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Method for reducing endotoxin in *Moraxella catarrhalis* UspA2 protein preparations

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Abstract

The UspA2 protein from the bacterium *Moraxella catarrhalis* is a potential vaccine candidate for preventing human diseases caused by this organism. Before a vaccine can be administered parentally, the level of endotoxin must be reduced as much as possible. However, in this case the endotoxin was very tightly complexed with the UspA2 protein and could not be dissociated with Triton X-100. It was found that it dissociated from the protein with the zwitterionic detergents Zwittergent 3-12 and Zwittergent 3-14. The endotoxin could then be separated from the protein by either ion-exchange or gel filtration chromatography. Using the limulus amoebocyte lysate assay for quantitation, the endotoxin was reduced approximately 20 000-fold. The removal of residual endotoxin from UspA2 preparations had no detrimental effect on the immunological properties of the protein. Mouse antisera raised against UspA2 prior to, and following endotoxin reduction exhibited comparable antibody and bactericidal titers against the tested strains. Further, mice immunized with both preparations, followed by pulmonary challenge with either a homologous or a heterologous isolate, exhibited comparable levels of clearance.

Keywords: *Moraxella catarrhalis*; Endotoxins; UspA2 protein preparations

1. Introduction

Endotoxin is comprised of lipopolysaccharide (LPS) or lipooligosaccharide (LOS) present in the cell wall of gram-negative bacteria. Endotoxins induce pyrogenic reactions and shock in mammals upon intravenous injection at concentrations as low as a few ng/ml [1]. For this reason, proteins prepared from gram-negative bacteria must be as free as possible of endotoxin in order not to induce adverse reactions when administered to animals or people. A number of approaches are typically utilized to reduce endotoxin contamination of protein preparations. These include ion-exchange chromatography [2,3]; affinity adsorbents, such as immobilized L-histidine, poly-L-lysine, poly(γ-methyl L-glutamate), and polymyxin B [4–6]; gel filtration chromatography; ultrafiltration; and Triton X-114 phase separation [7,8]. Two important factors influencing the success of any approach are the affinity of the endotoxin and protein antigen for the chromatography support or media used and the affinity of the endotoxin for the protein antigen. A third factor is whether the affinity of the endotoxin for the protein

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can be modified by factors such as temperature, pH, detergents, solvents and denaturants.

The surface-exposed UspA2 protein from *Moraxella catarrhalis* is a potential vaccine candidate for preventing otitis media and other diseases caused by this organism [9]. The protein from the O35E strain has a molecular mass of 240 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and reacts with a monoclonal antibody (MAb) designated 17C7 [9]. The earliest indication of its vaccine potential was that this MAb exhibited complement-dependent bactericidal activity [10] and passive administration of the antibody to mice promoted pulmonary clearance of a *M. catarrhalis* challenge [11]. The *uspA2* gene from this strain encodes a protein with a molecular mass of 62 483, a size much smaller than that determined by SDS–PAGE [12]. Confirmation of the smaller subunit size came from matrix-assisted laser desorption ionization time-of-fight (MALDI-TOF) mass spectrometric analysis. By this method, the predominant species has an average molecular mass of 59 518 [9]. Subsequent studies demonstrated that purified UspA2 elicits bactericidal antibodies in mice to both homologous and heterologous disease isolates [9], and that mice immunized with it followed by pulmonary challenge with either the homologous or a heterologous isolate clear the bacteria more rapidly than mock-immunized mice [9].

UspA2 was first purified in our laboratory utilizing Triton X-100 (TX-100) detergent extraction of whole cells followed by ion-exchange and hydroxyapatite chromatography [9]. The UspA preparations purified using this procedure, however, contained a residual level of contaminating endotoxin that was capable of eliciting a pyrogenic response in rabbits. We examined a number of approaches to reduce the level of endotoxin, and in this report, we describe the use of the zwitterionic detergents Zwittergent 3-12 (Z3-12) and Zwittergent 3-14 (Z3-14) for the dissociation of endotoxin from the purified UspA2 protein and the subsequent separation of endotoxin from UspA2 using either ion-exchange or gel filtration chromatography. The resulting preparations of UspA2 exhibited immunological properties comparable to those observed before the endotoxin level was reduced.

2. Experimental

2.1. Bacteria

Dr. Eric Hansen of the University of Texas Southwestern Medical Center provided the TTA24 and O35E isolates. Additional isolates were obtained from Dr. Dwight Hardy of the University of Rochester and the American Type Culture Collection. The bacteria were routinely passaged on Mueller–Hinton agar (Difco, Detroit, MI, USA) incubated at 35°C with 5% carbon dioxide. The bacteria used for the purification of the protein were grown in sterile broth containing 10 g casamino acids (Difco) and 15 g yeast extract (BBL, Cockeysville, MD, USA) per liter. The isolates were stored at −70°C in Mueller–Hinton broth containing 40% glycerol.

2.2. Purification of UspA2

UspA2 was purified from *M. catarrhalis* O35E as described previously [9]. Briefly, the method entailed extracting the UspA2 protein from whole *M. catarrhalis* O35E cells with 0.03 *M* Tris–HCl (pH 8.0) containing 1.0% TX-100 followed by trimethylaminoethyl (TMAE) ion-exchange chromatography using the same buffer system. UspA2 was eluted from the TMAE column with 1.0 *M* NaCl. The UspA2 fractions were pooled, exchanged into 0.01 *M* sodium phosphate buffer (pH 7.0) containing 0.1% TX-100, and applied to a ceramic hydroxyapatite column. The protein was eluted using a sodium phosphate gradient (0.01–0.5 *M* sodium phosphate, pH 7.0) with 0.1% TX-100.

2.3. Purification of LOS

LOS was purified from *M. catarrhalis* by the method of Westphal and Jann [13]. Briefly, The bacteria were lyophilized and resuspended in 45% phenol and incubated at 68°C for 30 min. After cooling, the slurry was centrifuged at low speed to separate the phases. The upper water phase was saved and exhaustively dialyzed against water to remove residual phenol. The volume was reduced to one eighth of the starting volume by rotary evaporation at 40°C under reduced pressure. The concen-

trated liquid was then centrifuged at 3000 *g* and the supernatant lyophilized. The crude lyophilized LOS was dissolved in water to give a 3% solution. This was centrifuged at 105 000 *g* for 4 h. The pellet was saved and resuspended in water. The high-speed centrifugation step was repeated two more times discarding the supernatant each time. The final pellet from the high-speed centrifugations was suspended in water and centrifuged at 3000 *g*. The supernatant of purified LOS was saved and lyophilized.

2.4. LOS reduction using Superose-12 chromatography

Approximately 14 mg of purified UspA2 in 13.5 ml phosphate-buffered saline (PBS) containing 0.1% TX-100 (J.T. Baker, Phillipsburg, NJ, USA) was combined with 1.5 ml PBS containing 10.0% Z3-12 (Calbiochem, La Jolla, CA, USA) to create a 1.0% Z3-12 solution. The UspA2 was applied at a flow-rate of 1.5 ml/min to a 500-ml Superose-12 gel filtration column (950×26 mm, preparative grade, Pharmacia Biotech, Piscataway, NJ, USA) equilibrated in PBS containing 1.0% Z3-12. Fractions of 9.0 ml were collected for analysis.

2.5. LOS reduction using TMAE Fractogel chromatography

Approximately 20 mg of purified UspA2 in PBS containing 0.1% TX-100 was applied at a flow-rate of 2.0 ml/min to a 10.0-ml TMAE Fractogel anion-exchange column [50×16 mm, Model 650(S), particle size 0.025–0.4 mm; EM Separations, Gibbstown, NJ, USA] equilibrated with a 0.03 *M* Tris–HCl buffer (pH 8.0) containing 5.0% Z3-12. The protein was washed onto the column with five bed volumes of equilibration buffer and the UspA2 eluted using a 10-bed volume linear NaCl gradient (0–1.0 *M*) in a 0.03 *M* Tris–HCl buffer (pH 8.0) containing 5.0% Z3-12. Fractions of 4.0 ml were collected for analysis.

2.6. Polymyxin B chromatography

A 1.0-ml Acticlean Etox column (55×5 mm; Sterogene Bioseparations, Arcadia, CA, USA) was treated with 0.1 *M* sodium hydroxide, as described by the manufacturer, and equilibrated in 0.05 *M* sodium acetate buffer (pH 5.0) containing 0.1% TX-100. Approximately 4.0 mg of purified UspA2 in the same buffer was applied to the column at a flow-rate of 0.2 ml/min. The protein was washed through the column with 10 bed volumes of equilibration buffer at a flow-rate of 0.2 ml/min and 1.0-ml fractions were collected for analysis.

2.7. SDS–PAGE and Western blot analysis

SDS–PAGE was performed as described by Laemmli [14] using 4 to 20% (w/v) gradient acrylamide (Zaxis, Huson, OH, USA). Proteins were visualized by staining the gels with Pro-Blue (Owl Separation Systems, Portsmouth, NH, USA). Gels were scanned with the Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA, USA). Transfer of proteins to polyvinylidene difluoride (PVDF) was accomplished with a semidry electroblotter and electroblot buffers (Owl Separation Systems). The PVDF membranes were probed with a *M. catarrhalis* LOS-specific monoclonal antibody followed by goat anti-mouse alkaline phosphatase conjugate as the secondary antibody (Bio-Source International, Camarillo, CA, USA). Western blots were developed with the 5-bromo-4-chloro-3-indoylphosphate-nitroblue tetrazolium phosphatase substrate system (Kirkegaard and Perry Labs., Gaithersburg, MD, USA).

2.8. Protein estimation

Protein concentrations were estimated by the bicinchoninic assay (Pierce, Rockford, IL, USA) using bovine serum albumin as the standard.

2.9. Limulus Amoebocyte Lysate (LAL) assay

LAL assay was performed as described by Bang et al. [15].

2.10. Rabbit pyrogen test

The US Food and Drug Administration (FDA) pyrogen test was performed on samples of UspA2 before and after each procedure as described in CFR

610.13 [21]. Each sample was tested in the abbreviated form of the assay, using groups of three rabbits of similar mass. Each rabbit received the same amount of protein and volume of solution, based on the average mass of the group. The temperature of each rabbit was recorded at 1.0, 1.5, 2.0, 2.5 and 3.0 h post-administration. The highest recorded temperature rise for each of the rabbits was registered as the test value. A temperature increase of more than 0.5°C above that for the resting rabbit was considered indicative of a pyrogenic response.

2.11. Monoclonal antibodies

The lipooligosaccharide-specific MAb Mcat73-11 was prepared as described previously [10]. This Mab reacts with the ''A'' and ''C'' LOS serotypes of *M. catarrhalis* [16].

2.12. Vaccinations

Groups of 10 female BALB/c mice (Taconic Farms, Germantown, NY, USA) were administered vaccine subcutaneously twice 4 weeks apart with 5 μg of the UspA2 preparations with 100 μg 3-*O*-deacylated monophosphoryl lipid A (MPL; Ribi ImmunoChem Research, Hamilton, MT, USA) and 100 μg of aluminum phosphate. The mice were bled 2 weeks after the last immunization. Control mice were immunized subcutaneously with 5 μg of a genetically detoxified diphtheria toxin (CRM_{197}) with the same adjuvants on the same schedule.

2.13. Enzyme-linked immunosorbent assay (ELISA) procedures

The whole cell ELISA was performed as described previously [9].

2.14. Complement-dependent bactericidal assay

This assay was performed as described previously [10]. Relative killing by immune sera was calculated as the percent reduction in the number of colony forming units (CFUs) in the sample relative to that in a sample in which heat inactivated complement was replaced with active complement. The results are reported as the highest dilution of serum at which greater than 50% of the bacteria were killed.

2.15. Murine model of M. catarrhalis pulmonary clearance

The mice immunized as described above were challenged by instilling the bacteria into their lungs. The degree of pulmonary clearance was assessed 6 h after challenge as described previously [10]. The reported clearance values and statistical comparison were relative to a set of 10 mice immunized with CRM_{197}. The data were analyzed by the Wilcoxon signed-rank test. A probability (P) equal to or less than 0.05 was considered significant.

3. Results

3.1. Purification of UspA2

We previously described a large-scale, high-yield process for extracting and purifying UspA2 from *M. catarrhalis* cells [9]. Briefly, the method consisted of extracting the UspA2 protein from whole bacterial cells with 0.03 *M* Tris–HCl (pH 8.0) containing 1.0% TX-100 followed by TMAE ion-exchange chromatography using the same buffer system. UspA2 was eluted with 1.0 *M* NaCl. UspA2-enriched fractions were subsequently buffer exchanged by Sephadex G-25 chromatography and applied to a ceramic hydroxyapatite column and eluted using a sodium phosphate gradient with 0.1% TX-100. UspA2 purified by this method yielded a single band of ~240 000 molecular mass on SDS–PAGE by Coomassie blue staining, its purity was greater than 95% based on scanning densitometry (Fig. 1A). The preparations of purified UspA2 were examined for the presence of LOS endotoxin by Western blot analysis using the *M. catarrhalis* LOS-specific MAb Mcat73-11. Analysis of several lots of purified UspA2 detected a small amount of low-molecular-mass LOS by Western blot analysis (Fig. 1B). Based on extrapolation from lanes loaded with known amounts of purified *M. catarrhalis* LOS, the level of LOS detected was estimated at between 5 and 10 ng per μg of protein in the UspA2 preparations before our attempts to reduce it (data not shown).

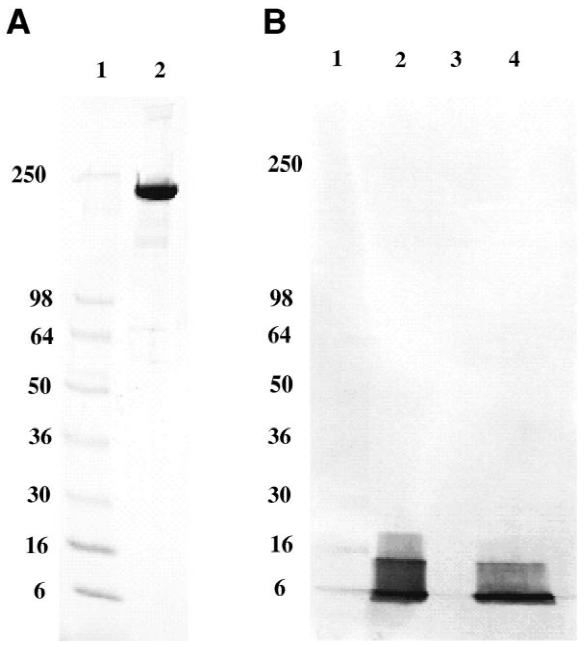

Fig. 1. SDS–PAGE and Western blot of purified UspA2. (A) Coomassie blue stained gel of purified UspA2 loaded at 3 μg (lane 2). Molecular size markers in kilodaltons (lane 1). (B) Western blot probed with *M. catarrhalis* LOS specific Mab Mcat73-11. Purified UspA2 loaded at 3 μg protein (lane 4). *M. catarrhalis* LOS standard loaded at 1 μg of LOS (lane 2). Blank lane loaded with deionized H_2O (lane 3). Molecular size markers in kilodaltons (lane 1).

3.2. Endotoxin removal from UspA2 preparations

Our initial attempts at removing residual LOS from purified UspA2 preparations were done in the presence of TX-100 and included gel filtration, anion-exchange, and polymyxin B chromatography. No separation was achieved using Superose-6 in the presence of either 0.1% and 1.0% TX-100; anion-exchange chromatography on TMAE Fractogel at varying pH values in the presence of 1.0% TX-100; or chromatography on polymyxin B. Polymyxin B is a peptide antibiotic with high binding affinity for the lipid A moiety of most endotoxins [17]. These data suggested that the LOS endotoxin is tightly associated with UspA2 in the presence of TX-100.

In our attempt to dissociate the LOS from UspA2, we examined both Superose-6 gel filtration and TMAE Fractogel chromatography in the presence of the zwitterionic detergents Z3-12 and Z3-14. Superose-6 chromatography in the presence of 1.0% Z3-12 resulted in complete separation of the high-molecular-mass UspA2 protein from the low-molecular-mass LOS endotoxin (Fig. 2A). Coomassie blue staining of the Superose-6 column fractions run on SDS–PAGE indicates the UspA2 protein was most concentrated in fractions 21–24 (Fig. 2B). Western blot analysis of the same column fractions using the *M. catarrhalis* LOS-specific MAb Mcat73-11, shows the LOS primarily in fractions 33–36 (Fig. 2C). The inclusion of Z3-12 in the Superose-6 chromatography buffer resulted in the dissociation of the UspA2–LOS complex and allowed for separation based on size difference. Chromatography in the presence of 1.0% Z3-14 gave the same result (data not shown). TMAE Fractogel chromatography in the presence of 5.0% Z3-12, and elution of UspA2 with a linear NaCl gradient, resulted in the separation of the UspA2 protein from LOS (Fig. 3A). Coomassie blue staining of TMAE column fractions run on SDS–PAGE shows the UspA2 protein primarily in fractions 29–32 (Fig. 3B). Western blot analysis of the same column fractions using MAb Mcat73-11, shows the LOS primarily in fractions 4–16 (Fig. 3C). The inclusion of Z3-12 in TMAE Fractogel chromatography buffers resulted in the dissociation of the UspA2–LOS complex and allowed for their separation based on charge differences. Lower concentrations of Z3-12 were less effective for dissociating the UspA2–LOS complex.

Following reduction of endotoxin by either Superose-6 or TMAE Fractogel chromatography, the UspA2 was exchanged back into 0.1% TX-100 by passage over a G-25 column equilibrated in 0.03 *M* Tris–HCl (pH 8.0) containing 0.1% TX-100 and then reapplied to a TMAE Fractogel column. Following extensive washing of the column, with at least 10 column volumes of 0.03 *M* Tris–HCl (pH 8.0) containing 0.1% TX-100, the UspA2 protein was eluted with the same buffer containing 1.0 *M* NaCl.

3.3. LAL gel-clot assay and rabbit pyrogenicity test

UspA2 preparations before and after LOS reduction using TMAE Fractogel chromatography in the presence of 5.0% Z3-12 were analyzed for endotoxin

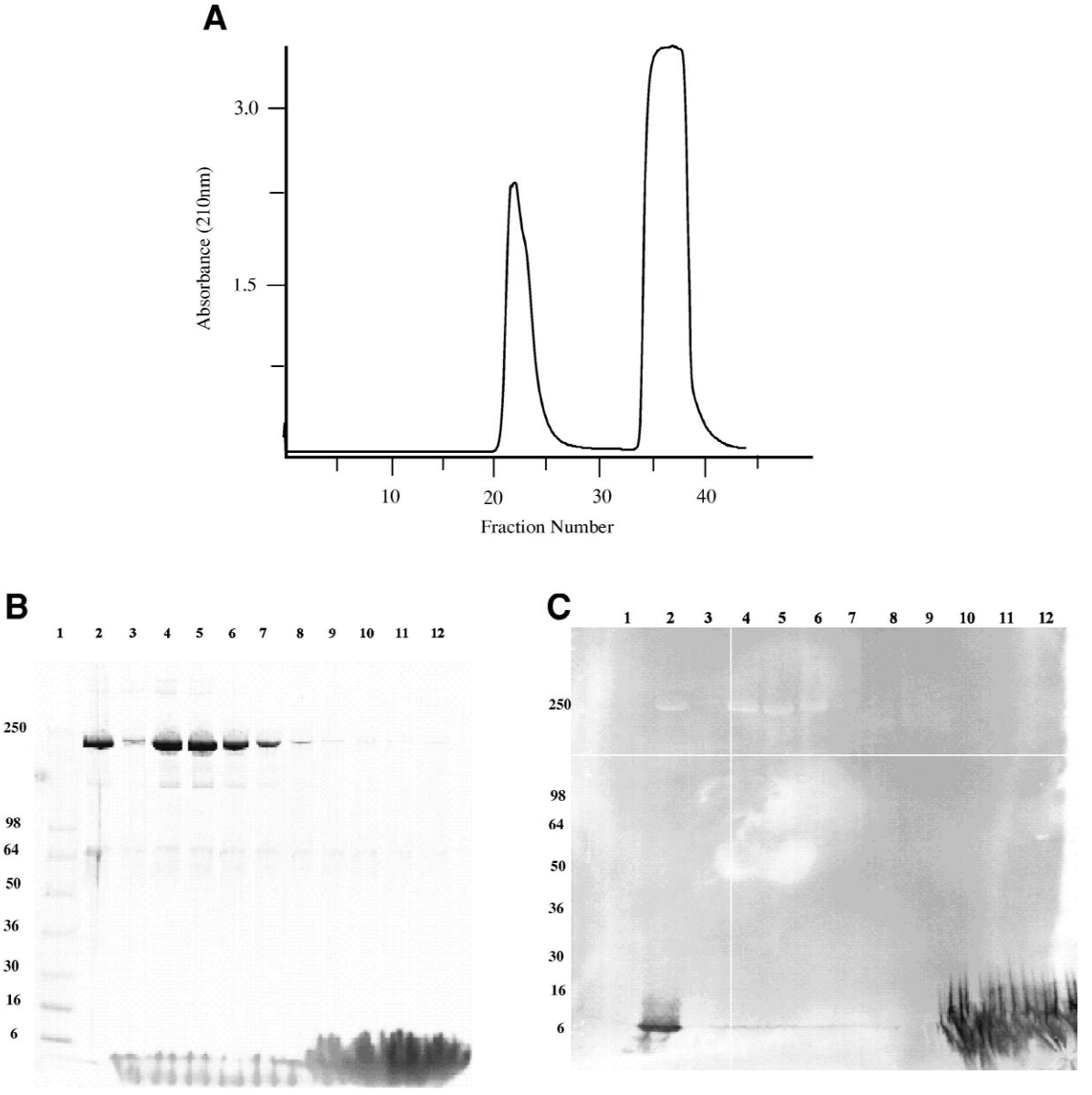

Fig. 2. Reduction of endotoxin in UspA2 by Superose-12 Chromatography in the presence of Z3-12. (A) Chromatography of UspA2 on a 500-ml Superose-12 column in PBS–1.0% Z3-12. (B) Coomassie blue stained SDS–PAGE of Superose-12 column fractions. Lanes: 1=molecular size markers (in kilodaltons), 2=UspA2 starting material, 3=fraction 20, 4=fraction 21, 5=fraction 22, 6=fraction 23, 7=fraction 24, 8=fraction 26, 9=fraction 29, 10=fraction 33, 11=fraction 35, 12=fraction 36. (C) Western blot of Superose-12 column fractions probed with *M. catarrhalis* LOS MAb 73-11. Lanes loaded as described for (B). The Coomassie blue staining material at the dye front in (B) is Z3-12. The cross-reactive material at the dye front in (C) lanes 2, 10, 11, and 12 is LOS. LOS mobility is distorted in the presence of 1.0% Z3-12.

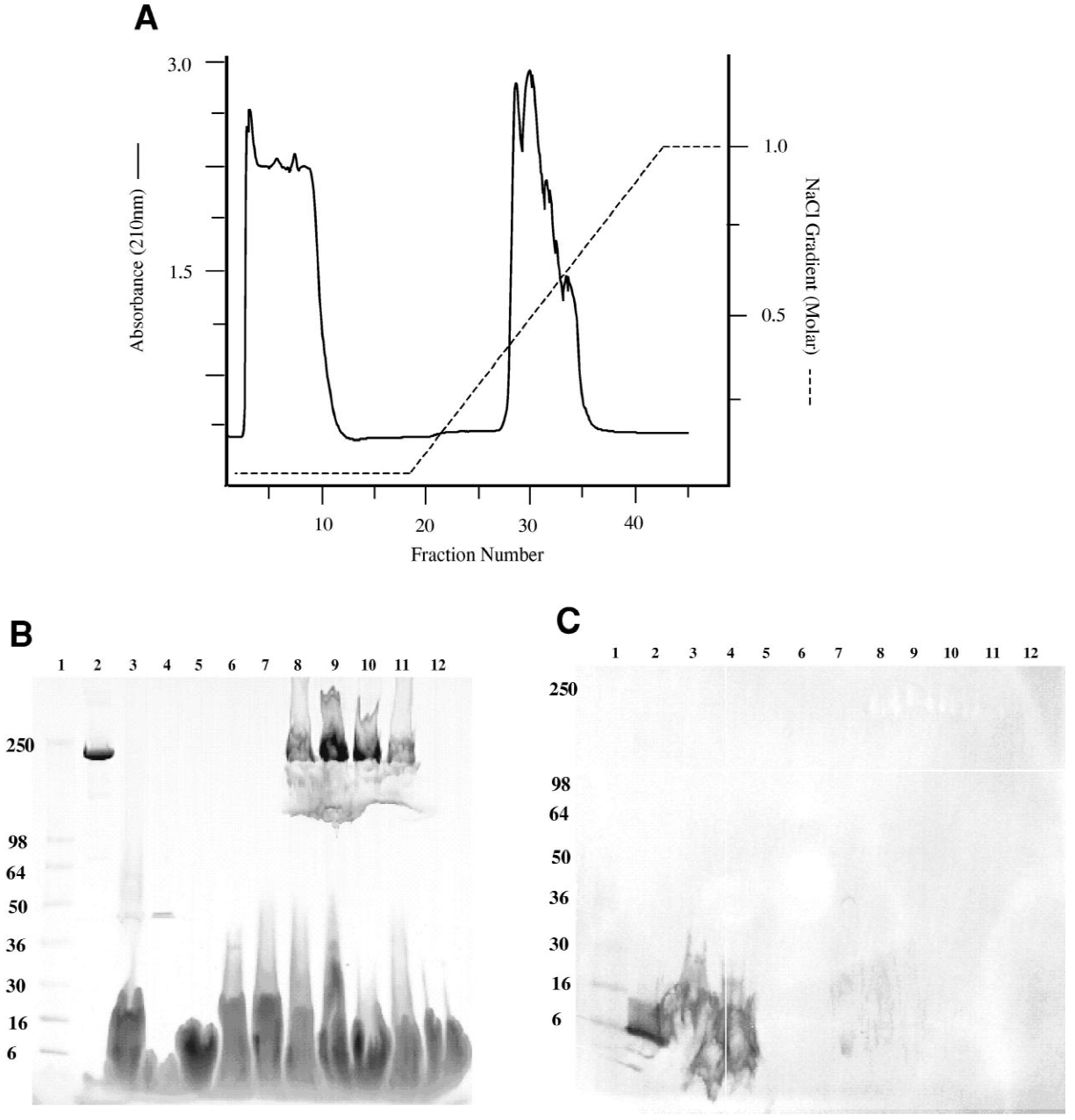

Fig. 3. Reduction of endotoxin in UspA2 by TMAE Fractogel Chromatography in the presence of Z3-12. (A) Chromatography of UspA2 on a 10-ml TMAE Fractogel column in 0.03 *M* Tris–HCl–5.0% Z3-12. (B) Coomassie blue stained SDS–PAGE of TMAE column fractions. Lanes: 1=molecular size markers (in kilodaltons), 2=UspA2 starting material, 3=fraction 4, 4=fraction 8, 5=fraction 16, 6=fraction 20, 7=fraction 24, 8=fraction 29, 9=fraction 30, 10=fraction 31, 11=fraction 32, 12=fraction 40. (C) Western blot of TMAE column fractions probed with *M. catarrhalis* LOS MAb 73-11. Lanes loaded as described for (B). The Coomassie blue staining material at the dye front in (B) is Z3-12. The cross-reactive material at the dye front in (C) lanes 2–5 is LOS. Both UspA2 and LOS mobility is considerably distorted in the presence of 5.0% Z3-12.

Table 1
Reduction in endotoxin as measured with the LAL and rabbit pyrogenicity assays

UspA2 sample	LAL assay (EU/μg protein)	Rabbit pyrogenicity[a]
Before LOS reduction	26.4	0.2, 0.4, 0.6 (fail)
After LOS reduction	0.0012	0.0, 0.2, 0.4 (pass)

[a] Values are the temperature rises (°C) for individual rabbits above that seen for a normal resting animal. The Federal Code protocol requires testing in three rabbits. Then, if it does not pass, the sample is to be tested in an additional five rabbits [11]. In this instance the sample failed because one of the rabbits had a temperature rise above 0.5°C. It is likely this sample would have passed if it had been tested in the additional rabbits.

by the LAL gel-clot assay. Prior to endotoxin reduction, the UspA2 preparation exhibited an endotoxin level of 26.4 EU (endotoxin units)/μg (Table 1). Following chromatography on TMAE Fractogel, the endotoxin was reduced approximately 20 000-fold to 0.0012 EU/μg. UspA2 preparations were tested for rabbit pyrogenicity at a dose of 6 μg of protein per kg. The reduction in endotoxin correlated to reduced pyrogenicity in rabbits (Table 1).

3.4. Antibody reactivity to whole bacterial cells and bactericidal activity

Antisera prepared against the UspA2 before and after endotoxin reduction, were assayed in the whole cell ELISA against the homologous O35E strain and two heterologous isolates (Table 2). Following the removal of endotoxin, the titers elicited by UspA2 were comparable to the titers elicited prior to endotoxin reduction.

The bactericidal activities of the antisera to UspA2 prior to, and following endotoxin reduction were determined against the same isolates (Table 2). Sera raised against both protein preparations exhibited similar bactericidal titers in the range of 400–800 against each tested isolate. Negative control sera and pre-immune sera had titers of <100 against each strain tested (data not shown).

3.5. Pulmonary challenge

Immunized mice were given a pulmonary challenge with the homologous O35E strain or the heterologous TTA24 strain. Relative to control mice immunized with CRM_{197}, enhanced clearance of both strains was nearly equivalent for the mice immunized with UspA2 preparations prior to and following endotoxin removal (Table 3).

4. Discussion

The method we described previously for the purification of UspA2 from *M. catarrhalis* resulted in a preparation that had greater than 95% homogeneity based on Coomassie Brilliant Blue stained SDS–PAGE followed by scanning densitometry [9]. Although they often marginally passed the rabbit pyrogen test, the level of endotoxin detected by the LAL assay was consistently unacceptable. The high level of endotoxin as LOS in the UspA2 preparations was confirmed by western blots probed with a MAb specific for *M. catarrhalis* LOS. Therefore, we deemed it necessary to develop a method to reduce

Table 2
Whole bacterial cell ELISA titers and complement dependent killing titers toward three bacterial isolates for sera elicited by the UspA2 preparations

Isolate	Whole cell ELISA titer[a]		Bactericidal titers[b]	
	Before LOS reduction	After LOS reduction	Before LOS reduction	After LOS reduction
O35E[c]	379 329	158 670	800	800
430:345	9856	9782	800	400
1230:359	25 634	25 875	400	800

[a] ELISA titers were determined for a pool of sera from 10 immunized mice. The titers for the pool of sera drawn from the same mice before immunization was <50, the lowest dilution tested.

[b] Complement dependent bactericidal titers were determined for the same pool of sera as the whole cell ELISA titers. The bactericidal titers for the pool of sera drawn before immunization was <100, the lowest dilution tested.

[c] The UspA2 was prepared from the O35E isolate.

Table 3
Comparison of bacterial pulmonary clearance of the homologous (O35E) and a heterologous (TTA24) isolate of the bacteria by mice immunized with UspA2 preparations before and after LOS reduction

Challenge isolate	Before LOS reduction		After LOS reduction	
	% Clearance	*P*	% Clearance	*P*
O35E[a]	28.6	0.049	56.1	0.0053
TTA24	38.0	0.022	60.3	0.0047

[a] The UspA2 was prepared from the O35E isolate.

endotoxin without destroying the protein's biological properties before it could be evaluated as a vaccine for human use.

The challenge was the strong association of the LOS with the UspA2 protein. We found the LOS could not be separated from UspA2 in Triton X-100 containing buffers. This was based on our attempt to separate them by charge using ion-exchange chromatography, by size using gel filtration chromatography, and specific affinity using polymyxin B chromatography. Yet, it was clear that the LOS was not covalently bound to UspA2 because they could be separated by SDS–PAGE. We speculate that this tight binding may have an important biological function. UspA2 has been placed among a group of proteins classified as autotransporters [18] and is not an integral outer membrane protein. Immuno-electron microscopy using a MAb specific for both UspA1 and UspA2 indicates that both proteins are present in the corona-like material surrounding the bacterium and not in the membrane itself (unpublished results). One hypothesis is that its tight association with LOS serves to anchor it to the bacterial surface. Another is that the tight binding lessens the host's inflammatory response to LOS which may play a role in the pathogenicity of *M. catarrhalis*. This would explain why preparations often lacked or exhibited a low level of pyrogenicity in the rabbit test despite the presence of high concentrations of LOS. It was because of the marginal pyrogenicity that we thought it necessary to reduce LOS as much as possible before using it in a human vaccine.

The use of detergents, such as octyl-β-D-glucopyranoside and Triton X-114, has been shown to successfully aid in the dissociation of endotoxin from protein [7,19]. The approach that proved successful for the dissociation of endotoxin from UspA2 was to change the detergent from TX-100 to a zwitterionic detergent. Using either Z3-12 or Z3-14, separation was achieved by both ion-exchange chromatography and gel filtration. The inability of TX-100 to dissociate the endotoxin–UspA2 complex while both Z3-12 and Z3-14 proved successful may reside in the charge characteristics of the detergents. Triton X-100 is a non-ionic detergent containing no charged moieties while the Zwittergents contain zwitterionic head groups with both negatively and positively charged moieties. Most zwitterionic detergents are effectively neutral; however, in some cases strong polarization exists [20]. The charge characteristics of Z3-12 and Z3-14 and the interaction of the detergent with either the endotoxin and/or the protein may aid in the dissociation of the endotoxin from UspA2. Structural differences between the detergents may also play a role in effective dissociation of endotoxin and protein. Whatever the mechanism, the use of the Zwittergent detergents proved quite suitable for the removing LOS from UspA2 without disrupting the immunogenic properties of the protein. For both subunit as well as cell-derived vaccines from gram-negative bacteria, the reduction of pyrogenic material is critical to the safety of the product. We have described an efficient method using zwitterionic detergents to remove substantial amounts of endotoxin without affecting the potency of the product. An endotoxin limit of 5.0 EU/kg, based on LAL assay, has been defined as the approximate threshold pyrogen dose for humans and rabbits (21). Prior to endotoxin reduction, the UspA2 preparations contained as much as 158 EU/kg. However, following chromatography in the presence of Z3-12 we achieved levels of approximately 0.0072 EU/kg. The endotoxin removal process has been successfully implemented in our GMP process to produce UspA2 subunit vaccine for clinical trials. We are currently

exploring the use of Zwittergent detergents for the removal of LPS or LOS from other protein preparations, as this approach may be applicable to other proteins that strongly associate with endotoxin.

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