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

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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, Haemophilus 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 anti-viral 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 induced detectable immune responses in 75% (live of eight) of newborn baboons (4). Studies with mice (5–7; reviewed in Ref. 8) and lambs (9, 10) also confirmed that the newborn is capable of responding to vaccination. Recently, we reported that in utero DNA vaccination of fetal lambs induced immune responses in all vaccinated fetuses, suggesting a new approach to the prevention of vertical disease transmission from mother to newborn infant (11). The induction of protective immune responses in utero could have a significant impact on survival and quality of life for the large number of infants infected during or shortly after birth.

Vertical disease transmission in newborns occurs primarily through mucosal surfaces of the eyes, upper and lower respiratory tract, and the gastrointestinal tract. Thus, in utero induction of mucosal immune responses would significantly enhance disease protection in the neonate. Furthermore, the induction of immunological memory is an important feature of the adaptive immune system that has been effectively exploited to enhance vaccine efficacy. Therefore, in the present study, we investigated the capacity of in utero DNA vaccination to induce immune memory and protective immunity in the neonate. Neonatal immune memory was addressed by analyzing immune responses to either a secondary DNA vaccination or a viral infection within the first 10 days after birth. In utero induction of protective immunity in neonates was investigated with two different DNA vaccines. Immune protection against a respiratory viral infection was assessed in an experimental challenge with infectious bovine herpesvirus (BHV)-1. Immune protection against HBV, an important pathogen of newborn infants (12, 13), was also evaluated following in utero immunization with either a HB surface Ag (HBsAg) protein or DNA vaccine. This comparison provided an opportunity to evaluate the efficacy of fetal immunization with an oral DNA vaccine relative to conventional delivery of a protein Ag.
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 syndromechorial placentation 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 R. Braun and S. van Drunen Littel-van den Hurk (Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, Canada). Plasmid pMC1-HBsAg was a generous gift from H. L. Davis and R. Weeratna (Loeb Research Institute, Ottawa, Ontario, Canada) in pyrogen-free PBS was given to all fetuses. The recombinant DNA plasmid solution or sterile, pyrogen-free PBS (Sigma-Aldrich) 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 with three absorptive swabs (Merocel; Solan Kenomed, Jacksonville, FL) after spraying 150 μl of PBS into each nostril. The swabs were placed proximal to the external nares to absorb fluid without disrupting the nasal mucosa. Nasal swabs were placed in 1.5-ml Eppendorf tubes (Brinkmann Instruments, Mississauga, Ontario, Canada) and kept on ice. Tubes were pierced at the bottom, placed inside a second tube containing 10 μl of 0.1 M PMSF (Sigma-Aldrich), and centrifuged for 30 s at 15,850 × g.

Challenge infection

Each lamb was challenged with 5–7 × 10^7 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 DeVILBISS nebulizer (model 099HC; DeVILBISS, Somerset, PA).

Flow cytometry and clinical pathology

Flow cytometry was performed using the Virology Laboratory, Royal University Hospital (Saskatoon, Saskatchewan, Canada).

Flow cytometry and clinical pathology

mAb specific for sheep IgM (clone Plg45A), IgG1 (clone Big71SA), CD25 (CACT116), and MHC II (clone TH14B) were purchased from VMRA (Pullman, WA). The CD5 (clone ST1a), CD4 (clone 17D-13), CD8 (clone E95), and y6 TCR (clone 86D)-specific mAbs were purchased from Southern Biotechnology Associate (Birmingham, AL). Flow cytometric analyses were restricted to viable cells by excluding cells stained with propidium iodide (2.5 μg/ml;
FL3). Specific mAb staining was determined by subtracting cells reacting with isotype-matched and concentration-matched (1–10 μg/ml) irrelevant mAbs (Caltag Laboratories, Burlingame, CA). All samples were analyzed with a FACSscan (BD Biosciences, Mountain View, CA) flow cytometer, and the CellQuest program (BD Biosciences) was used for data acquisition and analysis. The analysis of total white blood cell counts and differential counts of lymphocyte, monocyte, and polymorphonuclear cell populations was performed by Prairie Diagnostic Services (Western College of Veterinary Medicine, Saskatoon, Saskatchewan, Canada).

gD-specific LPRs

Blood was collected in EDTA-treated vacutainer tubes (BD Biosciences), and mononuclear cells were isolated, as described previously (11). Briefly, 3 × 10^5 cells/well (96-well microtiter plates; Naïge Nunc International, Naperville, IL) were cultured in a final volume of 200 μl of AIM-V medium (Life Technologies) supplemented with 2% FBS (Life Technologies) and 50 μM 2-ME (Sigma-Aldrich). Cells were stimulated with either purified gD protein (2.5 or 5 μg/ml), 1 μg/ml Con A (Sigma-Aldrich), or medium alone. After a 72-h incubation, the cells were incubated with 0.4 μCi/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 lymphocyte and monocyte populations of vaccinated and naive lambs were analyzed for each time point using a two-tailed t test, which returned the probability associated with a Student’s t 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 oligodeoxynucleotides 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 I 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 I). 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+ CD5+ ) 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.

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 μg 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 ear, with 50 μg 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

| Table 1. Immune responses of newborn lambs to in utero oral DNA vaccination |
|--------------------------|-----------------|------------------|
| Expt. | In Utero Immunization | Vaccine Response | Serum Ab Titer |
| I | pSLIA-tgD | 7/8 | 5589 ± 3650 |
| PBS | 0/7 | 112 ± 57 |
| II | pSLIA-tgD | 5/7 | 1083 ± 352.4 |
| PBS | 0/4 | 186 ± 143 |
| III | pMCG-16-HBsAg | 3/4 | 95.6 ± 68.9 |
| Engerix-B | 1/4 | 66.1 ± 64.3 |
| PBS | 0/6 | 0 |

*Each experiment was performed independently.

†Fetal 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.

‡Vaccine 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 lymphocyte proliferative 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 an 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.
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).
A). 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 μg of plasmid pSLIA-tgD, 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. Virus shedding in nasal secretions following BHV-1 challenge of newborn lambs. Fetal lambs were orally immunized in utero with 500 μg of either pSLIA-tgD plasmid (DNA; n = 5) or PBS (PBS; n = 4). Between 7 and 10 days of age, newborn lambs were aerosol challenged with 5–7 x 10^7 infectious particles of 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.](http://www.jimmunol.org/cgi/content/full/188/11/5883/DC1/Fig3)

![FIGURE 4. In utero DNA immunization induced both systemic and mucosal immune responses. Fetal lambs were orally immunized in utero with 500 μg of either pSLIA-tgD plasmid (DNA; n = 5) or PBS (PBS; n = 4). Between 7 and 10 days of age, newborn lambs were aerosol challenged with 5–7 x 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.](http://www.jimmunol.org/cgi/content/full/188/11/5883/DC1/Fig4)
utero DNA immunization (Expt. II, Table I) did develop gB- and gD-specific serum Abs following viral infection (data not shown). The gD-specific Ab response in these lambs provided further evidence that in utero DNA immunization did not induce immune tolerance.

To further clarify the role of systemic and mucosal immune responses in preventing and clearing viral infection, we compared the immune responses of lambs (Fig. 3, 232 and 233) that shed the least virus after infection. Before challenge, lamb 233 had high gD-specific IgG serum Ab (titer = 6089), gD-specific LPR (SI = 60), and SLgA in nasal secretions (titer = 2750), but on day 12 p.i. SLgA (titer = 230) and LPR (SI = 42.3) had decreased. The gD-specific serum Ab titer did increase (titer = 24460), but the absence of gB-specific Abs p.i. indicated sterile immunity. In contrast, before infection, lamb 232 had low gD-specific serum Ab (titer = 383), SLgA (titer < 10), and LPR (SI = 4.6) but displayed a marked increase in gD-specific LPR (SI = 63.1) p.i. Furthermore, on day 12 p.i., gD-specific serum Ab titer increased slightly (titer = 1312), and a low level of gB-specific serum Ab was detected (data not shown). These observations suggest that for lamb 232 viral clearance was mediated primarily by cell-mediated immune responses. Collectively, this analysis of immune responses indicates that in utero DNA immunization induced both systemic and mucosal immune responses, and both types of immunity may contribute to disease protection.

**Oral immunization in utero with the HBsAg**

To test the broader validity of oral DNA immunization in utero, it was important to assess the efficacy of other plasmid vectors and other vaccine Ags. HBsAg was selected because HBV is an important human pathogen that is vertically transmitted from mother to newborn infant. Furthermore, the efficacy of oral DNA vaccination in utero could be compared with that of the recombinant protein, as there exists a known correlation between serum Ab titer (>10 mIU/ml) and disease protection (31).

Four fetuses were orally immunized with 500 µg of pMCG-16 plasmid-encoding HBsAg (pMCG-16-HBsAg), and four fetuses were injected i.m. with 10 µg of recombinant, purified HBsAg formulated in alum (Engerix-B), the recommended dose for newborn infants. Seven 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 Ig 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 secondary 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

**FIGURE 5.** Oral DNA immunization in utero induces protective HB serum Ab titers. Fetal lambs were orally immunized in utero with 500 µg of pMCG-16-HBsAg plasmid (DNA; n = 4) or injected i.m. with 10 µg of rHBsAg (Engerix; n = 4). Serum samples were collected biweekly for the first 3 mo after birth. HBsAg-specific serum Ab titers (mIU/ml) were determined with a commercial microparticle enzyme immunoassay, and protective Ab titers (>10 mIU/ml) are indicated by the horizontal line.
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 GM-CSF (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. 4). 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 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.
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