirosis, Amsterdam, the Netherlands (Table 1). The organisms were maintained and cultivated in the EMJH medium (Difco Laboratories, Detroit, Mich.) at 37°C in a shaking incubator for 2 ~3 weeks to yield a cell density of about 10^7 cells/ml. About 10 liters of culture were prepared for each serovar for the antigenic analysis. The organisms were killed by heatng at 80°C for 30 min and then harvested by centrifugation at 15,000 *g* for 30 min. After washing the organisms twice with phosphate buffered saline (PBS), pH 7.2, the pellet was suspended in a minimal volume of distilled water (DW) and lyophilized before measuring the dry weight.

Table 1. Serovars and sources of *L. interrogans*

Serogroups	Serovars	Sources
Icterohae-morrhagiae	*icterohae-morrhagiae*	NIH, Korea
	copenhageni	NIH, Korea
	lai	NIH, Korea
	strain 30R	NIH, Korea
Canicola	*canicola*	WHO/FAO
Pomona	*pomona*	WHO/FAO
Grippotyphosa	*grippotyphosa*	WHO/FAO

Preparation of lipid antigens

Lipids were extracted from the lyophilized whole cells of each serovar with $CHCl_3$: CH_3OH (2 : 1) solution (30 ml/g dry weight) at 50°C overnight twice (Brennan *et al.* 1978). The extract solution were pooled and dried under N_2 flow.

The lipid extracts were then washed using $CHCl_3$: CH_3OH : H_2O (4 : 2 : 1) solution and the organic phase was collected and dried under N_2. The washed lipid was used as the total lipid antigen for detecting antibodies.

The washed lipid extracts were dissolved in $CHCl_3$ and applied to silical gel (70~230 mesh; Aldrich Chemical Co., Milwaukee, Wis.) column. Lipids were then eluted first with two bed volumes of $CHCl_3$, followed by 5%, 10%, 20% 30%, 40%, 50 % CH_3OH in $CHCl_3$, and finally with CH_3OH. Each lipid fraction was dried under N_2 flow and examined for the presence of glycolipids by thin-layer chromatography (TLC) using various developing solutions. Lipids were located by spraying with 10% H_2SO_4 in ethanol.

Preparaton of lipopolysaccharide antigens

From the residues of the whole cells after extracting lipids, lipopolysaccharides (LPS) were isolated by the modified Westphal phenol extraction method (Keleti and Lederer, 1974). Briefly, the residue of each serovar was suspended in DW (35 ml/g) at 68°C and mixed vigorously with the equal volume of 90% phenol (preheated to 68°C) in a shaking water bath. After cooling to about 10°C in an ice bath, the mixture was centrifuged at 12,000 *g* for 45 min. The upper layer was then collected, and dialyzed against DW for three days to remove phenol. After dialysis, the LPS containing solution was centrifuged at 5,000 *g* for 15 min and finally, the supernatant was lyophilized. The carbohydrate concentration of the LPS antigens was determined by phenol-sulfuric assay using glucose as a standard

(Keleti and Lederer, 1974)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The LPS antigens were separated on 10% gel by a discontinous SDS-PAGE as reported by Laemmli (1970). Briefly, the LPS antigens were suspended in a sample buffer containing 30 mM Tris HCl, pH 6.8, 1% SDS, 10% glycerol, 0.1% (v/v) 2-mercaptoethanol, and bromophenol blue and heated at 100°C for 3 min. The antigen lysate (20 μl containing 15 μg of antigen) was then applied to each lane of the stacking gel consisted of 4% acrylamide-0.13 % bisacrylamide, 0.1% SDS, 0.1% ammonium persulfate, 0.1% (v/v) TEMED in 10 mM Tris-HCl, pH 6.8. The resolving gel contained 10% acrylamide-0.33% bisacrylamide, 0.1% SDS, 0.1% ammonium persulfate, 0.04% TEMED in 0.4 M Tris-HCl, pH 8.8. Electrophoresis was carried out in 25 mM Tris, 250 mM glycine buffer, pH 8.3, containing 0.1% SDS at 40 mA for about 16 h in a vertical electrophoresis system (Bio-Rad, Richmond, Calif.). The LPS in the gel were then stained with a silver staining kit (Bio-Rad).

Preparation of rabbit antiserum to *L. interrogans*

The whole cell sonicates of each serovar were used for the immunization of rabbits to prepare the hyperimmune antisera to the corresponding serovr. A portion of 0.5 ml of the whole cell sonicates (2 mg/ml) was mixed with the equal volume of Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, Mo.) using a double-end needle. The mixture (1 ml) was injected into a rabbit intramuscularly, and booster immunizations were given periodically to maintain high level of antibodies.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA protocol desribed by Voller *et al.* (1979) was employed with minor modifications reported as previously (Cho *et al.* 1983 & 1988). Briefly, the whole cell sonicate and LPS antigens were diluted in carbonate-bicarbonate buffer, pH 9.6 and the lipid antigens were dissolved in ethanol. A 50 μl portion of diluted antigens (1~10 μg/ml for the whole cell and lipid antigens; 0.1~1.0 μg/ml for the LPS antigens) of each serovar was added to wells of microtitier plates (Dynatech Laboratories, Inc., Alexandria, Va.), and incubated at 37°C for overnight. Wells were then washed with phosphate buffered saline solution (PBS), pH 7.4, containing 0.05% (v/v) tween 20 (PBST) and blocked by adding 100 μl of PBST-0.05% (w/v) bovine serum albumin (BSA) at 37°C for 1 h. After emptying the wells, 50 μl of corresponding rabbit antisera were added and incubated at 37°C for 90 min. After washing the wells with PBST, 50 μl of peroxidase-conjugated anti-rabbit immunoglobulin G (Organon Teknika Co., West Chester, Pa.) diluted 1 : 2,000 in PBST-5 % (v/v) normal goat serum (NGS) were add and incubated at 37°C for 1 h. After another washing, 50 μl of substrate solution containing o-phenylenediamine and H_2O_2 were added to wells and incubated at room temperature for about 15 min. The reaction was stopped with 50 μl of 2.5 N H_2SO_4 and the absorbance was then read at 490 nm, For lipid antigens, tween 20 was not added to any of the solutions above. Each assay was run in triplicate and the mean absorbance was used in the analysis of the results.

RESULTS

SDS-PAGE analysis of lipopolysaccharides

Lipopolysaccharides from each serovar were examined by SDS-PAGE to find any difference in their electrophoretic patterns between serovars. As shown in Fig. 1, most of the LPS antigens ran closely to each other corresponding to the protein molecules of 21~31 kDa. However, the molecular weights of these LPS antigens could not be derived

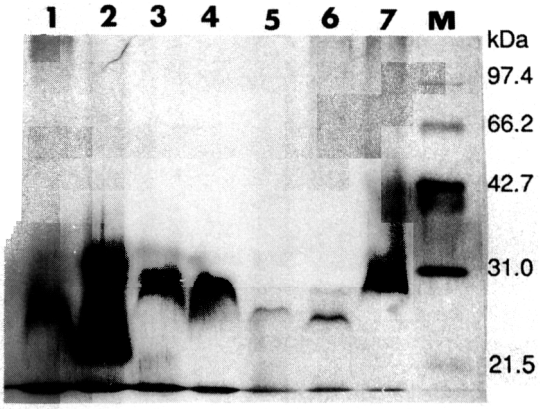

Fig. 1. SDS-PAGE of the LPS antigens of L. interrogans *serovars* grippotyphosa *(lane 1),* icterohaemorrhagiae *(lane 2),* canicola *(lane 3),* lai *(lane 4),* copenhageni *(lane 5),* pomona *(lane 6), and the Korean isolate 30R, Molecular size markers are given on the lane M.*

due to the different behavior in SDS-PAGE from the proteins. The LPS of serovars *grippotyphosa* and *icterohaemorrhagiae* were distinct from those of other serovars. Interestingly, there was a similarity in the electrophoretic pattern of LPS among serovars *canicola, lai,* and the Korean strain 30R. LPS of serovars *pomona* and *copenhageni* ran closely between each other and were somewhat similar to that of serovar *grippotyphosa*.

TLC analysis of lipids

To compare the lipid profiles among the serovars, lipids eluted from the silica gel column with different solvent polarity were analyzed in TLC. In the 5 % CH_3OH in $CHCl_3$ fraction (Fig. 2A), there were minor differences in the lipid profile between serovars. For example, a similar lipid pattern was

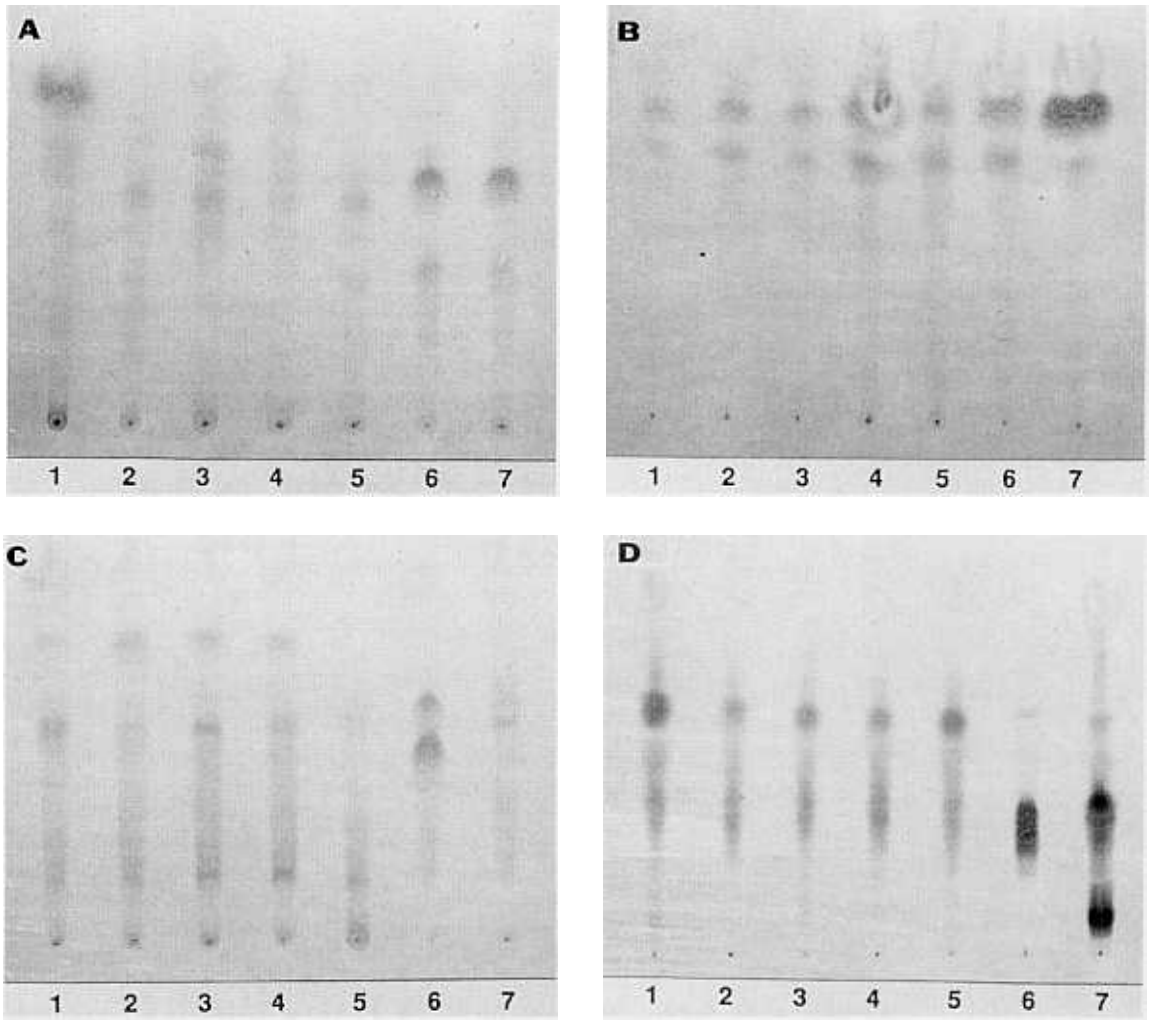

Fig. 2. TLC of the lipids of L. interrogans *serovars* canicola *(lane 1),* icterohaemorrhagiae *(lane 2),* lai *(lane 3), the Korean strain 30R (lane 4),* grippotyphosa *(lane 5),* copenhageni *(lane 6), and* pomona *(lane 7). The total lipids were applied to a silica gel column and eluted with 5% (A), 10% (B), 30% (C), and 50% (D) CH_3OH in $CHCl_3$. Chromatogram was developed in $CHCl_3 : CH_3OH : H_2O$ (A, 90 : 10 : 1; B, 60 : 12 : 1; C, 65 : 25 : 4; D, 60 : 35 : 8) and lipids were located with 10% H_2SO_4 in ethanol.*

Table 2. Seroreactivity of the whole cell antigens of *L. interrogans* with homologous and heterologous antisera

Antiserum against serovars	Whole cell antigens from serovars						
	icterohae-morrhagiae	*copenha-geni*	*lai*	Strain 30R	*canicola*	*pomona*	*grippo-typhosa*
icterohaemorrhagiae	**1.39***	1.24	1.30	1.20	1.14	1.28	1.30
copenhageni	1.13	**1.66**	1.54	1.42	1.30	1.62	1.33
lai	0.99	1.42	**1.52**	1.44	1.23	1.83	1.29
Strain 30R	1.45	1.64	1.79	**1.55**	1.52	1.74	1.65
canicola	0.89	1.28	1.26	1.37	**1.43**	1.44	1.07
pomona	0.93	1.11	1.42	1.10	0.99	**1.46**	1.16
grippotyphosa	1.05	1.14	1.42	1.08	1.02	1.65	**1.32**

* Numbers indicate the mean absorbance of three wells and the average of the standard deviations was 2.9±1.6% of the mean values.

Table 3. Seroreactivity of the LPS antigens of *L. interrogans* with homologous and heterologous antisera

Antiserum against serovars	LPS antigens from serovars						
	icterohae-morrhagiae	*copenha-geni*	*lai*	Strain 30R	*canicola*	*pomona*	*grippo-typhosa*
icterohaemorrhagiae	**1.69***	0.11	0.09	0.06	0.02	0.09	0.05
copenhageni	0.08	**1.45**	1.66	1.67	1.78	1.44	0.02
lai	0.01	0.44	**1.49**	1.57	1.12	0.90	0.01
Strain 30R	0.13	1.02	1.61	**1.72**	1.30	1.26	0.07
canicola	0.05	0.67	1.71	1.00	**1.72**	1.67	0.01
pomona	0.21	0.06	0.31	0.05	0.75	**1.47**	0.01
grippotyphosa	0.15	0.12	0.09	0.06	0.03	0.10	**1.11**

* Numbers indicate the mean absorbance of three wells and the average of the standard deviations was 2.9±1.6% of the mean values.

found between serovars *copenhageni* and *pomona*, between *lai* and the strain 30R, and between *icterohaemorrhagiae* and *grippotyphosa*. However, there was no difference in the lipid pattern of 10% CH_3OH in $CHCl_3$ fraction among the serovars (Fig. 2B). In the 30% CH_3OH in $CHCl_3$ fraction (Fig. 2C), the lipid profiles of serovars *grippotyphosa*, *copenhageni*, and *pomona* were different from the other serovars; meanwhile, the same lipid patterns were found among the serovars *canicola*, *icterohaemorrhagiae*, *lai* and the strian 30R. Finally, in the more polar 50% CH_3OH in $CHCl_3$ fraction (Fig. 2D), the lipids of serovars *copenhageni* and *pomona* were different from those of other serovars.

Seroreactivtiy of LPS and lipid antigens

The seroreactivity of the LPS and lipid antigens from each serovar were examined by ELISA first using the homologous rabbit antiserum to determine proper antigen concentration and serum dilution. Subsequently, each antigen was reacted with the heterologous antiserum to examine the cross-reactivity between serovars under study. When the whole cell sonicate antigens were used, there were marked cross-reactivity among the serovars with the heterologous antisera (Table 2), although there were some difference in the reactivity between the homologous and heterologous antisera in certain serovars. For example, the whole cell antigen of serovar *lai* reacted strongly with antisera against all other serovars as well as serovar *lai* in ELISA. Likewise, antisera against the Korean strain 30R reacted strongly with the antisera against all serovars under study.

In contrast, LPS antigens of serovars *icterohaemorrhagiae* and *grippotyphosa* showed reactivity

Table 4. Seroreactivity of the lipid antigens of *L. interrogans* with homologous and heterologous antisera

Antiserum against serovars	Lipid antigens from serovars (Strain)						
	icterohae-morrhagiae	*copenha-geni*	*lai*	230R	*canicola*	*pomona*	*typhosa*	*grippo-*
icterohaemorrhagiae	**1.40***	0.29	0.46	1.02	0.50	0.23	0.21
copenhageni	0.09	**1.81**	0.69	0.82	0.56	0.68	0.07
lai	0.06	1.37	**1.31**	1.22	0.78	1.06	0.13
Strain 30R	0.07	1.39	1.04	**1.05**	0.69	0.52	0.01
canicola	0.11	1.17	0.79	0.19	**1.85**	1.05	0.33
pomona	0.09	0.52	0.75	0.63	0.19	**1.34**	0.04
grippotyphosa	0.08	0.19	0.32	0.08	0.10	0.51	**0.59**

* Numbers indicate the mean absorbance of three wells and the average of the standard deviations was 2.9±1.6% of the mean values.

only with the homologous antisera (Table 3). In addition, antisera against these two serovars had ELISA reactivity only to LPS antigens of the corresponding serovars. Interestingly, however, LPS antigens of serovars *copenhageni, lai, canicola* seemed reactive to the heterologous antisera of these serovars. Particularly, the LPS antigens of serovars *lai* and the strain 30R showed a similar reactive pattern between each other to the homologous and heterologous antisera, thus indicating the close relatedness in its antigenicity. The LPS antigen of serovar *pomona* reacted strongly with antisera against serovars *pomona* and *canicola,* and moderately with antisera against serovar *lai.*

The seroreactivity of lipid antigens showed a moderate discrimination among the serovars under study. For example, lipids of serovar *icterohaemorrhagiae* showed strong reactivity only with antisera to the organism, indicating the serovar specificity (Table 4). However, the antiserum against the serovar showed a minor or moderate reactivtiy with the lipid antigens of other serovars. In addition, the lipid antigens of serovars *copenhageni, lai, canicola* and *pomona* showed the strongest reactivity with the antisera to the corresponding serovars, although there was a moderate reactivity with the heterologous antisera among those serovars. The lipid antigens of the strain 30R showed a little difference in the reactive pattern from serovar *lai* and seemed reactive with antisera against all serovars belonging to the serogroup Icterohaemorrhagiae.

DISCUSSION

Although the cross-absorption MA tests have been used as the standard test for serotyping of *L. interrogans* (Alexander, 1985), the nature of the serovar or serogroup specific antigens remains largely unknown. Recently, Terpstra and Schoone (1983) identified at least 49 precipitating antigens of *L. biflex* by the crossed immunoelectrophoretic analysis. Considering this complexity of antigens and the existence of more than 180 serovars in *L. interrogans* (Johnson and Faine, 1984), the isolation of serovar or serogroup specific antigens would be of great value in the serotyping of *L. interrogans* and in the serodiagnosis of leptospirosis. In the advent of a wide use of LPS as serogroup antigens in gram-negative bacteria (LeMinor, 1984), and of glycolipids as serotype or species specific antigens in mycobacteria (Brennan *et al.* 1978; Brennan, 1984), we examined the LPS and lipid antigens of *L. interrogans* for seroreactivity.

For the isolation of LPS, we used the residue of whole cells after the extraction of lipids. Therefore, only LPS tightly bound to the cell wall skeleton were left and isolated by the subsequent hot phenol-water extraction. The resulting LPS of each serovar showed one or two bands stained with silver nitrate in SDS-PAGE. Using a similar procedure, Shinagawa and Yanagawa (1972) isolated a type-specific main (TM) antigen which gave one major precipitating band in immunodiffusion. However, Oh *et al.* (1991) reported 3~4 major LPS bands in

SDS-PAGE when LPS were isolated directly from the whole cells by the phenol extraction. Therefore, the removal of lipids before phenol extraction seemed important for the isolation of homogeneous LPS, although the importance of other LPS antigens was not evaluated.

Of interest was that SDS-PAGE patterns and seroreactivity of LPS antigens of *L. interrogans* were closely related. For examples, serovars *grippotyphosa* and *icterohaemorrhagiae* showed distinct SDS-PAGE patterns and exclusive serovar specificity. This strongly supports the presence of the TM antigen which had the nature of LPS and was specific only to a Kyoto strain of the Hebdomadis serogroup (Shinagawa and Yanagawa, 1972). A close relatedness was also found between serovar *lai* and the Korean strain 30R, thus indicating that the strain 30R may belong to the same serogroup or serotype. Therefore, the LPS antigens isolated in this study may confer the serovar or serogroup specificity in certain serovars of *L. interrogans* and may be correspond to the polysaccharide complement-fixing antigen described by Schneider (1954a & 1954b) and the TM antigen (Shinagawa and Yanagawa, 1972). However, a moderate or considerable cross-reactivity of LPS antigens of serovars *canicola, lai, pomona, copenhageni* with the heterologous antisera may indicate that the LPS antigens also contain the cross-reacting immunodeterminants in certain serovars.

The lipid antigens of *L. interrogans* also showed a limited degree of serovar specificity. Particularly, the lipids of serovar *icterohaemorrhagiae* showed the exclusive specificity. However, the lipid antigens of the other serovars contained cross-reactive antigens binding moderately to the heterologous antisera. This may reflect the numerous glycolipids which behaved similarly in the TLC analysis. Although we didn't have the chance to examine the seroreactivtiy of each glycolipid in this study, Ono *et al.* (1987) showed the evidence of serovar specific glycolipid antigen in serovar *canicola* using monoclonal antibodies. Therefore, it would be of great interest to determine which glycolipid(s) retains the specificity in future studies.

Interestingly, there was a minor difference in the seroreactivity of lipids between serovar *lai* and the Korean isolate 30R despite the similarity of LPS between the two strains. This may indicate that the strain 30R is different serologically from the serovar *lai*. In fact, Cho *et al.* (1989) reported that the strain 30R is different in it seroreactivity from other Korean isolates and Oh *et al.* (1991) did not include the strain 30 in any of the newly proposed serovars *yeonchon* and *hongchon* from Korea. Therefore, this study suggests that the LPS and lipid antigen of the LPS and lipid antigen of *L. interrogans* may retain serovar or serogroup specific antigens. Further chemical analysis is, however, essential for explanation of the specificity of the LPS and lipid antigens.

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