Efficacy of Recombinant Leukotoxin in Protection against Pneumonic Challenge with Live Pasteurella haemolytica A1

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The recombinant leukotoxin (rLKT) of the bacterium Pasteurella haemolytica A1 was examined for its ability to protect cattle from experimental challenge with logarithmic-phase P. haemolytica. Six different vaccines were utilized in the experiment: P. haemolytica culture supernatant, P. haemolytica culture supernatant enriched with rLKT, rLKT alone, P. haemolytica culture supernatant enriched with Escherichia coli supernatant not containing LKT, E. coli supernatant alone, and phosphate-buffered saline. rLKT alone showed no protective capacity against development of clinical signs of respiratory disease or against development of postmortem lung lesions after experimental challenge. It was, however, shown to enhance the efficacy of the culture supernatant vaccine and decrease clinical signs and pneumonic lesions. The complexity of protective immunity in this disease is emphasized in this study, and, although LKT is an important virulence factor of the organism, an immune response to LKT alone does not protect animals against disease.

Pasteurella haemolytica A1 is the principal etiologic microorganism isolated from bovine pneumonic pasteurellosis (shipping fever). This common disease of feedlot cattle is responsible for major economic losses in the cattle industry (20). Some of the virulence factors associated with the organism are a heat-labile exotoxin, which is specific for ruminant leukocytes (13, 29), and surface structures, including capsular polysaccharide (1, 8, 21, 22) and fimbriae (21). Leukotoxic activity is present in bacterium-free culture supernatant from logarithmic-phase cultures, and vaccination of calves with P. haemolytica culture supernatant has been shown to induce resistance to experimental challenge (7, 23, 24, 31). However, this crude culture supernatant contains other soluble antigens, including siaialoglycoprotease (25), neuraminidase (6), and various other antigens (3a).

P. haemolytica is a normal inhabitant of the ruminant nasopharynx (5). Under stressful conditions such as shipment or because of primary respiratory viral infection, pulmonary clearance mechanisms are impaired, resulting in colonization of the lung by P. haemolytica and disease (18, 26, 36). The leukotoxin (LKT) is felt to play a major role in pathogenesis by further impairment of primary lung defense, subsequent immune response, and induction of inflammation as a consequence of leukocyte lysis. In experimental challenge studies, calves depleted of neutrophils by intravenous administration of hydroxurea remained well while neutrophil-sufficient controls developed the fibrinous broncho-pneumonia typical of shipping fever (32). The presence of a naturally occurring antitoxic response is related to disease resistance in the field (19, 30), and a commercial culture supernatant vaccine (Respontion; Langford Inc., Guelph, Ontario, Canada) containing LKT has shown efficacy in reducing the incidence and severity of pneumonia following experimental challenge (28) and in the feedlot (12). In addition to inducing antitoxic immunity, this vaccine stimulates an immune response to the other soluble antigens present in the culture supernatant, including agglutinating antigens specific to P. haemolytica serotype 1. Thus, the role of an immune response to toxin alone in protection is unknown.

In an attempt to better understand protective immunity, we have endeavored to produce by recombinant DNA technology a clone bank in Escherichia coli of the genes coding for the soluble antigens of P. haemolytica A1. We previously reported the successful isolation of the genes encoding the LKT (16, 17, 35) and a serotype 1-specific surface antigen (9). Subsequent analysis of the LKT gene revealed its similarity to the alpha hemolysin of E. coli (34) and led to the designation of the RTX family of toxins (14). This similarity has been exploited in the production of recombinant LKT (rLKT). rLKT is synthesized from an expression system in which the LKT is expressed from the tac promoter and secreted into the culture supernatant by the E. coli HlyB-HlyD secretion system (35a).

We report here the first experimental challenge trial in cattle in which rLKT was used as a vaccine. We believe this is also the first trial employing an RTX toxin as a vaccine in the target species for the disease.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. E. coli HB101 containing the recombinant plasmids pLKT60 and pWAM716 were as described elsewhere (35a). Briefly, pLKT60 is a recombinant plasmid in which lktC and lktA are placed behind the inducible tac promoter in the vector pT7Q18 (33). pWAM716 was obtained from R. A. Welch. It contains the hlyB and hlyD secretion genes of the E. coli alpha-hemolysin determinant (4). The leukotoxin supernatant was prepared by the following method. E. coli HB101 containing the recombinant plasmids was cultured at 37°C for 18 h in LB broth containing the antibiotics chloramphenical and ampicillin, as previously described (15). The culture was then diluted 1/200 in the same broth as described above and incubated on a shaking platform at 37°C for 4 h. Following centrifugation (8,000 x g for 10 min), the organisms were resuspended in the same volume of LB broth with antibiotics containing the promoter inducer isopropyl β-D-thiogalactopyranoside (Sigma Chemical Co., St. Louis, Mo.)
to a concentration of 0.5 mM. This culture was incubated for 2 h at 37°C on a shaking platform and centrifuged (8,000 × g for 10 min). The supernatant was recovered, filtered through a 0.22-μm-pore-size filter, dialyzed against distilled water for 48 h, and lyophilized.

Logarithmic-phase cultures of *P. haemolytica* A1 (ATCC 43270) in brain heart infusion broth were prepared for challenge as previously described (31).

**Vaccine preparations and trial design.** Six groups of five holstein-friesian calves ranging in age from 2 to 5 months were utilized in the trial. Each calf received one of six vaccines intramuscularly twice at a 3-week interval. Three weeks after the last vaccination, all calves were challenged by the intrabronchial instillation of 25 ml of logarithmic-phase *P. haemolytica* A1 in phosphate-buffered saline (PBS) (optical density at 525 nm = 1; approximate concentration, 10^11 CFU/ml) (31). Clinical signs were monitored and scored daily for 5 days prechallenge and 5 days postchallenge (Table 1; 31). Six days after challenge, all calves were euthanized with intravenous barbiturate and the lungs were examined and scored for the percentage of lung tissue that was pneumatic (11).

The six vaccines used were PBS (group 1), the *P. haemolytica* culture supernatant vaccine Presponse (group 2), Presponse enriched with *E. coli* supernatant containing rLKT (group 3), Presponse enriched with supernatant harvested from *E. coli* HB101 without the plasmids (mock LKT) (group 4), rLKT alone (group 5), and mock LKT alone (group 6). Mock LKT was used as a control for the effects, if any, of endotoxin in the *E. coli* preparations, since preparations were known to contain approximately 10^3 endotoxin units per ml by the *Limulus* amebocyte lysate assay (Whittaker Bioproducts Inc., Walkersville, Md.). Presponse vaccine is endotoxin-free by the *Limulus* assay. Vaccines for groups 2, 5, and 6 were given in 2-ml doses. Vaccines for groups 3 and 4 contained 2 ml of Presponse plus 2 ml of the recombinant product. Group 1 calves received 4 ml of PBS.

The rLKT preparation was used at a protein concentration of 3.2 mg/ml. This is 10 times the protein concentration estimated to be in Presponse. Quil A (1 mg per calf; Cedarlane, Hornby, Ontario, Canada) in aluminium hydroxide (Cedarlane) was used as an adjuvant at a antigen-to-adjuvant ratio of 1:3.

Mock LKTA/C was used at a protein concentration estimated to represent the quantity of *E. coli* proteins present in the rLKT preparation and was used with the adjuvant as described above.

**TABLE 1. Evaluation and scoring of clinical signs**

<table>
<thead>
<tr>
<th>Clinical sign</th>
<th>Score*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough</td>
<td></td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>0.5</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>0.5</td>
</tr>
<tr>
<td>Off feed</td>
<td>1.0</td>
</tr>
<tr>
<td>No hay</td>
<td>0.5</td>
</tr>
<tr>
<td>No hay or grain</td>
<td>1.0</td>
</tr>
<tr>
<td>Weak, lethargic</td>
<td>1.0</td>
</tr>
<tr>
<td>Down, unable to rise</td>
<td>1.0</td>
</tr>
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</table>

*Maximum daily score = 5.*

**Statistical analysis.** Data were analyzed by the Kruskal Wallis technique (SAS, Cary, N.C.) by using a nonparametric paired comparison (27). The probability level for significance was 95%.

Vaccine efficacy was determined by using Abbot’s correlation (2), which examines the proportion of calves protected in the vaccine group relative to the proportion of calves protected in the comparison or control group. Protection was defined by the use of clinical scores. A calf was considered to be protected if for at least 4 of the 5 days of the postchallenge period its clinical score was less than or equal to its mean prechallenge clinical score.

**RESULTS**

**Evaluation of clinical scores.** The mean 5-day clinical score for each of the groups is shown in Fig. 1. Clinical scores in the group of calves which received Presponse enriched with rLKT (group 3) were significantly (*P ≤ 0.05*) lower than those of calves receiving PBS (group 1), rLKT alone (group 5), or mock LKT alone (group 6). The presence of Presponse in any vaccine was significantly (*P ≤ 0.05*) related to decreased clinical signs. The lowest clinical scores were observed in calves receiving Presponse enriched with rLKT, while vaccination with rLKT alone showed no beneficial effects over PBS.

**Evaluation of postmortem findings.** The mean percent pneumatic tissue for each group is shown in Fig. 2 (11). Calves in group 3 (Presponse plus rLKT) had significantly less pneumatic tissue than calves in groups 1, 2, 5, and 6. Although not statistically different, this group also had markedly decreased pneumatic tissue compared with calves receiving mock LKT. As for clinical scores, vaccination with rLKT alone resulted in no beneficial effect over PBS.

**Vaccine efficacy.** The efficacy of each of the six vaccines was calculated by using Abbot’s correlation and is shown in Table 2. In this trial, Presponse enriched with rLKT showed greater efficacy than the five other vaccines. It was 50% more efficacious than PBS alone and 33% more efficacious than either Presponse alone or Presponse enriched with mock LKT. Use of rLKT alone afforded no protective advantage over PBS.

**DISCUSSION**

The importance of *P. haemolytica* LKT in the pathogenesis of pneumonia is acknowledged, yet the role of a toxin-specific immune response alone has not been clearly established. Previous experimental and field studies have shown that both antisurface (agglutinating) and antitoxic responses can be related to resistance (30, 31). The failure of classical bacterins to protect vaccinated cattle has been attributed to the absence of LKT in these preparations and the consequent inability to induce an antitoxic response (31). More efficacious live vaccines are thought to confer protection through in vivo production of antibodies among the antigen mix. Its efficacy has been attributed to its ability to induce toxin-neutralizing antibodies.

Prior to this time, difficulties encountered in attempts to purify native LKT (3, 10) made confirmation of its role in protection impossible. Development of rLKT permitted examination in the trial reported here of the protective capacity of LKT free of other *P. haemolytica* antigens. The inability
FIG. 1. Group mean clinical score during 5 days postchallenge. Maximum score = 25 (maximum daily score = 5; n = 5). Like letters indicate significant differences between groups (P < 0.05).

of rLKT to protect cattle against experimental challenge even though it was used at a concentration greatly exceeding that in the complex Presponse vaccine emphasizes the complexity of protective immunity, which apparently involves the interplay of responses to several P. haemolytica antigens. This trial confirms that, among these, LKT is a key component. Enrichment of the mix of soluble antigens present in Presponse with rLKT resulted in a marked enhancement of protective efficacy. However, other soluble antigens are apparently equally crucial. Among these, fac-

FIG. 2. Group mean percent pneumonic tissue. Like letters indicate significant differences between groups (n = 5; P < 0.05).
TABLE 2. Vaccine efficacy

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>% Efficacy versus group a</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
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<td>5</td>
<td>0</td>
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<td>6</td>
<td>0</td>
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a Vaccine efficacy = (PV - P)c/(1 - P)c, where PV is the proportion of calves protected in the vaccinated group and Pc is the proportion of calves protected in the comparison group. Protection was defined by ≤1 day of increased postchallenge clinical score, compared with the mean prechallenge clinical score.

tors related to the bacterial surface, including capsule and various adhesins, are likely candidates. In particular, the virtually exclusive association of serotype 1 with bovine pneumonia pasteurellosis strongly suggests that type-specific antigens of P. haemolytica have a role in virulence and hence protection. In one small previous challenge study, it was found that vaccination with culture supernatant from serotype 1 protected two of three calves, while vaccination with culture supernatant from the nonpathogenic serotype 11 was not protective even though both vaccines induced similar LKT-neutralizing activity (31). From our clone bank, we have isolated the genes of a serotype 1-specific antigen (9) as well as protease genes (unpublished data) and neuraminidase genes (unpublished data), and we shall use the corresponding recombinant antigens to further test our hypothesis of immunity.

The findings of this protection study have broader scope when one recognizes the relationship of P. haemolytica rLKT to homologous toxins produced by the other pathogenic gram-negative bacteria Bordetella pertussis, Morganella morganii, Proteus vulgaris, Proteus mirabilis, Actinobacillus equuli, A. suis, A. pleuropneumoniae, and A. actinomycetemcomitans. LKTs/hemolysins produced by all of these bacteria belong to the RTX gene family and possess toxin determinants homologous to that of E. coli alpha-hemolysin (14, 35b). Their common ancestry, so evident in their specialized set of secretion genes and in repeat domains (RTX) of toxin structural protein, also suggests common mechanisms of actions for these toxins. It is reasonable to expect that aspects of protective immunity will likewise be similar. Use of one member of the RTX family in its recombinant form in this vaccine trial should provide valuable information of benefit to other researchers whose interests include the study of immunity to the gram-negative organisms listed above.

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