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Short communication

Intranasal vaccination of calves with *Mannheimia haemolytica* chimeric protein containing the major surface epitope of outer membrane lipoprotein PlpE, the neutralizing epitope of leukotoxin, and cholera toxin subunit B

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ABSTRACT

This study was done to determine if intranasal vaccination of weaned beef calves with a chimeric protein containing the immunodominant surface epitope of *Mannheimia haemolytica* PlpE (R2) and the neutralizing epitope of leukotoxin (NLKT) covalently linked to truncated cholera toxin (CT) subunit B (CTB) could stimulate secretory and systemic antibodies against *M. haemolytica* while enhancing resistance of cattle against *M. haemolytica* intrabronchial challenge. Sixteen weaned beef calves were intranasally vaccinated with CTB-R2-NLKT chimeric (SAC102) or with R2-NLKT-R2-NLKT chimeric (SAC89) protein with or without native CT on days 0 and 14 and were challenged intrabronchially on day 28. *In vitro*, SAC102 bound the CT receptor molecule, GM_1-ganglioside. Mean IgA antibodies to *M. haemolytica* whole cells (WC) and to LKT were high on day 0. A small, yet significant increase ($p < 0.05$) was found in mean nasal antibodies to *M. haemolytica* WC for the SAC89 + CT and SAC102 vaccinates after the second vaccination. SAC102 stimulated significant ($p < 0.05$) mean serum antibody responses to all three antigens by day 28. Following challenge, mean antibodies to WC and LKT significantly increased ($p < 0.05$) for the SAC102, SAC89 and SAC89 + CT groups with the mean antibody responses to rPlpE stimulated by SAC102 vaccination being significantly higher ($p < 0.05$) than for the other vaccinated and control groups. On day 1 after challenge, mean clinical score for the control group was significantly higher ($p < 0.05$) than for the SAC102 and SAC89 + CT vaccinates, and by day 2 after challenge, clinical score for the control group was significantly higher ($p < 0.05$) than for all three chimeric vaccinated groups. Therefore, intranasal vaccination with CTB-R2-NLKT (SAC102) and R2-NLKT-R2-NLKT (SAC89) chimeric proteins enhanced resistance against intrabronchial challenge with the bacterium as well as stimulating antibody responses to *M. haemolytica* antigens.

1. Introduction

A major cause of severe bacterial pneumonia in feedlot cattle is *Mannheimia haemolytica* serotype 1 (S1) (Rice et al., 2007). Current vaccines against *M. haemolytica* are only moderately efficacious against shipping fever pneumonia of beef cattle (Rice et al., 2007). Shewen and Wilkie (1988) demonstrated that immunity against *M. haemolytica* requires antibodies against bacterial cell-surface antigens as well as leukotoxin (LKT) neutralizing antibodies. Our laboratory demonstrated that a major 45 kDa, surface-exposed, outer membrane lipoprotein PlpE is an

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immunologically important surface protein (Pandher et al., 1999). Cattle vaccinated with commercial *M. haemolytica* vaccines along with recombinant *M. haemolytica* S1 PlpE (rPlpE) had significantly greater resistance against experimental challenge with *M. haemolytica* S1 or S6 than did cattle vaccinated with the commercial vaccine alone (Confer et al., 2003, 2006).

Epitope-mapping studies in our laboratory found that the major epitope region of *M. haemolytica* S1 PlpE (designated R2) consists of eight imperfect hexapeptide repeats of QAQNAP located near the N-terminal region (Ayalew et al., 2004). For LKT, the epitope involved in neutralization is localized to a 32 amino acid region near the C-terminus (Lainson et al., 1996; Rajeev et al., 2001). Therefore, we recently reported several PlpE-LKT chimeric constructs containing the R2 epitope of PlpE and the neutralizing epitope of LKT (NLKT) and demonstrated that subcutaneous vaccination with these chimeric proteins stimulated complement-fixing anti-PlpE and LKT-neutralizing antibodies and enhanced resistance against challenge (Ayalew et al., 2008; Confer et al., 2009a).

Because upper respiratory colonization is the first step in the pathogenesis, mucosal routes of immunization, both intranasal and oral, are being studied (Shewen et al., 2008). Intranasal vaccination of young dairy calves with a PlpE/NLKT chimeric protein supplemented with cholera toxin (CT) as a mucosal adjuvant, stimulated mucosal anti-PlpE and anti-LKT antibodies (Confer et al., 2009b). Cholera toxin is composed of a homopentameric subunit, cholera toxin B subunit (CTB), and one polypeptide A subunit (Lycke, 2005; Sanchez and Holmgren, 2008). Non-toxic CTB mediates binding to eukaryotic cell receptors through interaction with cell-surface GM_1-ganglioside, thus facilitating induction of mucosal immune responses. To enhance mucosal immunization with PlpE/NLKT chimeric proteins and to prevent reliance on potentially toxic CT, we developed a *M. haemolytica* chimeric protein, designated SAC102 that is composed of one copy each of truncated CTB (i.e. CTB without signal peptide), R2, and NLKT. In this study, we report the structure of SAC102 and its binding to GM_1-ganglioside. In addition, we vaccinated weaned beef calves intranasally with SAC102, determined antibody responses, and quantified clinical signs following intrabronchial challenge.

2. Materials and methods

2.1. Cattle

Sixteen weaned crossbred beef calves were housed initially in one pen outdoors at the Wendal Wallace Bovine Research Center, Oklahoma State University. The calves had been screened for anti-*M. haemolytica* whole cell antibodies and found to have antibody concentrations of $<$0.5 ng of antigen-specific IgG as determined by ELISA, which was found previously to be a normal background concentration for calves susceptible to challenge with *M. haemolytica* S1 (A.W. Confer, unpublished data). Upon arrival at the research facility, all calves were vaccinated with a 7-way clostridial and leptospiral vaccine, and treated with an anthelmintic. Prior and subsequent to challenge, cattle were housed indoors in pens of two calves each in a BSL-2 isolation barn. The calves received free-choice native grass hay supplemented with grain ration while housed outdoors and a complete pelleted maintenance diet when housed indoors. The Institutional Animal Care and Use Committee approved all studies (protocol #182).

2.2. Enzyme-linked immunosorbent assay (ELISA)

Sera were assayed for anti-*M. haemolytica* IgG antibodies using ELISA against partially purified native LKT, formalin-killed whole bacterial cells (WC), and rPlpE as previously described (Confer et al., 1997, 2003, 2006). Ninety-six-well microtiter plates were coated with WC at a concentration equivalent to 10^8 CFU/well or 50 ng/well each of LKT or rPlpE diluted in coating buffer. Serum dilutions for the various assays were 1:800 for WC, 1:1600 for LKT, or 1:400 for PlpE in PBS Tween 20 + 1% BSA, which were in the linear range of established dilution curves. Affinity-purified horseradish peroxidase-conjugated goat anti-bovine IgG [H + L] (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) diluted at 1:400 was used as secondary antibody, and o-phenylenediamine tablets (Amresco, Inc., Solon, OH) were used as substrate. Antibody responses were expressed as nanograms of immunoglobulin binding based on a set of IgG standards on each plate.

For determination of anti-*M. haemolytica* IgA antibodies in nasal secretions, the previous antigen-coating steps were repeated (Confer et al., 2009b). Nasal secretions were diluted 1:2, with PBS Tween 20 + 1% BSA, and antibodies determined using HRP-conjugated goat anti-bovine IgA (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) secondary antibody at a dilution of 1:400. Due to small sample sizes, only enough was available to conduct ELISA for mucosal IgA against WC and LKT. Antibody responses were expressed as nanograms of immunoglobulin binding based on a set of IgA standards on each plate.

2.3. Immunogens

Recombinant *M. haemolytica* R2-NLKT-R2-NLKT chimeric protein (SAC89) has a calculated molecular mass of 46,864.5 Da. The design, construction, expression, and purification of SAC89 were recently described (Ayalew et al., 2008).

The construction and purification of the *M. haemolytica* CTB-R2-NLKT chimeric protein (SAC102) was done as follows (Fig. 1). The DNA coding for the CTB lacking the leader peptide was amplified from genomic DNA of two *Vibrio cholerae* strains (ATCC 39315D and 51394D) by polymerase chain reaction (PCR) using a forward primer (5′-CAccatggGCACACCTCAAAATATTAC T-3′) that anneals between positions 64 and 81 and reverse primer (5′-GTATagatctATTTGCCATACTAATTGC-3′) that binds to 372–355 of the *ctB* gene (GenBank accession number ABQ65166). The size of the amplimer was confirmed by agarose gel electrophoresis and cleaned with QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). The cleaned PCR product was digested with NcoI and BglII, gel purified and