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Research paper

Challenges in mucosal vaccination of cattle

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ABSTRACT

Recognition of the mucosal portal of entry for many infectious diseases and of the relevance of mucosal immune response to protection has encouraged the development of vaccines administered by mucosal routes, principally oral and intranasal, for stimulation of intestinal and nasopharyngeal lymphoid tissues respectively. The oral route is problematic in cattle and other ruminants where antigen degradation in the rumen is likely, prior to transit to the intestine. On the other hand, rumination can be exploited for exposure of nasopharyngeal tissues during cuddling if vaccine antigen is expressed by a fibrous feed like alfalfa. An increase in anti-leukotoxin (Lkt) IgA was demonstrated in nasal secretions of calves following feeding of alfalfa expressing a truncated Lkt50 from *Mannheimia haemolytica*, and there is evidence suggesting that such vaccination may protect against experimentally induced pneumonia. Intranasal vaccination is an alternative approach for use in pre-ruminating calves. Intranasal administration of ISCOMs carrying soluble antigens of *M. haemolytica*, including native Lkt, induced Lkt specific IgA in nasal secretions after vaccination at 4 and 6 weeks of age. Subcutaneous (s.c.) administration of the same vaccine induced Lkt specific IgG in both serum and nasal secretions, whereas s.c. administration of a commercial *M. haemolytica* vaccine did not. Regardless of the vaccination strategy employed it is difficult to assess the immunogenicity of mucosally administered vaccines because production of secreted antibodies tends to be transient, and they do not persist on the mucosal surface in the absence of ongoing antigenic stimulation. An additional challenge is demonstration of vaccine efficacy in response to experimental infection. Protection of the mucosally vaccinated animal will most probably result from recall response, which may not amplify sufficiently to counter the effects of experimental pulmonary delivery of a large bolus of virulent bacteria, even though the response would suffice over the more prolonged and gradual infection that occurs in natural induction of pneumonia.

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1. Introduction

The vast majority of infectious diseases in all species are initiated by colonization of, or entry across, mucosal

surfaces of the respiratory, intestinal or urogenital tracts. There has, therefore, been a great deal of interest in immune response at these sites and in development of vaccines that target these portals of entry (Hodgins et al., 2005). The reality is that most current vaccines for such infections are delivered parenterally and act thorough induction of systemic rather than mucosal immunity. Protection is typically mediated by “spill-over” of mediators onto mucosal surfaces or by blocking of infection once the mucosal surface is breached; examples include vaccines for influenza viruses, *Vibrio*, *Salmonella*, and

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Abbreviations: DCs, dendritic cells; ISCOMs, immune stimulating complexes; Lkt, leukotoxin; Lkt50, truncated leukotoxin.

Shigella bacteria. In cattle, parenteral vaccines against pneumonia caused by *Pasteurella*, *Mannheimia* and *Haemophilus* bacteria or abortion caused by *Campylobacter* or *Brucella* successfully protect deeper mucosal organs like the lung and uterus, respectively, where IgG is the dominant immunoglobulin and there is ready access to systemic mediators in blood (Corbeil et al., 1981; Wilkie and Markham, 1981). Despite these successes, development of mucosally delivered vaccines remains an area of active investigation in many laboratories, for both human and veterinary pathogens.

Why vaccinate mucosally? Immune mediators, both immunoglobulins and effector T cells generated by mucosal exposure to antigens differ from those generated by systemic immunization (Boyaka et al., 2005). Certainly, where the goal is prevention of infection, the presence of mediators on the mucosal surface is needed. Memory cells generated at mucosal sites and in draining lymph nodes, home preferentially to other mucosal locations providing a primed response at all potential portals of exposure (Youngman et al., 2005). There are also non-immunological reasons for seeking vaccines that are delivered without injection, including ease of delivery and the absence of injection site reactions. Vaccination of food producing animals would be facilitated by mass delivery of vaccine in feed, water or by aerosol, meaning less labor cost for producers and reduced stress on the animals. Additionally, carcass condemnation due to needle breakage or injection site reactions would be avoided (Roeder et al., 2002). Increasing consumer pressure for organically produced food and a natural approach to disease management is more compatible with disease prevention using non-invasive methods of vaccine delivery.

Mucosal delivery of antigens triggers immune response in mucosa-associated lymphoid tissues including the Peyer's patches of the small intestine, the tonsil and associated pharyngeal lymphoid tissues, the bronchus associated lymphoid tissues of the lung and diffuse lymphoid aggregates lining the urogenital tract. Induction sites for mucosal immunity have been most thoroughly described for the intestinal tract where antigens are delivered to underlying Peyer's patches by specialized membranous non-ciliated epithelial cells, M cells, located in villous crypts, and by dendritic cells (DCs) that send processes to the surface between ciliated columnar epithelial cells of the villi. These DCs deliver antigen to Peyer's patches and to draining mesenteric nodes (Meeusen et al., 2004). Similar mechanisms for antigen acquisition exist at other mucosal sites, including in tonsillar crypts and in BALT where both M cells and epithelial DCs have been identified (Gebert and Pabst, 1999; Stanley et al., 2001), and presumably also at genital sites (Hodgins et al., 2005). Experimentally, calves have been immunized by the vulvovaginal and rectal routes (Loehr et al., 2000, 2001), but most mucosal vaccines deliver antigens by oral administration, which targets intestinal induction, or intranasally which targets pharyngeal and, depending on particle size, deeper respiratory tissues.

There are many challenges inherent in mucosal antigen delivery. Intranasal vaccines must be delivered in dosages sufficient to overcome innate clearance mechanisms and

facilitate uptake in the pharynx. Oral vaccines must be protected against degradation by digestive processes, mechanisms are needed to facilitate antigen adherence to the mucosal epithelium and avoid clearance with the mucociliary blanket coating the gut, and the potential for induction of tolerance rather than active immunity must be considered (Mestecky et al., 2005). In mouse models, oral delivery of auto-antigens may lead to oral tolerance and reversal or reduction of autoimmunity. However, oral tolerance has not yet been reported in studies where plant expressed antigens have been delivered as vaccines in mice or other monogastric animals (Arntzen et al., 2005; Rice et al., 2005). Successful oral vaccines exist; vaccines for polio and rabies are excellent examples. Vaccines using avirulent live bacteria (e.g. *Salmonella*, *Bordetella*) or viruses (e.g. adenovirus, coronavirus, reovirus) have been shown to be quite effective in stimulating mucosal immune responses and in cases where the vaccine organism is at least minimally invasive, systemic responses as well.

Perhaps the greatest challenge for mucosal immunization lies in vaccination with non-replicating antigens. Such preparations are difficult to protect against digestion, tend not to adhere to mucosal surfaces, and generally fail to trigger the danger signals needed to initiate appropriate cellular stimulation for active immune response. Experimentally several strategies have been employed to overcome these inherent challenges. Vaccine antigens have been enclosed within microspheres, linked to bacterial toxin subunits such as CT or LT, or administered with molecules that induce danger signals, like CpG motifs, or with cytokines to stimulate lymphocyte activation (Holmgren et al., 2003).

2. An edible vaccine for ruminating cattle

Ruminating animals pose a particular challenge for development of orally administered vaccines. Encapsulation of antigens in polymer microparticles or microspheres that resist digestion in the rumen is one approach that shows promise for stimulation of mucosal immune response in Peyer's patches by oral delivery (Bowersock et al., 1999). An alternative approach is to exploit the process of rumination for exposure of the pharyngeal lymphoid tissues including the tonsil during cudging. This could be a particularly valuable approach for vaccination against both respiratory and intestinal diseases, since it is recognized that memory cells produced in tonsillar lymphoid tissue migrate preferentially to the lung and intestine, priming these sites for subsequent natural exposure (Brandtzaeg and Johansen, 2005). With this in mind, we hypothesized that delivery of protective antigens in a palatable fibrous feed, such as alfalfa, could lead to repeated exposure of pharyngeal lymphoid tissue by cudging during rumination. Because of its relevance as an economically important disease of cattle, we selected bovine pneumonic pasteurellosis as the target disease for study. Our extensive experience with immunity to the principal causative bacterium *Mannheimia haemolytica* and its protective antigens made this both an appropriate and convenient model system to test both the immunogenicity

and efficacy of edible vaccines comprised of transgenic alfalfa.

The first antigen targeted in this research was the leukotoxin (Lkt). Many studies have demonstrated Lkt's importance in pathogenesis and the correlation between the presence of anti-Lkt antibodies in serum and protection against pneumonia; however, most suggest that an anti-Lkt response is essential but not sufficient alone to provide immunity (Shewen and Wilkie, 1988; Jeyaseelan et al., 2002). The transgenic alfalfa used in these experiments expressed a truncated form of leukotoxin (Lkt50) that contained the neutralizing epitope (Lee et al., 2001). The concentration of Lkt50 was at least 90 µg/g of dried plant material, estimated as a percentage of total soluble protein. Posttranslational modification of proteins occurs in plants (Gomord and Faye, 2004), including both N-linked and O-linked glycosylation, but plant glycosylation patterns can differ from those found in bacteria. Since the extent of glycosylation and type of glycan added to proteins can alter their immunogenicity, an early step in this work was demonstration of the immunogenicity of plant expressed Lkt50 following intramuscular inoculation of rabbits, and verification that antisera from immunized rabbits recognized both recombinant and native Lkt (Lee et al., 2001).

Use of transgenic alfalfa as a vehicle provides an efficient means for delivery of antigen that also furnishes protection against immediate dilution and destruction in the rumen. The natural process of cudging means that the fibrous feed is regurgitated, chewed slowly and held as a cud in the posterior oral cavity. Typically this activity occurs 10–100 times over a period of several days, spraying the pharyngeal lymphoid tissues with antigen during each cycle, before it is finally digested and passed on. With this system, concerns about delivery, avoidance of innate clearance mechanisms and antigen destruction are addressed, but at least two major challenges remain as barriers to vaccine development. The first is demonstration of immunogenicity when the anticipated response is predominately mucosal and therefore inherently both difficult to sample and transient in the absence of continual antigen stimulation. It is also quite possible that pharyngeal exposure will merely prime the lung for an anamnestic response on infection, rather than lead to production of mucosal antibodies in response to the levels of antigen delivered by vaccination. The second related challenge is demonstration of efficacy, given that protection is most likely derived from a recall response. Such response should suffice during natural exposure since this is gradual and continual over a period of hours or days, but can easily be overwhelmed in experimental challenge where the successful challenge model uses intrabronchial delivery of a large number of organisms, sufficient to cause pneumonia, as a single bolus.

To determine the extent of these challenges and to address related questions of dose and duration of feeding needed for immunization, we have conducted a series of pilot studies feeding transgenic alfalfa to small groups of calves, typically two vaccinates and two controls that receive an equal amount of wild type alfalfa in lieu of the transgenic feed. For initial studies we opted to use colostrum deprived animals, reared in an isolation facility,

to avoid interference by passive maternal antibodies and to minimize commensal colonization by *M. haemolytica* that might confound demonstration of response to vaccination. The animals were not germfree, nor were they caesarian derived, thus there was a low level of colonization and a low baseline antibody titer in serum at the time of first feeding, typically about 5 months of age. All experiments were conducted under approval of the University of Guelph Animal Care Committee and adhered to the Canadian Council of Animal Care Guidelines for Use of Animals in Research. Growth, processing, storage and feeding of the transgenic alfalfa as well as disposal of animals and animal waste were as specified in letters of permission from the Canadian Food Inspection Agency, which is responsible for regulation of GMOs and use of experimental vaccines.

In early trials, calves were fed in two rounds, 300 g of dried alfalfa, each day for 5 days, at a 2-week interval. They were then challenged approximately 3 weeks after the second feeding by intrabronchial administration of 25 ml of *M. haemolytica* (ATCC 43270) at approximately 10^5 CFU/ml. This dose was estimated to cause infection sufficient to elicit recall response, but not produce pneumonia in controls. Calves were euthanized 5 or 6 days post-challenge and mononuclear cells were harvested from blood, tonsil, spleen, and retropharyngeal, bronchial and mesenteric lymph nodes. Serum and nasal swabs were collected at various points throughout the trial, and the presence of antibodies was determined by an Lkt specific ELISA using either alfalfa expressed Lkt50 or native leukotoxin, in log phase serum-free culture supernatant, as the antigen. The latter ELISA was further adapted as an ELISpot assay for detection of antibody secreting cells in mononuclear cell preparations following incubation with native leukotoxin. Mononuclear cell culture supernatants were also assayed for production of interferon gamma using a commercial kit (Bovigam, Pfizer).

During these studies it was noted that feeding of transgenic Lkt50 alfalfa led to an increase in Lkt specific IgA in nasal secretions 1 week following the second feeding (Fig. 1). This increase was transient and by the time of challenge levels of specific nasal IgA were similar in vaccinates and controls. No changes in serum antibodies were observed prior to challenge. Low level intrabronchial challenge with *M. haemolytica*, $<10^7$ CFU/ml, resulted in an increase in both serum and nasal antibodies in all calves at 5 days post-challenge. These observations illustrate the difficulty in demonstrating humoral immune response to mucosal vaccination. The response is likely to be local, not systemic, and transient. Thus sampling site and timing become critical. A similar response to vaccination was recently observed in a larger scale feeding trial in colostrum sufficient calves (20 vaccinates and 10 controls) (Fig. 2). Baseline serum antibodies to Lkt were higher in all calves than previously observed in colostrum deprived animals, and feeding did not result in a noticeable change in serum antibodies. Several calves fed recombinant alfalfa maintained elevated nasal IgA to Lkt from 1 week after the second feeding up to the day of challenge. That the response was sustained in these animals may be a characteristic of conventional calves, since continuous

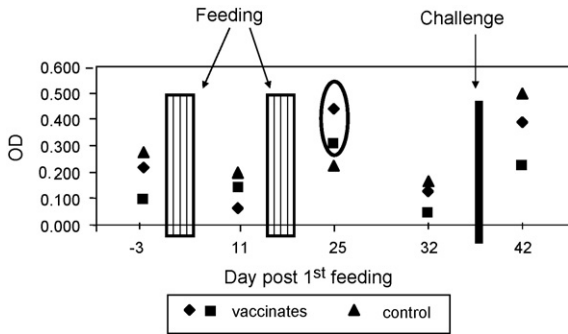


Fig. 1. IgA antibodies to leukotoxin (Lkt) in nasal secretions, determined in an ELISA assay using alfalfa expressed Lkt 50 as antigen. OD = optical density against Lkt50 alfalfa minus OD using a mock antigen preparation derived from alfalfa expressing GFP. Grids span feeding days, solid vertical bar indicates day of challenge, oval indicates increase in specific IgA 7 days after feeding in vaccinated calves (2 vaccinates, 1 control).

antigen stimulation due to natural exposure could help maintain the response that was enhanced by vaccination. In this experiment where the challenge, 25 ml of *M. haemolytica* at $10^{9.3}$ CFU/ml, was intended to induce pneumonia, a correlation between the level of anti-Lkt nasal IgA and protection against pneumonia was observed. However, there was also a correlation between the level of anti-Lkt IgG in serum and protection, even though we could not demonstrate an effect of vaccination on the circulating level of those antibodies. Thus, despite the encouraging nasal antibody results, we cannot confidently associate protection with vaccination.

During one pilot study the challenge dose, at 10^7 CFU/ml, was sufficient to induce pneumonia in the two control calves (lesion scores 5/10 for both, 11% and 27% pneumonic tissue). Both calves fed alfalfa expressing Lkt50 had no clinical signs of pneumonia and no lesions at necropsy. Although caution must be exercised in interpreting this as evidence of vaccine efficacy, since calf numbers are small,

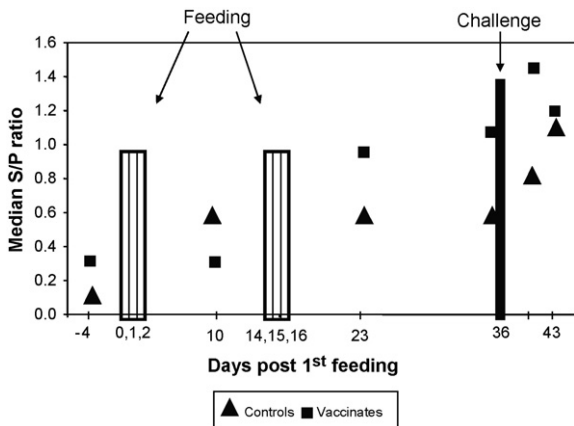


Fig. 2. Group means for IgA antibodies to leukotoxin in nasal swabs obtained from 20 calves fed alfalfa expressing Lkt50 and 10 control calves fed wild-type. Median sample to positive (S/P) ratios determined in an ELISA assay using native Lkt as antigen. Grids span feeding days, solid vertical bar indicates day of challenge.

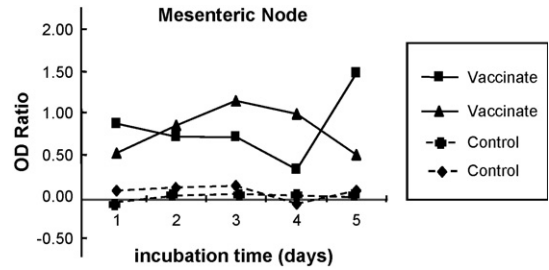


Fig. 3. In vitro IFN γ production by lymphocytes harvested from mesenteric lymph nodes post challenge then cultured with native leukotoxin. Measured using an IFN γ test kit (limit of detection 80 pg/ml, Bovigam, Pfizer). OD ratio = ratio in culture supernatant to IFN γ positive standard. Incubation time = time in culture prior to sampling.

several interesting observations were made with respect to the response of mononuclear cells in susceptible versus resistant calves. Vaccinated calves had Lkt specific antibody producing cells in 2 and 4 day old cultures of cells derived from blood and bronchial nodes harvested at necropsy and there was evidence of class switching since IgG1 and IgA producing cells were present as well as cells producing IgM. Each of the control calves had only a single blood cell (1 in 10^5) producing antibodies, one IgM and one IgA. There was no interferon gamma production by blood mononuclear cells of any calves, response in tonsil cells was very weak, and there was no differential between vaccinated and control calves in IFN γ response in bronchial node cells. Spleen and retropharyngeal node cells from vaccinates produced IFN γ rapidly within 24 h of incubation, whereas cells from control calves responded later or not at all. The most striking difference was observed on culture of cells from mesenteric nodes with Lkt. Cells from vaccinates responded by producing IFN γ , controls did not (Fig. 3). This was especially interesting since the challenge was pulmonary, not intestinal; thus, one would not expect activation of lymphocytes in these nodes. Does this confirm trafficking of antigen-specific memory cells following exposure of the nasopharynx to transgenic alfalfa or does this suggest that vaccine antigen survived rumination and sensitized GALT by transport across the intestinal epithelium? These questions remain to be addressed but at least we have identified a tissue to target in ongoing investigations.

3. Intranasal vaccination of neonatal calves using ISCOMs

Is the edible format the future for veterinary vaccines in ruminanting animals? Perhaps, but even if that approach succeeds it will not address the question of pneumonia in younger calves that are not yet ruminating. Bacteria in the Pasteurellaceae family are major contributors to enzootic pneumonia that occurs at 6–8 weeks of age in both veal calves and replacement dairy heifers (van Donkersgoed et al., 1993). Mucosal delivery of vaccine is also a relevant goal in these neonates, in particular intranasal delivery has appeal for both logistical (ease of delivery) and immunological (targets nasopharyngeal lymphoid tissues) per-

spectives. The added difficulty in calves of this age is the potential for interference in active immune response by passive maternal antibodies. In fact, pneumonia, at 6–8 weeks of age, occurs precisely during the period when passive immunity has waned to the extent that it is no longer protective, but the effects of interference with active immune response have delayed generation of protective immunity (Prado et al., 2006). Previously we demonstrated that calves do not produce leukotoxin neutralizing antibodies in response to parenteral vaccination with a commercial *M. haemolytica* culture supernatant vaccine (Prepsponse, Wyeth/Fort Dodge) prior to 6 weeks of

age (Hodgins and Shewen, 1998). Earlier induction of active immunity may be possible through selection of an appropriate adjuvant or delivery by a mucosal immune route.

Immune stimulating complexes (ISCOMs) are an antigen delivery and adjuvant system (Morein et al., 1984) wherein many antigens can be incorporated within or on the surface of small (30–100 nm) cage like structures formed of cholesterol, saponin and phosphatidylcholine. Antigens contained in multivalent subunit ISCOM vaccines are found both within the cytosol and endosomic vesicles of antigen presenting cells (Villacres et al., 1998). ISCOM

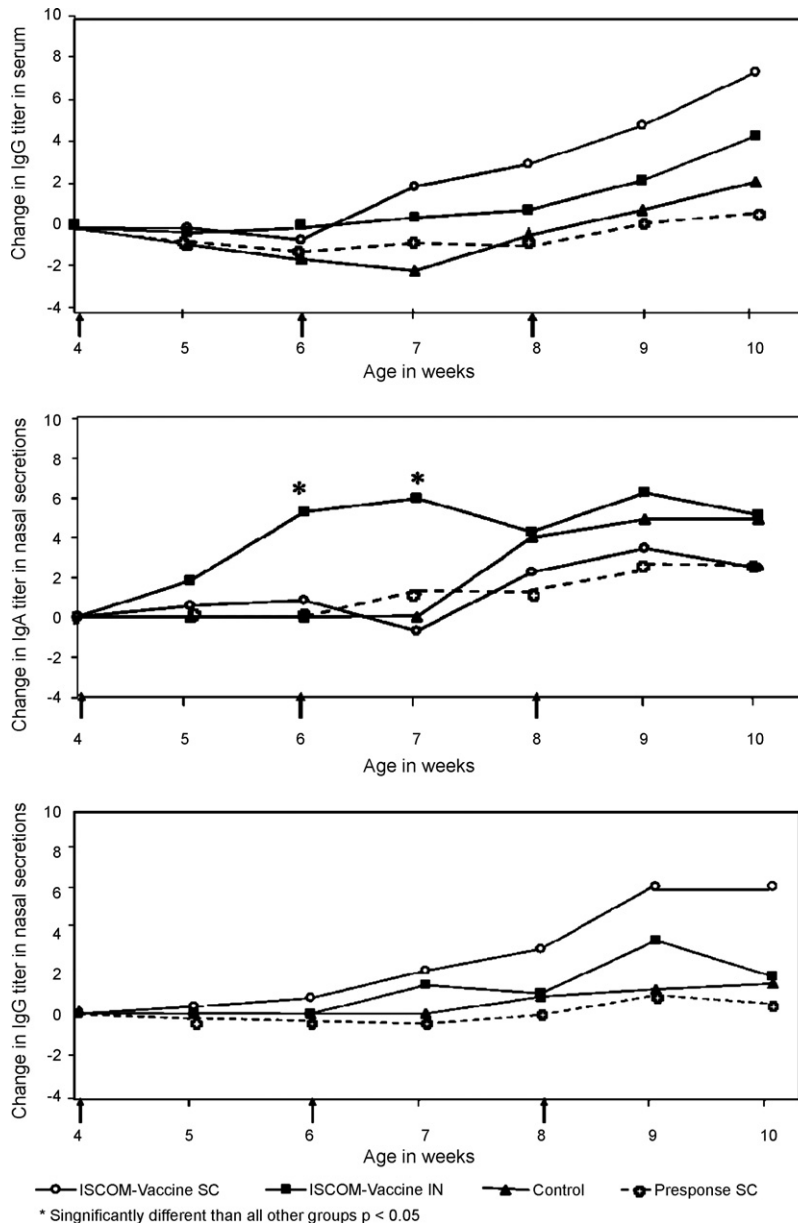


Fig. 4. Mean change in titers ($-\log_2$) to leukotoxin (Lkt) determined by ELISA, for calves vaccinated at 4 and 6 weeks of age with ISCOMs containing Lkt, s.c. ($n = 3$) or i.m. ($n = 3$), or with Prepsponse SQ, s.c. ($n = 3$), compared to unvaccinated controls ($n = 4$). All vaccinated calves also received Prepsponse SQ at 8 weeks of age, to evaluate recall response. Top panel: IgG in serum. Mid Panel: IgA in nasal secretions. Lower Panel: IgG in nasal secretions.

vaccines have been shown to stimulate both humoral and cell mediated immune responses and are claimed to override the down-regulatory effects of passively acquired maternal antibodies (Nordengrahn et al., 1996; Hagglund et al., 2004). ISCOMs, prepared using supernatants from log-phase cultures, were shown to contain native Lkt, as the target antigen, as well as other soluble antigens of *M. haemolytica*. ISCOM vaccines were used to vaccinate groups ($n = 4$ or 5 per group) of colostrum fed dairy heifers at 4 and 6 weeks of age, by either the intranasal or subcutaneous routes. Response to vaccination was compared to that in unvaccinated controls and a group of calves that received a commercial vaccine, Presponse SQ (Wyeth/Fort Dodge) subcutaneously at 4 and 6 weeks. All vaccinated calves were challenged to assess recall response by subcutaneous vaccination with the commercial vaccine at 8 weeks of age, an age where response to vaccination might normally be expected. All three vaccines were standardized by antigen capture ELISA to contain concentrations of Lkt equivalent to that present in the commercial vaccine. Sera and nasal swabs were collected weekly from 1 to 10 weeks of age and antibody responses were determined by direct and indirect agglutination, for antibodies to bacterial surface antigens, and by ELISA for isotypic response to Lkt.

Antibody responses were expressed as the change in titer from that at week 4, the time of initial vaccination, to adjust for antibodies present due to passive transfer. Subcutaneous vaccination with ISCOMs induced an increase in the direct agglutination titer in serum at week 7, one week after the second vaccination and the titer remained elevated for the duration of the study (data not shown). Vaccination with the commercial s.c. vaccine had no effect on titer by any assay before 8 weeks of age. The earlier i.m. formulation of the same vaccine had been shown previously to induce agglutinating antibodies and IgM to capsular polysaccharide, but not Lkt neutralizing responses, in calves vaccinated at 2 and 4 weeks of age (Hodgins and Shewen, 1998). Both ISCOM vaccines induced an increase in serum IgG to Lkt 1 week following the second dose (Fig. 4, top panel). The rate of decline in titer, from week 4 to 7, was less in calves receiving Presponse compared to controls. Titers in control calves rose by week 8, consistent with a naturally induced active immune response to commensal colonization. As hoped, intranasal vaccination induced a significant change in Lkt specific IgA in nasal secretions 1 week following first vaccination at week 4, the earliest age at which we have succeeded in inducing active immunity to Lkt to date. This may be particularly important given the correlation between nasal IgA to Lkt and protection, recently demonstrated in our edible vaccine trial (above). By 8 weeks of age nasal IgA titers had begun to increase in all groups (Fig. 4, middle panel). Interestingly, subcutaneous vaccination with ISCOM vaccine induced an increase in Lkt specific IgG in nasal secretion, which could relate to spill-over from systemic response, but may reflect homing of memory cells to the nasopharynx permitting enhanced response to natural exposure locally (Fig. 4, lower panel). Thus ISCOM vaccines, but not the commercial vaccine, induced immune response to Lkt in serum and nasal

secretions following vaccination at 4 and 6 weeks of age. This preceded responses arising from natural exposure that were evident at 8 weeks of age. The hope would be that this earlier response would protect should the calves receive a challenge sufficient to induce pneumonia in the 6–8 week old period, but that was not assessed in this trial.

4. Conclusions

These studies demonstrate that it is possible to detect immune response to mucosal vaccination targeted at the nasopharyngeal lymphoid tissue by examining antibodies in nasal secretions. We have also collected saliva and feces from many of these animals and will analyze those to determine their utility as alternative indicators of mucosal response. We could demonstrate enhanced response, compared to controls, in sera from some calves vaccinated mucosally using an edible vaccine, but only post-challenge. This would be important as a protective response to natural exposure, but is not useful for demonstration of immunogenicity in response to vaccination per se. Additionally, though it may be adequate to stave off pneumonia during the course of natural infections, the recall response was not adequate to provide protection against an experimental challenge sufficient to induce pneumonia using a single bolus exposure. Therefore, it may be necessary to further refine challenge protocol to enable differentiation, for example by adjusting challenge dose to animal weight, prechallenge serum titer or other criteria. Additionally it is important to continue to examine immune response to mucosal antigen exposure, to improve our understanding of factors that lead to its stimulation and those parameters that reflect stimulation. This will assist in finding new and innovative means to enhance responsiveness, including adjuvants and delivery systems, and improved methods for detection of immunogenicity.

Conflict of interest

Author P. Shewen is co-author on patents that protect the commercial vaccine Presponse and receives loyalty revenue from Wyeth/Fort Dodge related to this vaccine. She currently holds a contract with Dow AgroSciences that funds an unrelated project. With these exceptions, she and her co-authors have no financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper entitled "Challenges in Mucosal Vaccination of Cattle".

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