Enteric Immunization Induces Active Mucosal and Systemic Immunity in Neonatal Ruminants

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Abstract

Neonatal animals are highly susceptible to infectious diseases, many of which gain entry through mucosal surfaces. Although immunization is a cost-effective way of controlling newborn disease, vaccination of newborn ruminants is not common. Vaccines are thought to be ineffective in newborn ruminants due to interference by maternal antibodies and a less reactive, immature immune system. These conclusions were based on observations made following the use of vaccines injected parenterally. However, recent work at VIDO indicates that oral vaccination of newborns is successful.

The overall objective of our study was to determine if enteric immunization of newborn ruminants would induce active local and systemic immune responses. Our vaccine was comprised of the Human Adenovirus 5 vector with the gene for the gD antigen of Bovine Herpes virus-1 inserted in the E3 region (HAd5-gD/E3). Our animal model was lambs in which gut-loops were surgically prepared *in utero* that contained either ileal or jejunal Peyer's patches. Mucosal and systemic immune responses were evaluated by determination of gD-specific antibody secreting cells (ASC), interferon gamma secreting cells (IFN-γ SC), and lymphocyte proliferation response (LPR) in the gut, lung lymph node (LN) and spleen. Specific antibodies were also evaluated in intestinal contents and in the serum.

To determine if gut-associated lymphoid tissues (GALT) was immunocompetent in ruminants we compared immune responses in newborn lambs with responses in 5-6 weeks old lambs. Similar mucosal and systemic responses were detected in the two groups of lambs with regard to ASC, IFN-γ SC, LPR and antibody in intestinal contents and in serum. Thus, GALT is immunocompetent in ruminants at birth.

To determine if maternal antibody specific for vaccine antigen interferes with induction of mucosal and systemic responses in neonatal lambs, we compared immune responses of lambs fed colostrum from immunized ewes with the responses of lambs fed colostrum from nonimmunized ewes. Similar mucosal (LPR, IFN-γ SC and ASC in gut) and systemic (LPR and IFN-γ SC in spleen) responses were found in both groups of lambs. This indicated that maternal antibody did not interfere with induction of immunity by the adenovirus vaccine vector.

To determine if enteric immunization of neonates induced long term mucosal memory in the respiratory system, groups of neonatal lambs were immunized with HAd5-gD/E3 either in gut-loops or intradermally. Secondary immunization with gD in liposomes was done intratracheally after 5 months. There were strong immune responses in the regional lymph nodes (LPR, IFN-γ SC, ASC) and in the spleen (LPR, IFN-γ SC) of both groups of lambs. Interestingly, gD-specific ASC were present in lung LN only in lambs that had a primary oral immunization.

We conclude that mucosal and systemic immunity was induced in neonates following enteric immunization with an adenovirus vector. Maternal antibody specific for the vaccine antigen did not appear to interfere with the development of the immune responses. In addition, enteric immunization induced long term memory. Oral immunization has the potential to protect against respiratory pathogens.

Introduction

Neonatal animals are highly susceptible to infectious diseases. A contributing factor to this increased disease susceptibility is the fact that vaccination is not commonly practiced with newborn animals. Vaccines are thought to be ineffective in newborn animals due to several reasons, including interference by maternal antibodies and a less reactive, immature immune system. Evidence is accumulating that neonatal animals are capable of mounting immune responses to antigens (Sarzotti *et al.*, 1996; Forsthuber *et al.*, 1996). Furthermore, vaccinologists have showed that human infants

developed protective immunity following vaccination within the first week after birth with BCG, oral poliovirus vaccine, or hepatitis B virus vaccines. In this regard, it has been recognized that the immune system of newborn humans and sheep is more mature compared to that in rodent neonates (cited in Bona and Bot, 1997). These developmental differences may be a contributing factor to some of the failures in experimental neonatal immunization observed in studies with rodents.

The majority of pathogens that cause disease in ruminants enter the body via mucosal surfaces such as the gastrointestinal tract, the respiratory tract and the urogenital tract. Induction of protective immune responses at the mucosal sites is critical for protection against mucosal pathogens. A common mucosal immune system, in which exposure of one mucosal site to an immunogen induces immunity at several mucosal sites, has evolved to protect the body against these mucosal pathogens. For example, exposure of the gut associated lymphoid tissue (GALT) to an antigen induces the development of immune effector cells that seed other mucosal surfaces such as the respiratory tract (Dunkley *et al.*, 1995). The mucosal immune system is distinct but not separate from the systemic immune system. In this regard, immune responses at mucosal sites are more effectively induced if the vaccine is delivered via these sites, than if the vaccine is administered parenterally. Mucosal immunization has the ability to induce systemic immune responses as well. It is likely that passively acquired maternal antibodies may interfere with vaccine antigens after parenteral administration, but less so if administered via mucosal routes. We reasoned that since GALT is functionally mature at birth in sheep, it would be possible to induce good immune responses in the gut of newborn lambs by enteric immunization.

The overall objective of our study was to determine if enteric immunization of newborn ruminants would induce active local and systemic immune responses.

Materials and Methods

Vaccine

The vaccine used was a human adenovirus 5 vector with the gene for the gD antigen of bovine herpes virus-1 inserted in the E3 region (HAd5-gD/E3. The recombinant was generated as previously described (Mittal *et al.*, 1996). This is a replication-competent adenovirus vector. Preliminary experiments with intradermal injections corfirmed that the HAd5-gD/E3 expressed gD protein in sheep and induced a gD-specific immune response.

Animals

Suffolk lambs were used for all the experiments and were obtained from the Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, Canada. Our animal model was lambs in which intestinal-segments (gut-loops) were surgically prepared *in utero* that contained either ileal or jejunal Peyer's patches. The surgical procedure was done as described by Reynolds and Morris (1983), modified such that an end-to-end anastomosis was used to re-establish the continuity of the intestine.

Measurement of mucosal and systemic immune responses

Mucosal immune responses were evaluated by determining gD-specific immune responses in the Peyer's patches, lung lymph node (LN), and intestinal contents. Systemic immune responses were evaluated by assaying serum antibody and splenocyte proliferation responses.

Antibody secreting cells (ASC), and interferon-γ secreting cells (IFN-γ SC) were determined by ELISPOT. For antibody secreting cell ELISPOT, cells were isolated and plated in a 12 h assay. The frequency of gD-specific ASC per 1 X 10^6 cells was calculated by substracting the number of ASC detected in the absence of gD from the number of ASC in the presence of gD coated plates. Three replicate cultures were counted for each lamb and data presented as mean values for individual lambs.

For IFNγ secreting cell ELISPOT, cells were cultured for 24 h with 0.2 μg/ml purified gD antigen or medium alone (background) prior to determining IFNγ secreting cell frequency. The gD-specific IFNγ secreting cell frequency was calculated by substracting the number of spots in wells with background cells from the number of spots in wells with gD stimulated cells. Three replicate cultures were counted for each lamb and data presented as mean values for individual lambs.

For assaying lymphocyte proliferation responses (LPR), cells were cultured for 72 h with 0.2 μg/ml of purified gD antigen or medium alone (background). Cells were pulsed with 0.4 μCi/ml of [H^3]-thymidine during the final 6 h of culture. Thymidine incorporation was evaluated by scintillation counting. Proliferation responses were expressed as a stimulation index (counts per minute with gD antigen /counts per minute with background cultures).

The titer of gD-pecific antibodies was evaluated in intestinal contents and in serum by ELISA. Microtiter plates were coated overnight with gD antigen at a concentration of 0.5 μg/ml. Four-fold dilutions of the test sera were then added to the wells. An alkaline phosphatase-conjugated rabbit anti-sheep was added and the plates developed with a substrate.

Is GALT immunocompetent at birth in lambs?

To determine if gut-associated lymphoid tissues (GALT) was functional in newborn ruminants, we compared the immune responses of 2 day-old lambs with

those of 5-6 weeks old lambs. For this experiment, HAd5-gD/E3 vector was injected into intestinal-segments and four weeks later, tissue samples were assayed for the presence of gD-specific immune responses.

Does maternal antibody interfere with the induction of mucosal and systemic responses in neonatal lambs?

For these experiments we compared the immune responses of lambs fed colostrum from ewes immunized with HAd5-gD/E3 and gD protein with the responses of lambs fed colostrum from nonimmunized ewes. The HAd5-gD/E3 vector was injected into the intestinal segments of newborn lambs that had received gD-specific maternal antibody or had not received maternal antibody. Three weeks after immunization, tissue samples were collected from the lambs and assayed for gD-specific immune responses.

Does enteric immunization induce long term mucosal memory in the respiratory tract?

To determine if enteric immunization of neonates induced long term mucosal memory in the respiratory system, one group of neonatal lambs was immunized with HAd5-gD/E3 in gut-loops and a second group was immunized intradermally. A secondary immunization was done with gD protein in a liposome preparation, injected intratracheally after 5 months. Samples were collected and assayed for gD-specific immune responses 6 weeks after the last immunization.

Results

Is GALT immunocompetent at birth in lambs?

Similar mucosal and systemic responses were detected in the newborn and 5 week-old lambs by gD-specific ASC, IFN-γ SC, LPR and antibody titers in intestinal contents and in serum.

Figure 1 shows gD specific LPR with cells isolated from Peyer's patches of lambs immunized as newborns or at 5-6 weeks of age. Antigen specific immune responses were induced at intestinal mucosal sites in both age groups of lambs (a stimulation index of >2.5 is considered a positive response).

Figure 2 shows gD specific LPR in spleen cells from lambs immunized at birth and at 5-6 weeks of age, indicating that enteric immunization induced both mucosal and systemic immune responses.

Does maternal antibody interfere with the induction of mucosal and systemic responses in neonatal lambs?

Similar mucosal (LPR, IFN-γ SC and ASC in gut) and systemic (LPR and IFN-γ SC in spleen) responses were found in both groups of lambs. Figure 3 shows similar gD-specific LPR with cells from Peyer's patches of lambs that had not received (- passive ab) or had received (+ passive ab) maternal antibody. This indicates successful induction of mucosal responses in the presence of maternal antibody.

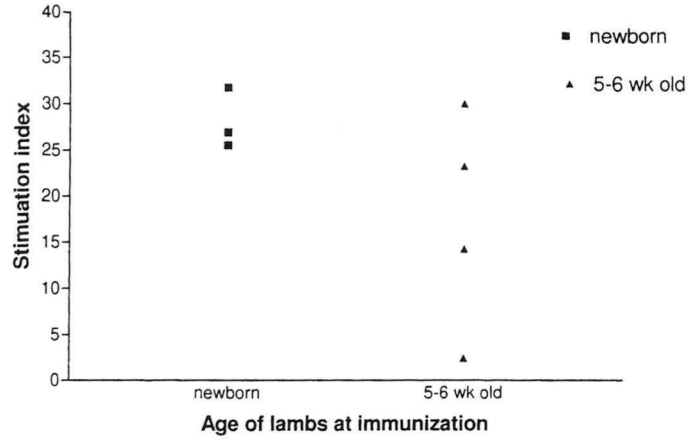

Figure 1. gD-specific lymphocyte proliferation responses in Peyer's Patch intestinal-segments of lambs immunized as newborns or at 5-6 weeks of age. The HAd-gD/E3 vector (2 x 10^8) was injected into intestinal segments. PP cells were collected four weeks after immunization and proliferation responses determined by H^3-thymidine incorporation (stimulation indices shown).

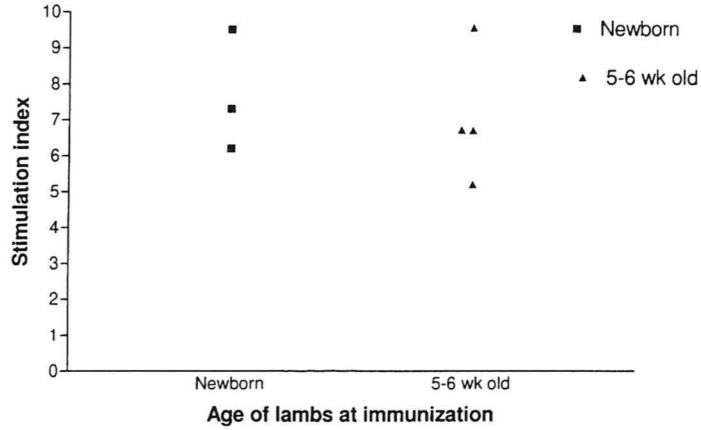

Figure 2. gD-specific lymphocyte proliferation responses in spleen cells of lambs immunized as newborns or at 5-6 weeks of age. The HAd-gD/E3 vector (2 x 10^8) was injected into intestinal segments. Spleen cells were collected four weeks after immunization and proliferation responses determined by H^3-thymidine incorporation (stimulation indices shown).

Figure 4 shows similar gD-specific LPR with cells from spleens of lambs that had not received (- passive ab) or had received (+ passive ab) maternal antibody. This confirms that enteric delivery of a vaccine can induce immunity at the systemic level even in the presence of maternal antibody.

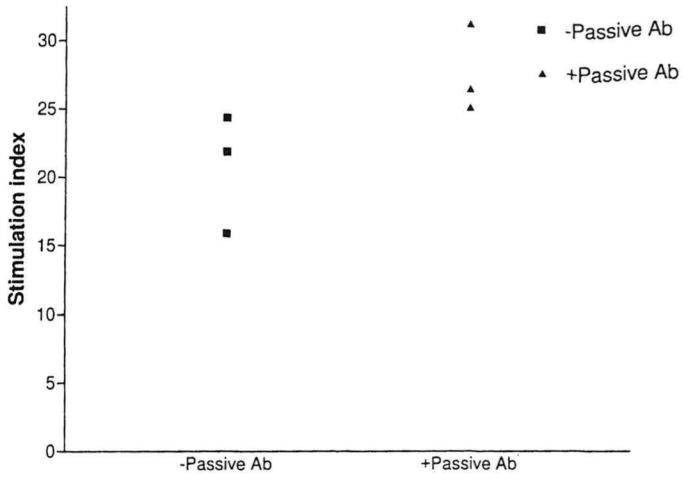

Figure 3. gD-specific lymphocyte proliferation responses in Peyer's Patches of lambs that had not received (-Passive Ab) or had received (+Passive Ab) maternal antibody specific for gD. The HAd-gD/E3 vector (2×10^8) was injected into intestinal segments. Peyer's Patch cells were collected three weeks after immunization and proliferation responses determined by H^3-thymidine incorporation.

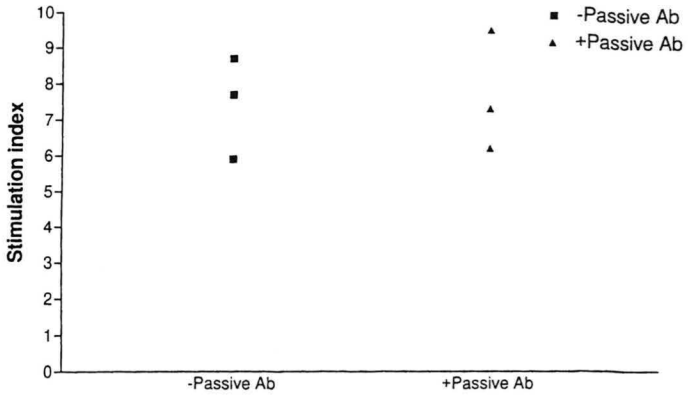

Figure 4. gD-specific lymphocyte proliferation responses in splenocytes of lambsthat had not received (-Passive Ab) or had received (+Passive Ab) maternal antibody specific for gD. The HAd-gD/E3 vector (2×10^8) was injected into intestinal segments. Spleen cells were collected three weeks after immunization and proliferation responses determined by H^3-thymidine incorporation.

Does enteric immunization of the neonate induce long term mucosal memory in the respiratory tract?

Figures 5a and 5b show that gD-specific ASC and IFNγ SC were detected in Peyer's patches 5 months af ter primary immunization in the gut. Six weeks after a secondary intratracheal immunization of the 5-month old lambs, gD-specific ASC were present in lung LN only

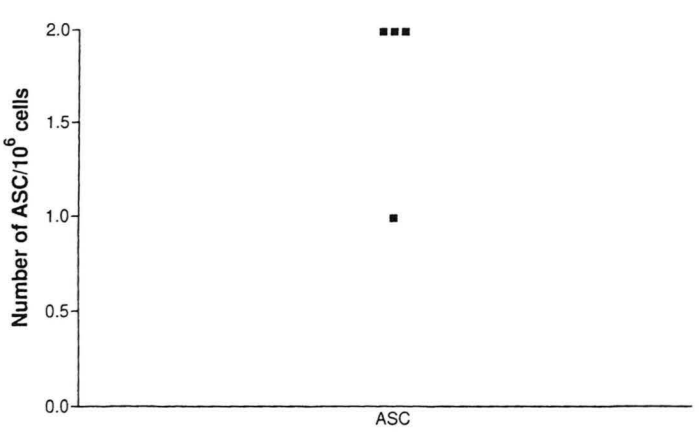

Figure 5a. gD-specific antibody secreting cells (ASC) in Peyer's patches after primary immunization either in gut (intestinal segments) or intradermally with HAd5-gD/E3 followed by a secondary immunization 5 months after. Cells were assayed for ASC by ELISPOT 6 weeks after secondary immunization.

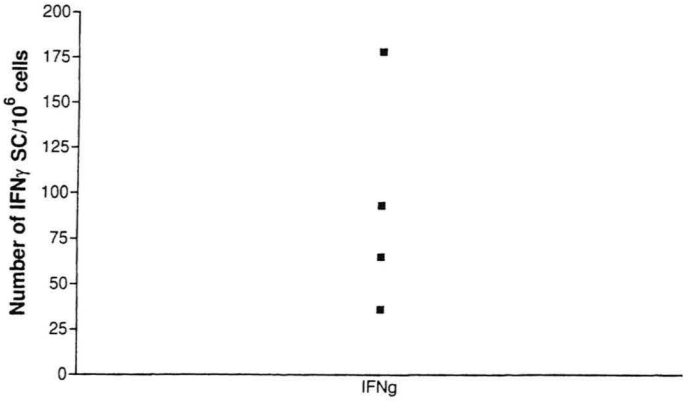

Figure 5b. gD-specific IFNγ secreting cells in Peyer's patches after primary immunization either in gut (intestinal segments) or intradermally with HAd5-gD/E3 followed by a secondary immunization 5 months after. Cells were assayed for IFNγ secreting cells by ELISPOT 6 weeks after secondary immunization.

in lambs that had a primary enteric immunization (Figure 6). Thus priming of a secondary mucosal immune response in the lung was best achieved by enteric immunization of the neonate.

Figure 7 shows gD-specific IFNγ SC in spleen cells 5 months after primary immunization of neonatal lambs in the gut or intradermally, and 6 weeks after a secondary intratracheal immunization. This indicates that primary enteric immunization followed by intratracheal boost induces systemic immune responses as well as primary immunization by the parenteral (intradermal) route.

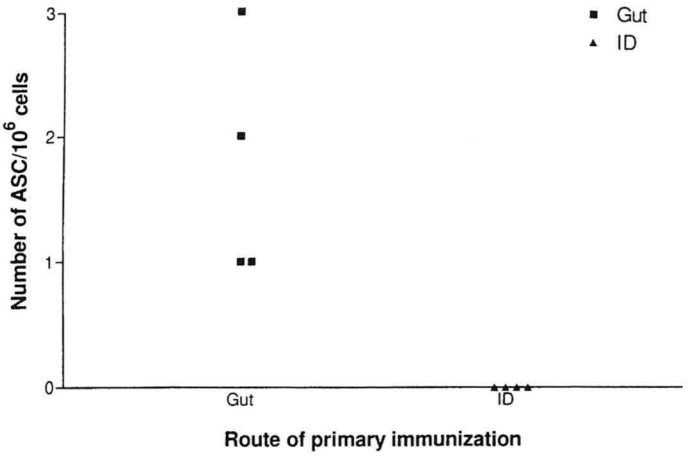

Figure 6. gD-specific antibody secreting cells (ASC) in lung lymph node after primary immunization either in the gut (intestinal segments) or intradermally followed by a secondary immunization intratracheally 5 months after. Lung lymph nodes were collected 6 weeks after secondary immunization and the number of ASC determined by ELISPOT assay.

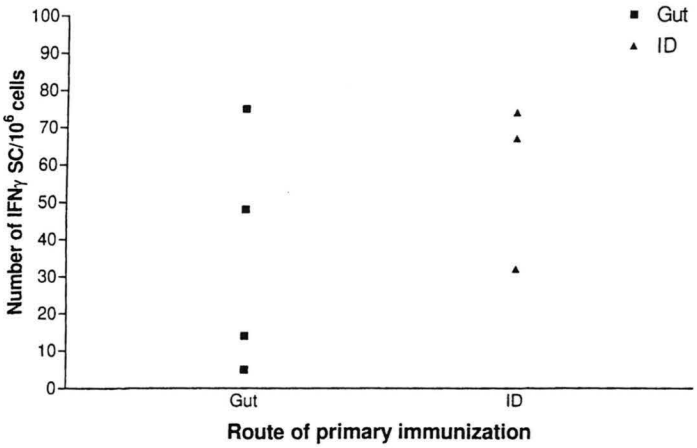

Figure 7. gD-specific IFNγ secreting cells (IFNγ SC) in the spleen of lambs after primary immunization with HAd/gD-E3 vector either in the gut (intestinal segments) or intradermally followed by a secondary immunization 5 months after. Spleen cells were assayed for IFNγ SC by ELISPOT 6 weeks after the secondary immunization.

Discussion

A major observation in this study was that a single enteric administration of the HAd-gD/E3 vaccine vector induced gD-specific mucosal and systemic immune responses in 2-day old lambs. Furthermore, these responses were qualitatively similar to those responses observed in similarly vaccinated 5-6 week old lambs. This indicated that GALT is immunocompetent in lambs at birth. Since the immune systems of sheep and cattle are developmentally and functionally similar, our results would suggest that newborn calves would likely mount immune responses to vaccine antigens delivered in a similar manner. Furthermore, systemic immune responses were also induced by mucosal delivery of the vaccine. Systemic immune responses would be critical for protection and recovery from infections if the mucosal defenses fail to contain the infection. The fact that we could induce both mucosal and systemic immune responses at birth strongly suggests that there is potential for vaccinating newborn ruminants.

A major barrier to vaccination of newborn animals is perceived to be the interference with the induction of immunity by passively acquired maternal antibodies. In our study, maternal antibodies did not appear to interfere with immunization, when the vaccine was delivered mucosally. In domestic species such as dogs, young animals are given multiple vaccinations during the first few months of life. This approach has achieved a reasonable degree of success. However, multiple vaccinations are not practical or economical in ruminants. Thus a vaccine that would be efficacious in the presence of maternal antibodies, after a single immunization would be a major advantage. One way to circumvent the interference of maternal antibodies may be to deliver a vaccine that achieves sustained antigen production after immunization. This sustained antigen production may enhance the clearance of maternal antibodies (Bona and Bot, 1997). It is possible that the replicating vaccine vector used in our study worked in a similar slow release mechanism. Alternatively, in ruminants there are high levels of maternal antibodies in the gut only during the first 48-72 h *post partum*. Thereafter, maternal antibodies are maintained in the circulatory system and these do not seem to interfere with the induction of a mucosal immune response. The optimum immune protection would be achieved if the vaccine induced active immunity even in the presence of maternal antibodies. This would mean that the vaccine would provide additional protection, especially in cases where there is insufficient protection from maternal antibodies.

Enteric immunization induced long term mucosal memory in the respiratory tract. Almost 7 months after primary immunization (and 6 weeks after secondary immunization), gD-specific ASC were present in lung LN only in lambs that had a primary immunization in the gut , but not in those that were primed intradermally. It thus appears that enteric immunization effectively primes for a secondary immune response in the lung, whereas intradermal immunization does not. This suggests that there is potential to deliver multivalent vaccines via the gut to protect against enteric and respiratory pathogens, and probably other mucosal surfaces.

In summary, our experimental model establishes the following concepts: (1) GALT is immunocompetent in neonatal ruminants, (2) Mucosal vaccination induces both mucosal and systemic immune responses, (3) Maternal antibodies do not interfere with induction of mucosal and systemic immunity and (4) Enteric immunization induces long term mucosal memory. More work is required to determine if the immune responses induced are protective against infection. Vaccines that can protect newborn animals against infection will have a great impact in the animal health industry.

References

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