Protective activity of glycolipid antigen against infection by *Leptospira interrogans* serovar *canicola*

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A protective glycolipid antigen (PAG) was extracted from *Leptospira interrogans* serovar *canicola* with chloroform/methanol/water (1:2:0.8, by vol.) and partially purified by silica gel column chromatography. The PAG elicited a protective response in hamsters and in cyclophosphamide-treated mice subsequently challenged with homologous *Leptospira*. The PAG band was detected as a single smear-like band, corresponding to a protein of 23–30 kDa, by silver-staining in SDS-PAGE. In immunoblots, this band reacted with a monoclonal antibody, A5, which agglutinated serovar *canicola* and recognized a serovar-specific antigen. Furthermore, the PAG did not migrate on silica gel TLC, but was detected at the origin as a ninhydrin- and naphthol-positive spot. This suggests that PAG is a hydrophilic molecule with a carbohydrate chain that contains amino groups, possibly as amino sugars.

Introduction

Vaccination is thought to be the most effective way to control leptospirosis and chemically inactivated bacterins are used in many countries (Bey & Johnson, 1986). Previous studies have shown that the outer-envelope fraction of *Leptospira* induces better protection than lyophilized whole cells (Auran *et al.*, 1972; Bey *et al.*, 1974). Monoclonal antibody (mAb) against leptospiral 'lipopolysaccharide' (LPS) protects newborn guinea pigs from experimental infection by *Leptospira* (Jost *et al.*, 1986). The partial purification and protective activity of a glycolipid antigen (PAG) of serovar *lai* (Icterohaemorrhagiae serogroup) has been reported (Masuzawa *et al.*, 1989b).

Ono *et al.* (1987) purified the glycolipid antigen from *L. interrogans* serovar *canicola* (Canicola serogroup) and characterized its chemical properties. By using mAb A5 against *L. interrogans* serovar *canicola*, it appeared that antigenic determinants of the agglutinin of *Leptospira* were present in the carbohydrate moiety of the outer envelope and that mAb A5 protected hamsters from infection (Yanagihara *et al.*, 1988). In the present study, the purification of the PAG of serovar *canicola* was attempted. Its protective activity in hamsters and cyclophosphamide-treated mice was evaluated and its

chemical nature characterized by TLC and SDS-PAGE.

Methods

Organisms. For the preparation of antigen, *L. interrogans* serovar *canicola* strain Moulton was cultured in Baseman-Cox medium (Baseman & Cox, 1969) at 30 °C for 7 d. A virulent strain was maintained by passage in hamsters; it was not subcultured more than twice.

Preparation of PAG. PAG was extracted from lyophilized cells as previously described (Masuzawa *et al.*, 1989b). The extract was separated into two fractions: a chloroform fraction and a methanol/water fraction. No differences in SDS-PAGE profiles (data not shown) nor in protective activity were observed between the chloroform and methanol/water fractions (Table 1). The two fractions were therefore pooled [the chloroform/methanol/water (CMW) extract]. The CMW extract obtained was further purified by silica gel (silica gel 60; Merck) column chromatography. Its elution profile was monitored by SDS-PAGE, and two fractions – fraction I (Fr I) and PAG – were obtained. Fr I was eluted with chloroform/methanol/water (1:2:0.6, by vol.); PAG was eluted with chloroform/methanol/water (1:2:0.8, by vol.). After evaporation of the solvent under reduced pressure below 40 °C, each fraction was suspended in a small amount of distilled water and lyophilized.

SDS-PAGE and immunoblot. The purity of Fr I and PAG was monitored by SDS-PAGE using a 15% (w/v, acrylamide) gel according to the method of Laemmli (1970). After electrophoresis, gels were either stained by the silver staining method of Tsai & Frasch (1982) and with Coomassie brilliant blue R-250 or transferred onto a nitrocellulose membrane (pore size 0.2 µm; Toyo-roshi Co.) by the method of Towbin *et al.* (1979). The antigen bands on the membrane were immunostained with mAb A5 (Masuzawa *et al.*, 1989a), which was raised against the agglutinin of *L. interrogans* serovar *canicola*.

Abbreviations: CMW, chloroform/methanol/water; EIA, enzyme immuno-assay; Fr I, fraction I; LPS, lipopolysaccharide-like substance; mAb, monoclonal antibody; PAG, protective antigen.

Fig. 1. Antigen profiles of *L. interrogans* serovar *canicola* as shown by SDS-PAGE and immunostain. (a) Silver-stained gel; (b) immunostaining with mAb A5 as primary antibody against serovar *canicola* (right). Lanes: 1, whole cells; 2, CMW extract; 3, fraction I; 4, PAG; 5, residual fraction.

Fig. 2. Antigen profiles of *L. interrogans* serovar *canicola* as shown by TLC and TLC/EIA. Each antigen (50 µg) was spotted on the TLC plate and developed in a solution of methyl acetate/1-propanol/chloroform/methanol/0.25% potassium chloride (25:20:20:20:17, by vol.). Antigen spots were detected by Dittmer reagent (phospholipid), ninhydrin reagent (amino group) and naphthol reagent (carbohydrate) on TLC plates. In the immunostain, each antigen was spotted from the left with 0.2, 0.4, 0.6, 0.8 and 1.0 µg of material and developed with the above solution. Spots of specific antigen were visualized by immunostaining with mAb A5 as the primary antibody.

Protection tests. The protective immune response induced by each fraction and by whole cells was evaluated in hamsters and mice. Male Golden hamsters (4-week-old) and BALB/c mice were vaccinated intraperitoneally with the preparations of serovar *canicola* on day 0 and 7, respectively. The BALB/c mice were also injected intraperitoneally (300 mg kg⁻¹) with cyclophosphamide (Sigma) on day 12, in order to suppress antibody production (Adler *et al.*, 1976, 1977). These animals were challenged intraperitoneally with virulent serovar *canicola* culture on day 14. The survival of hamsters and mice was observed for 14 d after the challenge, at which time the survivors were killed and their kidneys removed. The kidneys were inoculated into Korthof medium and incubated at 30 °C for 14 d to detect the presence of *Leptospira*.

TLC and TLC/enzyme-immuno assay (EIA). Fr I or PAG were spotted onto plastic TLC plates (Polygram Sil G; Marchery-Nagel) and developed with methyl acetate/1-propanol/chloroform/methanol/0.25% (w/v) potassium chloride (25:20:20:20:17, by vol.). Phosphati-

dylethanolamine, lyso-phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine and sphingomyelin (Sigma) were used as standard phospholipids. Each spot was detected by Dittmer reagent for phospholipids, naphthol reagent for carbohydrates and ninhydrin reagent for amino groups. TLC/EIA was done with mAb A5 as the primary antibody by the method of Higashi *et al.* (1984).

Results

SDS-PAGE, immunoblot, TLC and TLC/EIA

After silver staining of SDS-PAGE gels, the CMW extract and PAG of *L. interrogans* serovar *canicola* appeared as a smear-like band, corresponding to protein

Table 1. *Protective effect of PAg against L. interrogans serovar canicola infection*

Hamsters were injected intraperitoneally with the stated immunizing agent on day 0 and 7 and challenged intraperitoneally with *L. interrogans* serovar *canicola* (1.25×10^8 cells per hamster) on day 14. NT, Not tested.

Immunizing agent	Dose per hamster (μ g)	Survivors after 14 d/no. of hamsters tested (% survivors)	Kidney culture (positive/survivors)
Whole cells	5.0	4/5 (80)	0/4
	1.0	4/5 (80)	0/4
Chloroform fraction	20.0	5/5 (100)	0/5
	5.0	4/5 (80)	0/4
	1.0	4/5 (80)	0/4
Methanol/water fraction	20.0	5/5 (100)	0/5
	5.0	3/5 (60)	0/3
	1.0	3/5 (60)	0/3
Residual fraction	20.0	0/5 (0)	NT
	5.0	0/5 (0)	NT
Saline (control)	0	0/5 (0)	NT

Table 2. *Protective effect of PAg against L. interrogans serovar canicola infection*

Hamsters were injected intraperitoneally with the stated immunizing agent on day 0 and 7 and challenged intraperitoneally with *L. interrogans* serovar *canicola* (5.6×10^7 cells per hamster) on day 14. NT, Not tested.

Immunizing agent	Dose per hamster (μ g)	Survivors after 14 d/no. of hamsters tested (% survivors)	Kidney culture (positive/survivors)
Whole cells	10.0	5/5 (100)	0/5
	1.0	5/5 (100)	0/5
Fraction I	10.0	0/5 (0)	NT
	1.0	0/5 (0)	NT
PAg	10.0	4/5 (80)	0/4
	1.0	5/5 (100)	0/5
Residual fraction	10.0	0/5 (0)	NT
	1.0	0/4 (0)	NT
Saline (control)	0	0/5 (0)	NT

of molecular mass about 23–30 kDa (Fig. 1). Coomassie blue staining failed to reveal the band in the CMW, Fr I and PAg lanes (data not shown). Furthermore, PAg of *L. interrogans* serovar *canicola* only reacted with mAb A5 in the immunostain. The band found in PAg was not detected in the extraction residue or in Fr I.

TLC (Fig. 2) showed that Fr I consisted of two spots of Dittmer- and ninhydrin-positive material with R_F

Table 3. *Protective effect of PAg against L. interrogans serovar canicola infection in cyclophosphamide-treated mice*

Mice were injected intraperitoneally with the stated immunizing agent on day 0 and 7 and injected intraperitoneally with cyclophosphamide (300 mg kg^{-1}) on day 12 and challenged intraperitoneally with *L. interrogans* serovar *canicola* (1.0×10^8 cells per mouse) on day 14. NT, Not tested.

Immunizing agent	Dose per mouse (μ g)	Mean survival time $\pm$ SD (d)	Survivors after 14 d/no. of mice tested (% survivors)	Kidney culture (positive/survivors)
Whole cells	10.0	15.0 ± 0	5/5 (100)	0/5
	1.0	15.0 ± 0	5/5 (100)	0/5
	0.1	11.2 ± 3.5	0/3 (0)	NT
PAg	10.0	15.0 ± 0	5/5 (100)	0/5
	1.0	13.4 ± 3.6	4/5 (80)	0/4
	0.1	9.8 ± 5.0	2/5 (40)	0/2
Saline (control)	0	10.0 ± 1.4	0/5 (0)	NT

values of 0.65 and 0.52, which were identified as phosphatidylethanolamine and lyso-phosphatidylethanolamine, respectively, by comparison with standard phospholipids. Only one spot of ninhydrin- and naphthol-positive material was observed in PAg at the origin of the chromatogram and it was immunostained by mAb A5. The Fr I sample was not immunostained.

Protective activity in hamster and in cyclophosphamide-treated mice

Both the chloroform fraction and the methanol/water fraction exhibited the same degree of protective activity as whole cells (Table 1). However, no protective effect was elicited by the residual fraction. These results indicated that the protective antigen was efficiently extracted by the CMW method from dried cells of serovar *canicola*.

The CMW extract of serovar *canicola*, after further purification by silica gel column chromatography, had a protective activity which was comparable with that of lyophilized whole cells (Table 2). Whole cells and PAg appeared to protect hamsters from infection by homologous *Leptospira*: *Leptospira* were not detected in the kidneys of any survivors. On the other hand, Fr I and the extraction residue did not show any protective activity.

Protective activity in cyclophosphamide-treated mice (antibody production suppressed) was compared using whole cells and PAg (Table 3). Immunization with PAg or whole cells at doses of 1 to $10 \mu\text{g}$ per animal elicited protective activity such that live leptospires were not recovered from the kidneys of the survivors. Administration of 0.1 μg whole cells per animal was ineffective

against homologous infection, but the same dose of PAg produced a weak protective effect.

Discussion

A similar electrophoretic profile to that found in this study was observed in a PAg preparation of serovar *lai* (Masuzawa *et al.*, 1989b). Electrophoretic profiles of PAg from serovar *canicola* were quite similar to that of the 'lipopolysaccharide-like substance, LLS' of serovar *canicola* prepared by Shimizu *et al.* (1987) and that of 'LPS' of serovar *copenhageni* (data not shown) provided by Dr B. Adler (Jost *et al.*, 1986). The yield of purified LLS, extracted from leptospiral cells by the hot-phenol/water method, was only a small percentage of the starting material (Shimizu *et al.*, 1987). The PAg prepared by the CMW method in this study gave yields from dried cells as high as about 20% and it was rarely contaminated with protein. The residual fraction after CMW extraction did not contain the band which corresponded to PAg by silver stain. Furthermore, the PAg showed a potent protective effect, but the residual fraction was ineffective against leptospiral infection. The evidence demonstrated that the PAg was satisfactorily extracted from leptospiral cells by CMW extraction and that it was the principal protective antigen of leptospire. In experimental lethal infection of cyclophosphamide-treated mice, which were more sensitive than hamsters to leptospiral infection, 0.1 µg of PAg per mouse gave partial protection, and it appeared that the protective effect of PAg was more potent than that of whole cells on a weight for weight basis.

Our study confirms that PAg can be effectively extracted from leptospiral cells by CMW treatment and can be purified by silica gel column chromatography. Furthermore, PAg was immobile on silica gel TLC: it remained at the origin and was detected by ninhydrin and naphthol. This suggests that PAg might have hydrophilic carbohydrate chains with amino groups, which could be amino sugars.

Further studies on the chemical composition and LPS-like activity of PAg are now in progress.

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