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*J Immunol* 2002; 168:1877-1885; doi: 10.4049/jimmunol.168.4.1877

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Oral DNA Vaccination In Utero Induces Mucosal Immunity and Immune Memory in the Neonate

Volker Gerdts, Marlene Snider, Robert Brownlie, Lorne A. Babiuk, and Philip J. Griebel

Infectious diseases are a major cause of neonatal morbidity and mortality in humans; pathogens involved include HSV, HIV, hepatitis B virus (HBV), group B streptococcus, *Hemophilus* sp., and *Chlamydia* sp. (reviewed in Refs. 1 and 2). Transmission of these diseases from mother to infant frequently occurs shortly before, during, or after birth by early rupture of the amniotic membranes or direct contact with infectious secretions during labor. Infection can also result from nonsterile delivery techniques, by breastfeeding, or during the first days of life in a perinatal nursery. Improved neonatal care together with cesarean sections, antibiotic treatments, and maternal antiviral therapy have been used with limited success to prevent vertical disease transmission. Thus, there is a significant need for alternative therapeutic approaches to prevent neonatal infection.

Vaccination has provided a very cost-effective approach to prevent infectious disease, but the induction of tolerance or a state of nonresponsiveness was previously thought to preclude vaccination as an effective therapy in the fetus or newborn (3). Increasing evidence, however, indicates that fetal immunization can induce active immunity in the newborn. Three in utero immunizations with a hepatitis B (HB) vaccine induce detectable immune responses in 75% of immunized lambs. Thus, the present investigation confirms that oral DNA immunization in utero can induce both mucosal and systemic immune responses in the neonate and that this immunity has the potential to prevent vertical disease transmission. The Journal of Immunology, 2002, 168: 1877–1885.

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Received for publication August 8, 2001. Accepted for publication December 12, 2001.

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The fetal lamb provides an appropriate animal model for evaluating in utero DNA vaccination and the induction of both systemic and mucosal immunity in newborn infants. The syndromesmoschial placentalization of sheep prevents prenatal transfer of maternal Ab, and surgical manipulation of the fetal lamb and pregnant ewe is associated with a low risk of abortion (14). Furthermore, there are many developmental similarities between the ovine and human immune systems (15, 16). In particular, mucosal-associated lymphoid tissues develop in the fetuses of both species (17), and it has recently been shown that the gut-associated lymphoid tissue (Peyer’s patches) of newborn lambs can respond to enteric vaccination (18). Furthermore, if pregnant ewes lack detectable serum Ab titers for a specific vaccine Ag, then the postnatal transfer of maternal Ab does not interfere with an evaluation of humoral immune responses in the newborn lamb (11, 18). Therefore, it is possible to evaluate both cell-mediated and humoral immune responses in the newborn lamb following fetal vaccination.

Materials and Methods

Cells and viruses

Bovine viral diarrhea virus-free Madin-Darby bovine kidney cells were cultured in MEM (Life Technologies, Burlington, Ontario, Canada) supplemented with 5% FBS (Life Technologies). BHV-1 strain 108 is a virulent field isolate (19) and was propagated in Madin-Darby bovine kidney cells.

Plasmids

Plasmid pSLIA-tgD (20), encoding the truncated form of glycoprotein D (tgD) of BHV-1 under the control of the human CMV immediate early promoter/1A region, was kindly provided by H. L. Davis and R. Weeratna (Loeb Research Institute, Ottawa, Ontario, Canada). Plasmid pMCG-16-HBsAg was a generous gift from H. L. Davis and D. Weeratna (Loeb Research Institute, Ottawa, Canada) and contained the open reading frame of the HBV S gene (21). Plasmids were purified with the Qiagen kit (Qiagen, Mississauga, Ontario, Canada). Plasmid DNA used for immunizations was dissolved in sterile pyrogen-free PBS (pH 7.3; Sigma-Aldrich, Oakville, Ontario, Canada).

Animals

Suffolk sheep were obtained from the Department of Animal and Poultry Science (University of Saskatchewan, Saskatoon, Saskatchewan, Canada) and were cared for in accordance with Guidelines of the Canadian Council for Animal Care. The timed mating of ewes and confirmation of pregnancy were performed as previously described, and pregnant ewes were seronegative for glycoprotein D (gD)-specific Abs to ensure there was no passive transfer of gD-specific Ab to newborn lambs (11). The newborn lambs were performed as previously described, and pregnant ewes were seronegative for any detectable serum Ab or proliferative responses to in utero DNA immunization were evaluated for gD-specific immune tolerance by immunization with an inactivated, commercial BHV-1 vaccine (Triangle 3; Ayerst Veterinary Laboratory, Guelph, Ontario, Canada). The vaccine was injected i.m. following the manufacturer’s guidelines, and each lamb received the equivalent of three bovine doses.

Collection of nasal secretions

Nasal secretions were collected daily for 8 days postinfection (p.i.) by placing a sterile cotton swab in the left nostril. The cotton swab was saturated with normal saline and then placed into 1 ml of MEM (Life Technologies) and stored at −70°C. Infectious virus recovered from each swab was quantified by plaque titration, as described previously (23).

Challenge infection

Each lamb was challenged with 5–7 × 107 PFU of BHV-1 strain 108 by covering the nostrils and the oral cavity with an inhalation mask and aerosolizing virus for 4 min with a DeVILBIS nebulizer (model 099HC; DeVILBIS, Somerset, PA).

Virus isolation

Nasal secretions were collected daily for 8 days postinfection (p.i.) by placing a sterile cotton swab in the left nostril. The cotton swab was saturated with normal saline and then placed into 1 ml of MEM (Life Technologies) and stored at −70°C. Infectious virus recovered from each swab was quantified by plaque titration, as described previously (23).

ELISAs and Western blot analysis of gD-, gB-, and HBsAg-specific Abs

Polyethylene microtiter plates (Immulon 2 HB; Dynex Technologies, Chantilly, VA) were coated with 0.1 µg of recombinant gD or the truncated version of glycoprotein B (gB) per well and incubated with serially diluted fetal, lamb, or ewe sera. Biotin-conjugated rabbit anti-sheep IgG (1/6,000 dilution; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used to detect total gD- or gB-specific IgG. Rabbit anti-sheep IgG Ab-specific Ab (Be- thyl, Montgomery, TX; 1/2,000 dilution) was purified, biotinylated, and used to detect gD-specific IgA in nasal secretions. The reaction was amplified using the alkaline phosphatase-streptavidin complex (1/10,000 dilution; Life Technologies) and visualized with p-nitrophenyl phosphate (Sigma-Aldrich). The presence of gD- and gB-specific Abs in the sera of fetal lambs was also assessed by Western blotting. Briefly, purified gD or gB was transferred to nitrocellulose after electrophoretic separation on a 8.5% polyacrylamide gel. After blocking, filters were incubated overnight in serum (1/50 dilution in TBS), and bound Ab was visualized with alkaline phosphatase-conjugated goat anti-mouse (1/5,000; Kirkegaard & Perry Laboratories) or rabbit anti-sheep antiserum (diluted 1/5,000; Kirkegaard & Perry Laboratories) using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich). BHV-1 gB- and gD-specific mAbs (24) were a generous gift from S. van Drunen Littel-van den Hurk (Veterinary Infectious Disease Organization). HBsAg-specific Ab tiers were assayed using a Microparticle Enzyme Immunoassay (Abbot IMX AUSSAB; Abbott Laboratories, Mississauga, Ontario, Canada), and the assay was read using the Abbot IMX kit and analyzer (Abbott Laboratories). The assay was conducted in the Virology Laboratory, Royal University Hospital (Saskatoon, Saskatchewan, Canada).

Flow cytometry and clinical pathology

mAb specific for sheep IgM (clone P1g145A), IgG1 (clone Blg715A), CD25 (clone CACTI116), and MHC II (clone TH14B) were purchased from VMRA (Pullman, WA). The CDS (clone ST1a), CD4 (clone 17D-13), CD8 (clone E95), and γδ TCR (clone 86D)-specific mAbs were produced by hybridomas generously provided by W. Hein (AgResearch, Wallaceville, New Zealand). FITC-conjugated and PE-conjugated, isotype-specific goat anti-mouse Ig Abs were purchased from Southern Biotechnology Associates (Birmingham, AL). Flow cytometric analyses were restricted to viable cells by excluding cells stained with propidium iodide (2.5 µg/mL;
ever, as expected from previous investigations (15), the number of lymphocytes, monocyte, and polymorphonuclear cell populations was performed by Prairie Diagnostic Services (Western College of Veterinary Medicine, Saskatoon, Saskatchewan, Canada).

\[ gD \text{-specific LPRs} \]

Blood was collected in EDTA-treated vacutainer tubes (BD Biociences), and mononuclear cells were isolated, as described previously (11). Briefly, \( 3 \times 10^5 \) cells/well (96-well microtiter plates; Naigle Nunc International, Naperville, IL) were cultured in a final volume of 200 \( \mu l \) of AIM-V medium (Life Technologies) supplemented with 2% FBS (Life Technologies) and 50 \( \mu M \) 2-ME (Sigma-Aldrich). Cells were stimulated with either purified gD protein (2.5 or 5 \( \mu g/ml \)), 1 \( \mu g/ml \) Con A (Sigma-Aldrich), or medium alone. After a 72-h incubation, the cells were incubated with 0.4 \( \mu Ci/well \) (methyl-[3H]thymidine; Amersham Pharmacia Biotech, Baie de Urfe, Quebec, Canada) for 16 h. Cells were harvested following standard liquid scintillation protocols, and proliferative responses were calculated as the mean of triplicate cultures and expressed as a stimulation index (SI = cpm in the presence of Ag/cpm in the absence of Ag).

**Statistical analysis**

Data for serum Ab titers and virus shedding in nasal secretions were transformed to \( \log_{10} \) before performing a one-way ANOVA for data at each time point. Data for lymphocyte proliferative responses were also analyzed with one-way ANOVA to compare responses within assays conducted at each time point. When ANOVA indicated a significant difference among means, then Tukey’s multiple comparison test was used to identify groups that were significantly different. Data for leukocyte and lymphocyte populations of vaccinated and naive lambs were analyzed for each time point using a two-tailed test. All statistical analyses were performed using GraphPad Prism 2.01 Software (GraphPad Software, San Diego, CA).

**Results**

**Efficacy and safety of in utero DNA vaccination**

It is now evident that bacterial DNA can have a wide variety of effects on cells involved in both innate and adaptive immune responses (reviewed in Ref. 25). For example, CpG motifs present in bacterial DNA or synthetic oligodeoxyribonucleotides can enhance B cell survival, affect dendritic cell differentiation, and induce the secretion of a variety of cytokines by B cells, monocytes, NK cells, and dendritic cells (26–30). Therefore, several parameters were evaluated to determine whether oral exposure to plasmid DNA had adverse effects on fetal development or altered immune system development.

Table 1 is a summary of data obtained from all in utero manipulations. All fetuses immunized with plasmid DNA (\( n = 19 \)) or protein vaccine (\( n = 4 \), or exposed to PBS placebo (\( n = 17 \)) displayed normal fetal development and were born alive and without assistance. None of the ewes showed clinical signs of distress or illness following the surgical procedure. Plasmid DNA transfection and expression were confirmed by the presence of gD-specific serum Ab (detected by ELISA and Western blot) and gD-specific lymphoproliferative responses (LPR) in 12 of 15 (80%) newborn lambs (Table 1). The number of various blood leukocyte subpopulations was compared between lambs orally exposed to either plasmid DNA or PBS in utero. There were no significant differences between these two groups of lambs (\( n = 5 \)) when values for blood mononuclear cells, polymorphonuclear cell, or monocytes were compared at birth and at 3 and 6 wk of age. Briefly, the number of polymorphonuclear cells per milliliter of blood and the number of monocytes per milliliter of blood remained relatively constant throughout the neonatal period. However, as expected from previous investigations (15), the number of lymphocytes per milliliter of blood almost doubled between birth (PBS, 1.2 ± 0.5; DNA, 2 ± 1.5; mean ± SD; \( n = 5 \)) and 6 wk of age (PBS, 4.3 ± 0.8; DNA, 3.5 ± 0.9; mean ± SD; \( n = 5 \)). Furthermore, in utero DNA immunization did not induce significant differences in the number of B lymphocytes (surface IgM and surface IgG1), T lymphocyte populations (CD4⁺, CD8⁺, and γδTcR⁺), or activated T lymphocytes (CD25⁺ CD56⁺) present in blood at birth and 6 wk of age. Thus, oral exposure in utero to plasmid DNA did not appear to affect fetal gestation or neonatal viability, or to significantly alter blood leukocyte populations.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>In Utero Immunization</th>
<th>Vaccine Response in Neonates</th>
<th>Serum Ab Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>pSLIA-tgD</td>
<td>7/8</td>
<td>5589 ± 3650</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>0/7</td>
<td>112 ± 57</td>
</tr>
<tr>
<td>II</td>
<td>pSLIA-tgD</td>
<td>5/7</td>
<td>1083 ± 352.4</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>0/4</td>
<td>168 ± 143</td>
</tr>
<tr>
<td>III</td>
<td>pMCCG-16-HBsAg</td>
<td>3/4</td>
<td>95.6 ± 68.9</td>
</tr>
<tr>
<td></td>
<td>Engerix-B</td>
<td>1/4</td>
<td>66.1 ± 64.3</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>0/6</td>
<td>0</td>
</tr>
</tbody>
</table>

* Each experiment was performed independently.

* bFetal lambs were immunized on days 121–123 of gestation by injecting DNA vaccine or PBS into the oral cavity. Engerix-B vaccine was injected i.m.

* cVaccine response defined as serum Ab- or Ag-induced proliferative responses to the vaccine Ag. The numerator represents the number of newborn lambs with vaccine responses and the denominator represents the number of fetuses immunized per group. gD-specific serum Ab and lymphoproliferative responses were assayed during the first week after birth. Positive gD-specific Ab titers were defined as the highest reciprocal of the serum dilution that gave a OD reading 2-fold greater than the average values for sera from naive fetuses. A positive proliferative response was defined as a SI exceeding 3.5. HB vaccine responses were defined as serum Ab >10 mIU/ml at 3 wk of age.

Data presented are the mean ± SE of the mean for values from responding animals.

**In utero immunization induces long-term immune memory in the neonate**

An important concern regarding fetal immunization is the possible induction of immune tolerance, which would prevent the induction of specific immunity in the neonate. To analyze the induction of immune memory, fetal lambs were orally immunized with 500 \( \mu g \) of either pSLIA-tgD plasmid DNA (\( n = 8 \)) or PBS (\( n = 7 \)). Before suckling colostrum, seven of eight newborn lambs immunized in utero with pSLIA-tgD had detectable gD-specific serum Abs (Expt. I, Table I; Fig. 1A). In contrast, none of the lambs immunized in utero with PBS had detectable gD-specific serum Abs (Expt. I, Table I; Fig. 1A). Subsequently, at 3 days of age, three lambs from each group were injected intradermally, on the lateral aspect of the lower mandible, with 500 \( \mu g \) of pSLIA-tgD plasmid secondary DNA immunization of newborn lambs induced significantly (\( p < 0.01 \)) elevated gD-specific serum Ab titers relative to lambs that had received no in utero DNA immunization and newborn lambs that received a primary DNA immunization (Fig. 1A). A single in utero DNA immunization induced serum Ab titers that were significantly (\( p < 0.05 \)) elevated, relative to naive lambs (PBS/PBS), for 6 wk after birth. However, after 1 wk of age, the gD-specific serum Ab titers of lambs that received a single in utero DNA immunization did not differ significantly from the Ab titers of lambs receiving a primary DNA immunization at birth (PBS/DNA). Thus, these data confirmed that in utero DNA immunization had induced gD-specific immune memory.

The anamnestic humoral immune response observed for gD was confirmed by assaying gD-specific proliferative responses of blood mononuclear cells (Fig. 1B). Seven of eight newborn lambs, immunized in utero with pSLIA-tgD plasmid, displayed gD-specific
LPR (SI > 3.5), and these responses were significantly \( (p < 0.05) \) different from naive lambs. Furthermore, secondary DNA immunization at birth induced a significant \( (p < 0.01) \) increase in gD-specific LPR relative to all other groups of lambs. Thus, both humoral and cellular gD-specific immune responses clearly indicated that in utero DNA immunization induced immune memory that responded strongly to secondary DNA immunization at birth. In addition, the primary response following fetal immunization (DNA/PBS) was significantly higher than that following neonatal immunization (PBS/DNA) (Fig. 1). Thus, in utero oral immunization was more effective than intradermal DNA vaccination in the neonate.

The one lamb with no detectable gD-specific response following in utero immunization (Expt. I, Table I) could be explained by either a vaccine delivery failure or the induction of immune tolerance. To address this question, lambs at 13 wk of age were immunized with an inactivated BHV-1 vaccine. Within 2 wk of BHV-1 vaccination, lamb 120, a previous nonresponder to in utero and neonatal DNA immunization, had a gD-specific Ab response, as measured by both ELISA and Western blot (Fig. 2). The ELISA titer was within the same range as naive lambs and lambs that had received a single intradermal DNA immunization at birth (Fig. 2A, lamb 120). Therefore, there was no evidence that either in utero or neonatal DNA immunization had induced gD-specific immune tolerance.

We also addressed the duration of specific immune memory following a single in utero immunization. At 13 wk after birth, the majority of lambs had low titers of gD-specific Ab (Fig. 2A), and lambs were then vaccinated with an inactivated BHV-1 vaccine. Following immunization, lambs that received a secondary DNA immunization at birth produced the highest gD-specific serum Ab titers, and this response was significantly \( (p < 0.01) \) greater than that of naive lambs and ~4-fold greater than that of lambs that received a primary DNA immunization at birth. Furthermore, lambs that received only a primary oral DNA exposure in utero also had significantly \( (p < 0.05) \) elevated Ab titers when compared with naive lambs and an ~3-fold greater response than that of lambs that received a primary DNA immunization at birth (Fig. 2B).

FIGURE 1. In utero immunization induces immune memory in neonatal lambs. Fetal lambs were orally immunized with either pSLIA-gD (DNA; \( n = 6 \)) or PBS (\( n = 6 \)). Between 3 and 5 days of age, each treatment group was subdivided into two equal groups (\( n = 3 \)) that then received a secondary immunization with either PBS (DNA/PBS; PBS/PBS) or pSLIA-gD (DNA/DNA; PBS/DNA). A, gD-specific serum Ab responses. Differences in Ab titer were statistically significant between DNA-immunized and PBS-treated lambs at birth (\(^{**}, p < 0.001\)) and following secondary immunization between DNA/DNA and both PBS/PBS and PBS/DNA groups (\(+, p < 0.01\)) and between the DNA/PBS and the PBS/PBS group (\(*, p < 0.05\)). B, gD-specific LPR of blood mononuclear cells. In utero DNA-immunized lambs had significantly increased LPR in comparison with PBS-treated lambs at birth (\(^{**}, p < 0.01\); \(*, p < 0.05\)). Following secondary immunization, the LPR of the DNA/DNA group was significantly \( (***, p < 0.01; *, p < 0.05) \) increased in comparison with all other treatment groups.

FIGURE 2. Ab responses to inactivated BHV-1 vaccination. A, Sera from all lambs of the four treatment groups were analyzed in gD-specific ELISA at 13 wk of age (pre-BHV), before an i.m. injection of inactivated BHV-1 vaccine (BHV), and at 15 wk of age. Significant differences between DNA/DNA/BHV and PBS/PBS/BHV \( (**, p < 0.01) \) as well as between DNA/PBS/BHV and PBS/PBS/BHV \( (*, p < 0.05) \) are indicated. Lamb 120 had no detectable serum Ab response (ELISA) following in utero and neonatal DNA immunization. B, Western blot with purified gD protein. Serum samples were collected: a, at birth; b, 10 days following secondary DNA immunization at birth; and c, 10 days following immunization with BHV-1 protein. Lane 1, Incubation with gD-specific mAb provided a positive control for protein detection; lane 2, sera from a lamb immunized with DNA in utero and at birth; lane 3, sera from a lamb immunized in utero, but not at birth with DNA; lane 4, sera from a lamb receiving PBS in utero and a primary intradermal DNA injection at birth; lane 5, sera from a naive lamb that received only the BHV-1 vaccination; lane 6, sera from lamb 120.
There was no significant difference when gD-specific Ab titers were compared between lambs receiving a single in utero immunization and lambs that received a secondary DNA immunization at birth. Therefore, data were pooled for these two groups \((n = 6)\) and compared (\(t\) test) with the responses of lambs that received a single DNA immunization at birth \((n = 4)\). This analysis revealed a highly significant \((p < 0.005)\) difference between these two groups and further supported the previous conclusion that in utero immunization induced immune memory more effectively than did neonatal immunization. The present data also demonstrate that a single in utero exposure to plasmid DNA could induce immune memory that persisted for at least 3 mo after birth.

In utero immunization induces mucosal immunity and reduces viral infection

We previously observed that oral DNA immunization in utero induced gD-specific immune responses in the retropharyngeal lymph nodes, which drain the oral cavity \((11)\). This localized immune response suggested that oral DNA immunization might be an effective approach to induce mucosal immunity and protect against vertical disease transmission. Therefore, we analyzed mucosal immune responses in newborn lambs before and after challenge with BHV-1.

Seven fetuses were orally immunized with 500 \(\mu\)g of plasmid pSLIAtgD, and four were injected orally with PBS (Expt. II, Table I). At 7–10 days of age, these lambs were challenged with BHV-1 strain 108, and Fig. 3 summarizes viral shedding data for the five of seven lambs that responded to in utero DNA immunization. Lambs immunized in utero with DNA shed, on average, one to two logs less infectious virus during the first 4 days p.i. In fact, two of the DNA-immunized lambs \((232\) and \(233\); Fig. 3) shed very little infectious virus at any time following challenge. Thus, in utero DNA vaccination induced immune responses that could limit viral replication at the site of primary infection.

To identify the immune responses responsible for reduced viral shedding, the systemic and mucosal immune responses were analyzed before and after viral challenge. Lambs injected orally with PBS in utero had no detectable gD-specific immune responses before viral infection and did not develop significant increases in gD-specific serum Ab titer (Fig. 4A), secretory IgA (SIgA) titer (Fig. 4B), and gD-specific LPR (Fig. 4C) by day 12 p.i. In contrast, lambs immunized in utero with DNA had detectable gD-specific serum Ab titers (Fig. 4A), SIgA titers (Fig. 4B), and LPR (Fig. 4C) before viral infection, and there was a significant increase in gD-specific serum Ab titers (Fig. 4A) and LPR (Fig. 4C) p.i. Thus, fetal immunization induced both systemic and mucosal immune responses and immune memory that responded to viral infection. Furthermore, both lambs that lacked a detectable response to in

![FIGURE 3](http://www.jimmunol.org/)  
**FIGURE 3.** Virus shedding in nasal secretions following BHV-1 challenge of newborn lambs. Fetal lambs were orally immunized in utero with 500 \(\mu\)g of either pSLIAtgD plasmid (DNA; \(n = 5\)) or PBS (PBS; \(n = 4\)). Between 7 and 10 days of age, these lambs were challenged with BHV-1 strain 108, and virus shedding in nasal secretions was assayed in vitro with a plaque assay. Data presented are values for individual lambs for the first 4 days p.i., and values for two lambs \((232\) and \(233\)) in the DNA group are indicated by placing the animal number in adjacent parenthesis. The horizontal bar indicates arithmetic mean of values for each group.

![FIGURE 4](http://www.jimmunol.org/)  
**FIGURE 4.** In utero DNA immunization induced both systemic and mucosal immune responses. Fetal lambs were orally immunized in utero with 500 \(\mu\)g of either pSLIA-tgD plasmid (DNA; \(n = 5\)) or PBS (PBS; \(n = 4\)). Between 7 and 10 days of age, these lambs were aerosol challenged with 5–7 \(\times\) \(10^7\) infectious particles of BHV-1 strain 108. gD-specific serum Ab titers \((A)\), SIgA titers in nasal secretions \((B)\), and LPR in blood lymphocytes \((C)\) were assayed at the day of challenge (prechallenge) and 12 days after challenge with BHV-1 (postchallenge). \(*\), \(p < 0.01\).
were injected i.m. with 10 μg of plasmid-encoding HBsAg (pMCG-16-HBsAg), and four fetuses were injected orally with PBS, to serve as negative controls for HB ELISA specificity, and none of these lambs developed detectable HBsAg Ab titers throughout the experimental period (data not shown). All newborn lambs were seronegative for HBsAg, but at 3 wk of age three of the four lambs (75%) orally immunized with pMCG-16-HBsAg plasmid had protective Ab titers, but only one of four lambs (25%) injected in utero with Engerix-B vaccine developed a protective Ab titer (Fig. 5). The marked difference in protective Ab titers between DNA- and protein-immunized groups persisted for at least the next 8 wk. Thus, a single oral DNA vaccination of fetuses induced more rapid seroconversion and a higher frequency of disease protection in neonates than a single in utero vaccination with recombinant protein vaccine.

**Discussion**

Neonates of all species are highly susceptible to infectious diseases, and this increased disease susceptibility is reflected in high morbidity and mortality rates during the first year of life (reviewed in Refs. 1 and 2). Maternal IgG and other factors in milk, such as complement components and soluble CD14, provide some disease protection in the neonate (32, 33), but this is not sufficient to protect them from many diseases. Thus, vaccination is an attractive approach to enhance disease protection in the neonate. There is increasing evidence that neonates of many species can respond to vaccination with the induction of appropriate adaptive immune responses (8, 10, 34–39). The present investigation extends this evaluation of neonatal immune competence by demonstrating that adaptive immune responses induced in utero are functional in the neonate. The data clearly demonstrate that a single in utero oral injection of a DNA vaccine induced both systemic and mucosal immune responses and immune memory that responded to second-ary immunization and viral infection. Furthermore, there was no evidence to validate immune tolerance in nonresponders (Fig. 2). Therefore, the present investigation supports the conclusion that a competent immune system is present before birth and adaptive immune responses induced in utero are functional in the neonate.

Ovine and human fetuses display similar patterns of lymphoid development in thymus, spleen, lymph nodes, and Peyer’s patches, and share similarities in the appearance of circulating leukocytes in blood (15–17). Because of these developmental similarities, the fetal lamb has frequently been used as a model system to study prenatal development of the mammalian immune system (14, 15). Thus, we believe that the present data provide significant evidence for a new therapeutic approach to significantly reduce the risk of vertical disease transmission from an infected mother to the newborn infant. The absence of a detectable immune response in 12–25% of immunized fetuses, however, clearly indicates the necessity to optimize vaccine delivery.

The present results not only confirm that fetal lambs, in the last trimester of gestation, are immunocompetent (11) but also revealed that the fetus responded exceptionally well to oral DNA vaccination. Previous investigations indicated that oral vaccination was less efficacious than other routes of DNA immunization (40–43). The strong response of fetal lambs to oral DNA vaccination, however, suggests that some unique aspect of fetal physiology might enhance DNA transfection or gene expression. Our previous investigations indicated that the oral cavity was the primary site of
transfection and gene expression following oral DNA immunization (11). One possible explanation for the enhanced efficacy of oral immunization in utero might be the much lower turnover rate for mucosal epithelial cells in the fetus (44). A reduced rate of epithelial cell attrition could prolong the duration of plasmid expression and thereby increase antigenic exposure in the fetus. Thus, induction of immune memory in utero might be greatly enhanced by using a DNA vaccine that facilitates Ag expression over an extended interval (reviewed in Ref. 45).

The developmental state of the fetal immune system might also contribute to a more efficient induction or immune memory. A large pool of naive recirculating T cells, with a diverse TCR repertoire, is present in the fetal lamb (46). These fetal T cells have a relatively long life span when compared with T cells in the neonate. Thus, differences in fetal lymphocyte physiology might also contribute to increased memory T cell survival following Ag stimulation. The very high gD-specific LPR, observed following secondary immunization of newborn lambs (Fig. 1B), confirmed that memory T cells induced in utero can survive the functional transition that occurs in the immune system following birth (46). Furthermore, immune memory induced in utero persisted for at least 3 mo after birth, even in the absence of a secondary antigenic stimulation (Fig. 2A). It is now evident that immune memory relies on the persistence of functionally distinct B and T lymphocytes (reviewed in Refs. 47 and 48), and this is the first report to confirm that memory T cells, induced during fetal development, survive and function in the neonate. Thus, the present animal model provides a unique opportunity to further define the functional differences between naive and memory T cells.

Another unique characteristic of mammalian development is fetal production of cortisol, which then induces parturition. In the ovine fetus, beginning at day 125 of gestation, there is a gradual elevation in serum cortisol that reaches peak levels at birth (49). Glucocorticoids have dramatic effects on peripheral T cell function and trafficking, alter thymocyte commitment to the CD4 lineage, and decrease the survival of activated T cells and immature lymphocytes (reviewed in Ref. 50). Thus, the induction of an adaptive immune response in the presence of a rising cortisolemia would appear to represent a major challenge for fetal immunization. The presence of high gD-specific serum Ab titers and gD-specific LPR at birth and the induction of strong anamnestic immune responses in newborn lambs (Figs. 1 and 4), however, clearly indicate that cortisolemia did not prevent the induction of either a primary or secondary immune response.

The induction of immune tolerance is thought to be a potential risk when immunizing fetuses and newborns with a DNA vaccine (51, 52). There are numerous reports, however, of successful DNA immunization in neonates of several species, including mice (53–59), rats (60), sheep (9), pigs (61, 62), chimpanzees (63, 64), and baboons (65, 66). To date, only immunization with a plasmid DNA, encoding the circumsporozoite protein of Plasmodium yoelii, induced nonresponsiveness in newborn mice (51, 52). This neonatal tolerance, however, was prevented by coadministration of plasmid-encoding γ-IFN (53). Thus, for DNA vaccines administered to neonates, the role is an active immune response rather than tolerance (8). We have now advanced this observation by inducing fetal immune responses that can potentially reduce the high risk of vertical disease transmission at birth (11). A total of 34 fetal lambs, between days 121 and 124 of gestation, were immunized with DNA vaccines, and ~90% of all fetuses developed detectable immune responses. These responses corroborate that, in sheep, immunological competence has developed during the last trimester of gestation, and there was no evidence of immune tolerance following a secondary protein immunization (Fig. 2). Fetal responses to vaccination will probably be influenced by several factors, including fetal age and development, the nature of the Ag itself, and vaccine formulation, dose, and route of delivery route. Thus, a full investigation of each vaccine might be wise before considering mass in utero vaccination strategies.

Mucosal surfaces are the primary site of entry for infectious agents during birth and the neonatal period. Thus, the induction of both systemic and mucosal immune responses is considered necessary for optimal disease protection (67, 68). The presence of gD-specific SIgA in nasal secretions of newborn lambs confirmed that oral immunization in utero induced mucosal immune responses (Fig. 4B). It was difficult, however, to correlate mucosal immunity with viral clearance following BHV-1 challenge, because both humoral and cell-mediated systemic immune responses were present (Fig. 4). The present observations did provide clear evidence, however, that in utero DNA immunization can enhance both viral clearance and the secondary immune response following a respiratory tract infection. Thus, oral DNA immunization in utero should reduce the risk of vertical disease transmission through mucosal surfaces.

The efficacy of oral DNA vaccination in utero was further substantiated by the study with HBsAg DNA and protein vaccines. A single, oral DNA vaccination induced higher protective Ab titers in more lambs and with less delay than did a single i.m. injection of the recombinant protein vaccine (Fig. 5). The relatively poor response to a single in utero injection of protein vaccine is consistent with a previous investigation in chimpanzees that reported low Ab titers (~20 mIU/ml) at 16 wk of age following three in utero immunization with 1 mg of rHBsAg protein (63). In the present investigation, neither protein nor DNA vaccination induced protective levels of HBsAg-specific serum Ab until lambs were 3 wk of age (Fig. 5). Thus, a 7-wk interval was required for protective Ab responses to develop following a single in utero oral DNA vaccination (Fig. 5). Thus, it might be necessary to alter the timing of in utero DNA immunization to achieve protective immunity at the time of birth.

The practice of in utero immunization in infected, pregnant women would require a thorough assessment of benefits relative to the risks associated with this medical treatment. A major concern regarding the use of DNA vaccines is the possibility that bacterial DNA, through CpG motifs, can have numerous effects on both innate and adaptive immune responses (reviewed in Ref. 25). This raises the possibility that DNA vectors may directly alter the development or function of the fetal immune system. Plasmid incubated in amniotic fluid was degraded 50% after 2–4 h and totally degraded within 8 h (data not shown). Therefore, there is a limited time interval for both DNA transfection and a direct interaction between bacterial DNA and the innate immune system. Experiments are in progress to determine whether fetal exposure to plasmid vectors, not expressing Ag, alters gene expression in lymphoid tissues.

Achieving efficient DNA transfection in vivo is one of the major limitations of DNA vaccination and gene therapy. It is conceivable, however, that oral immunization in utero might be performed by using ultrasound-guided vaccine delivery. A similar technique was recently used to inject a DNA analog into specific sites and organs of fetal lambs (69). Thus, it will be important to determine exactly where DNA transfection occurs within the oral cavity and to optimize delivery parameters, such as DNA concentration and volume. If efficient and consistent oral DNA delivery can be achieved in utero, then this route of delivery might provide new strategies for both vaccination and gene therapy.
Acknowledgments

We thank Dr. Don Wilson, Carolyn Olson, Jamie Mamer, and Tracey Bru- neau for assistance with surgery and animal care. We also thank Donna Dent and Terry Beskoroyne for their excellent technical assistance. Special thanks to Dave Dixon (Department of Animal Science and Poultry, University of Saskatchewan) for providing pregnant ewes.

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