Stability, antigenicity, and aggregation of *Moraxella bovis* cytolysin after purification and storage

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Objectives—To compare stability, antigenicity, and aggregation characteristics of *Moraxella bovis* cytolysins among isolates from geographically diverse areas.

Study Population—8 isolates of *M bovis*.

Procedure—Filter-sterilized broth culture supernatants of *M bovis* were concentrated, diafiltered, and chromatographed. The endotoxin and cytolysin activities in samples were measured. Chromatographed cytolysins of *M bovis* were examined by immunoblotting. Hemolytic and leukotoxic activities were measured from samples collected at each step of purification and before and after storage. Hemolysis was measured directly by use of washed bovine erythrocyte targets. Leukotoxicity was measured by use of a ^{51}Cr release assay.

Results—Cytolysin was retained by a filter with 100-kd nominal molecular weight limit. Hemolytic activity, leukotoxic activity, and endotoxin were eluted together in void volume of a gel-filtration column (molecular mass exclusion limit = 4×10^7 d). Gel-column chromatographed diafiltered retentate had the greatest specific cytolytic activity and the highest endotoxin-to-protein ratio. Frozen diafiltered retentate(–80°C, 4 months) was cytolytic after thawing. Immunoblots of gel-column chromatographed cytolysin contained 4 proteins with molecular masses between 90 and 68 kd. Fractions with high lytic activities also had additional protein bands with molecular masses of 98 and 63 kd. Immunoblots of gel-column chromatographed diafiltered retentate revealed proteins with molecular masses between 90 and 68 kd.

Conclusions and Clinical Relevance—Diafiltered *M bovis* cytolysin is aggregated with endotoxin. Antigenicity and cytolytic activities in diafiltered retentate are conserved among *M bovis* isolates. Diafiltration could be useful for bulk semipurification of *M bovis* cytolysin. Cytolysin-enriched vaccines of *M bovis* could be contaminated by endotoxin. (*Am J Vet Res* 2004;65:977–983)

Moraxella bovis, a major cause of **infectious bovine keratoconjunctivitis (IBK)**, produces a leukotoxic hemolytic cytolysin that belongs to the **repeats-in-structural-toxin (RTX)** group of bacterial exoproteins.[1-5] The cytolysin of *M bovis*, the MbxA protein, is an important pathogenesis factor[6] that consists of 927 amino acids and 6 nonapeptide consensus sequences of glycine rich repeats.[2] Studies[5,7–11] performed prior to the sequencing of the *MbxA* gene examined separate hemolytic or leukotoxic activities of *M bovis*, but a subsequent investigation with anti-recombinant MbxA revealed that the single protein was a true cytolysin with diverse affinities for target cells.[1,2] The gene encoding MbxA has been cloned, and small quantities of the active cytolysin have been expressed in *Escherichia coli*.[1,2]

The MbxA structural protein is exported into culture supernatants while attached to cell membrane fragments of *M bovis*.[5] The greatest rate of toxin export occurs during the log-phase of growth.[5] Despite a theoretic molecular mass of 98.8 kd, biologically active *M bovis* cytolysin was retained by filters with a **nominal molecular weight limit (NMWL)** as large as 300 kd.[12] Filters with high NMWL could, in a single step, concentrate nondenatured aggregated cytolysin from large quantities of culture supernatant. Additional means of purification by use of diafiltration could remove smaller unbound contaminants.

Concentration and purification of cytolysin from culture supernatants by use of high NMWL filters may have relevance to vaccine development for IBK. The cytolysin is an important pathogenesis factor of *M bovis*, and in a previous study,[12] vaccination with the protein induced protection against homologous challenge in calves. Calves that were vaccinated with retentate from ultrafiltered culture supernatants of *M bovis* were resistant after challenge; comparable calves with natural infections developed heterospecific antihemolytic antibodies.[12,13] The scale-up production of a cytolysin-based vaccine has not been attempted, however, because production of sufficient quantities of active protein has been difficult and MbxA is denatured rapidly in impure culture supernatants.[1,5,6,8] The immunogenicity of functionally inactive MbxA has not been examined.

Cytolysin in culture supernatants is adherent to high molecular mass fragments of bacterial membranes, which are coincidentally concentrated during ultrafiltration.[5] Gram-negative bacterial **lipopolysaccharides (LPSs)** contain endotoxin; concentration of these membranes during ultrafiltration could be disadvantageous if the retentate were to be used as an IBK vaccine. Consequently, in the study reported here, endotoxin concentrations were measured at all stages of cytolysin purification. For the cytolysin to serve as a clinically useful immunogen, the protein should initiate antitoxic responses that are effective against all *M bovis* isolates. The specificity of serologic reactions to cytolysin from *M bovis* has not yet been examined.

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The purpose of the study reported here was to compare the stability, antigenicity, and aggregation characteristics of *M bovis* cytolysins among isolates from geographically diverse areas.

Materials and Methods

Bacteria—A leukotoxic hemolytic strain of *M bovis* (T+) was isolated from a beef cow in Georgia with IBK. During serial subculture of the T+ strain, a nonpiliated nonhemolytic variant, designated T-, was recovered. The T- strain retained the other biochemical and cultural features of the parent T+ strain. Other isolates[a] of hemolytic leukotoxic *M bovis* were recovered during our clinical investigations of infected cattle that were pastured in regions of the United States that included the following areas: southern Georgia, northern California, and North Carolina. All isolates were identified as *M bovis* by use of biochemical criteria for nonfermenting bacteria.[14,15] Aliquots of frozen (–80°C) stock cultures of each isolate were maintained in a sterile mixture of skim milk and glycerol (1:1 vol/vol). Thawed stock cultures were inoculated onto trypticase soy agar plates that contained 5% sheep blood and were incubated (36°C; 18 hours) to evaluate purity.

Cytolysin production—Subcultures of *M bovis* were prepared by heavy inoculation of bacterial colonies from sheep blood agar plates onto trypticase soy agar plates, followed by incubation (36°C, 18 hours) until bacterial growth reached confluence. After incubation, bacteria were scraped from the agar plates and suspended into 10 mL of trypticase soy broth (1 plate/10 mL of broth). Calcium chloride was added to broth shaker flasks (500 mL) that contained heart infusion broth[b] in pyrogen-free water to obtain a final concentration of 1.5mM. Broth shaker flasks were inoculated with 10 mL of the bacterial suspension. Inoculated flasks were incubated at 35.5 ± 0.5°C on a rotary shaker[c] set at 200 oscillations/min until the **optical density** (**OD**; at 420 nm) of the medium reached 1.85. Broth cultures then were divided into 250-mL bottles and centrifuged for 1 hour at a relative centrifugal force of 13,000 × *g* (4°C) supernatants were then filtered through a sterile 0.2-µm-pore-diameter hydrophilic polyethersulfone membrane.[d] Permeate (designated bacterial filtered permeate) was concentrated 100-fold by ultrafiltration by use of spiral wrapped regenerated cellulose filters (100-kd NMWL)[e] according to the manufacturer's recommendations and a previous procedure.[f] Operating parameters for the filter included 30-psi pressure, 300 to 500 mL/min flow rate, and a 5 to 10-psi pressure differential.

The retentate (designated ultrafiltered retentate) was collected and diafiltered against 45 volumes of chilled (4°C) buffer that consisted of 50mM Tris, 500mM sodium acetate, 1.5mM $CaCl_2$, and 20% glycerol (pH, 8.2). The diafiltration was discontinued when the OD (at 280 nm) of the permeate (designated diafiltered permeate) reached ≤ 0.01. After diafiltration, the retentate (designated diafiltered retentate) was further pumped across the filter until the final volume reached approximately 40 mL. Leukotoxic and hemolytic activities at each stage of purification were measured. Aliquots from each stage of purification were frozen (–80°C), whereas others were refrigerated (4°C) for 120 days before hemolytic and leukotoxic activities were remeasured. All experiments were performed in triplicate.

Chromatography—Diafiltered retentate of *M bovis* isolates was chromatographed through a 1.5 × 30-cm gel-filtration column[g] (molecular mass exclusion limit of 4×10^7 d) that was equilibrated with a buffer that contained 50mM Tris, 0.5M sodium acetate, 1.5mM $CaCl_2$, and 20% glycerol (pH, 8.0). The column was loaded with 200 µL of diafiltered retentate and was eluted with buffer at a flow rate of 0.5 mL•min^{-1}. Absorbance (at 280 nm) of column effluent was measured continuously. Column chromatographed diafiltered retentate fractions (3.0 mL) were collected and examined for leukotoxic, hemolytic, and endotoxic activities. The elution profile of the column was calibrated by use of globular proteins with defined molecular weights.[h] The **void volume** (V_o) of the column was 3.5 mL, and the **total bed volume** (V_t) was 16.9 mL. For the standard curve, the **partition coefficient** (K_{av}) was plotted versus $\log_{10}$ of the molecular mass of each standard. The **elution volume** (V_e) for each sample and standard was measured from the chromatographic profile. The K_{av} was calculated by use of the following formula[16]:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

Leukotoxicity—Leukotoxicity was measured by ^{51}Cr (sodium chromate) release from immortalized bovine lymphocytes (BL-3 cells)[i] that were seeded from frozen stock cultures in Leibovitz's medium L-15[j] and minimal essential medium (1:1, vol/vol)[k] with penicillin (100 U/mL), streptomycin (100 µg/mL)[j], and 15% heat inactivated fetal bovine serum.[j] After 4 days of incubation (37°C, 5% CO_2), cells were harvested by centrifugation and the pellets were washed 3 times in Dulbecco's PBS solution.[j] After the final wash, cells were resuspended in Dulbecco's PBS solution qto a final concentration of 2×10^7 cells/mL, labeled with 200 µCi of ^{51}Cr[k] for1 hour at 37°C, and then washed 3 times in McCoy's 5A medium.[j] After the final wash, cells were diluted to a final concentration of 1×10^6 cells/mL in McCoy's 5A medium. Labeled BL-3 cells (500 µL) were added to 500 µL of sample and incubated for 1 hour (37°C). Labeled cells (500 µL) that were incubated in 500 µL of McCoy's 5A medium, with and without 0.1% octylphenoxypolyethoxyethanol,[l] served as respective positive and negative controls. After incubation, cell suspensions were centrifuged and the radioactivity in **counts per min (CPM)** was measured in 500 µL of supernatant. The percentage of lysis was calculated by use of the following formula:

$$\% \text{ Lysis} = \frac{\text{CMP speciman} - \text{CPM media control}}{\text{CMP100\%} - \text{CMP media control}} \times 100$$

A unit of leukotoxic activity was defined as the amount of leukotoxin that was required to release 1% of the total amount of ^{51}Cr from the labeled BL-3 cells. Specific activity was defined as the number of leukotoxin units per microgram of protein.

Hemolysis—Hemolytic activity was measured by use of a direct assay. Bovine erythrocytes were harvested from 10 mL of heparinized bovine blood. Whole blood was centrifuged, and the packed erythrocytes were washed 3 times with **Tris-buffered saline and calcium chloride buffer** (**TBS $CaCl_2$**; 50mM Tris, 150mM NaCl, 1.5mM $CaCl_2$; pH, 8.0). For the hemolysin assay, 1 µL of packed erythrocytes was added to 100 µL of 10-fold serial dilutions of test sample. The mixtures were incubated for 6 hours (37°C) before final evaluation of hemolytic activity. Washed erythrocytes in TBS $CaCl_2$ incubated with and without 0.1% octylphenoxypolyethoxyethanol served as the respective positive and negative controls. After incubation, the cell suspension was centrifuged and absorbance (at 450 nm) of the supernatants was measured by use of an automated reader.[m]

The percentage hemolysis was calculated by use of the following formula:

$$\% \text{ Hemolysis} = \frac{\text{OD speciman} - \text{OD mediacontrol}}{\text{OD speciman} - \text{OD media control}} \times 100$$

A unit of hemolytic activity was defined as the dilution of sample that released 50% of the total hemoglobin from the

packed erythrocytes. Specific activity was defined as the number of hemoglobin units per microgram of protein.

Antisera and endotoxin—Antisera against the cloned expressed carboxy-terminal peptide of MbxA (amino acids 590 to 927) were prepared by a commercial laboratory.[2] The activity of endotoxin in each purification stage was measured by use of a timed gel formation limulin assay.[n]

Electrophoresis—Proteins were examined by use of a discontinuous polyacrylamide gel electrophoresis method.[17] For electrophoretic examination of column chromatographed diafiltered retentate, fractions were collected from 3 replicate chromatograms. Corresponding fractions from each replicate were pooled and concentrated by vacuum to a final volume of 2.5 mL.[o] Each pooled and concentrated sample was applied to a desalting gel column,[p] which was eluted with a buffer composed of Tris (0.02M; pH, 8.2), sodium chloride (150mM), and calcium chloride (1.5mM). After buffer exchange, samples were again concentrated by centrifugation under vacuum to a 0.5-mL volume prior to electrophoresis. For electrophoresis, the concentrated column fractions were mixed with an equal volume of 2X loading buffer composed of 62.5mM Tris (pH, 6.8), 0.7mM β-mercaptoethanol, 20% glycerol, 4.1% **sodium dodecyl sulfate (SDS)**, and 0.2 mg of bromophenol blue/mL. The solutions were heated at 100°C for 5 minutes and electrophoresed on a discontinuous polyacrylamide gel. Stacking and running gels consisted of 3.9% and 7% polyacrylamide, respectively. Gels were either washed and silver stained, or proteins were transferred onto polyvinylidene fluoride membranes[q] for immunoblotting.[18,19]

Immunoblotting—To reduce nonspecific binding, blots were blocked in a Tris SDS buffer that contained 0.1% Tween 20, 20mM Tris (pH, 7.4), 150mM NaCl, and 5% teleost gelatin[r] for 12 hours. The membranes were then washed once and incubated with antiserum that was diluted 1:400 in blocking buffer (20 mL) for 12 hours. Afterwards, blots were washed 4 times and then incubated with 0.5 µCi of ^{125}I-labeled protein A[s] in 20 mL of Tris SDS buffer. The ^{125}I-labeled protein A blots were incubated for 2 hours and then washed 3 times (4 h/wash) in Tris-NaCl (20mM Tris [pH, 7.4], 150mM NaCl, 0.1% Tween 20) buffer. After washing, the blots were air-dried and autoradiographed.

Silver staining—Gels were stained by use of an ammoniacal silver staining procedure.[19] After electrophoresis, gels were bathed overnight in a mixture of 50% ethanol and 10% acetic acid solution and then subjected to 3 cycles of rinsing in water that contained 20% ethanol alternated with 5% glutaraldehyde. The fixed gels were rinsed in distilled water and sensitized by addition of 1.4 mL of NH_4OH (14.8M) dissolved in 100 mL of distilled water. Addition of sodium hydroxide (190 µL, 10N) was followed by drop-wise addition of 1.0 mL of silver nitrate (0.8 g). After 30 minutes of incubation, the solution was removed and the gel was washed 3 times in deionized water (20 min/wash). The gels were developed by addition of a solution that contained 0.005% citric acid and 0.019% formaldehyde; the reaction was stopped at an appropriate time by the addition of a mixture of 10% acetic acid and 20% ethanol. Stained gels were washed for 3 more cycles in distilled water, sealed in porous plastic wrappers, and then dried over vacuum.

Protein measurement—Protein concentrations were measured by use of a commercially adapted bicinchoninic acid assay.[20,t] Protein concentrations from column chromatography were measured by use of a UV spectrophotometer[u] set at a wavelength of 280 nm because protein concentrations in these specimens were less than the sensitivity of the bicinchoninic acid assay. All protein measurements were performed in triplicate.

Statistical evaluation—Differences between means were evaluated for significance by use of an ANOVA for repeated measures and a Student *t* test. Values of $P < 0.05$ were considered to represent a significant difference from the null hypothesis.

Results

Leukotoxicity—The specific activity of leukotoxin at each stage of purification was determined (**Table 1**). The specific activity of whole cells at harvest was 0.11 ± 0.04 U/µg of protein, which was less than that of the column chromatographed diafiltered retentate (3.18 ± 0.37 U/µg). The specific activity of leukotoxin in diafiltered retentate was 0.39 U/µg of protein, which was significantly greater (by 3.5-, 9.8-, and 5.6-fold) than the respective concentrations of 0.11, 0.04, and 0.07 U/µg of protein in whole cells, bacterial filtered permeate, and ultrafiltered retentate. The specific activity of leukotoxin in the column chromatographed diafiltered retentate was 3.18 U/µg of protein, which was significantly greater (by 29-, 80-, 45-, and 8-fold) than the respective values of 0.11, 0.04, 0.07, and 0.39 U/µg of protein in whole cells, bacterial filtered permeate, ultrafiltered retentate, and diafiltered retentate, respectively. An increase in leukotoxin specific activity was found in the diafiltered retentate and column chromatographed diafiltered retentate after storage, but the differences from day 0 measurements were not significant. Culture supernatants from the *M bovis* T- strain were not leukotoxic (data not shown).

Hemolysis—The 50% end points were not evident in specimens that contained high hemolytic activity. Serial dilutions from the same specimen often produced 50% hemolysis. The variability of the endpoints resulted in a lack of significant differences of hemolytic activities (**Table 2**). Both frozen and refrigerated column chromatographed diafiltered retentate retained hemolytic activity for 120 days. Hemolytic activity also was detected in bacterial filtered permeate, whole cells, and ultrafiltered retentate after refrigeration or freezing. Culture supernatants from the *M bovis* T- strain were not hemolytic (data not shown).

Table 1—Mean (± SD) specific leukotoxic activities (U/µg of protein) during purification and storage

		4 Months of storage	
Sample type	**Day 0**	**4°C**	**–80°C**
Whole cells	0.11 ± 0.04[a]	0.09 ± 0.10[a]	0.12 ± 0.05[a]
Bacterial filtered permeate	0.04 ± 0.00[a]	0.03 ± 0.30[a]	0.03 ± 0.001[a]
Ultrafiltered retentate	0.07 ± 0.03[a]	0.02 ± 0.03[a]	0.05 ± 0.03[a]
Diafiltered retentate	0.39 ± 0.43[b]	0.72 ± 0.30[b]	1.18 ± 0.36[b]
Chromatographed diafiltered retentate	3.18 ± 0.37[c]	15.3 ± 14.00[b,c]	10.80 ± 6.00[b,c]

[a,b,c]Superscript letters that differ indicate values that are significantly ($P < 0.05$) different within the same sample type.

Gel chromatography—The elution profile for diafiltered retentate of the *M bovis* T+ strain was determined (**Figure 1**). In the gel-column chromatography, globular protein standards with molecular masses of 66 and 150 kd eluted at respective values of K_{av} of 0.35 and 0.31, whereas MbxA immunoreactive proteins eluted from the column at a K_{av} of 0.01, which corresponded to a globular protein with a molecular mass of approximately 4×10^7 d. Leukotoxic and hemolytic activities were eluted together with endotoxin in the V_o of the gel-filtration column. The predominant hemolytic and leukotoxic activities were eluted coincidentally with a protein peak that had a K_{av} of 0.01 (molecular mass of 4×10^7 d). Two smaller peaks, which had values of K_{av} of 0.18 and 0.31 (fractions 8 and 9, and 11 and 12, respectively), were leukotoxic but not hemolytic. Fractions 13 to 17 were neither hemolytic nor leukotoxic.

When probed with MbxA carboxy-terminal antiserum, western blots of fractions 6 to 9 contained 4 proteins that migrated with apparent molecular masses of 90, 80, 75, and 68 kd. Fraction 5, which corresponded to the peak protein activity and eluted at a K_{av} of 0.01, contained these 4 bands as well as additional immunoreactive proteins with molecular masses of 98 and 63 kd. A single faintly recognized protein was found with a molecular mass of 90 kd in fraction 11.

The protein elution profile of the *M bovis* T- strain, as measured at an OD of 280 nm, was similar to that of the T+ strain, but none of the fractions had discernible hemolytic or leukotoxic activities. Proteins were not visible in the immunoblots from any of the fractions from the gel-column chromatographed diafiltered retentate of the T- strain, but numerous proteins were detectable by silver staining (**Figure 2**). Silver stained gels of cytolytic fractions from T+ strain contained a 90-kd molecular mass protein that was absent in corresponding fractions of the T- strain.

Concentrations of endotoxin in fractions from column chromatographed diafiltered retentate were determined. A small amount of endotoxin activity was found in fraction 4 (0.01 µg/mL). Peak endotoxin activity was seen in fractions 5 and 6 (0.5 µg/mL each); progressively smaller amounts of activity were seen in fractions 7 to 9 (0.02, 0.01, and 0.01 µg/mL, respectively) and in fraction 11 (0.01 µg/mL).

Endotoxin measurements—Endotoxin concentrations at each purification stage were determined (**Table 3**). Ultrafiltered retentate had 7 µg of endotoxin/mL, which was 10-fold greater than that of whole cells (0.7 µg/mL) and 4-fold greater than that of the bacterial filtered permeate (2.9 µg/mL). Diafiltered permeate contained 0.02 µg of endotoxin/mL. The endotoxin concentration of the column chromatographed diafiltered permeate was 0.6 µg/mL, which was less than that of diafiltered retentate (2.6 µg/mL).

Table 2—Mean (± SD) specific hemolytic activities (U/µg of protein) during purification and storage.

Sample type	Day 0	4 Months of storage	
		4°C	–80°C
Whole cells	9.08 ± 12.3	0.11 ± 0.08	3.48 ± 1.73
Bacterial filtered permeate	6.41 ± 8.40	0.15 ± 0.11	0.07 ± 0.04
Ultrafiltered retentate	0.96 ± 0.69	0.29 ± 0.43	0.23 ± 0.15
Diafiltered retentate	3.04 ± 2.01	3.64 ± 4.84	1.75 ± 2.31
Chromatographed diafiltered retentate	14.13 ± 8.27	23.86 ± 29.61	11.97 ± 9.97

No significant differences were found between corresponding samples measured on day 0 and at 4 months.

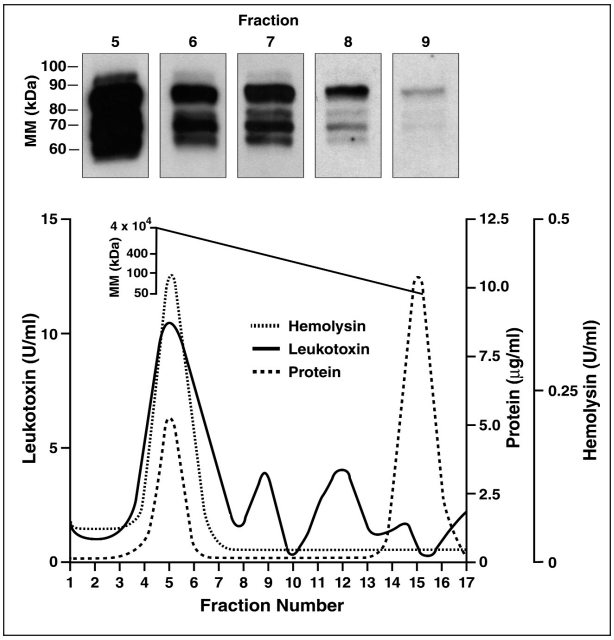

Figure 1—Chromatographic profile of diafiltered retentate. Fraction size was 3.0 mL. Diafiltered retentate of *Moraxella bovis* isolates was chromatographed through a 1.5 × 30-cm gel-filtration column (molecular mass [MM], 4×10^7 d). The elution buffer consisted of 50mM Tris, 0.5M sodium acetate, 1.5mM $CaCl_2$, and 20% glycerol (pH, 8.0). The column was loaded with 200 µL of diafiltered retentate and was eluted at a flow rate of 0.5 mL•min^{-1}. Molecular masses based on the partition coefficient (K_{av}) of each fraction are shown above the elution curves. Immunoblots of fractions with cytolytic activities are shown. Primary antibody for immunoblots was a 1:250 dilution (in PBS solution) of rabbit anti-carboxy-terminal antiserum against recombinant MbxA.

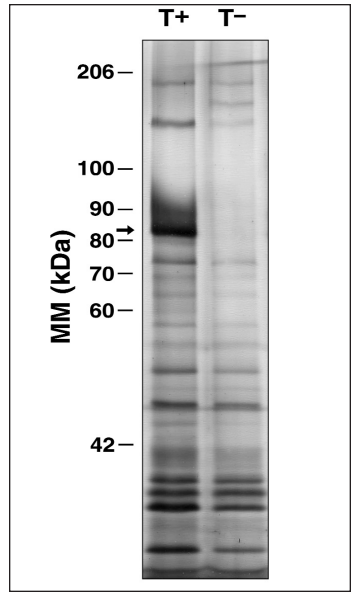

Figure 2—Silver stained gel of peak cytolytic fraction of gel-column chromatographed diafiltered retentate from *M bovis* T+ and T- strains. Notice the 90-kd protein (arrow) that is exclusive to the T+ strain.

The protein adjusted concentration of endotoxin in diafiltered retentate and chromatographed diafiltered retentate was, respectively, 0.5 and 0.1 µg of endotoxin/µg of protein, which was 50- and 10-fold greater than the endotoxin concentration of bacterial filtered permeate (0.01 µg of endotoxin/µg of protein; Table 3). The ultrafiltered retentate contained 0.07 µg of endotoxin/µg of protein.

Antigenic studies—All isolates of *M bovis* except for the T- strain were leukotoxic and hemolytic. Immunoblots of pooled column chromatographed diafiltered retentate from all hemolytic and leukotoxic isolates contained 4 protein bands with apparent molecular masses of 90, 80, 68, and 65 kd (**Figure 3**). Bands of 1 strain (isolate H from Georgia) reacted less intensely to the probe than those of other isolates and contained only 3 bands with molecular masses of 90, 80, and 68 kd. Four of the other isolates had a fifth immunoreactive band that had an apparent molecular mass of 160 kd. Isolates designated as isolate D and isolate G also had protein bands with molecular mass of 98 kd; isolate G also had a protein with a molecular mass of 58 kd. Immunoreactive bands were not seen in the lanes that contained gel-column chromatographed diafiltered retentate of the T- strain. The leukotoxic activities in the diafiltered retentate that was prepared from the various strains ranged between 0 and 0.24 U/mL, and the corresponding hemolytic activities ranged between 0 and 5.7 U/mL.

Table 3—Mean (± SD) endotoxin and protein-adjusted endotoxin concentrations during purification of *Moraxella bovis* cytolysin.

	Endotoxin concentrations	
Sample type	**µg/mL**	**µg/µg of protein**
Whole cells	0.70 ± 1.50	0.001 ± 0.010
Bacterial filtered permeate	2.90 ± 1.80	0.01 ± 0.04
Ultrafiltered retentate	7.00 ± 42.00	0.07 ± 0.09
Diafiltered retentate	2.6 ± 22.0	0.5 ± 0.6
Diafiltered permeate	0.02 ± 0.01	0.00
Chromatographed diafiltered retentate	0.6 ± 0.2	0.1 ± 0.1

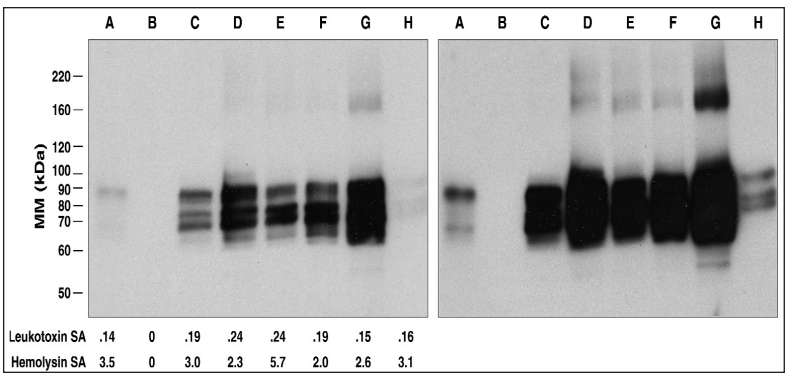

Figure 3—Photograph of an autoradiogram of immunoblots of the gel-column chromatographed diafiltered retentate from each of 8 *M bovis* isolates. Exposures of the labeled immunoblot were 3.5 hours (left panel) and 5.5 hours (right panel) for the blots on the left and the right, respectively. Diafiltered retentate was chromatographed on a gel-column, and the first protein peak (K_{av} = 0.01) from each chromatography was pooled, concentrated to 2.5 mL, desalted, and concentrated again to a 0.5-mL volume by vacuum centrifugation. After electrophoresis and a transblotting process, the immunoblots were exposed to a primary antibody consisting of a 1:250 dilution of anti-carboxy-terminal recombinant MbxA. All isolates except the T– strain were hemolytic on 5% sheep blood agar plates. Origin of isolates included the following: A = T+ strain (from northern Georgia); B = T– strain (derived from the T+ strain from northern Georgia); C = northern Georgia; D = California; E = Iowa; F = North Carolina; G = Iowa; and H = southern Georgia. Numbers at the bottom reflect the specific activity (SA) of leukotoxin and hemolysin. Isolates A and H had weak binding affinity to the antibody probe.

Discussion

Findings in our study reveal the efficacy of a large-capacity high exclusion limit filter for nondenaturing concentration and semipurification of the *M bovis* cytolysin. Our data indicate that the filter could be useful for experimental vaccine production. In our study, approximately 10 hours were required to concentrate and diafilter the cytolysin; the filtration procedure achieved a final approximate 125-fold concentration with an approximate 10-fold increase of leukotoxin specific activity.

Vaccines produced from filter-concentrated culture supernatants of *M bovis* may stimulate protective immunity against IBK. Findings in a previous study[12] with experimentally challenged calves revealed that calves were resistant to challenge with *M bovis* after immunization with ultrafiltered retentate of hemolytic culture supernatants. Although adverse systemic reactions were not described for calves in the previous study, findings in our study indicate that the ultrafiltered retentate vaccine would probably have contained a large and potentially toxic concentration of endotoxin. This would have occurred because in the filtration step we found that endotoxin, leukotoxin, and hemolysin were concentrated together. In our study, endotoxin was not removed by the sequential diafiltration step, and the protein-adjusted endotoxin concentration of the diafiltered retentate was 0.5 µg of endotoxin/µg of protein. This concentration was greater than that of bacterial filtered permeate or ultrafiltered retentate. Consequently, if 10 and 20 µg of specific MbxA were administered, the vaccine would also have delivered between 0.05 and 0.1 µg of endotoxin/kg. This concentration of endotoxin would initiate nonfatal yet clinically visible responses in calves that weigh 100 kg.[21] Although the protein-adjusted concentration of endotoxin increased during diafiltration, the purification process could actually reduce the overall potential for clinical endotoxic reaction by increasing the specific activity of the cytolysin and, in turn, reducing the amount of vaccinal protein that is required for induction of an effective immune response. Endotoxin removal, which should be a major goal for any parenterally administered gram-negative bacteria derived vaccine, was not attempted in our study because the addition of detergents or chaotropic agents to the diafiltered retentate could have reduced the overall immunogenicity of cytolytic protein. For vaccine production, additional purification steps would increase the unit costs.

In our study, the leukotoxic activities of frozen diafiltered retentate at 4 months were greater than corresponding values on day 0. The importance of the increased

activity was unclear because the differences between the day 0 and 4-month samples were not significant. The increased activity could have been merely related to interassay variability or, conversely, to a type II error that masked the presence of a true storage-related increase in leukotoxicity. If this were the case, then increased specific activity of leukotoxin could have been caused by removal of proteolysins or other inhibitory substances. Similar increases have been seen in culture supernatants of *M bovis* after addition of the serine protease inhibitor, phenylmethyl sulfonyl fluoride.[7]

The lack of MbxA in the column chromatographed diafiltered retentate from T- was consistent with the results of a study that revealed the deletion of the *mbx* operon in nontoxigenic *M bovis* strains.[1] In our study, minor immunoblot differences were found among the cytolytic *M bovis* isolates, but all isolates, except the T-strain, produced immunoreactive proteins with molecular masses that ranged between 90 and 68 kd. The 160-kd protein that appeared in 4 of the column chromatographed diafiltered retentate samples could have been produced by the other isolates at concentrations that were undetectable by the immunoblot probe. The weak protein signal on immunoblot analysis of isolate H could have been related to a low MbxA expression or a sequence variation that reduced binding affinity. Because of the similarities of protein migration in the immunoblots, we suspect that the various staining intensities were most likely related to differing amounts of MbxA expression by *M bovis*. The methods for genetic regulation of expression of the *mbx* operon are unknown, but iron- and heat- sensitive promoters that are upstream from the *lktA* gene have been identified in *Pasteurella haemolytica* and a regulatory factor, named RfaH, that serves as a transcriptional elongation protein has been found in *E coli*.[14,22] A JP-2 like promoter has been found in *Actinobacillus actinomycetemcomitans*.[23] Because of the similarities between the *MbxA* gene and genes found in other RTX-producing bacteria, the existence of such promoters in *M bovis* would seem to be likely. In our study, constituent proteins with molecular masses of < 90 kd appeared in the V_o fractions of gel-column chromatographed diafiltered retentate (molecular mass of 4×10^7 d). Physical attachment between endotoxin and cytolysin was indicated by the coelution of the lysins, by the retention of cytolysin by a high NMWL filter, by failure to separate endotoxic and cytolytic activities by use of diafiltration, and by identification of low concentrations of endotoxin in noncytolytic fractions. The attachment between endotoxin and cytolysin could have occurred between the fatty acyl groups of the endotoxin and the putative lysyl acylation sites at positions 536 and 660 of MbxA.[2] The hydrophobic inner core of the bacterial cell wall of gram-negative bacteria is composed of a fatty acylated backbone that consists of a disaccharide of phosphorylated β-(1→6)-linked D-glucosamine that may bind proteins by hydrophobic interactions.[24-27] Similar mechanisms of attachment between endotoxin and RTX proteins have been postulated for β-hemolytic *E coli* and *P haemolytica*.[28-30]

Aggregation between LPS and MbxA also probably explains the results of a previous study that revealed outer membrane localization of the *M bovis* cytolysin.[13] Dissociation and regeneration experiments of MbxA and LPS, as have been reported for other bacteria, were not conducted in our study because earlier attempts to disrupt the aggregates by use of octylphenoxypolyethoxyethanol or SDS resulted in an irreversible loss of leukotoxicity.[29-31]

We postulate the existence of a pathogenic synergy between LPS and MbxA. Inflammation from *M bovis* infection is probably enhanced by LPS binding to transmembrane signaling proteins, which include a toll-like receptor-2 (CD14) and heat shock receptors, that are clustered on specific detergent resistant islands on the cell membranes.[32-35] The LPS binding to these domains may serve to concomitantly deliver MbxA to the cell membrane. Endotoxin activation of chemokine receptors could attract neutrophils and enhance nutrient acquisition by lysis of neutrophils that have migrated into the avascular cornea. Micellar aggregates of MbxA and LPS could also stabilize RTX proteins to prolong cellular necrosis in a manner similar to that described for the LktA (ie, leukotoxin)-LPS aggregates of *P haemolytica*.[29]

The largest of the proteins on immunoblots of cytolytic column fractions had a molecular mass of 90 kd, which was less than the predicted 98.8 kd of the complete MbxA. Because of the high specificity of the antiserum probe for cytolysin, the 4 diafiltered retentate proteins and the cytolytic gel-column fractions probably represented enzymatic digests of the full-length MbxA. *Moraxella bovis* produces and exports proteolysins, which include C8 esterase-lipase, phosphoamidase, leucine and valine aminopeptidases, gelatinase, and C4 esterase.[36] The proteolysins would have been active prior to diafiltration, which would have reduced the size of the native expressed MbxA.

The inactivation of LktA denaturation by aggregation into particulates with a molecular mass of > 8,000 kd, as occurs in *P haemolytica*,[29] seems to be unlikely in the case of MbxA. In our study, the apparent molecular mass of MbxA aggregates exceeded 4×10^7 d; yet despite the large size, the proteins were cytolytic for as long as 4 months. The denaturation of the MbxA in the stored whole cells and the nondiafiltered retentate appears to be similar to that seen with HlyA protein of *E coli,* where hemolytic aggregates with molecular masses > 8,000 kd were inactivated rapidly by trypsinization.[30] A finding in an earlier study[7] in which the addition of the serine protease inhibitor, phenylmethyl sulfonyl fluoride, to sterile culture supernatants of *M bovis* prolonged and enhanced cytolytic activities further supports our hypothesis of an enzymatic MbxA denaturation. Denaturation of cytolysin on whole cells could have corresponded to cellular inactivation that occurred during storage.

In our study, the elution of endotoxin and cytolysin together in gel-column fractions 5 to 9 and also in fraction 11 indicates that there is a potential size heterogeneity of the aggregates. Proteins in fraction 11 eluted at a K_{av} of 0.31, which corresponded to a molecular mass of 98 kd, yet immunoblot analysis of the fraction revealed a single protein with a molecular mass of 90 kd, suggesting that the protein in that frac-

tion consisted of MbxA and LPS oligomeres. The lack of hemolysin in this fraction was attributed to a lower sensitivity and greater variability of the hemolytic assay, compared with the ^{51}Cr test for leukotoxicity.

Results of our study indicate that cell-free supernatants of *M bovis* broth cultures contain aggregates of cytolysin and LPS. Our results also indicate that diafiltration could be useful for bulk semipurification of the cytolysin. Antigenicity and cytolytic activities of MbxA in the diafiltered retentate were conserved among isolates of *M bovis* and, when refrigerated or frozen, were stable for as long as 4 months.

[a]Two strains, GLN 63 and EPP 63, were provided by George W. Pugh, National Animal Disease Control, Ames, Iowa. Others were recovered and identified by the corresponding author; isolates are available from the corresponding author upon request.
[b]Difco Laboratories, Detroit, Mich.
[c]Queue Orbital Shaker, Queue Systems, Asheville, NC.
[d]Gelman Sciences, Ann Arbor, Mich.
[e]Amicon S1Y100 spiral wound membrane cartridge, Millipore Inc, Billerica, Mass.
[f]O'Connell KA. *The development and testing of a vaccine for the prevention of infectious bovine keratoconjunctivitis*. PhD dissertation, University of California, Davis, Calif 1995.
[g]Superose 6 HR column, Amersham Pharmacia Biotech, Uppsala, Sweden.
[h]Gel filtration molecular weight markers, Sigma Chemical Co, St Louis, Mo.
[i]Courtesy of Gordon Theilen, Department of Surgery and Radiology University of California, Davis, Calif.
[j]Gibco BRL Life Technologies Inc, Gaithersburg, Md.
[k]Sodium^{51}Chromate, New England Nuclear Life Science Products, Boston, Mass.
[l]Triton X-100, Sigma Chemical Co, St Louis, Mo.
[m]Spectramax 250 spectrophotometer, Molecular Devices Corp, Sunnyvale, Calif.
[n]Associates of Cape Cod Inc, Falmouth, Mass.
[o]Speed Vac Centrifugal Concentrator, ThermoSavant, Holbrook, NY.
[p]PD-10 desalting column, Amersham Pharmacia Biotech, Uppsala, Sweden.
[q]Immobilon-P, Millipore Inc, Bedford, Mass.
[r]Teleost gelatin, Sigma Chemical Co, St Louis, Mo.
[s]^{125}I-Protein A, New England Nuclear Life Science Products, Boston, Mass.
[t]Pierce BCA protein assay kit, Pierce Inc, Rockford, Ill.
[u]61105 Shimadzu UV160U, Shimadzu Inc, Columbia, Md.

References

1. Angelos JA, Hess JF, George LW. An RTX operon in hemolytic Moraxella bovis is absent from nonhemolytic strains. *Vet Microbiol* 2003; 92:363–377.

2. Angelos JA, Hess JF, George LW. Cloning and characterization of a *Moraxella bovis* cytolysin gene. *Am J Vet Res* 2001;62:1222–1228.

3. Gray JT, Fedorka-Cray PJ, Rogers DG. Partial characterization of a *Moraxella bovis* cytolysin. *Vet Microbiol* 1995;43:183–196.

4. Beard MK, Moore LJ. Reproduction of bovine keratoconjunctivitis with a purified haemolytic and cytotoxic fraction of *Moraxella bovis*. *Vet Microbiol* 1994;42:15–33.

5. Ostle AG, Rosenbusch RF. *Moraxella bovis* hemolysin. *Am J Vet Res* 1984;45:1848–1851.

6. Clinkenbeard KD, Thiessen AE. Mechanism of action of *Moraxella bovis* hemolysin. *Infect Immun* 1991;59:1148–1152.

7. Hoien-Dalen PS, Rosenbusch RF, Roth JA. Comparative characterization of the leukocidic and hemolytic activity of *Moraxella bovis*. *Am J Vet Res* 1990;51:191–196.

8. Kagonyera GM, George L, Miller M. Effects of *Moraxella bovis* and culture filtrates on ^{51}Cr-labeled bovine neutrophils. *Am J Vet Res* 1990;50:18–21.

9. Sandhu TS, White FH. Production and characterization of *Moraxella bovis* hemolysin. *Am J Vet Res* 1977;38:883–885.

10. Nakazawa M, Nemoto H. Hemolytic activity of *Moraxella bovis*. *Jap J Vet Sci* 1979;41:363–367.

11. Kagonyera GM, George LW, Munn R. Cytopathic effects of *Moraxella bovis* on cultured bovine neutrophils and corneal epithelial cells. *Am J Vet Res* 1989;50:10–17.

12. Billson FM, Hodgson JL, Egerton JR, et al. A haemolytic cell-free preparation of *Moraxella bovis* confers protection against Infectious Bovine Keratoconjunctivitis. *FEMS Microbiol Lett* 1994;124:69–74.

13. Ostle AG, Rosenbusch RF. Immunogenicity of *Moraxella bovis* hemolysin. *Am J Vet Res* 1985;46:1011–1014.

14. Weyant RS, Moss CW, Weaver RE, et al. *Identification of unusual pathogenic Gram-negative aerobic and facultatively anaerobic bacteria*. 2nd ed. Baltimore: The Williams & Wilkins Co, 1996;393–394.

15. Bovre K, Froholm LO. Variation of colony morphology reflecting fimbriation in *Moraxella bovis* and reference strains of *Moraxella nonliquefaciens*. *Acta Pathol Microbiol Scand* 1972;80B:629–640.

16. Anonymous. *Gel filtration: principles and methods*. Piscataway, NJ: Amersham Biosciences, 2004;79–80.

17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.

18. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979;76:4350–4354.

19. Harlow E, Lane D. *Antibodies: a laboratory manual*. New York: Cold Spring Harbor Laboratories, 1988;485–510.

20. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;150:76–85.

21. Cullor JS, Fenwick BW, Williams WR, et al. Active immunization with *E. coli* J5 and its protective effects from endotoxic shock in calves. In: Szentivani A, Friedman H, Nowotny A, eds. *Immunobiology and immunopharmacology of bacterial endotoxins*. New York: Plenum Press, 1986;265–268.

22. Marciel AM, Highlander SK. Use of operon fusions in *Mannheimia haemolytica* to identify environmental and *cis*-acting regulators of leukotoxin transcription. *Infect Immun* 2001;69:6231–6239.

23. Brogan JM, Lally ET, Poulsen K, et al. Regulation of *Actinobacillus actinomycetemcomitans* leukotoxin expression: analysis of the promoter regions of leukotoxin and minimally leukotoxic strains. *Infect Immun* 1994;62:501–508.

24. El-Samalouti VT, Hamann L, Flad HD, et al. The biology of endotoxin. In: Holst O, ed. *Methods in molecular biology. Bacterial toxins: methods and protocols*. Totowa, NJ: Humana Press, 2000;287–309.

25. Zahringer U, Lindner B, Reitschel ET. Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. *Adv Carbohydr Chem Biochem* 1994;50:211–276.

26. Brandenberg K, Mayer H, Koch M, et al. Influence of the supramolecular structure of free lipid A on its biological activity. *Eur J Biochem* 1993;218:555–563.

27. Seydel U, Labischinski H, Kastowsky M, et al. Phase behavior, supramolecular structure and molecular conformation of lipopolysaccharide. *Immunobiology* 1993;187:191–211.

28. Bauer ME, Welch RA. Pleiotropic effects of a mutation in *rfaC* on *Escherichia coli* hemolysin. *Infect Immun* 1997;65:2218–2224.

29. Li J, Clinkenbeard KD. Lipopolysaccharide complexes with *Pasteurella haemolytica* leukotoxin. *Infect Immun* 1999;67:2920–2927.

30. Ostolaza H, Bartolome B, Serra J. α-Haemolysin from *E. coli*. Purification and self-aggregation properties. *FEBS Lett* 1991;280:195–198.

31. Clinkenbeard KD, Clinkenbeard PA, Waurzyniak BJ. Chaotropic agents cause disaggregation and enhanced activity of *Pasteurella haemolytica* leukotoxin. *Vet Microbiol* 1995;45:201–209.

32. Yang RB, Mark MR, Gray A, et al. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* 1998;395:284–288.

33. Cinek T, Horejsi V. The nature of large noncovalent complexes containing glycosyl-phosphatydylinositol-anchored membrane glycoproteins and protein tyrosine kinases. *J Immunol* 1992;149:2262–2270.

34. Triantafilou M, Trantafilou K. Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. *Trends Immunol* 2002; 23:301–304.

35. Triantafilou K, Triantafilou M, Dedrick RL. Interactions of bacterial lipopolysaccharide and peptidoglycan with a 79 kDa and an 80 kDa protein on the surface of CD14+ and CD14- cells. *Hum Immunol* 2001;62:50–63.

36. Frank SK, Gerber JD. Hydrolytic enzymes of Moraxella bovis. *J Clin Microbiol* 1981;13:269–271.