Effect of \textit{Fusobacterium necrophorum} Leukotoxoid Vaccine on Susceptibility to Experimentally Induced Liver Abscesses in Cattle\textsuperscript{1}

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\textbf{ABSTRACT:} The efficacy and the optimum dose of \textit{Fusobacterium necrophorum} crude leukotoxoid vaccine required to immunize and protect steers against experimentally induced liver abscesses were evaluated. The vaccine consisted of cell-free culture supernatant of a high leukotoxin-producing strain of \textit{F. necrophorum}, inactivated with formalin and homogenized with an adjuvant. Twenty-five steers were assigned randomly to the following five treatment groups: control; three doses (1.0, 2.0, and 5.0 mL) of the culture supernatant; and 2.25 mL of the concentrated supernatant (equivalent to 5 mL of the original supernatant). Vaccine was injected subcutaneously on d 0 and 21. Blood samples were collected weekly to monitor antileukotoxin antibody titers. Three weeks after the second vaccination (d 42), all steers were injected intraportally with \textit{F. necrophorum} culture to induce liver abscesses. Three weeks later (d 63), steers were euthanatized and necropsied; livers were examined and protection assessed. Antileukotoxin antibody titers in the control steers generally did not differ from the baseline (wk 0) titers. The titers in the vaccinated groups increased, more so after the second injection, and the increase was generally dose-dependent. Necropsy examination revealed that all steers in the control group had abscesses in the liver. In the vaccinated groups, two of five steers in the 1.0-mL group and one each in the 2.0-, 5.0-, and 2.25-mL (concentrated) groups had liver abscesses. Antileukotoxin antibody titers were higher (\(P < .05\)) in steers that did not develop abscesses than in steers that developed abscesses. The difference suggested a protective effect of antileukotoxin antibodies against experimentally induced liver abscesses.

Key Words: Liver Abscesses, Steers, \textit{Fusobacterium necrophorum}, Vaccines

\textbf{Introduction}

Liver abscesses in feedlot cattle are an economic concern because they cause liver condemnation and reduced feed efficiency and weight gain (Brink et al., 1990). \textit{Fusobacterium necrophorum}, is the primary etiologic agent of bovine hepatic abscesses (Scanlan and Hathcock, 1983; Tan et al., 1996). The incidence of bovine hepatic abscesses is related to feeding high-grain diets; rapid fermentation of grain results in ruminal acidosis and rumenitis, which are predisposing factors for liver abscesses (Smith, 1944; Jensen et al., 1954). \textit{Fusobacterium necrophorum}, a normal inhabitant of the rumen, colonizes the ruminal epithelial wall, reaches the liver via the portal circulation, and induces infection (Scanlan and Hathcock, 1983; Tan et al., 1996).

The pathogenicity of \textit{F. necrophorum} is attributed mainly to a leukotoxin that is cytotoxic to leukocytes, macrophages, ruminal epithelial cells, and possibly hepatocytes (Roberts, 1967, 1970; Garcia et al., 1975; Emery et al., 1984; Tan et al., 1992, 1994b). Previous studies have indicated that antileukotoxin immunity reduced the incidence of hepatic abscesses and interdigital necrobacillosis (Garcia et al., 1974; Clark et al. 1986). However, the literature on the prospect of producing an effective vaccine containing leukotoxoid is conflicting (Emery and Vaughn, 1986; Emery et al., 1986b). In our previous study, \textit{F. necrophorum} cell-free culture supernatant containing leukotoxoid elicited a high antileukotoxin antibody titer in steers.
and provided protection against experimentally induced hepatic abscesses (Saginala et al., 1996). This study was conducted to determine the optimum dose of F. necrophorum crude leukotoxoid required to immunize steers and provide protection against experimentally induced liver abscesses.

Materials and Methods

Preparation of the Vaccine. Fusobacterium necrophorum subsp. necrophorum strain A25, previously isolated from a liver abscess (Lechtenberg et al., 1988) was grown in prerduced, anaerobically sterilized, brain heart infusion broth (BHI; Difco Laboratories, Detroit, MI; Tan et al., 1992). A late-log phase culture (7 h) was centrifuged at 13,500 \( \times g \) for 30 min at 4°C, and the supernatant was filter-sterilized through a .45-μm membrane filter (Micron Separation, Westborough, MA). An aliquot of the supernatant was concentrated 5.2-fold at 4°C using a hollow fiber concentration/desalting system with a 3,000-Da molecular exclusion filter (Amicon, Danvers, MA). Leukotoxin in the original and concentrated supernatants was inactivated by adding formalin at a concentration of .3% and gently stirring at 4°C for 24 h. Ribi (synthetic trehalose dicorynomycolate in light mineral oil; Ribi Immunochem, Hamilton, MT), an oil emulsion adjuvant, was added at 10% (vol/vol) of the antigen, and the mixture was homogenized (Silverson L4R, Silverson Machines Ltd., Buckinghamshire, U.K.).

Leukotoxin and Protein Assays. The leukotoxicity of the original and concentrated culture supernatants, before and after formalin inactivation, was tested with a tetrazolium (MTT) 3-[4, 5-(dimethylthiazoyl-2-yl)-2, 5-dimethyletetrazolium bromide] dye reduction assay using bovine polymorphonuclear neutrophil (PMN) leukocytes as target cells (Vega et al., 1987; Tan et al., 1992). The leukotoxin unit was calculated as the reciprocal of the culture supernatant dilution causing a 10% loss in viability of bovine PMN cells (Tan et al., 1992). Leukotoxin concentrations of the original and concentrated supernatants also were quantified with a sandwich ELISA with purified monoclonal antibody (MAb F7b10) as the capture antibody, biotinylated mAb as the indicator antibody, and affinity-purified leukotoxin from F. necrophorum subsp. necrophorum strain A25, as the standard (Tan et al., 1994c). Protein concentration was estimated with the Pierce BCA protein assay (Pierce, Rockford, IL).

Immunization Schedule. Twenty-five Holstein steers (mean body weight 390 kg) given ad libitum access to a diet of alfalfa hay and with low baseline serum titers of leukotoxin-neutralizing antibodies were used. Steers were divided randomly into five treatment groups of five animals each: control, three doses (1.0, 2.0, and 5.0 mL) of the culture supernatant, and a dose of the concentrated supernatant (2.25 mL) equivalent to the leukotoxin concentration in 5.0 mL of the original culture supernatant (Table 1). The purpose of using the concentrated supernatant was to determine whether the process of concentration would reduce immunogenicity or protective effects. The concentrated supernatant was diluted with PBS to 4.5 mL in order to keep the adjuvant concentration similar to the 5.0-mL dose. Steers in the control group received 5 mL per animal of PBS (pH 7.4) mixed with Ribi adjuvant. All injections were given subcutaneously on d 0 and 21.

Serological Evaluation. Jugular blood samples were collected on d 7, 14, 21, 28, 35, and 42 after the first vaccination to monitor the serological immune response. Serum samples were assayed for antileukotoxin activity with an MTT dye reduction neutralization assay with bovine PMN as target cells and F. necrophorum subsp. necrophorum, strain A25 culture supernatant (200 units/mL) as the source of leukotoxin (Tan et al., 1994a). The serum neutralizing antibody titer was defined as the reciprocal of the serum dilution that caused 50% neutralization of the standard leukotoxin (Tan et al., 1994a). Serum samples also were evaluated for antileukotoxin IgG antibodies with an ELISA using affinity-purified leukotoxin as the coating antigen. Affinity purification

Table 1. Treatment groups and leukotoxin concentration in the culture supernatant and concentrated supernatant of Fusobacterium necrophorum

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose per animal, mL</th>
<th>Protein concentration, mg/dose</th>
<th>Leukotoxin concentration per dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>—</td>
<td>4.5</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>Culture supernatant</td>
<td>.9</td>
<td>—</td>
<td>.5</td>
</tr>
<tr>
<td>3</td>
<td>Culture supernatant</td>
<td>1.8</td>
<td>—</td>
<td>.2</td>
</tr>
<tr>
<td>4</td>
<td>Culture supernatant</td>
<td>4.5</td>
<td>—</td>
<td>.5</td>
</tr>
<tr>
<td>5</td>
<td>Concentrated supernatant</td>
<td>—</td>
<td>2.25</td>
<td>.5</td>
</tr>
</tbody>
</table>

*Culture supernatant concentrated 5.2-fold with a 3,000-Da exclusion hollow fiber concentration/desalting system.
of the leukotoxin was done using monoclonal antibody, MAb F7b10 (Tan et al., 1994c). Briefly, the ELISA procedure consisted of coating an Immulon 2 (Dynatech Laboratories, Alexandria, VA) plate with 100 μL of affinity-purified leukotoxin per well at a protein concentration of .1 μg/mL. The plate was incubated at 37°C for 2 h and washed three times with PBS containing .05% of Tween 20 (PBS-Tween). To prevent nonspecific binding, the plate was blocked with 3% gelatin in PBS and incubated at 37°C for 1 h. After the plate was washed three times with PBS-Tween, 100 μL of serially diluted serum was added to each well, and the plate was incubated at 37°C for 2 h. The plate was washed four times with PBS-Tween, 100 μL of rabbit antobody IgG, conjugated with horseradish peroxidase (Zymed Laboratories, San Francisco, CA) was added to each well, and the plate was incubated at 37°C for 1 h. After the plate was washed four times with PBS-Tween, 100 μL of ABTS (2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid; Sigma Chemical, St. Louis, MO) and hydrogen peroxide substrate solution was added to each well, and the plate was incubated at room temperature for 30 min. Absorbance was measured at 405 nm. The titer was defined as the reciprocal of the serum dilution at which the absorbance was five times the absorbance of the negative control (i.e., fetal bovine serum) wells.

Experimental Induction of Hepatic Abscesses. Fusobacterium necrophorum subsp. necrophorum, strain A25 was grown in anaerobic BH1 as previously described. Ten milliliters of log-phase culture (7 to 8 h) with an absorbance of .65 at 600 nm, as measured in a spectrophotometer, and a cell concentration of 2.2 × 10^8 cfu/mL, as determined with the spread plate method with anaerobic blood agar plates in an anaerobic glove box (Forma Scientific, Marietta, OH), was used to infect each animal. The culture was inoculated intraperitoneally with an ultrasound-guided, percutaneous, catheterization procedure described by Lechtenberg and Nagaraja (1991). Jugular blood samples were collected before (wk 6) and after (wk 7, 8, and 9) intraportal challenge. Serum samples were collected before (wk 6) and after (wk 7, baseline titer throughout the 6-wk sampling period. The control steers following injection with PBS emulsified with Ribi adjuvant generally did not differ from the vaccinated groups (Figure 1). A significant treatment × week interaction (P < .01) occurred. The serum antileukotoxin IgG antibody, as measured by ELISA, increased markedly following the first vaccination and continued after the second injection. Addition of formalin reduced the activity of leukotoxin by about 83% based on MTT assay; however, the concentration based on sandwich ELISA remained unaffected. Apparently, formalin inhibits the biological activity but not the antigenicity of the leukotoxin.

Immune Response Following Vaccination. All steers had low serum leukotoxin-neutralizing antibody titers (mean 13) and antileukotoxin ELISA titers (mean 3,023) on d 0 of vaccination. Antibody titers in the control steers following injection with PBS emulsified with Ribi adjuvant generally did not differ from the baseline titer throughout the 6-wk sampling period. The titers in the vaccinated steers increased (P < .01) following the first vaccination, and the magnitude of increase was much greater after the second injection (Figure 1). A significant treatment × week interaction (P < .01) occurred. The serum antileukotoxin IgG antibody, as measured by ELISA, increased markedly (P < .01) in all vaccinated groups immediately following the first vaccination and continued after the second injection. Although some increase in the IgG antibody titers occurred in the control group, the magnitude of increase was much greater in the vaccinated groups (Figure 2). A treatment × week interaction did not occur for antileukotoxin ELISA titers. Generally, the antibody titers seemed to be related to the dose of leukotoxoid; the 1.0-mL dose elicited the lowest and the 5.0-mL dose elicited the highest antibody titers. The mean serum leukotoxin-neutralizing titers were 66, 93, 282, and 144 for 1.0, 2.0, 5.0 mL of culture supernatant and 2.25 mL of concentrated supernatant, respectively. The antibody response to 2.25 mL of the concentrated supernatant
LEUKOTOXOID VACCINE FOR LIVER ABSCESES

Figure 1. Serum leukotoxin-neutralizing antibody titers in control or steers vaccinated with 1.0, 2.0, or 5.0 mL of culture supernatant and 2.25 mL of the concentrated supernatant. SEM = 1.6, Treatment effect $P < .01$, week effect $P < .01$, and treatment $\times$ week interaction $P < .05$.

Figure 2. Serum antileukotoxin antibody (ELISA) titers in control or steers vaccinated with 1.0, 2.0, or 5.0 mL of culture supernatant and 2.25 mL of concentrated supernatant. SEM = 1.4, Treatment effect $P < .01$, week effect $P < .01$.

Figure 3. Mean serum antileukotoxin antibody (ELISA) titers in control or vaccinated steers. Means not sharing the same superscript differ ($P < .01$).

Experimentally Induced Hepatic Abscesses. All steers were free of hepatic abscesses as observed by ultrasonography on d 0 of the intraportal challenge with F. necrophorum. Necropsy examination on d 21 after the intraportal challenge showed that all five steers in the control group had abscesses in the liver. In the vaccinated groups, two of five steers in the 1.0-mL group, and one each in the 2.0-, 5.0-, and 2.25-mL (concentrated) groups had liver abscesses (Table 2). Based on Fisher’s exact test (2-tail), the incidence of liver abscesses in the vaccinated groups (all four doses) was lower ($P < .01$) than that in the control. There were no differences in the severity of abscesses, based on size and number, between control and vaccinated groups. Fusobacterium necrophorum subsp. necrophorum was isolated from all abscesses.

Antibody Response Following Intraportal Challenge. Following intraportal challenge (wk 7, 8, and 9), neutralizing antibody and antileukotoxin ELISA titers increased in the control and the vaccinated groups. There was a trend ($P = .09$) for serum leukotoxin neutralization titers to be higher in the vaccinated groups than in the control group. However, in the case of ELISA titers, the control and the 5.0-mL dose groups had higher titers than the other groups (Table 3).

Steers that developed abscesses ($n = 10$) had lower leukotoxin neutralizing ($P < .05$) and ELISA ($P = .05$) titers during wk 1 to 6 than those steers ($n = 15$) that did not develop abscesses in the liver, regardless of the treatment (Figures 4 and 5). Also, serum antileukotoxin ELISA (following intraportal challenge) titers tended ($P = .07$) to be higher in steers with liver abscesses than in those that had no
Table 2. Experimental induction of liver abscesses in control or vaccinated steers

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of steers</th>
<th>Necropsy</th>
<th>Incidence, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>5</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Culture supernatant, 1.0 mL</td>
<td>5</td>
<td>2/5</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>Culture supernatant, 2.0 mL</td>
<td>5</td>
<td>1/5</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Culture supernatant, 5.0 mL</td>
<td>5</td>
<td>1/5</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>Concentrated supernatant, 2.25 mL</td>
<td>5</td>
<td>1/5</td>
<td>20</td>
</tr>
</tbody>
</table>

\(^a\)Fisher's exact test (2-tail), control vs vaccinated P < .01.

\(^b\)Culture supernatant concentrated 5.2-fold with a 3,000 Da exclusion hollow fiber concentration/desalting system.

Liver abscesses. However, the leukotoxin neutralization titers were not different between the groups (abcessed vs nonabcessed) following the intraportal challenge.

Discussion

Leukotoxin of F. necrophorum is considered to be the primary virulence factor involved in the onset of hepatic and interdigital necrobacillosis in cattle (Emery et al., 1984; Tan et al., 1992). This is supported by observations that strains that produced leukotoxin caused more abscesses in mice than strains that did not produce leukotoxin (Coyle-Dennis and Laureman, 1979) and that nonleukotoxin-producing strains failed to induce foot abscesses in cattle following intradermal inoculation (Emery et al., 1985). Leukotoxin may help the organism directly by protecting it from the host defense and indirectly by causing hepatic parenchymal damage resulting from the release of cytolytic products and oxygen metabolites (Styrt et al., 1990). Several studies have suggested that immunity against leukotoxin may be related to protection against F. necrophorum infection (Roberts, 1970; Garcia et al., 1974; Garcia and McKay, 1978; Emery and Vaughan, 1986; Emery et al., 1986b). Although antigenic preparations containing leukotoxin induced protective immunity in mice, results in sheep and cattle have been variable. Roberts (1970) showed that in rabbits formalin-killed F. necrophorum cells induced humoral bactericidal antibodies that had no influence on growth once the organism was established and, therefore, proposed that effective protection required vaccination that would elicit antibodies able to neutralize the leukotoxin. Garcia et al. (1974) were able to reduce the incidence of liver abscesses in feedlot steers and heifers by approximately 70% compared with the control by immunization with a toxoid, possibly leukotoxoid, prepared from the cytoplasmic fractions.

Table 3. Antileukotoxin antibody titers in control and vaccinated steers following intraportal challenge with Fusobacterium necrophorum

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Leukotoxin neutralization(^b) ELISA(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>454</td>
</tr>
<tr>
<td>Culture supernatant, 1.0 mL</td>
<td>637</td>
</tr>
<tr>
<td>Culture supernatant, 2.0 mL</td>
<td>1,055</td>
</tr>
<tr>
<td>Culture supernatant, 5.0 mL</td>
<td>2,505</td>
</tr>
<tr>
<td>Concentrated supernatant, 2.25 mL(^b)</td>
<td>637</td>
</tr>
<tr>
<td>SEM</td>
<td>1.6</td>
</tr>
</tbody>
</table>

\(^a\)Mean of wk 7, 8, and 9.

\(^b\)Treatment effect of P = .09.

\(^c\)Treatment effect of P < .05.

\(^d\)Culture supernatant concentrated 5.2-fold with a 3,000 Da exclusion hollow fiber concentration/desalting system.

\(^e\)Means in a column that do not have common superscripts differ (P < .05).

Figure 4. Serum leukotoxin neutralizing antibody titers in steers that developed (n = 10) or did not develop (n = 15) liver abscesses. SEM = 1.4, abscess effect P < .05, week effect P < .01.
of F. necrophorum. Antibodies in rabbits induced by injecting partially purified leukotoxin were able to prevent the cytolytic effect of the toxin on peripheral blood leukocytes (Emery et al., 1986a). Also, cattle immunized against F. necrophorum culture supernatant containing leukotoxin had a lower incidence of interdigital necrobacillosis (Clark et al., 1986). In contrast, passive immunization of mice with rabbit antisera having a high leukotoxin neutralizing antibody titer failed to protect mice from experimental infection (Emery and Vaughan, 1986). Earlier studies in cattle have suggested that culture supernatant was more immunogenic (Takeuchi et al., 1984) and more protective (Clark et al., 1986) than other antigenic components of F. necrophorum. In our previous study involving comparison of culture supernatant and whole-cell culture to immunize cattle, culture supernatant induced higher antileukotoxin titers and was more protective than whole-cell culture (Saginala et al., 1996). Leukotoxin of F. necrophorum is an exotoxin and is not associated with the cells (Tan et al., 1994b). Although the concentration of leukotoxin in whole-cell culture would be expected to be similar to that in cell-free culture supernatant, the immune response was lower with whole-cell culture than with cell-free culture (Saginala et al., 1996). Possibly, the immune system was overwhelmed with a multitude of antigens contained in the whole-cell culture. Therefore, cell-free supernatant was chosen for vaccine production.

In this study, the antibody response to leukotoxoid seemed to be generally dose-related. The second vaccination, administered 3 wk after the first, elicited a classic secondary response to the antigenic stimulation. The difference in titers between the leukotoxin-neutralizing antibody assay and indirect ELISA reflects the sensitivity of the ELISA procedure. Among the doses evaluated, 5 mL elicited the highest antibody titers and the 1.0-mL dose elicited the lowest. Although the 5.0-mL dose of culture supernatant and 2.25 mL of concentrated supernatant had similar leukotoxin titers (based on the biological assay), the antibody response seemed to be greater with the 5.0-mL than with the 2.25-mL dose. The dose of 2.25-mL of concentrated supernatant was chosen to equalize the concentration of leukotoxin with that of the 5.0-mL dose. However, measurement by sandwich ELISA indicated that the leukotoxin concentration was higher in the 2.25-mL concentrated supernatant than in the 5.0-mL culture supernatant (22.4 vs 11.5 µg, respectively). Sandwich ELISA is based on the binding of a monoclonal antibody to a specific determinant of a leukotoxin molecule, whereas the MTT assay measures the biological activity (cytotoxicity to PMN cells) of the toxin (Vega et al., 1987). Perhaps the filtration process allowed degradation of the leukotoxin molecule, thus reducing the biological activity but not affecting, or possibly enhancing, the concentration of antigenic determinants. The purpose of using concentrated supernatant was to determine whether an additional processing step of the culture supernatant to reduce the injection volume would alter its immunogenicity or protective effect. Apparently, concentrating the culture supernatant reduced its immunogenicity, as evidenced by lower neutralization and ELISA titers. Possibly, the filtration with a 3,000-Da exclusion hollow fiber reduced small molecular weight proteins that might have contributed to the immunogenicity of the supernatant.

The leukotoxin-neutralizing and ELISA titers increased considerably in vaccinated and control steers following intraportal inoculation of live F. necrophorum culture. The increase was not surprising because intraportal challenge exposure and subsequent abscess development (in the control) would have served as antigenic stimulus (Tan et al., 1994a).

**Implications**

Results of the present study indicated that Fusobacterium necrophorum culture supernatant was capable of eliciting antileukotoxin immunity that provided some degree of protection against experimentally induced liver abscesses. The differences in serum antileukotoxin titers between the steers that developed liver abscesses and steers that did not following the intraportal challenge was indicative of the protective effect of antileukotoxin antibodies. However, further studies are required to determine the efficacy,
optimal dose, and number and timing of injections of the vaccine in feedlot cattle with naturally developing liver abscesses.

Literature Cited


