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Characterization of, and immune responses of mice to, the purified OmpA-equivalent outer membrane protein of *Pasteurella multocida* serotype A:3 (Omp28)

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Abstract

Pasteurella multocida A:3 is a major cause of bovine pneumonia. A major antigenic heat-modifiable 28 kDa outer membrane protein (Omp28) was previously identified. The purpose of this study was to purify and characterize Omp28 immunologically and structurally. Omp28 was extracted from *N*-lauroylsarcosine-insoluble protein preparations by a combination of detergent fractionation with Zwittergent 3-14 and chromatography. Partial N-terminal amino acid sequence confirmed Omp28 as a member of the OmpA-porin family. However, porin activity could not be demonstrated in a lipid-bilayer assay. Heat modifiability of purified Omp28 was demonstrated, and Omp28 was found in outer membrane fraction of *P. multocida*. Surface exposure of Omp28 was demonstrated by partial protease digestion of intact bacteria, by binding of anti-Omp28 polyclonal ascites fluid to the bacterial surface, and by partial inhibition of anti-outer membrane antiserum binding by previous incubation of the bacteria with anti-Omp28 serum. CD-1 mice vaccinated with purified Omp28 developed a significant antibody titer ($P < 0.05$) compared to the control treatment group but were not protected from a homologous intraperitoneal bacterial challenge. By contrast, treatment groups vaccinated with *P. multocida* outer membrane, formalin-killed *P. multocida* or a commercial vaccine were significantly protected from challenge. In vitro complement-mediated killing of *P. multocida* was observed in post-vaccination sera of outer membrane, formalin-killed *P. multocida*, and commercial vaccine-treatment groups, but not with sera from the Omp28-treatment group. In conclusion, although Omp28 is surface exposed and antigenic, it may not be a desirable immunogen for stimulating immunity to *P. multocida*. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Pasteurella multocida*; Outer membrane protein; Mice; Immunity; OmpA

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1. Introduction

Pasteurella multocida is an important pathogen for several animal species, including humans, and is associated with a variety of diseases (Confer, 1993). In cattle, *P. multocida* A:3 is one of the etiologic agents of pneumonia in the bovine respiratory disease (BRD) complex (Welsh, 1993). The pathogenesis of BRD involves environmental stressors, viral infections, modified host immune status, and bacterial infections (Frank, 1989). The lack of full comprehension of the disease pathogenesis and immunity has allowed the disease to prevail with significant economic burden to the cattle industry. In a recent study, the estimated annual economic loss to the cattle industry in the United States due to BRD is 640 million dollars (Bowland and Shewen, 2000).

Several studies have demonstrated the importance of outer membrane proteins (OMPs) in the development of a protective antibody response against Gram-negative bacteria. Vaccination of mice (Vasfi Marandi and Mittal, 1997), chickens (Zhang et al., 1994) and rabbits (Lu et al., 1991b) with *P. multocida* OMPs stimulated significant protection against challenge with the live bacteria. Protection has also been obtained by vaccination with selected and purified OMP (Luo et al., 1997) or by passive monoclonal antibody (mAb) reconstitution to purified OMPs (Lu et al., 1991a; Vasfi Marandi and Mittal, 1997). High antibody responses to several *P. multocida* OMPs correlated with resistance of cattle to experimental *P. multocida*-induced pneumonia (Confer et al., 1996). *P. multocida* OMPs have been examined in several in vitro assays for the ability to influence phagocytosis (Truscott and Hirsh, 1988), activation of neutrophils (Galdiero et al., 1998), and cytokine expression (Iovane et al., 1998).

Porins represent a group of channel forming proteins that traverse the outer membrane of several genera of Gram-negative bacteria. They have been extensively studied and share a basic structure containing a high proportion of antiparallel β-chains that takes on a barrel conformation (Nikaido, 1992; Chevalier et al., 1993). Depending on the type of porin, the β-barrel can associate in the outer membrane as a homotrimer or remain as a monomer. The channel formed allows for the passive passage of small solutes based on size, shape, hydrophobicity, charge, or substrate specificity (Hancock et al., 1982; Jap et al., 1991). In addition, porins have a role in bacteriophage attachment and structural integrity of the cell envelope (Lugtenberg and Van Alphen, 1983; Jap and Walian, 1996).

One of the major *P. multocida* OMPs is an antigenic, heat-modifiable 28 kDa OMP with some N-terminal amino acid sequence homology to *Escherichia coli* OmpA, a porin protein (Dabo et al., 1997; Marandi and Mittal, 1996). Cattle resistant to experimental *P. multocida*-induced pneumonia develop high antibody titers to the 28 kDa OMP, and studies revealed surface exposure of the protein (Confer et al., 1996; Dabo et al., 1997). Vasfi Marandi and Mittal (1997), however, failed to demonstrate protection of mice against *P. multocida*-infection using continuous delivery of a mAb to the 28 kDa OMP. Recently, the complete gene sequence for *P. multocida* strain Pm70 (an avian A:3 strain) was published (May et al., 2001), and the sequence of the *P. multocida* strain 232 (a cattle A:3 strain) OmpA-equivalent protein has been deposited in the GenBank database (Dabo and Confer, 2001, accession number AY035341.1).

In this report, we describe the purification and characterization of the 28 kDa OMP (heretofore referred to as Omp28) from *P. multocida* serotype A:3 strain 232, isolated from cattle, and determine that protein's potential for stimulating immunity in mice. Because

this OMP is highly antigenic in cattle and since failure of previous mouse protection by passive transfer of an Omp28-specific mAb could be due to the specificity of those antibodies for a non-surface exposed epitope, we undertook to purify and characterize this OmpA-equivalent protein of *P. multocida* and to determine its immunogenicity and potential role in inducing protective antibodies against the whole molecule.

2. Materials and methods

2.1. Bacterial cultures

P. multocida A:3 (strain 232), originally isolated from a case of bovine pneumonia and kindly donated by Dr. John Berg (University of Missouri, Columbia, MO), was grown on brain–heart infusion (BHI; Becton Dickinson, Sparks, MD) agar containing 5% bovine blood for 18 h at 37 °C in a 5% CO_2 environment (Dabo et al., 1997). An isolated colony was transferred to 3.0 ml of BHI broth in a 15 ml sterile polystyrene tube and grown at 37 °C with rotatory shaking at 120 oscillations/min for 4 h. A 200 μl culture aliquot was transferred to 2.0 l of BHI broth in a 4.0 l Erlenmeyer flask and grown at 37 °C with rotatory shaking at 120 oscillations/min for 14 h.

2.2. Bacterial outer membranes preparation

Bacterial envelopes were prepared by sonication and centrifugation as previously described (Simons et al., 1992). Outer membranes, heretofore referred to as OMP-enriched fraction, were extracted with 0.5% sodium *N*-lauroylsarcosine (Sarkosyl; Sigma, St. Louis, MO) in 0.01 M Tris buffer and collecting the insoluble fraction by centrifugation, whereas inner membranes were collected as the soluble fraction (Dabo et al., 1997).

2.3. Zwittergent 3-14 detergent fractionation

The OMP-enriched fraction was solubilized at 1% (w/v) Zwittergent® 3-14 (Calbiochem-Novabiochem Corp., La Jolla, CA), and 20 mM 3-[*N*-morpholino]propanesulfonic acid buffer (MOPS; Sigma), pH 7.5 for sonication for 15 s on ice with a 50% duty cycle (Bransonic 450, Danbury, CT) (Munson et al., 1983). Insoluble protein was sedimented by centrifugation at 150,000*g* for 60 min at 4 °C. The insoluble protein was solubilized for sonication as above in 0.1% Zwittergent 3-14, 50 mM ethylenediaminetetraacetic acid (EDTA; Sigma) in a 20 mM MOPS buffer at pH 7.5. Zwittergent–EDTA insoluble protein was centrifuged and solubilized for sonication as above in 0.1% Zwittergent 3-14, 0.4 M NaCl and 20 mM MOPS buffer at pH 7.5. The supernatant, designated Zwittergent fraction 3 (Zfr3), was retained and stored at −70 °C. The fourth Zwittergent fraction was obtained by solubilizing the insoluble pellet in 1.0% Zwittergent 3-14 and 20 mM MOPS at pH 7.5 and centrifuging, as described above.

In addition to Zwittergent extraction, isolation of Omp28 was attempted using *n*-octyl polyoxyl ethylene (OPOE; Bachem Bioscience Inc., Philadelphia, PA) as previously described (Mahasreshti et al., 1997).

2.4. Chromatography

Zfr3 was loaded onto an anion exchange chromatography (High-Q chromatography column (5.0 ml); Bio-Rad laboratories, Richmond, CA) previously equilibrated with buffer A (0.1% Zwittergent 3-14, 10% methanol, and 20 mM MOPS at a pH 7.3). Proteins were eluted by applying a 0–100% gradient of buffer B (0.1% Zwittergent 3-14, 10% methanol, 20 mM MOPS, and 1.0 M NaCl at a pH 7.3). Fractions containing Omp28 were collected and stored at −70 °C.

Pooled anion exchange fractions were concentrated using centrifugal concentrators (Centricon®; Millipore Corp., Bedford, MA) and loaded onto a gel filtration chromatography column (Superdex 75 HR; Amersham Pharmacia Biotech Co., Piscataway, NJ) previously equilibrated with buffer B (see anion exchange chromatography), and the protein eluted at 0.5 ml/min. A UV detector at 290 nm detected protein-containing fractions; the appropriate fractions were collected and stored at −70 °C.

2.5. N-terminal amino acid sequencing

Purified Omp28, subjected to SDS-PAGE, was transferred onto a polyvinylidene difluoride membrane (Bio-Rad), Coomassie brilliant blue stained, and the amino-terminal end sequenced as described previously (Dabo et al., 1997).

2.6. Electrophoresis and immunoblot analysis

OMP-enriched fractions were equilibrated to 1 mg protein/ml and subjected to discontinuous SDS-PAGE (Dabo et al., 1997). Gels were stained with Coomassie brilliant blue. Coomassie-stained gels were further analyzed by densitometry using a video densitometer in transmittance mode (Mosier et al., 1989). Analyses of peaks were performed, and data were expressed as total peak area optical density for each band (Multi-Analyst; Bio-Rad Co., Hercules, CA).

Proteins were transferred to nitrocellulose membranes, and antigens were identified immunologically, using bovine or mouse sera at a 1:25 and 1:250 dilutions, respectively (Confer et al., 1996). Immune complexes were detected with alkaline phophatase-conjugated, goat anti-bovine IgG or rabbit anti-mouse IgG (1:25,000) catalyzing precipitate formation by an alkaline phosphatase sensitive substrate (Sigma Fast™ BCIP/NBT; Sigma).

2.7. Enzyme-linked immunosorbent assay

Antibodies to outer membranes were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described with minor modifications (Confer et al., 1985). Wells of 96-well microtiter plates (Costar®; Corning Corp., Corning, NY) were coated with 100 ng of *P. multocida* OMP-enriched fraction. Preliminary assays were done to determine appropriate serum dilutions to use in the assay (Malvano et al., 1982). Results indicated that serum dilutions of 1:4000 were consistently in the titratable range regardless of whether the sera had high or low antibodies to *P. multocida*. Primary antisera were

subsequently tested in triplicate at a 1:4000 dilution in PBS containing 1% BSA and 0.5% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma). The extent of antibody binding to OMP preparations was detected using a 1:400 dilution of alkaline phosphatase-conjugated, affinity-purified goat anti-mouse IgG. Antibody responses were based on a colorimetric response using *p*-nitrophenyl phosphate substrate (Sigma Fast™; Sigma) and expressed as OD_{405}. All sera were assayed at one time. A control high responder serum and a low responder serum were present on each microtiter plate, and minor plate-to-plate variation adjusted as previously described (Voller et al., 1979).

2.8. Animals

Twelve-week-old BALB/c mice (Charles River Laboratories, Wilmington, MA) were used in the production of anti-Omp28 polyclonal antibodies, while vaccination studies utilized 5-week-old CD-1 mice. All mice were housed in a climate-controlled facility at 22 °C, ambient humidity and a 12/12 day/night cycle. The Oklahoma State University Institutional Animal Care and Use Committee approved animal protocols.

2.9. Production of polyclonal antibodies

Polyclonal antibodies (pAbs) for OMPs of *P. multocida* strain 232 were produced in CD-1 mice (Charles River Laboratories) by administering 40 μl subcutaneous inoculations of 10, 25, 50 or 100 μg of OMP-enriched fraction admixed with an equal volume of adjuvant (TiterMax®; CytRx Co., Norcross, GA) on days 0 and 14. On day 28, the mice were deeply anesthetized with methoxyflurane (Metofane®; Schering-Plough Corp., Union, NJ) and exsanguinated. The sera collected and stored at −70 °C.

Omp28-specific pAbs were produced in a hyperimmunized mouse ascites model (Donald et al., 1993). Briefly, *P. multocida* OMP-enriched fraction was separated on 10% SDS-PAGE. The Omp28 band excised from the gel, and the protein eluted using a buffer composed of 50 mM ammonium bicarbonate and 0.1% SDS. BALB/c mice were subcutaneously vaccinated with 50 μg of the eluted Omp28 on days 0, 14, 21, and 37. On day 37, mice were intraperitoneally injected with 10^6 T-180 sarcoma cells (ATCC TIB-66) to induce antibody-rich ascites fluid. The ascites fluid was collected and stored at −70 °C.

Bovine-anti-*P. multocida* outer membrane serum was prepared by subcutaneous vaccination of a weanling beef steer with 2 mg of Sarcosyl-extracted outer membrane in Freund's incomplete adjuvant. The vaccination was repeated once 14 days later, and serum harvested 28 days after the initial vaccination.

2.10. Omp28 surface exposure determination

Surface exposure of Omp28 from the outer bacterial membrane was examined by two different methods. The first method examined immunoblots of intact *P. multocida* after protease treatment of surface-exposed epitopes as described by Dabo et al. (1997). Trypsin, chymotrypsin, and *Staphylococcus* V8 protease were used as previously described (Dabo et al., 1997). The immunoblots were then probed with anti-Omp28 mouse ascites pAbs and examined for reduction in kDa in the Omp28 band. To determine if the capsule interfered

with protease digestion of surface-exposed regions, bacteria were suspended in either 500 μl of PBS or hyaluronidase 200 IU/ml (Sigma) at 37 °C for 1 h with gentle rocking. Bacteria were subsequently washed in PBS and resuspended in appropriate buffers for protease treatment.

Surface exposure was also examined by an inhibition ELISA (Rosenqvist et al., 1999). Live washed *P. multocida* were suspended in sterile PBS to $OD_{600} = 0.5$, and 1 ml of the *P. multocida* suspension added to one each of four centrifuge tubes. Ten microliters of one of the following were added to each tube: PBS, mouse anti-Omp28 serum, mouse anti-OMP serum, or mouse anti-*P. multocida* whole cell serum. Sera were obtained from the vaccination and challenge experiment described below. Tubes were incubated for 1 h at 37 °C on a rocking platform, and bacteria were washed three times in PBS. One-hundred microliters of bacterium–PBS mixture were added to 10 wells, and 100 μl of bacterium–sera mixtures were each added to five wells in a microtiter plate (Costar). Plates were incubated for 1 h at 37 °C, washed three times in PBS containing 1% BSA and 0.5% Tween 20 (PBS–BSA–Tween), and washed an additional three times in PBS. One-hundred microliters of a 1:2000 dilution of biotinylated mouse anti-Omp28 polyclonal ascites fluid were added to five wells of bacterium–PBS, and 100 μl of biotinylated bovine-anti-outer membrane serum added to five wells each of the bacterium–PBS or bacterium–serum mixtures. The reactions were incubated for 1 h at 37 °C. After three washes in PBS–BSA–Tween, 100 μl of a 1:6000 dilution of streptavidin-alkaline phosphatase was added and incubated for 1 h at 37 °C, followed by three washes in PBS. Control wells contained *P. multocida* treated with streptavidin-alkaline phosphatase. The intensity of binding of the biotinylated anti-Omp28 were based on a colorimetric response using *p*-nitrophenyl phosphate substrate and expressed as OD_{405}. This assay was done twice with five replicates per assay, and data are presented as the mean ± S.D. for the 10 replicates.

2.11. Porin activity determination

Membranes were made from 1% lipid (comprising 0.2% diphytanoyl phosphatidyl glycerol and 0.8% diphytanoyl phosphatidyl choline) in *n*-decane. Bilayers were painted across a 2 mm^2 hole in a Teflon divider separating two compartments containing 5.0–6.0 ml each of a bathing solution of 1.0 M KCl. At this time, the putative porin protein (1 nmol or less in 1% Triton X-100) was then added to the aqueous phase bathing the lipid membrane. Voltages were applied across this membrane through Calomel electrodes connected by a salt bridge and the resultant current boosted 109–1010-fold by a current amplifier, monitored on Tektronix model 7633 oscilloscope and recorded on Rikadenki R-01 strip chart recorder (Benz and Hancock, 1981).

2.12. Complement-mediated killing assay (CMK)

The CMK assay was modified from previously published protocols (Wijewardana and Sutherland, 1990; Wijewardana et al., 1990; Pandher and Murphy, 1996). The complement source was serum from a colostrum-deprived neonatal calf with undetectable antibodies against *P. multocida*, as determined by ELISA and Western blot analysis. To assay CMK,

sample mouse sera (12 μl), PBS (43 μl), and approximately 550 CFU of logarithmic phase *P. multocida* (5 h broth culture) were added in duplicate wells in sterile tissue culture microtiter plates (Elkay Products, Inc., Shrewsbury, MA). Forty microliters of the complement source were added to each replicate, except for internal controls, which lacked either a complement or antibody source. Each plate was sealed with an adhesive plastic film (Seal Plate™; ISC BioExpress, Kaysville, UT) and incubated at 37 °C with gentle rocking for 45 min. At the end of the time, using a modified Miles-Misra technique (1938), six 10 μl aliquots were removed from each well and spotted on BHI agar plates. The average CFU were determined for each sample after 12 h of growth at 37 °C in a 5% CO_2 environment.

2.13. Vaccination and challenge

To study the immunogenicity of Omp28, 108 female CD-1 mice were randomly divided among six groups of 18 mice each (Table 1). Pre-vaccination sera were collected from four mice and stored at −70 °C. Subsequently, mice were subcutaneously vaccinated on days 0 and 14 with 40 μl of PBS, PBS with adjuvant (TiterMax®), 75 μg of purified Omp28 with adjuvant, 75 μg of *P. multocida* strain 232 OMP-enriched fraction with adjuvant, or 75 μg of formalin-killed *P. multocida* strain 232 whole cells with adjuvant. As a known positive control, a commercial vaccine (Presponse HM®; Fort Dodge Animal Health, Fort Dodge, IA) was administered intraperitoneally at 1/20 the stated cattle dose as recommended by the US Department of Agriculture, Biological Division for *P. multocida* vaccine testing (Confer, 1993). On day 28, four mice from each group were deeply anesthetized with methoxyflurane and exsanguinated. Post-vaccination sera were collected and stored at −70 °C. The remaining mice were challenged with a 100 μl intraperitoneal injection of live *P. multocida* (1×10^8 CFU/ml) and monitored for death for 96 h.

Table 1
Evaluation of protection of CD-1 vaccinated mice against *P. multocida* challenge, corresponding antibody responses to *P. multocida* OMPs as measured by ELISA, and CMK activity of post-vaccination sera[a]

Experimental group	Immunization[b]	Route of immunization	Antibody response (OD_{650}) to *P. multocida* 232 OMPs		No. of dead/no. challenged	CMK (%)
			Day 0	Day 28		
1	PBS	Subcutaneous	0.000 ± 0.000 a	0.010 ± 0.004 a	10/10 a	12 a
2	PBS + adjuvant	Subcutaneous	0.008 ± 0.016 a	0.0135 ± 0.006 a,b	10/10 a	4.8 a
3	Omp28+adjuvant	Subcutaneous	0.000 ± 0.000 a	0.386 ± 0.128 c	10/10 a	4.7 a
4	OMPs + adjuvant	Subcutaneous	0.007 ± 0.012 a	0.475 ± 0.155 c	1/10 b	100 b
5	Formalin-killed *P. multocida* 232 + adjuvant	Subcutaneous	0.001 ± 0.001 a	0.121 ± 0.124 a,b,d	3/10 b	100 b
6	Commercial vaccine	Intraperitoneal	0.000 ± 0.000 a	0.201 ± 0.109 b,c,d	2/10 b	40.7 a

[a] Differences in letters designates non-relational treatment groups ($P < 0.5$).

[b] Adjuvant is TiterMax; CytRx Co., Norcross, GA. Commercial vaccine is Presponse HM®; Fort Dodge Animal Health, Fort Dodge, IA. OMPs are OMPs of *P. multocida* 232.

2.14. Statistical analyses

Mean antibody responses among the various vaccine groups were analyzed by a one-way analysis of variance and Tukey HSD post-hoc multiple comparisons. Antibody responses between days 0 and 28 were compared for each group by paired *t*-tests. Survival rates were compared among groups by chi-square analysis (Armitage and Berry, 1994).

3. Results

3.1. Extraction and purification of Omp28

Different combinations of detergents were tested for selective isolation of Omp28 from the OMP-enriched fraction. Attempts to solubilize and extract Omp28 with OPOE detergent were unsuccessful (data not shown). Using the Zwittergent 3-14 procedure, Omp28 was present in each fraction and in the remaining pellet as observed on SDS-PAGE and confirmed by Western blot analysis; Omp28 was most prevalent in Zfr3. Heat-modifiability was demonstrated for Omp28 as it migrated from 28 to 35 kDa when incubated at 37 and 100 °C, respectively (Fig. 1).

After Omp28 enrichment by Zfr3, Omp28 was further purified by anion exchange chromatography (Fig. 2). Densitometric analysis of the pooled Omp28-rich fractions separated on SDS-PAGE and then stained with Coomassie brilliant blue revealed purity was better than 95% (data not shown). Additional separation of proteins was achieved by

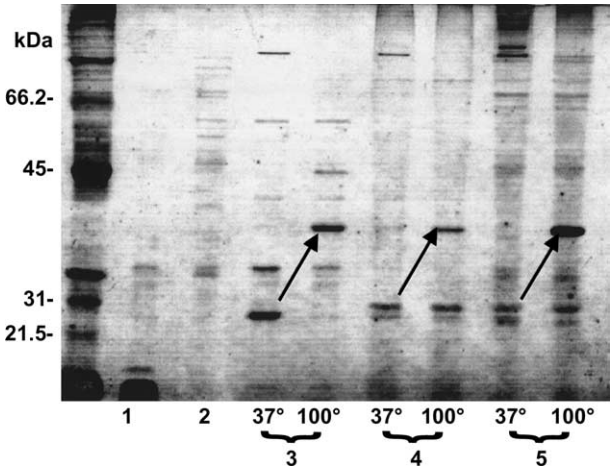

Fig. 1. SDS-PAGE (10% acrylamide gel, silver stain) showing the Zwittergent 3-14 fractions containing soluble OMP of *P. multocida*. Lanes 1–4 correspond to Zwittergent detergent fractions. Lane 1: Zwittergent fraction 1; lane 2: Zwittergent fraction 2; lanes 3: Zwittergent fraction 3 solubilized at 37 or 100 °C; lane 4: Zwittergent fraction 4 solubilized at 37 or 100 °C; lanes 5: the protein pellet remaining after the extraction procedure solubilized at 37 or 100 °C. Arrows indicate higher molecular weight migration of the 28 kDa band to 35 kDa when solubilized at 100 °C in loading buffer.

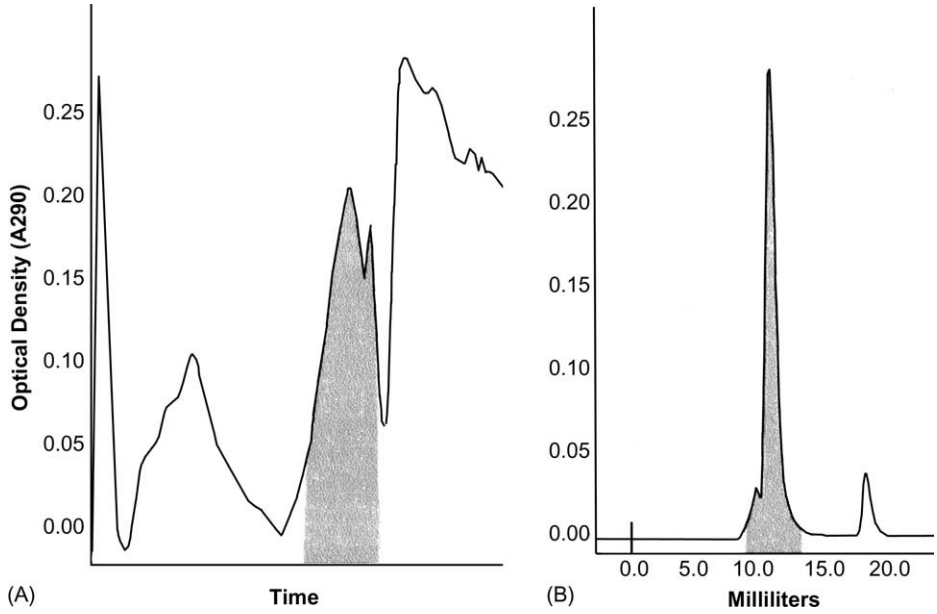

Fig. 2. Chromatographic isolation of Omp28. (A) Anion exchange chromatography elution profile (OD vs time). (B) Gel filtration chromatography elution profile (OD vs fraction). The areas shaded under the elution profiles correspond to fractions containing Omp28. Fractions were pooled, concentrated and analyzed for protein composition, as described in Section 2 and as shown in Fig. 3.

gel filtration chromatography, which revealed three absorbance peaks. The shaded area under the elution profile contained Omp28 (Fig. 2). Western blot analysis of the pooled Omp28 fractions probed with either hyperimmune sera from a calf vaccinated with *P. multocida* or from Omp28 ascites pAbs, identified two protein bands corresponding to 28 and 35 kDa (Fig. 3). N-terminal amino acid sequencing of both bands demonstrated identical sequences of APQPNTFYVGAKAGW, which is the same as previously published (Marandi and Mittal, 1996; Dabo et al., 1997; May et al., 2001).

3.2. Omp28 surface exposure

P. multocida Omp28 was demonstrated in outer membranes by immunoblotting of outer and inner membranes using anti-Omp28 pAb, mouse anti-purified Omp28 and mouse anti-*P. multocida* whole bacteria (Fig. 4). The latter two sera were obtained from the vaccination and challenge experiment described below. The pAb and anti-purified Omp28 serum recognized major protein bands at 28 and 35 kDa that were in highest concentration in the outer membrane fraction. Mouse anti-*P. multocida* whole cell serum faintly recognized the 35-kDa band in the outer membrane, but not in the inner membrane.

Dabo et al. (1997) demonstrated surface exposure of Omp28 (designated as Omp35 in that manuscript) using protease digestion of whole cells. Using those methodologies, we corroborated those findings by again demonstrating that treatment of *P. multocida* with chymotrypsin resulted in loss of the Omp28 protein band in encapsulated *P. multocida* or

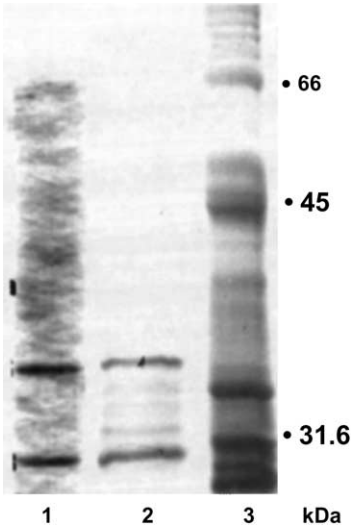

Fig. 3. SDS-PAGE and immunoblot analysis of pooled Omp28 containing fractions from gel filtration chromatography. Lane 1: Western blot probed with bovine sera hyperimmunized with *P. multocida*; lane 2: silver-stained SDS-PAGE gel; lane 3: molecular weight markers.

those that had capsule removed by preincubation with hyaluronidase. Omp28 was not susceptible to trypsin or to *Staphylococcus* V8 protease treatment.

Binding of anti-Omp28 pAB to whole bacteria resulted in OD_{405} of 0.80 ± 0.04, whereas binding of bovine anti-outer membrane serum to whole bacteria resulted in

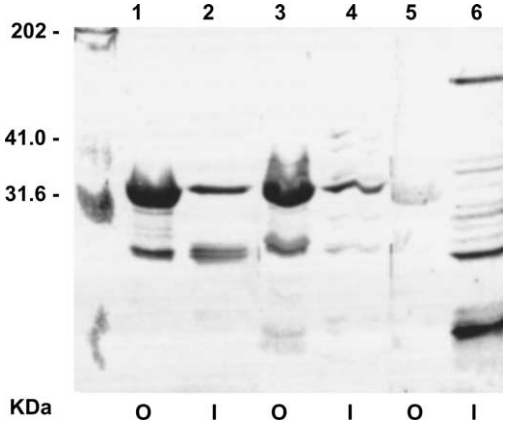

Fig. 4. Immunoblot of outer and inner membranes. Molecular weight (mw) markers and approximate values in kDa are on the left. *P. multocida* outer membranes (O) and inner membranes (I) alternate lanes. Lane 1: outer membrane reacted with mouse anti-Omp28 polyclonal ascites fluid; lane 2: inner membrane reacted with mouse anti-Omp28 polyclonal ascites fluid; lane 3: outer membrane reacted with mouse anti-Omp28 serum; lane 4: inner membrane reacted with mouse anti-Omp28 serum; lane 5: outer membrane reacted with mouse anti-whole *P. multocida* serum; lane 6: inner membrane reacted with mouse anti-whole *P. multocida* serum.

OD_{405} of 1.16 ± 0.12. Control wells had an OD_{405} of 0.15 ± 0.05. Pre-incubation of *P. multocida* with anti-whole cell, anti-outer membrane, or anti-Omp28 sera resulted in reductions in binding of bovine anti-outer membrane serum with mean OD_{405} values of 0.46 ± 0.03 (60.3% reduction), 0.36 ± 0.02 (69.0% reduction) and 0.40 ± 0.02 (65.5% reduction), respectively.

3.3. Omp28 porin activity

The ability of Omp28 to reconstitute channels in membranes was tested by the planar bilayer method, which has been successfully used to determine large numbers of non-specific, specific, and gated porin proteins from Gram-negative bacteria (Hancock et al., 1982). Control proteins (e.g. *P. aeruginosa* OprD and OprM) formed channels, but no porin activity was observed in the Omp28 preparation.

3.4. Mouse vaccination and challenge

Vaccination with Omp28, OMP-enriched fraction, and commercial vaccine resulted in a significantly $(P < 0.05)$ higher mean antibody responses against *P. multocida* OMP between days 0 and 28 when compared to the responses of the other treatment groups. Antibody responses for the Omp28- and OMP-enriched vaccinated groups were not significantly different $(P > 0.05)$ from each other (Table 1). Mean antibody response in formalin-killed whole cell vaccinates was not significantly different from responses for control groups. Vaccination with OMPs, formalin-killed whole cells, and the commercial vaccine resulted in significant protection $(P < 0.05)$ from challenge. Protection did not occur in control or Omp28 groups; all animals in these groups died within 52 h following challenge.

3.5. Anti-Omp28 antibodies in CMK of P. multocida

We examined the contribution of anti-Omp28 antibodies in CMK of *P. multocida*. As shown in Table 1, significant $(P < 0.05)$ CMK was achieved from day 28-sera from the OMP-enriched and formalin-killed whole bacteria vaccinates with 0% bacteria survivability. CMK activity of sera from the Omp28 and commercial vaccine groups resulted in 95.3 and 59.3% bacteria survivability, respectively, and were not significantly different $(P > 0.05)$ than that for the control group.

4. Discussion

With bacterial relatedness and structural similarities between Omp28 from *P. multocida* and PomA from *Mannheimia* (formerly *Pasteurella*) *haemolytica*, we unsuccessfully attempted to extract Omp28 using OPOE detergent fractionation, as previously described for PomA purification (Mahasreshti et al., 1997). Zwittergent 3-14 was successful in extracting Omp28, which had an N-terminal amino acid sequence identical with the sequence previously reported (Marandi and Mittal, 1996; Dabo et al., 1997; May et al.,

2001). The partial Omp28 N-terminal amino acid sequence had 80% homology to the OmpA porin protein of *E. coli* and 60% homology to other OmpA-family proteins from *Salmonella typhimurium* (Dabo et al., 1997). Most OmpA-family proteins are porins; however, repeated evaluation of Omp28 from *P. multocida* strain 232 in a lipid-bilayer assay did not demonstrate porin activity. It is possible that purification conditions were too harsh, resulting in partial denaturation of Omp28 with concurrent loss of porin activity. However, Zwittergent 3-14 is a mild non-denaturing detergent that has been shown to preserve isolated cell-bound receptor conformation (Zahidi et al., 1986), native epitopes from isolated P2 protein of *Haemophilus influenzae* (Munson et al., 1983), and allowed for reconstitution of native epitopes in OMPs of *Bacillus subtilis* after SDS detergent isolation (Idanpaan-Heikkila et al., 1996). Following Zwittergent 3-14 extraction, we further purified Omp28 using two chromatographic procedures. Those procedures could have affected the protein such that porin activity could not be demonstrated. Purified Omp28, however, retained heat modifiability indicating that denaturation was not obvious.

We confirmed that Omp28 is present in high concentrations in outer membrane fractions and corroborated previously demonstrated surface exposure based on susceptibility of Omp28 to chymotrypsin digestion of whole bacteria (Dabo et al., 1997). Surface exposure was further demonstrated by binding of anti-Omp28 polyclonal ascites to the surface of *P. multocida* in an ELISA. In addition, pre-incubation of *P. multocida* with anti-Omp28 serum resulted in a 65.8% reduction in binding of bovine anti-outer membrane serum to the bacterium. Marandi and Mittal (1996) failed to demonstrate surface exposure of Omp28 epitope identified by mAb MT4.1. That antibody perhaps recognized epitopes that might be buried in the membrane. Sequence data for the recently cloned *P. multocida* Omp28 gene predict regions of surface exposure (Dabo and Confer, 2001. Proc. 101st Gen. Mtg. Amer. Soc. Microbiol., Orlando, FL, H-68). However, the lack of protection of mice by Omp28 vaccination and failure to demonstrate CMK with serum against purified Omp28 may indicate that surface-exposed epitopes of Omp28 are not important in host defense against *P. multocida*.

Vaccination of mice with Omp28 stimulated a significantly higher ($P < 0.05$) antibody response than to formalin-killed whole cells (Table 1). However, Omp28 vaccination was neither protective from intraperitoneal challenge with *P. multocida* nor was sera from vaccination able to stimulate CMK. Vaccination with the OMP-enriched fraction, formalin-killed whole bacteria, and the commercial vaccine were able to stimulate significant ($P < 0.05$) protection against challenge. Although there is evidence that Omp28 is surface exposed, its surface exposure may be limited such that antibodies are ineffective in bactericidal activities in vivo and in vitro.

Several studies have successfully used antibodies against *P. multocida*, in ELISA, as a predictor of enhanced resistance against challenge. Those studies included cattle (Confer et al., 1996), buffalo (Chandrasekaran et al., 1994; Pati et al., 1996), rabbits (Klaassen et al., 1985; Lu et al., 1987), chickens (Hofacre et al., 1987) and mice (Wijewardana et al., 1990). In the present study, high antibody responses to *P. multocida* OMPs, in ELISA, were found in several groups of mice that were resistant to challenge. The Omp28-vaccinated group, however, had high antibody responses to OMP but were not resistant to challenge. Confer et al. (1996) showed that high antibody response to Omp28 as detected by quantifiable Western blots using cattle sera to probe OMP preparations correlated with resistance to

experimental challenge with virulent *P. multocida*. However, as observed in this study, a strong antibody response alone is not always a good predictor of protection. In fact, several studies have identified that antibodies to some bacterial antigens may be detrimental to the host defenses (Truscott and Hirsh, 1988; Hoffmann and Houle, 1995).

In conclusion, although Omp28 is a major antigenic OMP from *P. multocida* A:3 (strain 232) with some apparent surface exposure, antibodies to it do not appear to be protective in a mouse model. Therefore, Omp28 may not be a desirable immunogen for stimulating protection against *P. multocida* infection.

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