Effectiveness of a cytolysin-enriched vaccine for protection of cattle against infectious bovine keratoconjunctivitis

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Objective—To determine the immunogenicity of a *Moraxella bovis* cytolysin-enriched vaccine for prevention of infectious bovine keratoconjunctivitis (IBK).

Animals—104 mixed-breed beef calves ranging between 4 and 8 months of age.

Procedure—Vaccines were prepared by the diafiltration of broth culture supernatant from hemolytic *M bovis* or sterile media. The diafiltered retentate was combined with Quil A adjuvant. Calves were randomly assigned to receive either the cytolysin vaccine (n = 35) or, as controls, adjuvant (35) or saline (0.9% NaCl) solution (34). Eyes of all calves were examined weekly for signs of IBK for 15 weeks. Calves that developed severe IBK were treated SC with florfenicol.

Results—Cytolysin vaccine contained 4 proteins with molecular masses ranging between 65 and 90 kd. Cytolysin-vaccinated calves had fewer instances of IBK than control calves. The time of onset of corneal lesions in cytolysin-vaccinated calves that developed IBK was delayed, compared with that of calves in either control group. The cytolysin-Quil A vaccine contained endotoxin, but calves did not have clinical signs of illness after vaccination.

Conclusions and Clinical Relevance—Calves that were vaccinated with a cytolysin-enriched vaccine had some resistance to IBK. Vaccines containing concentrated diafiltered *M bovis* cytolysin could protect beef calves against IBK. (*Am J Vet Res* 2005;66:136–142)

Infectious bovine keratoconjunctivitis (IBK) is an acute ocular infection that is caused by *Moraxella bovis*.[1,2] Prevention of IBK is difficult because of the high attack rate and the sporadic lack of efficacy of commercially produced vaccines. Reasons for the inconsistent efficacy of vaccines include strain differences and lack of immunologic potency.[3-18] Vaccines that contain pilin proteins do not induce protective responses to heterologous strains of *M bovis*.[15-20] Live unattenuated hemolytic *M bovis* vaccines induce protective immunity in calves but are not used in clinical situations because they may evoke systemic reactions and the release of a live pathogen into livestock rearing environments could be dangerous.

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The *M bovis* cytolysin may play an important role in IBK pathogenesis and immunity.[21-28] Immunoblots and cross-neutralization studies[26,28] performed on *M bovis* isolates from different regions of the United States reveal that the cytolysin is antigenically conserved. During log-phase growth in broth culture, the *M bovis* cytolysin separates from the cell membrane while aggregated with bacterial lipopolysaccharide.[22,28] The aggregates can be retained by filters possessing a nominal molecular-weight limit of 100 kd.[28] Diafiltration of the retained cytolysin increases the stability and the specific activity, probably as a result of removal of proteolysins.

The present study was conducted because of the pathogenic importance of the cytolysin and the development of tangential flow filtration systems that enabled the concentration of large quantities of *M bovis* cytolysin. For the present study, calves that were located at a university field station were examined. The annual incidence of IBK in these calves exceeded 50%. The purpose of the study reported here was to test whether a vaccine consisting of Quil A adjuvant and diafiltered cytolysin of *M bovis* can protect calves against naturally occurring IBK.

Materials and Methods

Study site—The present study was conducted at a university field station located in Browns Valley, Calif, in the Northern Sacramento Valley, which is at 39.26N latitude and ranges from 67 to 615 m in elevation. The size of the field station was 2,315 hectares, and individual pastures ranged from 0.8 to 5 hectares. The summertime climate over the field station was warm and arid (0 inches of rainfall), with a peak summertime (July through September) temperature of 46.1°C. During the study, calves were confined to irrigated pastures that were seeded with trefoil; white clover; oats; and mixtures of rye, fescue, and Bermuda grasses. Calves were moved to different pastures during the summer depending on forage quality and quantity. The physical facilities at the field station were excellent and consisted of dampened corrals, a curved concrete-surfaced runway, a roofed examination area, a hydraulically operated squeeze chute, and ample fresh water. Calves on pastures had constant access to nonmedicated trace mineralized supplements and fresh water. Previous studies at the field station revealed an annual IBK prevalence in yearling calves that ranged from 57% to 98%[29-31] and a prevalence of *M bovis* infection that ranged from 33% to 64%.[29,31] Face flies were abundant and remained uncontrolled during the summer.

Animals—The experimental animals were 104 weaned yearling mixed-breed female beef calves ranging from 223 to 313 kg in weight. The cattle were owned by the Animal Science Department at the University of California, Davis. Calves, which were commingled during the entire study, were born during the months of November, December, and

January and, except for the experimental products, remained unvaccinated until the end of the study.

Examinations—The eyes of calves were first examined on May 19 (week 0) and then weekly thereafter for 16 weeks (until September 9). For examination, calves were restrained individually in a squeeze chute, where sterile fluorescein impregnated paper strips were inserted into the conjunctival fornix until moistened by the lacrimal secretions. Then, each eye was irrigated with sterile saline (0.9% NaCl) solution to remove unbound fluorescein. Corneal ulcers were reexamined under a focused, incandescent, high-intensity lamp without magnification and assigned a clinical score by use of the following criteria: 0 = normal eye, 1 = corneal ulcer < 0.5 cm in diameter, 2 = corneal ulcer > 0.5 but < 1 cm in diameter, 3 = corneal ulcer ≥ 1 cm in diameter, and 4 = corneal perforation.

Eyes with clinical scores ≥ 1 were photographed onto transparency film by use of a fixed camera lens that produced a 1:2 image reduction when focused. A ruled line that was marked in millimeter increments was incorporated into the photograph along with the calf identification. The resulting 2.3 × 3.5-cm transparencies were projected to 3-fold enlargements that were calibrated by measurement of the photographed line. The outlines of the corneal ulcers were traced, and the surface areas incorporated by the tracings were measured planimetrically.[30]

Study design—During the first examination (week 0), calves were randomly assigned to 1 of 3 experimental groups designated as 1, 2, or 3. Calves in group 1 (n = 34) were given 2 mL of saline solution, calves of group 2 (35) were given 2 mL of Quil A adjuvant and cytolysin vaccine, and calves of group 3 (35) were given 2 mL of Quil A adjuvant in diafiltered culture media. Injections were administered SC in the neck. The respective vaccinations were repeated 4 weeks later. During the study, calves with clinical scores ≥ 3 were treated with a dose of florfenicol[a] (40 mg/kg, SC) and excluded from further data collection. During the examinations, protective garments that included plastic aprons, obstetrical sleeves, and rubber gloves were worn. After each calf was examined, the exposed plastic surfaces were washed in water containing 1% chlorhexidine solution.[b] Examiners were unaware of the group assignments of calves until the end of the study. Staff that administered the vaccines did not participate in clinical monitoring or in surface area measurements of corneal ulcers.

Vaccine preparation—A cytolysin-enriched vaccine was prepared from a piliated and hemolytic isolate of *M bovis*, designated as Tifton 1. The Tifton 1 isolate was originally recovered from the ocular discharge of a clinically affected beef cow in Georgia. To reduce autoagglutination in broth shaker cultures, a nonpiliated variant of original isolate was selected for vaccine production. For selection of the nonpiliated strain, piliated *M bovis* was serially subcultured in trypticase soy broth[c] and aliquots were repeatedly removed and streaked for isolation onto the surface of blood agar plates.

During the serial subculturing, a variant strain that had nonpiliated morphology (ie, pitting of the agar surface, waxy colonial appearance, and autoagglutination in saline solution suspensions) was isolated. Lack of piliation of the variant strain was confirmed by transmission and scanning electron microscopy. Stock bacterial cultures of the nonpiliated variant were stored frozen (–80°C) in skim milk glycerol media until they were thawed for vaccine production. Vaccine was prepared by lawn inoculation of the variant onto 10% bovine blood agar plates. After 18 hours of aerobic incubation (36°C), *M bovis* was scraped from the surface of the blood agar plates and the growth was suspended in 10 mL of trypticase soy broth. The heavily inoculated broth was aseptically pipetted into 300-mL flasks of heart infusion broth[c] to which an extra $CaCl_2$ (1.5 mmol/L) was added. Flasks were incubated (35°C) on a shaking platform[d] set at 200 oscillations/min and then incubated until the **optical density** (**OD**; wavelength, 420 nm) reached 1.85. After incubation, the media were chilled while aliquots were centrifuged[e] (13 × *g* for 1 hour at 4°C). Chilled supernatant was filtered through hydrophobic polyethersulfone membranes[f] (mean pore diameter, 0.22µM) and then concentrated 100-fold by use of ultrafiltration with a filter with a nominal molecular-weight limit of 100 kd.[g] The operating parameters of the filter included a 30-psi pressure, flow rate of 300 to 500 mL/min, and 5- to 10-psi transmembrane pressure differential. The retentate (diafiltered retentate) was diafiltered by use of a buffer consisting of approximately 45 volumes of chilled (4°C) 50mM Tris (pH, 8.2), 500mM sodium acetate, 1.5mM $CaCl_2$, and 20% glycerol (Tris acetate buffer). Diafiltration was discontinued when the OD (wavelength, 280 nm) of the permeate reached < 0.01. Sterile media were concentrated and diafiltered in parallel to serve as an adjuvant control.

The cytolysin from the diafiltered retentate (cytolysin vaccine) and diafiltered sterile media (adjuvant control) were combined with Quil A adjuvant by use of a described procedure.[33,34] The diafiltered retentate of the *M bovis* was adjusted to a final concentration of 1.0 mg of protein/mL. The diafiltered retentate of media was diluted to the same extent, although the protein concentration before dilution was < 0.01 mg/mL. Cytolysin vaccine and adjuvant control vaccines were prepared in parallel by mixing 40 mL of the adjusted diafiltered retentate of cytolysin or media with 100 mg of decanoyl-N-methylglucamide[h] and agitating gently for 30 minutes at room temperature (approx 20°C). When the decanoyl-N-methylglucamide had dissolved, Quil A[i] was added to each specimen to achieve a final concentration of 0.1%. Mixtures were then pipetted into a dialysis bag[j] (nominal molecular-weight limit of 2 kd) and dialyzed against repeated changes of PBS solution (0.01M; pH, 7.2) for 3 days at 4°C. Following dialysis, contents of dialysis bags were harvested and sterilized by filtration through a polycarbonate membrane with a mean pore diameter of 0.22µM. Aliquots were collected for protein measurement, electron microscopy, PAGE, and immunoblotting.

Protein quantitation and hemolysin assay—Protein concentrations were measured by use of a colorimetric bicinchoninic acid assay.[34,k] Hemolytic activity of the diafiltered retentate was measured by mixing the retentate with 1 µL of washed, packed bovine RBCs. Mixtures were incubated for 6 hours (36°C) before the final evaluation of hemolytic activity was made. Washed, packed RBCs in Tris sodium acetate buffer incubated with or without 0.1% octylphenoxy polyethoxyethanol[l] (Triton) served as the respective positive and negative controls. After incubation (6 hours at 35°C), the RBC suspension was centrifuged and absorbance (450 nm) of each supernatant was measured by use of an automated reader. The percentage of hemolysis was calculated by use of the following formula:

$$\% \text{ Lysis} = \frac{\text{OD specimen} - \text{OD media control}}{\text{OD triton} - \text{OD media control}} \times 100$$

A unit of hemolytic activity was defined as the dilution of hemolysin that was required to release 50% of the total hemoglobin from the packed RBCs. Specific activity was defined as the number of hemoglobin units per microgram of protein.

Leukotoxin assay—Leukotoxic activities of the diafiltered retentate were measured by use of immortalized bovine lymphocytes as the target cells for a ^{51}Cr release assay. Cells were seeded from frozen stock cultures into flat-bottomed flasks containing Leibovitz L-15 and minimal essential medi-

a[m] (1:1 vol/vol) with penicillin (100 U/mL), streptomycin (100 µg/mL), and 15% heat-inactivated fetal bovine serum and incubated under 5% CO_2 for 5 to 10 days until sufficient density had been achieved. Cell suspensions were centrifuged, and the pellets were washed 3 times in Dulbecco PBS solution.[m] After the final wash, cells were resuspended in McCoy media[m] to a final concentration of 2×10^7 cells/mL, labeled with of ^{51}Cr[n] (200 µCi/10^7 cells) for 1 hour at 37°C, and then washed 3 times in McCoy 5A media. After the final wash, cells were diluted to a final concentration of 1×10^6 cells/mL in McCoy 5A and 500 µL of radioactively labeled cells was added to 500 µL of sample. After 1 hour of incubation, a 500-µL aliquot from each sample was removed and centrifuged for 5 minutes and the amount of radioactivity in each supernatant was measured. Samples containing 500 µL of labeled cells in 500 µL of media with and without 0.1% octylphenoxy polyethoxyethanol served as respective positive and negative controls. After incubation, cell suspensions were centrifuged and the radioactivity in **counts per minute (CPM)** was measured in 500 µL of supernatant. The percentage of lysis was calculated by use of the following formula:

$$\% \text{ Lysis} = \frac{\text{CPM specimen} - \text{CPM media control}}{\text{CPM trition} - \text{CPM media control}} \times 100$$

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

Antisera—New Zealand White rabbits were used to produce anticytolysin antisera for immunoblot probes. Rabbits were given 0.5 mL of either column-chromatographed diafilter retentate (50 µg of protein/dose) or peptide representing antiserum to amino acids 590 to 927 of recombinant cytolysin. The procedure for cloning, expression, and purification of the cytolysin from *Escherichia coli* has been previously described.[25] The column-chromatographed protein represented the void fraction of diafiltered retentate from a Superose 6HR gel-filtration column.[o] The procedure for fractionation and gel chromatography of the diafilter retentate included chromatography through a 1.5×30.0-cm gel-filtration column (Mr, 4×10^4 kd) that was equilibrated with a buffer containing 50mM Tris, 0.5M sodium acetate, 1.5mM $CaCl_2$, and 20% glycerol. The loading volume of the column was 200 µL of diafilter retentate. The elution flow rate was 0.5 mL/min. Absorbance (wavelength, 280 nm) of column effluent was measured continuously. Column-chromatographed diafilter retentate fractions (3.0 mL) were collected and examined for leukotoxic, hemolytic, and endotoxic activities.

For the primary immunization, the column-chromatographed diafilter retentate and Freund complete adjuvant were emulsified 1:1 (vol/vol) and injected (1.0 mL). For the booster injection, proteins and incomplete Freund adjuvant were emulsified and administered 21 days later. Antisera for immunoblots were harvested 3 weeks after the booster dose was administered. Rabbit antiserum to recombinant peptide from the carboxy terminus of MbxA protein representing amino acids 590 through 927 was produced by a commercial laboratory.[25,p]

Endotoxin measurement and gel electrophoresis and immunoblotting—The concentrations of vaccinal endotoxin were measured by a commercial laboratory with a timed gel-clot assay.[q] Aliquots of vaccine and adjuvant were examined by discontinuous PAGE and immunoblotting. For electrophoresis, samples were mixed with an equal volume of 2× loading buffer composed of 62.5mM Tris (pH, 6.8), 0.7mM β-mercaptoethanol, 20% glycerol, 4.1% SDS, and 0.2 mg of bromophenol blue/mL. The solutions were heated at 100°C for 5 minutes and electrophoresed by use of stacking and running gels composed, respectively, of 3.9% and 7% polyacrylamide. Proteins were electrophoretically transferred onto polyvinylidene fluoride membranes[r] for immunoblotting. To reduce nonspecific binding, blots were blocked in a Tris SDS buffer containing 20mM Tris (pH, 7.4), 0.1% Tween 20, 150mM NaCl, and 5% teleost gelatin[s] for 12 hours. 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. After the final wash, blots were incubated with 0.5 µCi ^{125}I-labeled protein A[t] in 20 mL of Tris SDS buffer. The protein A-^{125}I labeled blots were incubated for 2 hours and then washed 3 times (4 h/wash) in Tris-NaCl (20mM Tris [pH, 7.4], 150mM NaCl, and 0.1% Tween 20) buffer. After washing, blots were air-dried and autoradiographed.

Electron microscopy—Vaccine and adjuvant control specimens were examined by use of transmission and scanning electron microscopy.[u-w] Grids were negatively stained with 4% uranyl acetate in 70% ethanol and lead citrate (2 mg/mL) in 0.1N NaOH.

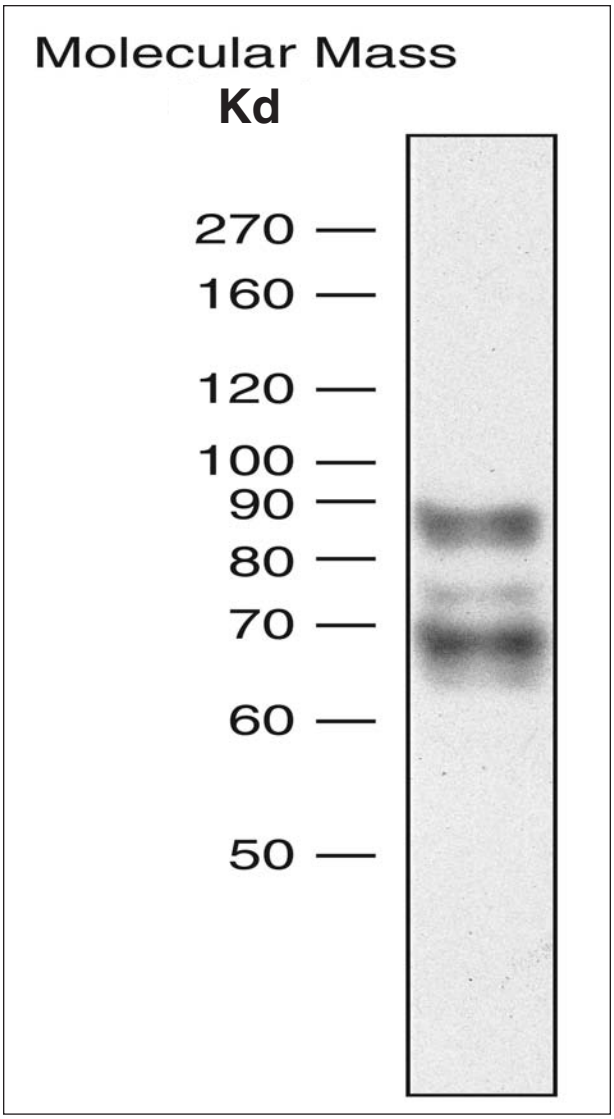

Figure 1—Immunoblot of cytolysin diafiltered retentate vaccine probed with antiserum to amino acids 590 to 927 of recombinant cytolysin. The blot was labeled with ^{125}I staphylococcal protein A. Notice the 4 immunoreactive proteins with apparent molecular masses ranging between 90 and 65 kd. The 4 bands that were recognized by use of the high-specificity probe were most likely cleaved from a parent molecule.

Statistical analysis—Categoric data (No. of calves affected) were compared by use of the Fisher exact test. The Kruskal-Wallis and Mann-Whitney *U* tests were used for comparison of clinical scores, healing times, and time to development of the first ulcer. The healing time was defined as the interval between the first and last time the ulcer was observed. Corneal ulcers were defined as lesions that retained fluorescein dye. A Cox regression model was used to compare healing rates and cumulative proportions of affected calves. Calves with ulcers that were not healed at the end of the study were included in the model as right-censored data. Data were not left-censored because no calves were found with corneal ulcers on day 0. Hazard ratios reflecting the rate of development of IBK in vaccinates, compared with control calves, were computed from the Cox regression coefficients. Surface area measurements of corneal ulcers were compared across groups by use of a 1-way ANOVA with repeated measures. For all analyses, a value of $P \leq 0.05$ was used to reject the null hypothesis.

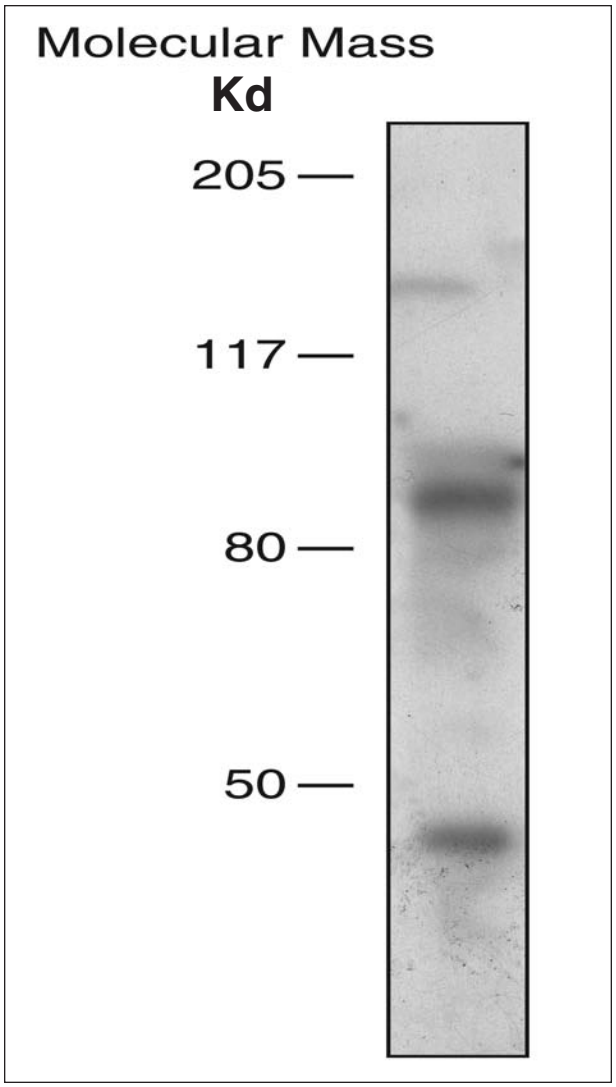

Figure 2—Immunoblot of cytolysin diafiltered retentate vaccine probed with antiserum to the Superose 6HR column chromatographed cytolysin. The blot was labeled with ^{125}I staphylococcal protein A. Notice the proteins with apparent molecular masses ranging between 90 and 65 kd and an additional band with an apparent molecular mass of approximately 40 kd. As a result of the low specificity of the antiserum, the relationship of the 40 kd band to the cytolysin structural toxin is unknown.

Results

Vaccine—The specific hemolytic and leukotoxic activities of the diafiltered retentate prior to dialysis were 0.7 and 0.2 U/µg of protein, respectively. Each dialysis bag yielded approximately 150 mL of fluid. The diafiltered retentate vaccine contained 30 µg of protein/mL. Immunoblots that were probed with the carboxy terminus antisera had 4 immunoreactive proteins with apparent molecular masses that ranged from 65 to 90 kd (**Figure 1**). Corresponding bands and additional low-molecular-weight proteins with molecular masses of approximately 40 kd were observed in immunoblots that were probed with anticolumn, chromatographed, diafiltered retentate serum (**Figure 2**). Immunologically reactive protein bands were not detected in immunoblots of the media control specimens. The endotoxin concentrations in the adjuvant and the diafiltered retentate vaccines were, respectively, 0.03 and 10.0 µg/mL. **Immunostimulating complexes (ISCOMs)** were not detected on electron microscopic examination of the negatively stained adjuvant cytolysin vaccine or the adjuvant control samples.

Field study—Weekly numbers of calves with IBK were recorded (**Figure 3**). Significantly fewer IBK-affected vaccinated calves than control calves were found on weeks 8, 10, and 12. Also, fewer IBK-affected vaccinated calves than control calves were found on weeks 7 and 9, but the differences were not significant. From weeks 0 to 9, no significant differences were found in the cumulative proportions among groups, but beginning on week 10 and continuing thereafter until week 16, the cytolysin-vaccinated calves had a significantly lower cumulative percentage of affected calves than either the saline solution or adjuvant control groups (P = 0.036 and 0.045 for groups 1 and 3,

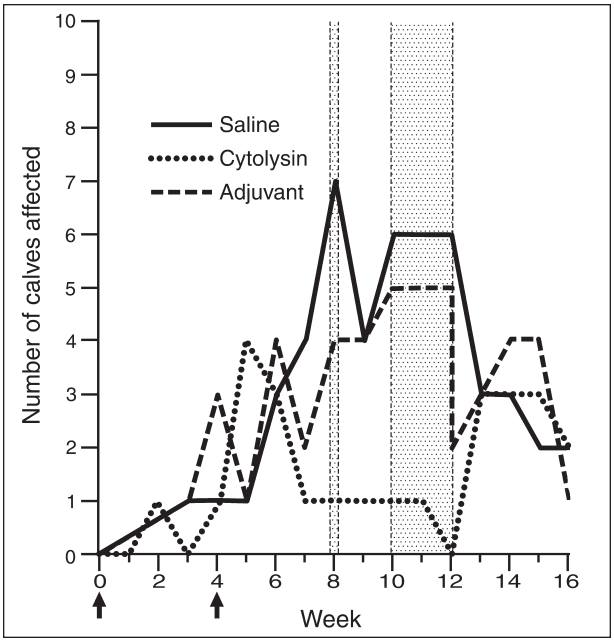

Figure 3—The number of calves affected with IBK each week. Arrows along the x-axis denote days that calves were vaccinated. Stippled areas represent periods when significantly ($P < 0.05$) fewer vaccinated calves (cytolysin) than control calves (adjuvant or saline [0.9% NaCl] solution) were affected with IBK.

respectively; **Figure 4**). Cumulative percentages of affected calves in groups 1 and 3 were not significantly different at any time. The respective hazard ratios for groups 2 and 3 were 0.399 and 1.255 (β–values were –0.918 and 0.227, respectively).

The surface areas of corneal ulcers at first observation and at peak were significantly smaller in group 2 calves than in group 1 and group 3 calves (**Table 1**). For calves with corneal ulcers, mean clinical ocular scores on the initial observation were significantly less in group 2 calves than in group 1 or group 3 calves. Mean clinical scores from later observations were not different among groups. Calves of group 2 developed corneal ulcers later in the summer, compared with calves of groups 1 and 3, but the mean healing time and number of recurrent ulcers were not significantly different among the 3 groups. The florfenicol treatments among calves of the 3 groups were not significantly (P = 0.1) different, but group 2 calves had numerically fewer treatments than control calves. Respective volumes of florfenicol that were administered to affected calves of groups 1, 2, and 3 were 208, 129, and 235 mL, respectively.

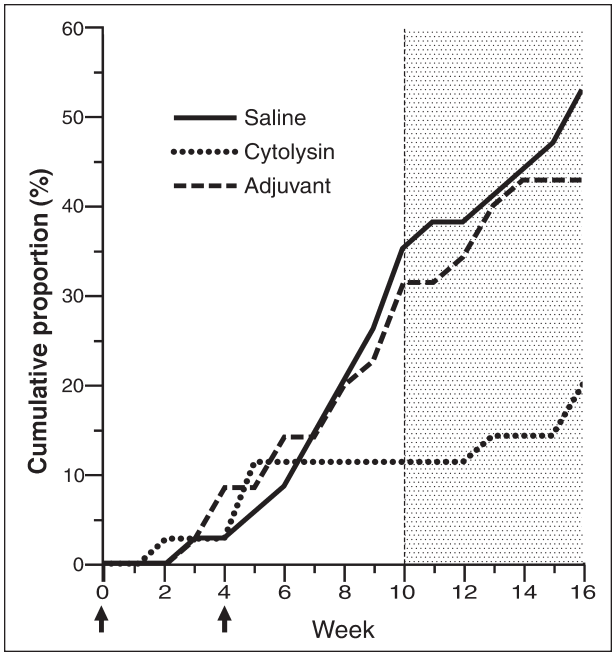

Figure 4—Cumulative proportion (%) of calves that developed IBK during the observation period. Arrows on the x-axis denote days that calves were vaccinated. Stippled area represents the period when the cumulative proportion of calves with IBK was significantly ($P < 0.05$) less for vaccinated calves (cytolysin) than for control calves (adjuvant or saline solution).

Discussion

The beneficial effects that were attributable to the cytolysin vaccine included fewer affected calves, smaller corneal ulcers, lower first observation clinical scores, and a 2.5-fold slower rate of IBK onset. The cytolysin-vaccinated calves required fewer antibiotic treatments than calves in either control group, but the differences were not significant. Because fewer cytolysin-vaccinated calves developed corneal ulcers, the florfenicol treatment of clinically affected calves would have selectively decreased corneal ulcer severity and shortened the mean healing time of the control group, as was found in our study (Table 1). The treatment probably decreased the intergroup differences of peak corneal ulcer surface areas, healing times, and weekly number of affected calves, as determined in previous treatment studies.[35,36] Intergroup comparisons without the confounding effect of treatment would have been interesting, but the severely affected calves were treated in our study because of economic and humanitarian concerns, which probably would have been similar to those encountered in a commercial herd that had IBK.

Our results were similar to those of a previous study[21] in which calves were resistant to challenge exposure following vaccination with cell-free retentate of homologous hemolytic *M bovis* culture supernatants. In our study, the antigenic relationships between the vaccine and the infecting strains are unknown. Given the diversity of calves and clinical nature of our study, calves were probably exposed to

Table 1—Mean (± SD) response data for vaccinated and control group calves.

Measurements	Treatment groups: Saline solution (34 controls)	Cytolysin (35 vaccinates)	Adjuvant (35 controls)
Ulcer surface area (cm^2)	—	—	—
First*	0.71 ± 1.8	0.08 ± 0.2§	1.23 ± 2.9
Maximum†	1.59 ± 4.0	1.00 ± 5.51§	1.28 ± 2.9
Mean clinical score‡	—	—	—
First	0.59 ± 0.7 (0.5)	0.171 ± 0.6 (0.3)§	0.71 ± 0.8 (0.5)
Subsequent	0.8 ± 0.3 (0.8)	0.71 ± 0.3 (0.5)§	0.75 ± 0.3 (1.0)
Healing time (d)	20 ± 13.2	25.6 ± 16.1	17.6 ± 15.0
No. of recurrences	5	3	6
First ulcer (d)	45.9	61.8§	49.6
No. of treated calves	11	4	10

Numbers in parentheses indicate the median value for the observation.
*First time that a corneal ulcer was recognized. †Reflects the largest surface area attained. ‡Mean value of clinical scores of both eyes. §Significantly ($P < 0.05$) different from mean values for both control groups.
Affected calves were treated with florfenicol (40 mg/kg) when corneal ulcers were ≥ 0.5 cm in diameter.

multiple strains of pathogenic *M bovis* and to environmental factors that could enhance the severity of IBK. Such factors included UV irradiation, dust, grass pollens, and face flies. Despite the potential for antigenic diversity between the vaccinal and clinical isolates and environmental factors, the cytolysin-vaccinated calves of our study had greater resistance to IBK than did either of the control group calves. The immunologic importance of the cytolysin was revealed by the lack of resistance of calves that were vaccinated with ultrafiltered retentate of nonhemolytic *M bovis*.[21]

The nominal molecular-weight limit filters of 100 kd in our study retained aggregates of cytolysin and bacterial lipopolysaccharide. Selective removal of peptides and small proteins probably resulted in an increase in specific cytolytic and endotoxic activities in the retentate. Despite the greater activities of cytolysin and endotoxin, the vaccinated calves of our study did not have clinical signs that were indicative of endotoxemia or tissue necrosis. No localized injection site swelling was found, and all of the cytolysin-vaccinated calves remained alert and responsive following vaccination. Our data therefore differed from those of a previous study[37] of endotoxemia in calves in which IV administration of endotoxin (0.05 µg/kg) resulted in clinical depression, tachycardia, and leukopenia. Calves in our study remained asymptomatic following vaccination despite 20 µg of endotoxin in the 2.0-mL vaccine dose. When delivered to smaller calves, that dose of endotoxin would have approximated 0.09 µg/kg. The reasons for the lack of clinically evident endotoxemia in the cytolysin-vaccinated calves are unknown; however, possibilities include recognition by investigators, delayed endotoxin absorption from subcutaneous tissues, low physiologic activity of the *M bovis* endotoxin, or a loss of potency that occurred during diafiltration and the addition of the adjuvant. Endotoxin may assume low toxic conformations under specific conditions of pH, buffer iconicity, concentration, and lipid saturation.[38] Although calves of our study did not develop gross clinical manifestations of endotoxemia, the vaccine was administered SC to calves in the early morning when the ambient temperature would have been low. Vaccine-related endotoxic effects could have been increased if vaccines were administered IV or were given under stressful or more environmentally harsh conditions. Because of the good environmental conditions at the time of vaccination and because body temperature and parameters of circulatory functions were not measured, we cannot with certainty conclude that the vaccine was nontoxic. We therefore recommend the measurement of endotoxin load prior to administration of any *M bovis* cytolysin-based vaccine. We also recommend that the endotoxin dose provided by cytolysin vaccine should not exceed 0.09 µg/kg, which is the approximate amount that was measured in our product.

Proteins that were observed in immunoblots of diafiltered retentate were similar to those that were identified previously[28] and considered to represent enzymatic cleavage products of the native toxin. The < 50-kd molecular-mass protein observed in immunoblots of our study was also most likely enzymatically cleaved fragments of the cytolysin that remained attached to the complexes of bacterial lipopolysaccharide and cytolysin following diafiltration. The relative contributions of lipopolysaccharide and cytolysin to the immunity of the vaccinated calves are unknown. Responses in the vaccinated calves could have been engendered by the lipopolysaccharide, cytolysin, or steric arrangement of the 2 molecules.

In our study, we attempted to incorporate the cytolysin antigen and Quil A into ISCOMs to enhance the humoral and mucosal immune responses. The ISCOMs increase major histocompatibility complex class II expression on antigen presenting cells; increase the expression of interleukin-1, interleukin-6, and interleukin-12[39]; and increase cellular, humoral, and local responses in immunized calves.[40-44] The method used in our study has been previously described for tachyzoite preparations of *Toxoplasma gondii*,[32,33] but unlike those investigations, our procedure did not result in the formation of ISCOMs. In our vaccine, neither phosphatidyl choline nor cholesterol was added exogenously. A low concentration of these molecules in the *M bovis* diafiltered retentate combined with the high pH of the diafiltration buffers may have inhibited formation of ISCOMs. We do not know whether any free Quil A would have remained after the extensive dialysis, but despite the lack of definitive ISCOMs and the possible adjuvant failure, calves that were given the cytolysin-based vaccine were more resistant to IBK than control calves. We therefore conclude that the clinical use of a diafiltered retentate-based vaccine dialyzed with Quil A as described herein may partially protect calves from naturally occurring IBK.

a. Nuflor, Schering-Plough Corp, Kenilworth, NJ.
b. Nolvasan, Wyeth Laboratories, Madison, NJ.
c. Difco Laboratories, Detroit, Mich.
d. Queue Orbital Shaker, Queue Systems, Asheville, NC.
e. Sorvall RC5C centrifuge, GS 3 Rotor, Sorvall Inc, Asheville, NC
f. Gelman Sciences, Ann Arbor, Mich.
g. Amicon S1Y100 spiral wound membrane cartridge, Millipore Inc, Billerica, Mass.
h. Mega-10 Ultrol Grade, Calbiochem, CN Biosciences Inc, La Jolla, Calif.
i. Superfos Biosector a/s, Frederikssund, Denmark, distributed by Accurate Chem, Westbury, NY.
j. SPECTRA/POR membrane tubing, Spectrum Medical Industries Inc, Los Angeles, Calif.
k. Pierce BCA protein assay kit, Pierce Inc, Rockford, Ill.
l. Triton X-100, Sigma Chemical Co, St Louis, Mo.
m. Gibco BRL Life Technologies Inc, Gaithersburg, Md.
n. Sodium ^{51}Chromate, NEN Life Science Products, Boston, Mass.
o. FPLC System, Amersham Pharmacia Biotech, Uppsala, Sweden.
p. Antibodies Inc, Davis, Calif.
q. Associates of Cape Cod Inc, Falmouth, Mass.
r. Immobilon-P, Millipore Inc, Billerica, Mass.
s. Teleost gelatin, Sigma Chemical Co, St Louis, Mass.
t. ^{125}I-Protein A, NEN Life Science Products, Boston, Mass.
u. Phillips EM400 with goniometer, transmission electron microscope, FEI Co, Hillsboro, Ore.
v. Phillips XL30TMP, scanning electron microscope, FEI Co, Hillsboro, Ore.
w. Phillips CM120 BioTWIN lens, transmission electron microscope, FEI Co, Hillsboro, Ore.

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