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Immunologic reactivity of a lipopolysaccharide-protein complex of type A *Pasteurella multocida* in mice

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The immunologic reactivity of a lipopolysaccharide (LPS)-protein complex isolated from a potassium thiocyanate extract of a *Pasteurella multocida* (capsular type A and somatic type 3) strain was evaluated in mice. The LPS-protein complex provided 100% protection in mice against a challenge with the homologous strain. However, when the complex was fractionated into LPS and protein moieties by phenol-water treatment, both components lacked immunogenicity. The complex and extracted components were mitogenic for mouse B lymphocytes with the protein moiety the most active. Although immune serum against the LPS-protein complex protected mice against challenge thereby indicating a role for humoral immunity, the LPS-protein complex of *P. multocida* was also found to induce cell-mediated immunity. This cell-mediated immunity was demonstrated in mice immunized with the complex by: (1) mitogenic responses of T lymphocytes, (2) induction of delayed type hypersensitivity reaction in the hind footpads, and (3) enhanced resistance to challenge infection with *Salmonella enteritidis*.

Key words: *P. multocida*, LPS-protein complex, Immunogenicity

Introduction

Pasteurella multocida belonging to Carter's capsular serotype A [8] can infect a variety of animal species. They are particularly important as an etiologic factor in the pneumonia of cattle and sheep, and in fowl cholera and a variety of clinical syndromes in rabbits used for biomedical research [7, 8, 24]. Killed and live bacterial vaccines have been utilized extensively for immunoprophylaxis in experimental and clinical circumstances [6, 9, 12, 23, 32]. However, these vaccines are of questionable efficacy and safety [6]. This has led to experimentation with various subcellular fractions of the organism in studies of bacterial pathogenesis and in the pursuit of an efficacious vaccine.

A number of subcellular fractions have been tested for the induction of immunity in experimental animals. These fractions are: a ribosomal fraction [4], purified or cloned outermembrane proteins [1, 3, 14, 25], a ribosomal-lipopolysaccharide (LPS) complex [33], purified LPS [37], LPS-protein complexes [16, 19, 20, 35, 36], and potassium thiocyanate (KSCN) extracts [30, 31, 38]. Many of these subcellular fractions provided a degree of protection in experimental animals and the role of humoral immunity against *P. multocida* infection has been suggested. However, it is not certain whether protection against *P. multocida* infection involves cellular immunity.

We previously reported that an LPS-protein complex isolated from a KSCN extract of a *P. multocida* strain (P-2383; capsular type A and somatic type 3) induced resistance in mice to challenge with the homologous strain [38]. Clinical signs in these mice following challenge infection suggested that the purified LPS-protein complex, P-2383-1, might be a better immunogen than the original KSCN extract. In this study, the immunologic reactivity of P-2383-1 was further characterized by fractionating the LPS-protein complex into LPS (P-2383-1-LPS) and protein (P-2383-1-PRO) moieties by phenol-water treatment.

Materials and Methods

Organism

Pasteurella multocida strain P-2383 (capsular type A and somatic type 3), used throughout this study, was originally isolated from a case of bovine pneumonia presented to the Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA. Culture conditions for growth of the organism, preparation of the KSCN extract and isolation of the LPS-

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protein complex (P-2383-1) from the KSCN extract have been described previously [38].

Fractionation of the LPS-protein complex

The LPS-protein complex was fractionated into LPS and protein moieties using an extended phenol-water procedure [42] as described previously [38]. Briefly, P-2383-1 suspended in 0.32 M NaCl/0.01 M tris-HC1 buffer was dialyzed against distilled water at 4°C before treatment with hot phenol (65°C). The LPS moiety (P-2383-1-LPS) was obtained from the water phase and the protein moiety (P-2383-1-PRO) was collected from the phenol phase by precipitation with cold ethanol (–20°C). Since P-2383-1-PRO was poorly soluble in water, it was solubilized by suspending 2 mg (dry weight) in 0.95 ml of distilled water and adding 0.05 ml of 0.1 N NaOH.

Preparation of the phenol-water extracted LPS (PW-LPS)

Phenol-water extracted LPS (PW-LPS) was prepared from *P. multocida* strain P-2383 using the method of Westphal and Jann (1965) with slight modification as described previously [38].

Chemical analysis

Total carbohydrate content was determined using a phenol-sulfuric acid based procedure [15] with glucose (Sigma Chemical Co., St. Louis, MO) as a standard. Protein content was determined colorimetrically by reacting the protein with Serva blue G dye (Serva Fine Chemicals Inc., Long Island, NY) using bovine serum albumin (Sigma) as a standard [34].

Active immunization and challenge of mice

White Swiss mice (Bio-Lab Corp., St. Paul. MN) of one sex, 8 weeks of age, and at least 20 g in weight were used throughout this study. The concentrations of the *P. multocida* fractions used for immunization were: P-2383-1 and P-2383-1-PRO, 1.0 mg/ml; and P-2383-1-LPS, 0.05 mg/ml. Mice were immunized by the subcutaneous route with 0.1 ml of *P. multocida* antigens for the active protection, and as well as for lymphocyte blastogenesis assay, induction of delayed-type hypersensitivity (DTH), and for the determination of the effect on the growth of *Salmonella enteritidis* (*S. enteritidis*). Mice were challenged with 0.1 ml of virulent *P. multocida* suspension intraperitoneally 2 weeks after the active immunization.

Passive immunization and challenge of mice

To produce hyperimmune serum, mice actively immunized with *P. multocida* antigens received a booster injection of 0.1 ml of the same antigen by the same route 2 weeks after the primary immunization. After 1 week, each mouse was bled from the ophthalmic venous plexus for preparation of antiserum. Antiserum from each immunizing group was pooled and the antibody titer of the pooled antiserum was determined by enzyme-linked immunosorbent assay (ELISA) as described below. For passive immunization, 0.2 ml of the antiserum was injected intraperitoneally into normal mice that were then challenged with 0.1 ml of virulent *P. multocida* suspension intraperitoneally 24 h later.

ELISA

One-tenth of an ml of P-2383-1-PRO (0.25 mg/ml protein concentration) or P-2383-1-LPS (0.5 mg/ml carbohydrate concentration) diluted in a buffer, containing 15 mM of sodium carbonate and 35 mM of sodium bicarbonate (pH 9.6), was added to each well of a flat bottomed microtiter plate (Immulon I; Dynatech Laboratories Inc., Alexandria, VA). The plate was incubated at 37°C for 24 h and stored at 4°C in a humidified chamber prior to use. The antigen-coated plate was rinsed 5 times with a cold washing containing 0.5 M NaCl, 0.5% Tween 80, 12 mM monobasic sodium phosphate and 5 mM dibasic sodium phosphate (pH 7.2). One-tenth of an ml of serial 1 : 3 dilutions of mouse serum was added to all the wells starting from a 1 : 20 dilution, and the plate was then incubated for 2 h at room temperature. The plate was washed, and 0.1 ml of peroxidase-conjugated goat anti-mouse IgG and IgM (Pel-Freez Biological, Rogers, AR) were added to each well. After incubation for 2 h, the plate was washed and 0.05 ml of a substrate solution (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) containing hydrogen peroxide and 2.2'-azinodi-(3-ethylbenzthiazoline sulfonic acid) was added to each well. After incubation for 10 min, the enzyme reaction was stopped by adding 0.1 ml of 0.5% hydrofluoric acid and the absorption at 405 nm was determined with an ELISA reader (Litton Bionetics, Charleston, SC). The titer was determined as the highest dilution with an optical density of 0.1 or greater.

Isolation of T and B lymphocytes from spleen

Between two and three weeks after the active immunization, mice from each experimental group were sacrificed and the spleens removed aseptically. Spleens were infused with 1 ml of RPMI 1640 medium (Gibco Laboratories, Grand Island, NY), containing 2 mM L-glutamine and 25 mM N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid supplemented with penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 10% fetal bovine serum (FBS; Hazleton Research Products, Denver, PA). Spleens were minced indivisually through a sterile 60-mesh stainless steel screen and the screen rinsed with 3 ml of medium. The spleen cell suspensions were transferred to screw cap tubes and the debris allowed to settle for 10 min. The non-sedimenting cell suspension was transferred

to another tube and centrifuged at 400 × g for 5 min. The pellet was washed twice with medium by centrifugation and resuspended in 2.5 ml of a filter-sterilized red cell lysing solution containing 0.645 M NH_4Cl, 0.01 M $KHCO_3$ and 0.1 mM disodium ethylenediamine tetraacetate (EDTA, Sigma). After incubation for 10 min in an ice bath with occasional shaking, the suspension was centrifuged through a layer of FBS. The supernatant was discarded and the pellet was washed twice by centrifugation. The spleen cells were counted and standardized to 10^7 cells per ml in a phosphate buffered saline solution (PBS, pH. 7.2) containing 5% FBS.

T and B lymphocytes from the spleen cell suspension were separated by a panning procedure [47], using a sterile petri dish (100 5 20 mm; Optilux, Falcon, Oxnard, CA) and rabbit anti-mouse immunoglobulins (RAM), a gift from Dr. M. J. Wannemuehler, Veterinary Medical Research Institute, Iowa State University, Ames, IA. Briefly, RAM were diluted in 10 ml of 0.05 M Trizma hydrochloride solution (Sigma), pH 9.5, and sterilized through a 0.2 μm membrane filter (Gelman Sciences Inc., Ann Arbor, MI). The final concentration of RAM was adjusted to 10 μg per ml and 10 ml of the solution was poured into the plate. After a 40 min incubation at room temperature, the solution was decanted and the plate was washed 4 times with 10 ml of PBS and once with 5 ml of PBS containing 1% FBS. To allow direct binding, 3 ml of the standardized spleen cell suspension was then poured into each RAM-coated plate. The plate was incubated on a level surface at 4°C for 70 min with unattached cells redistributed by swirling the plate for 40 min. The nonadherent cells (T lymphocytes) were removed by gently swirling and decanting the supernatant. Plates were then gently washed 5 times in 10 ml of PBS containing 1% FBS. To recover the adherent cells (B lymphocytes), 5 ml of PBS containing 5% FBS was added and the entire surface of the plate was vigorously flushed by pipet. Fluorescence microscopy, using fluorescein-conjugated goat anti-mouse IgG (Cooper Biomedical, Westchester, PA), was used to determine the purity of the pan-purified T and B lymphocytes.

Lymphocyte blastogenesis assay

Phytohemagglutinin (PHA, 20 μg/ml; Burroughs Welcome, Triangle Park, NC), concanavalin A (Con A, 25 μg/ml; Miles Laboratories, Elkhart, IN), PW-LPS of *P. multocida* (20 μg/ml, dry weight), P-2383-1 (100 μg/ml, protein concentration), P-2383-1-PRO (100 μg/ml protein concentration) and P-2383-1-LPS (40 μg/ml, carbohydrate concentration) were used for lymphocyte stimulation. Blastogenic responses of the pan-purified T and B lymphocytes were determined in a 96-well tissue culture plate (Costar, Cambridge, MA). Two hundred ml of the standardized cells (2×10^6) and 25 μl of mitogen, antigen or medium were added to each well in triplicate sets. After incubation at 37°C in a humidified CO_2 incubator for 48 h, 1 μCi of [^{3}H]-thymidine (Amersham Corp., Arlington Heights, IL) was added to each well. After an additional 16 h incubation the cells were harvested on a multiple well harvester (Katron Inc., Sterling, VA) and collected on filter pads. The cells were dried, placed in vials containing scintillation fluid and counted in a liquid scintillation counter.

Induction of DTH in the mouse footpad

The mouse footpad swelling technique described by Gray and Jenning (1955) was used to test the induction of DTH reaction against *P. multocida* fractions. Groups of mice were immunized with *P. multocida* fractions as indicated previously. Two weeks after the immunization, 0.03 ml of *P. multocida* fractions (of the same concentration used for the immunization) was injected into a hind footpad of one leg using a 27-gauge needle. The same volume of 0.32 M NaCl/0.01 M Tris-HCl buffer (Sigma) was injected into a hind footpad of the other leg as a control. The mouse footpads were observed at 2, 4, 6, 8 and 18 h and the swelling was measured after 1, 2, 3, 4, 5 and 6 days with a dial gauge caliper. Footpads were excised for histological examinations, fixed in 10% formalin, decalcified with EDTA, and paraffin sections stained with hematoxylin and eosin.

Effect on growth of *S. enteritidis* in mouse spleens

A *S. enteritidis* strain obtained from the National Veterinary Services Laboratory, USDA, Ames, IA was used in this experimentation. This strain did not react with antiserum against whole *P. multocida* cells or P-2383-1 by slide agglutination . The organism was passed five times through mice with reisolation from the spleen and the final isolate was grown for 4 h at 37°C in yolk material from 6-day old embryonated chicken eggs. The culture was divided into aliquots and stored at -70°C. To prepare the bacterial culture, the infected yolk material was thawed, inoculated on a 5% bovine blood agar plate and incubated for 24 h at 37°C. One colony was transferred to 30 ml of trypticase soy broth (TSB; Becton Dickinson and Co., Cockeysville, MD) and incubated for 18 h at 37°C. One-tenth of an ml of the culture was transferred to 5 ml of TSB and incubated for another 4 h. Mice were injected with approximately 1.5×10^4 colony forming units (c.f.u.) of the bacteria intraperitoneal. To obtain the growth curve of *S. enteritidis* in the spleen of normal mice, groups of mice were sacrificed at 1, 3, 5, 7 and 10 days after bacterial infection with the bacteria. Spleens were removed aseptically and placed in homogenization tubes containing 5 ml of PBS. They were dispersed with a Teflon pestle, serially diluted and plated on a well-dried MacConkey agar plate in duplicate. Colony counts were conducted

after incubation at 37 for 18 h. To determine the effect of the LPS-protein complex of (P-2383-1) and its fractions on the growth of *S. enteritidis*, groups of 5 mice were treated as follows: Day 0, primary immunization subcutaneously or no treatment; Day 14, secondary immunization by the footpad route or no treatment; Day 21, challenge infection with *S. enteritidis* intraperitoneally: Day 22 or 26, determination of c.f.u. per spleen.

Statistical analysis

Student's t-test was used to determine statistical significance.

Results

Chemical analysis

The carbohydrate and protein contents of P-2383-1 and its PW-extracted fractions were 12% carbohydrate and 27% protein for P-2383-1, 50% and 7% for P-2383-1-LPS, and less than 2% and 100% for P-2383-1-PRO, respectively.

Active protection

The intact LPS-protein complex (P-2383-1) provided 100% protection in mice against a challenge by strain P-2383 (Table 1). However, its fractions (P-2383-1-LPS, P-2383-1-PRO) or a mixture of the two fractions failed to provide the protection. The protein moiety (P-2383-1-PRO) mixed with 20% aluminum hydroxide induced 40% protection, but the surviving mice were clinically ill and had not completely recovered 10 days after the challenge, the last day of observation.

Antibody titer and passive protection

Pooled antiserum from the mice immunized and boosted with P-2383-1 had the highest antibody titer (1 : 43,740) to both ELISA antigens and provided 100% protection in mice (Table 2). The mice immunized and boosted with P-2383-1-LPS had an equivalent antibody titer (1 : 43,740) to the P-2383-1-PRO as the mice antiserum from P-2383-1, but a slightly lower titer (1 : 4,860) to P-2383-1-LPS.

Table 1. Immunogenicity of mice induced by a LPS-protein complex of *P. multocida* (P-2383-1) and its fractions against challenge exposure to strain P-2383

Immunizing preparation	Challenge infection	
	115 c.f.u.	103 c.f.u.
P-2383-1	5/5[a]	5/5
P-2383-1-PRO	0/5	ND
P-2383-1-PRO+20% Aluminum hydroxide	ND	4/10[b]
P-2383-1-LPS	0/5	ND
P-2383-1-PROP-2383-1-LPS	0/5	ND
Control	0/5	0/5

[a] : No. survival/ no. tested

[b] : These four surviving mice were clinically ill up to 10 days.

Table 2. Resistance of mice against challenge infection by *P. multocida* strain P-2383 following intraperitoneal administration of mouse antiserum

Antiserum[a]	ELISA antigen		Challenge infection	
	P-2383-1-PRO	P-2383-1-LPS	98c.f.u.	980c.f.u.
P-2383-1	1:43,740[b]	1:43,740	5/5[c]	5/5
P-2383-LPS	1:43,740	1:4,860	0/5	ND
P-2383-1-PRO	1:1,620	1:180	ND	ND
Control	1:60	1:60	0/5	ND

[a] Serum obtained from mice immunized with various antigens.

[b] Antibody titer

[c] No. survival/no. tested

However, this antiserum did not protect mice against the challenge. The antiserum from the mice immunized and boosted with P-2383-1-PRO had antibody titers of 1 : 4,860 to the P-2383-1-PRO and of 1 : 180 to the P-2383-1-LPS.

Mitogenic response of mouse spleen cells

Spleens from mice immunized with P-2383-1 were hyperplastic and yielded approximately 180 million cells, while spleens from the control mice and the mice immunized with other antigens were normal in size and yielded approximately 120 million cells. Thirty five percentage of the T and B lymphocytes were recovered by the panning procedure, of the total spleen cells. Membrane fluorescence indicated that at least 97% of the cells in the T and B lymphocyte preparations were homogeneous. The optimal mitogenic concentrations of *P. multocida* fractions were determined and used in blastogenesis assays except for P-2383-1-PRO. The protein content of P-2383-1-PRO was adjusted to that of P-2383-1, since higher concentrations were extremely mitogenic for B lymphocytes (data not shown).

As indicated in Table 3, the LPS-containing fractions of *P. multocida* (PW-LPS, P-2383-1, and P-2383-1-LPS) were found to induce mitogenic responses in the B lymphocytes of all groups. The protein moiety of the LPS-protein complex, P-2383-1-PRO, induced mitogenic response of B lymphocytes more as than purified PW-LPS, the original LPS-protein complex (P-2383-1) and its PW-extracted LPS fraction (P-2383-1-LPS). Although the protein content of P-2383-1 was the same as that of P-2383-1-PRO, the complex induced a much lower mitogenic response.

The well-known T lymphocyte mitogens (PHA, ConA) induced a high level of mitogenic response in T lymphocytes from all groups of mice (Table 4). However, only P-2383-1 induced a significant mitogenic response ($p<0.01$) in the T lymphocytes isolated from the mice immunized with the same antigen, but not in those isolated from mice immunized with LPS or protein fraction, in those isolated from the control mice.

Table 3. Mitogenic responses of the pan-purified B lymphocytes of mice immunized with the LPS-protein complex antigen of *P. multocida* (P-2383-1) or fractions (P-2383-1-PRO, P-2383-1-LPS) of the complex

	Immunizing preparation			
Mitogen	Control	P-2383-1	P-2383-1-PRO	P-2383-1-LPS
PHA	3.7 ± 0.2[a]	2.3 ± 1.2	3.0 ± 0.8	3.8 ± 0.6
Con A	2.1 ± 0.2	1.5 ± 1.0	1.5 ± 0.4	3.0 ± 0.9
PW-LPS	35.9 ± 4.7	41.4 ± 11.5	46.8 ± 11.9	63.5 ± 6.1**
P-2383-1	30.0 ± 6.1	30.4 ± 9.0	21.4 ± 6.4	28.0 ± 8.4
P-2383-1-PRO	68.0 ± 8.0	69.3 ± 11.5	79.1 ± 21.3	87.2 ± 17.9
P-2383-1-LPS	13.8 ± 2.3	14.8 ± 4.5	15.5 ± 5.3	23.5 ± 3.7**
None	1.0 ± 0.1	0.9 ± 0.2	1.0 ± 0.2	1.0 ± 0.1

[a]X 10^3 counts per min, mean SD (N = 5)

** P<0.01

Table 4. Mitogenic responses of pan-purified T lymphocytes of the mice immunized with the LPS-protein complex of *P. multocida* (P-2383-1) or fractions (P-2383-1-PRO, P-2383-1-LPS) of the complex

	Immunizing preparation			
Mitogen	Control	P-2383-1	P-2383-1-PRO	P-2383-1-LPS
-PHA	65.5 ± 7.8[a]	73.1 ± 18.2	63.8 ± 16.4	76.4 ± 6.7
Con A	90.8 ± 7.7	73.6 ± 11.0	66.4 ± 19.8	76.6 ± 14.5
PW-LPS	4.9 ± 0.4	4.5 ± 0.4	4.7 ± 1.7	4.1 ± 0.6
P-2383-1	5.1 ± 0.2	20.1 ± 7.5**	4.9 ± 2.0	3.6 ± 0.8
P-2383-1-PRO	5.3 ± 0.4	5.8 ± 1.5	8.1 ± 3.4	6.8 ± 2.9
P-2383-1-LPS	2.3 ± 0.1	2.7 ± 0.7	2.8 ± 0.4	3.4 ± 0.5
None	1.2 ± 0.5	1.4 ± 0.2	1.2 ± 0.3	1.4 ± 0.4

[a] X 10^3 counts per min, mean SD (N = 6)

** P<0.01

Induction of DTH in the mouse footpad

As illustrated in Fig. 1A, inciting antigens other than P-2383-1 induced a temporary swelling (up to 0.6 mm) 24 h after injection in the footpads of mice immunized with P-2383-1. By 48 h, the swelling had completely subsided. However, P-2383-1 induced a swelling that was visible 18 h after injection, reached peak swelling (up to 2.5 mm) at 48 h, and then slowly subsided until 7 to 10 days, indicating the induction of DTH. When mice were immunized with P-2383-1 or its fractions (Fig. 1B), only those mice immunized with P-2383-1 exhibited the DTH reaction to the inciting antigen P-2383-1 in the footpads.

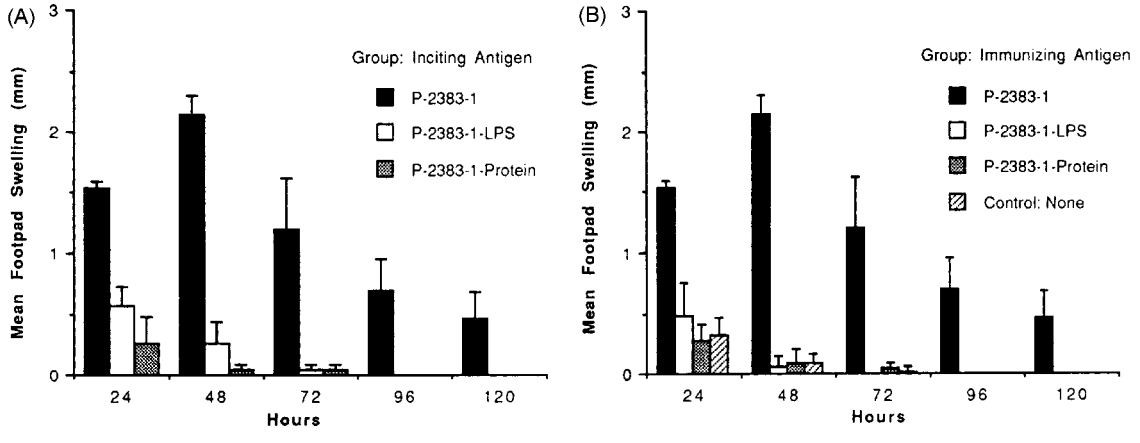

Fig. 1. A. Mouse footpad enlargement following an injection of inciting antigen into the footpads of an animal immunized 2 weeks earlier with P-2383-1. Values represent the mean differences between control and antigen-injected footpad thickness in the same animal (N = 10). B. Same as A except that P-2383-1 was administered to mice immunized 2 weeks earlier with several different antigen preparations.

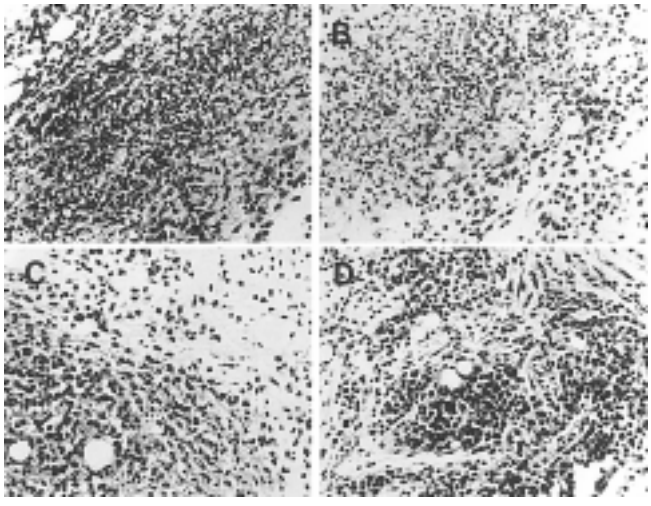

Fig. 2. Photographs showing the histopathologic changes in the footpads of mice immunized with P-2383-1. Tissues were obtained at various times following the introduction of P-2383-1 into the footpad 2 weeks after immunization: A, 24 h; B, 48 h; C, 72 h; D, 6 days.

Histological examination of the footpads in the mice immunized and incited with P-2383-1 revealed cellular infiltration characteristic of the typical DTH reaction. At 24 h, there were focally intense cellular infiltrates composed primarily of neutrophils, and a few macrophages (Fig. 2A). Increased numbers of mononuclear cells had diffusely infiltrated the footpads at 48 h and the ratio of neutrophils to mononuclear cells in the lesion was approximately 50 : 50 (Fig. 2B). At 72 h, the cellular infiltrates were composed predominantly of mononuclear cells, large foamy macrophages and lymphocytes (Fig. 2C). At day 6, few neutrophils were present but mononuclear cells were still in residence (Fig. 2D).

Growth of *S. enteritidis* in mouse spleens

A growth curve of *S. enteritidis* in the spleen of normal mouse is illustrated in Fig. 3. The numbers of bacteria in the spleen on day 1 were slightly less than the inoculum, but thereafter the organisms grew logarithmically up to day 5. No further dramatic increase in the numbers of bacteria was observed after day 7. However, hyperplasia of the spleen was noted between day 7 and 10 and multifocal necrosis in the spleen was observed on day 14, although the mice were clinically healthy (data not shown). Day 5 (log phase growth) was chosen to evaluate the immunizing effect of the LPS-protein complex, (P-2383-1), on the growth of *S. enteritidis* in the spleen. As indicated in Table 5, there was no difference on day 1 after-infection in the numbers of viable *S. enteritidis* in spleens of the control mice and the mice immunized with P-2383-1. However, the numbers in the spleens of the mice immunized with P-2383-1 on Day 5 were significantly lower ($p<0.01$) than in

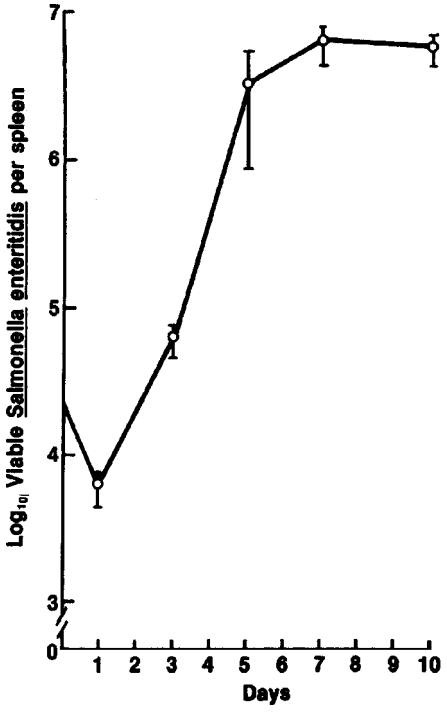

Fig. 3. Growth of *S. enteritidis* in spleens of normal mice (N = 5). Each mouse received 1.7×10^4 c.f.u. intraperitoneally and each point on the graph represents the mean bacterial counts in the spleens of 5 mice.

Table 5. Growth of *S. enteritidis* in the spleens of mice immunized with P-2383-1 and non-immunized control mice. Each mouse received 1.7×10^4 c.f.u. intraperitoneally one week after the secondary immunization (N = 5)

Primary immunization[a] (day 0)	Secondary immunization[b] (day 14)	c.f.u. per spleen, (mean SD) (day 22)	(day 26)
P-2383-1	P-2383-1	$7.3 \pm 1.6 \times 10^3$	$2.7 \pm 1.4 \times 10^4$**
None	None	$8.2 \pm 2.6 \times 10^3$	$1.3 \pm 0.5 \times 10^6$

[a]Mice were immunized subcutaneously.

[b]Mice were immunized via footpad.

** P<0.01

Table 6. Growth of *S. enteritidis* in spleens of non-immunized control mice and mice immunized with P-2383-1 or fractions of P-2383-1. Each mouse received 1.5×10^4 c.f.u. intraperitoneally at one week after the secondary immunization (N = 5).

Primary immunization[a] (day 0)	Secondary immunization[b] (day 14)	c.f.u. per spleen (mean $\pm$ SD) (day 26)
P-2383-1	None	$2.7 \pm 1.8 \times 10^4$**
P-2383-1-LPS	None	$4.6 \pm 1.1 \times 10^5$
P-2383-1-PRO	None	$5.9 \pm 2.3 \times 10^5$
P-2383-1	P-2383-1	$1.8 \pm 0.9 \times 10^4$**
None	None	$6.4 \pm 2.1 \times 10^5$

[a]Mice were immunized subcutaneously.

[b]Mice were immunized via footpad.

** P<0.01

the controls mice. The mice immunized with LPS (P-2383-1-LPS) or the protein (P-2383-1-PRO) fraction failed to suppress the growth of *S. enteritidis* in the spleen (Table 6).

Discussion

We previously reported on the immunogenic potential of an LPS-protein complex isolated from a KSCN extract of *P. multocida* [38]. The results of the active protection tests indicated that the LPS-protein complex was immunogenic confirming previous findings. However, the PW-extracted individual LPS (P-2383-1-LPS) and protein (P-2383-1-PRO) fractions of the complex and the mixture of the two fractions did not provide the same protection. This may indicate that intact LPS-protein complex is required to induce complete protection of mice against *P. multocida* infection. However, it is possible that phenol-water treatment of the LPS-protein complex may denature the protein or alter the antigenic determinant(s) of the complex associated with immunogenicity. Analysis of the complex and its fractions by SDS-PAGE and Western immunoblot indicated that the complex contains more and stronger reacted protein bands than indicated by its previously determined protein component [39]. In addition, a major protein band of approximately 23 kD present in the complex was apparently altered during fractionation since several weak bands were found rather than a single band. However, there is clear evidence that the immunogenic specificity of the LPS-protein complex is associated with the protein moiety. Since P-2383-PRO mixed with 20% aluminum hydroxide provided a degree of protection, this material apparently requires an adjuvant. The reason for the lack of immunizing activity of this material may be due to the absence of LPS that is known to be a potent adjuvant [21, 29]. The adjuvant effect of the LPS is indicated by the findings that a higher titer of antibody was induced against the protein moiety when P-2383-1-LPS (containing 7% protein) was used for immunization and that the antiserum obtained from mice immunized with P-2383-1-PRO without aluminum hydroxide had very low antibody titers.

It has been previously demonstrated that mice could be protected against *P. multocida* infection by passive immunization [10, 12, 20, 46]. Confirming previous findings, it was found that the passive transfer of immune serum against P-2383-1 protected mice against the challenge. However, the specificity of antibodies is of great importance. Antiserum against P-2383-1 provided protection while antiserum against P-2383-1-LPS did not, although both antisera had an equivalent antibody titer. It is also possible that the 7% protein moiety in P-2383-1-LPS may not contain the antigenic determinant(s) of the complex associated with immunogenicity.

LPS of gram-negative bacteria is known to be a potent mitogen for mouse B lymphocytes [29, 42, 43]. The results of our present study also indicated that the LPS-containing fractions of *P. multocida* (PW-LPS, P-2383-1 and P-2383-1-LPS) are be the potent mitogens for mouse B lymphocytes. The protein moiety, P-2383-1-PRO, induced far greater mitogenic responses in B lymphocytes than those of the original complex at the same protein concentration. In addition, P-2383-1-PRO was poorly soluble in water, but readily soluble in dilute alkali or phenol. These results indicate that P-2383-1-PRO might have similar characteristics to the classical endotoxin protein (or lipid-A associated proteins) of enterobacteria. The endotoxin proteins fractionated from butanol or trichloacetic acid-extracted LPS of enteric Gram negative bacteria by the phenol-water procedure were reported to exhibit similar characteristics [5, 29, 42, 43]. However, these endotoxin proteins exhibited additional biological activities, such as the activation of non-specific resistance to bacterial infection in mice [43], stimulation of human monocytes [28], and the induction of interferon and interleukin synthesis [29, 40]. In contrast to the classical endotoxin proteins of enterobacteria, P-2383-1-PRO was inconsistent in its immunologic reactivity. It lacked immunogenicity in mice as determined by challenge infection, and failed to induce DTH responses in mouse hind footpads and non-specific resistance to *S. enteritidis* infection. This indicates that the protein component possibly is a biologically variant of the endotoxin protein of enterobacteria, although exhibiting similar chemical and physical characteristics.

It has been suggested that the immune mechanism that protects mice against *P. multocida* infection is not cell-mediated [10, 12]. They found that transfer of peritoneal or spleen cells from immune mice to normal mice 1 h before challenge did not provide protection and that the cellular response of the passively immunized mice to the *P. multocida* organisms was predominantly polymorphonuclear in nature. In addition, a hyperimmune serum against *P. multocida* organisms did not influence *in vitro* bactericidal activity of peritoneal cells from normal or immune mice. Also, the inoculated bacteria multiplied, without losing virulence, for a long time (14 days) at the site of infection in mice. Moreover, bacteria were seldom found in any part of the body other than the site of infection, in passibly immunized mice. Therefore, Collins et al. suggested that the primary role of antibody against *P. multocida* infection is the inhibition of the rapid spread of the organism to the blood stream and other reticuloendothelial organs. However, evidence for the induction of cell-mediated immunity against *P. multocida* infection in mice was found in our observations. The mitogenic response of the pan-purified T lymphocytes in mice immunized with P-2383-1 indicated that these T lymphocytes were sensitized to this antigen. DTH reactions in the footpads of mice immunized

with P-2383-1 are further evidence of cell-mediated immunity. The size and duration of swelling and the histological changes in the footpad were very similar to the DTH reaction induced by *Mycobacterium tuberculosis*-purified protein derivative [17, 22]. It is known that mice can suppress the growth of facultative intracellular parasites including *Listeria monocytogenes*, *Brucella abortus* and *S. enteritidis* by cell-mediated mechanisms [2, 13, 26, 27]. Therefore, the suppressive effect of P-2383-1 on growth of *S. enteritidis*, which has no serologic relationship with P-2383-1, would support the induction of cell-mediated immunity by the LPS-protein complex of *P. multocida*.

For many years, the acquired resistance in mice to a facultative intracellular bacterium such as *L. monocytogenes* was thought to involve a systemic activation of mononuclear phagocyte bactericidal activity [26, 27]. However, Czuprynski et al. [13] suggested that the increased mobilization of neutrophils and mononuclear phagocytes into the site of infection is of prime importance to resistance in listeriosis. They came this conclusion since they could not demonstrate any difference in the bactericidal activities of the phagocytic cells in normal and immune mice. Also, the immune mice were able to recruit rapidly a large number of phagocytic cells at the site of infection due to release of lymphokines by T lymphocytes indicating a role for cell-mediated immunity. Like the other facultative intracellular parasites, *P. multocida* may be eliminated by neutrophils and macrophages if large numbers of these cells are present at the site of infection. However, mice may not recruit these phagocytic-cells at the site of *P. multocida* infection without the induction of cell-mediated immunity [2]. The induction of cell-mediated immunity, i.e., the production of lymphokines by T lymphocytes, may require significant time and appropriate antigenic stimulation. Antibodies against the organism may play a role in resistance by inhibiting the rapid spread of the organism through the body while certain surface material, such as the LPS-protein complex, may serve as a stimulatory agent for the later development of cell-mediated immunity.

The results of our study on immune mechanisms involved in protection of mice against *P. multocida* infection indicate that the LPS-protein complex of *P. multocida* may induce both humoral and cell-mediated immunity. Future studies must determine if the LPS-protein complex of *P. multocida* induces similar immunologic reactivity in cattle.

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