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Induction of Common Mucosal Immunity by Hormonally Immunomodulated Peripheral Immunization

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The study described in this report demonstrates that peripheral lymph nodes draining nonmucosal tissues can effectively serve as induction sites for the establishment of common mucosal immunity if the microenvironmental conditions are altered to mimic those normally present within mucosa-associated lymphoid tissues (e.g., Peyer's patches). Lymph node lymphocytes exposed in situ to the immunomodulatory influences of the hormone 1α,25-dihydroxy vitamin D_3 were found to produce less gamma interferon and interleukin-2 (IL-2) and far more IL-4, IL-5, and IL-10 than lymphocytes from control animals. When coupled with vaccination with hepatitis B surface antigen (HBsAg), the hormone-immunomodulated switch from a peripheral lymph node phenotype to a Peyer's patch-like pattern promoted the induction of both a systemic and a common mucosal immune response. This was determined by the observed increased concentrations of serum anti-HBsAg antibody and by finding that anti-HBsAg secretory antibodies were detectable in urogenital, lachrymal, fecal, and oral secretions only in the hormone-treated animals. In addition, specific antibody-secreting cells were detectable in the lamina propria of the lungs and small intestines of the hormone-treated animals subsequent to vaccination, indicating that the homing properties of antigen-specific B cells were being affected by the treatment procedure. The humoral and mucosal immune responses were further augmented if both 1α,25-dihydroxy vitamin D_3 and dehydroepiandrosterone were used together as hormonal immunomodulators. This novel immunization technique may afford new opportunities to effectively intervene in sexually transmitted diseases and other diseases caused by mucosal pathogens.

It has been long appreciated that T cells residing within the lymphoid organs which collectively constitute the mucosal immunity induction sites play unique roles in promoting the development of protective secretory immune responses (8, 35, 36). This concept was established long before it was formally recognized that products from activated T cells guide the development and differentiation of both themselves and other immunologically important cell types. The cellular controlling influences are effected through the pleiotropic activities of numerous species of cytokines, generally through autocrine- or paracrine-acting mechanisms. It is now generally accepted that the types of cytokines produced in response to cellular activation and their temporal sequence of cellular production within the microenvironment of the various immunity induction sites dictate the quantity and quality of the immune responses elicited to antigen exposure (19, 38, 45).

T cells residing within mucosal immunity induction and effector sites appear to be programmed for the predominant production of those species of lymphokines and cytokines important for promoting the development and effectuation of a secretory immune response. These include interleukin-4 (IL-4), IL-5, IL-6, and IL-10 and also include the activities of the bioactive form of transforming growth factor β (TGF-β) (5, 30, 52). IL-5 and IL-6 are important for the promotion of immunoglobulin secretion (7, 21, 39), while IL-10 and bioactive TGF-β are needed for appropriate isotype switching of B cells for immunoglobulin A (IgA) production (17). TGF-β may also affect T-cell homing properties through its capacity to promote changes in integrin expression (26, 47).

We recently determined that the patterns of T-cell lymphokines produced by activated lymphoid cells isolated from discrete lymphoid organs were somewhat lymphoid organ specific (5, 14). Type 1 cytokines, IL-2, and gamma interferon (IFN-γ) were found to represent the dominant secreted products produced by T cells residing in peripheral lymphoid organs receiving their major afferent lymphatic drainage from the skin. Although a bit less restricted, T cells residing within the spleen also produced a type 1-like pattern of cytokines following activation in vitro. Conversely, IL-4, IL-5, and IL-10 represented the major species of lymphokines produced by activated T cells residing within lymphoid organs draining the various mucosal tissues. These findings have been confirmed by others, who described the apparent dominance of a Th2-type response by T cells isolated from known induction and effector sites of mucosal immunity (52).

1,25-Dihydroxy vitamin D_3 [$1,25(OH)_2D_3$] represents a steroid hormone that is very important for the normal functioning of many physiologic processes. It has been implicated as being a natural immunomodulator, capable of altering a number of T-cell, B-cell, and macrophage responses (31, 37). When administered in vivo, $1,25(OH)_2D_3$ is capable of depressing the development of autoimmune diseases in genetically susceptible animal model systems (32, 33). T cells exposed to $1,25(OH)_2D_3$ in vitro demonstrate significantly altered responses following cellular activation with T-cell-specific mitogens. T-cell production of both IL-2 and IFN-γ have long be known to be markedly depressed in cells exposed to this steroid, a fact which has served to support the presently proposed role for $1,25(OH)_2D_3$ as a general immunosuppressant (10, 43).

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We previously reported that treatment of cloned murine T-cell lines with very low (<10^{-10} M) doses of 1,25$(OH)_2D_3$ enhanced their production of IL-4 after activation (3). 25-Hydroxy vitamin D_3 [25-$(OH)D_3$], the precursor to the active form of 1,25$(OH)_2D_3$, had no effect on T-cell cytokine production when tested over a similar range of doses. Further studies have now established that the biologic effects of 1,25$(OH)_2D_3$ on T cells is more complex than previously appreciated. Not only does low-dose treatment with 1,25$(OH)_2D_3$ augment the T-cell production of IL-4, but it also enhances activated T-cell production of IL-5 and IL-10 (12) and has been implicated as a positive influence in bioactive TGF-β production (42).

The present investigation demonstrates that the topical exposure of normal animals to 1,25$(OH)_2D_3$ can alter the synthesis of cytokines produced by activated T cells isolated from their draining lymphoid organs in a manner consistent with the cytokine patterns normally produced by T cells isolated from mucosal lymphoid organs (5). The results indicate that a localized hormonal manipulation of the lymphoid organ microenvironment with 1,25$(OH)_2D_3$ significantly enhances the intensity of the systemic humoral immune response following vaccination of normal adult animals. It was consistently observed that the coupling of conventional vaccination with a protein antigen with the immunomodulatory effects of 1,25 $(OH)_2D_3$ successfully promoted the induction of a common mucosal immune response to subcutaneously administered antigens. This was verified experimentally by the demonstration of significant titers of secretory immunoglobulins in vaginal, lung, fecal, and other external mucosal secretions following 1,25$(OH)_2D_3$-influenced peripheral vaccination plus the demonstrated presence of antibody-producing cells in the lamina propria of the lungs and intestines of only the animals who received vaccinations in combination with hormone treatment. Vaccines formulated to contain 1,25$(OH)_2D_3$ and dehydroepiandrosterone (DHEA) were significantly more effective than those with 1,25$(OH)_2D_3$ alone. Our results indicate that the hormone-immunomodulated systemic vaccination protocol could represent an attractive alternative to oral, respiratory, and intranasal routes of immunization. Antigen administration to mucosal tissues is considered essential for inducing common mucosal immunity, and the consistent stimulation of such responses to nonreplicating protein or polysaccharide antigens appears to require the use of toxic adjuvants such as cholera toxin (CT) (24). A protocol in which small amounts of vaccine protein are sufficient to elicit a demonstrable mucosal response would be especially useful when costly vaccine preparations, such as recombinant proteins or purified subunit vaccines, are used.

MATERIALS AND METHODS

Mice. Male and female C3H/HeN MTV^-, CBA, and CF-1 strains of mice were bred and housed in the University of Utah Laboratory Animal Facility from breeding stock originally purchased from the National Cancer Institute. Within each experiment, groups of four to eight age-, strain-, and sex-matched mice were used. All experiments presented in this report were repeated at least twice with similar results. The ages of the mice ranged between 12 and 25 weeks at the onset of the experimental vaccination protocols. The Institutional Animal Care and Use Committee and the Animal Resource Center at the University of Utah guarantee strict compliance with regulations established by the Animal Welfare Act.

Steroid compounds. 1,25$(OH)_2D_3$ was generously provided by Milan Uskoković, Hoffman-La Roche Inc. (Nutley, N.J.). The hydrophobic preparation was dissolved in 95% ethanol as a stock solution of 10^{-3} M and then stored at −20°C until used. DHEA was purchased from Sigma Chemical Co. (St. Louis, Mo.), dissolved in 95% ethanol as a stock solution, and stored at −20°C until used.

Culture conditions. Briefly, single-cell suspensions of lymphoid cells were prepared from appropriate lymphoid organs, washed twice in sterile balanced salt solution, and cultured, under serum-free conditions, at a density of 10^7 cells per ml per well with the T-cell-specific mitogen anti-CD3ε (1.5 μg/ml). Cells were placed in a 24-well Cluster culture plate (Costar, Cambridge, Mass.) at 37°C for a period of 24 h to elicit lymphokine secretion. The hybridoma clone producing the hamster anti-murine CD3ε monoclonal antibody 1452C-11.2 was obtained from J. Bluestone (University of Chicago). Cell-free culture supernatants were collected and stored at 4°C until assayed for lymphokine content. The culture period, cell concentrations, and culture medium, consisting of RPMI 1640 supplemented with 1% Nutridoma-SR (Boehringer Mannheim, Indianapolis, Ind.), antibiotics, 2 mM L-glutamine, and 5 × 10^{-5} M 2-mercaptoethanol, were all carefully evaluated to determine the optimal conditions for stimulating production of the lymphokines under evaluation. The use of serum-free culture conditions are essential to avoid the interference in lymphokine production caused by serum growth factors (15).

Capture ELISAs for lymphokine quantitation. Where indicated, the amounts of a specific lymphokine in test supernatants was quantitated by a capture enzyme-linked immunosorbent assay (ELISA), by use of our adaptation (5, 13, 15) of the method initially described by Schumacher et al. (48). Briefly, 100 μl of an appropriate capture monoclonal antibody (PharMingen, San Diego, Calif.) is added to the wells of a 96-well microtest plate (Corning no. 2581) at a concentration of 1 to 2 μg/ml in 0.05M Tris-HCl (pH 9.6). Following extensive washing and the blocking of reactive sites on the plastic with phosphate-buffered saline (PBS)–2% bovine serum albumin (BSA), test supernatants and twofold serial dilutions of a reference lymphokine (100 μl per well) were dispensed. After sufficient incubation and washing, 100 μl of the biotinylated detecting antibody, 1 to 2 μg/ml, was dispensed into each well. The ELISA was developed with avidin conjugated to horseradish peroxidase and 2,2′-azino-bis(3-ethylbenzthioline-6-sulfonic acid) (ABTS) (Sigma) substrate. Optical density readings were performed at 405 nm with a Vmax 96-well microtest plate spectrophotometer (Molecular Devices, Menlo Park, Calif.). The results of the analysis of each cytokine are reported in picograms per milliliter (± standard deviation). The sensitivity of detection of the lymphokines reported was 15 to 30 pg/ml.

Immunization regimens. One microgram of hepatitis B surface antigen (HB sAg) was delivered to each mouse in a 25-μl volume of an aluminum hydroxide solution (273 μg/ml) by immunization in the hind footpad. The antigen used for immunization (Recombivax-HB; Merck) was purchased from the University of Utah Medical Center pharmacy. In some experiments 1 to 2 μg of 1,25$(OH)_2D_3$, in a volume of 3.5 μl of 95% ethanol, was applied to the dorsal skin of the hind footpad (the site of immunization) 5 days after immunization. In experiments where 1,25$(OH)_2D_3$ was delivered directly into the vaccine, 0.05 to 0.1 μg of the hormone plus or minus 2 μg of DHEA was incorporated directly into the vaccine formulation. In all experiments where steroid hormones were incorporated directly into the vaccine formulations, an equivalent volume of 95% ethanol was added to the HBsAg-only vaccine preparations (0.35 μl of ethanol and 25 μl of vaccine).

Quantitation of specific antibody responses and isotypes. Quantitation of specific antibody in serum samples and mucosal secretions collected from HBsAg-vaccinated mice was performed by an indirect ELISA method with reagents purchased from Southern Biotechnology Associates (Birmingham, Ala.) and Zymed Laboratories, Inc. (San Francisco, Calif.). Briefly, HBsAg (received as a gift from William J. Miller, Merck Sharpe & Dohme, West Point, Pa.), diluted in 0.05 M Tris-HCl (pH 9.6) to a concentration of 0.5 μg/ml, was dispensed into 96-well plates. Following a minimum 2-h incubation at 37°C or overnight at 4°C, the plates were blocked with PBS–0.05% Tween 20–10% normal goat serum for another 2-h incubation at 37°C. Prior to adding the test serum samples, the plates were washed free of blocking buffer with three washes of distilled water and one wash with PBS–0.05% Tween 20. Individual serum or mucosal lavage samples were first diluted in PBS–0.05% Tween 20–10% goat serum and then diluted serially in the wells of the HBsAg-coated plates. The plates were incubated at 4°C overnight and then washed three times in distilled water and once in PBS–0.05% Tween 20. The detection antibody (horseradish peroxidase-linked goat anti-mouse immunoglobulin specific for IgG, IgG isotypes, or IgA) was diluted in PBS–Tween 20–10% normal goat serum at a dilution recommended by the manufacturer. After a final incubation and wash series, the ELISA was developed with ABTS-substrate. Optical density readings were recorded at 405 nm. A simple linear regression analysis of the immunoglobulin titration-generated reference curve was used to extrapolate the amount of specific antibody contained in the test samples. These data are reported in nanograms or picograms per milliliter (± standard error of the mean).

Microdot ELISA. Briefly, a modification of the protocol described by Obata and Cheng (41) was used to detect specific antibodies in various mucosal secretions of immunized animals. A suitable template containing 96 evenly spaced pinprick holes was produced on a sheet of nitrocellulose (0.45-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.). One microliter of antigen (20 to 50 μg/ml in PBS) was applied to the appropriate pinholes. The antigen-derivatized membranes were air dried, and the total sheet of nitrocellulose was blocked by incubation for 3 to 4 h in a solution of 10% nonfat dry milk. The membrane was then rinsed in double-distilled water several times and blotted between two pieces of filter paper to remove excess water.

Control and test samples were deposited onto individual pinpricks of the antigen-derivatized membrane with miniature cotton swabs. The swabs were made by twirling a small amount of cotton onto the end of a toothpick. To obtain test samples from a given mucosal surface, a swab was wetted in PBS–1% BSA,

blotted, and then gently wiped across the mucosal epithelium (oral, rectal, vaginal, or lachrymal) to adsorb a small volume of secretion. Positive control samples were derived from diluted serum obtained from hyperimmune mice that had been evaluated previously for their quantity of antigen-specific IgG and IgA.

The entire membrane was then incubated in a humidified chamber for 1 h. After rigorous washing with double-distilled water and three times with PBS–0.05% Tween 20, the membrane was incubated in a solution of PBS–1% BSA containing a biotinylated monoclonal rat anti-murine isotypic-specific antibody (PharMingen) at 2 μg/ml. Following a 60-min incubation in the isotype-specific antibody, the membrane was rinsed three times in water and three times in PBS–0.05% Tween 20 and then incubated in alkaline phosphatase-avidin D (1:1,000 dilution of 100 U/ml of stock; Vector Labs, Burlingame, Calif.). The microdot ELISA was developed after rigorous rinsing in water and in PBS–Tween 20 with a (bromochloroindolylphosphate-nitroblue tetrazolium) substrate. The substrate is made by adding 3.3 mg of bromochloroindolylphosphate and 1.7 mg of nitroblue tetrazolium to 10 ml of alkaline phosphate buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris [pH 9.5]). After 5 to 10 min, the dots reach maximum darkness, and the membrane is rinsed with water to stop further development.

ELISPOT analysis of antibody-forming cells. Lamina propria lymphocytes from the various mucosal lymphoid tissues of vaccinated animals were obtained by previously described procedures (11, 49). These isolation procedures deplete intraepithelial lymphocytes prior to collagenase digestion of the tissue fragments. Ninety-six-well nitrocellulose-backed microtiter plates (Millipore Corp., Bedford, Mass.) were coated with HBsAg (5 μg/ml) in 0.1 ml of carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. After extensive washing and blocking with PBS–5% BSA, serial dilutions of the lymphocyte-containing cell suspensions were added to tissue culture medium (RPMI 1640) and incubated at 37°C in 5% CO_2. After thorough rinsing with PBS–0.05% Tween 20, the spots were developed by sequentially adding goat anti-mouse IgA, IgG, or IgM antibodies conjugated to horseradish peroxidase. After an incubation and washing step, the spots were developed in a solution of 3-amino-9-ethylcarbazol (Sigma).

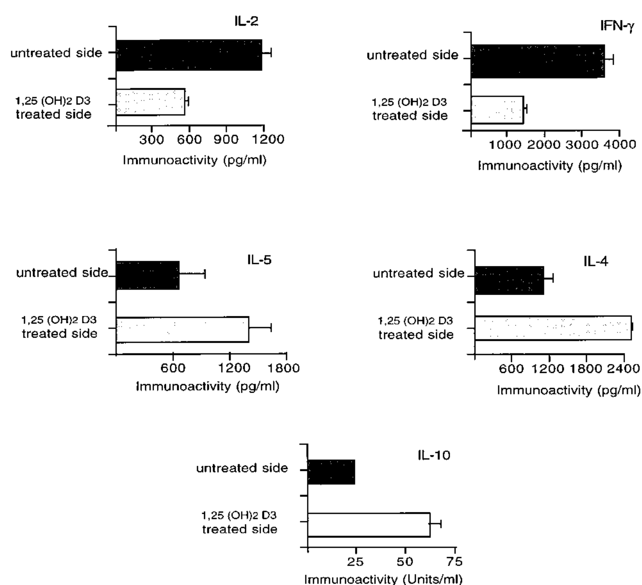

FIG. 1. Modulatory influence of topically applied $1,25(OH)_2D_3$ on cytokine production by T cells residing in the draining lymph nodes of normal mice.

RESULTS

Modification of the peripheral lymph node microenvironment with topically applied steroid hormones. The topical treatment of mice with different species of steroids (e.g., DHEA or dihydrotestosterone) alters the patterns of cytokines produced by T cells residing in the skin-draining lymph nodes. When appropriately low doses of hormone are used, T cells residing in the draining but not the contralateral lymph nodes represent the cells that are affected (4). Furthermore, the changes in T-cell function observed are totally consistent with the alterations previously reported to occur following an in vitro treatment of lymphocytes with the same steroids prior to their activation (3, 16). On the basis of results from in vitro studies with $1,25(OH)_2D_3$ (3), we questioned whether the application of this steroid to the forepaw of normal mice could influence the types of cytokines produced by T cells residing in the draining peripheral lymph nodes. Normal strain C3H mice were topically treated with 1 μg of $1,25(OH)_2D_3$ in ethanol. Three hours later, the animals were sacrificed, and the axillary and brachial lymph nodes were individually collected from the draining and contralateral sites. Lymphocyte suspensions were activated with anti-CD3ε in serum-free medium, and after a 24-h incubation, the supernatants were collected for cytokine analysis. The results of this study, showing a representative set of data, are presented in Fig. 1. Comparison of the cytokine responses (all analyzed quantitatively by capture ELISA) of lymphocytes residing in the lymph nodes draining the treated skin sites with those of the untreated skin sites demonstrated that a topical $1,25(OH)_2D_3$ treatment caused a marked enhancement in IL-4, IL-5, and IL-10 production while simultaneously depressing production of IL-2 and IFN-γ. This cytokine pattern is quite similar to the pattern of T-cell cytokines produced by cells isolated from mucosal draining lymph nodes, tissues established to represent the main inductive sites of mucosal immune responses (27, 52).

Induction of mucosal immunity to hepatitis B following systemic vaccination. Hepatitis B virus infections represent a major cause of chronic hepatitis, cirrhosis, and primary hepatocellular carcinoma (9). In developed countries, heterosexual and homosexual transmission of virus is the most common means to acquire hepatitis B virus infection (2, 23, 46). The presently available recombinant protein vaccine for hepatitis B, although effective in the general population after the required course of three systemic immunizations (51), fails to promote the induction of a local common mucosal immune response (6). We therefore questioned whether it would be possible to stimulate the induction of a common mucosal immune response to HBsAg with a subcutaneous or intramuscular vaccination protocol which included the technique of hormonal manipulation of the downstream peripheral lymph node with $1,25(OH)_2D_3$.

In the experiment presented, three groups of normal female strain CBA mice were vaccinated subcutaneously in one hind footpad with 1 μg of HBsAg in alum. One group received vaccine alone, a second group received vaccine plus a topical application of 1 μg of $1,25(OH)_2D_3$ 5 days later, and a third group received the vaccine with 0.1 μg of $1,25(OH)_2D_3$ incorporated directly into the vaccine preparation. All of the animals in each group were bled for serum samples and had vaginal lavage samples (75 μl of PBS) taken at the designated times. Animals were given a secondary dose of antigen only at day 50, which was followed by three additional serum and vaginal lavage fluid collections. The results (Fig. 2A and B) demonstrate that the $1,25(OH)_2D_3$ addition to the vaccination procedure is beneficial for development of systemic immunity, enhancing the titers of serum antibody obtained (both IgG and IgA) after the primary and the secondary antigen challenges. The recall systemic antibody response to antigen was found to be independent of further $1,25(OH)_2D_3$ treatment. Figure 2C and D present the results obtained from the ELISA analysis of the vaginal lavage fluids obtained from the same groups of HBsAg-vaccinated mice. Both secreted IgA (Fig. 2C) and secreted IgG (Fig. 2D) were analyzed. It is clear from these data that hormonal manipulation of the peripheral lymph node microenvironment with topical or directly incorporated 1,25-$(OH)_2D_3$ allows antigen-specific mucosal immunity to develop following a simple subcutaneous vaccination. This takes place

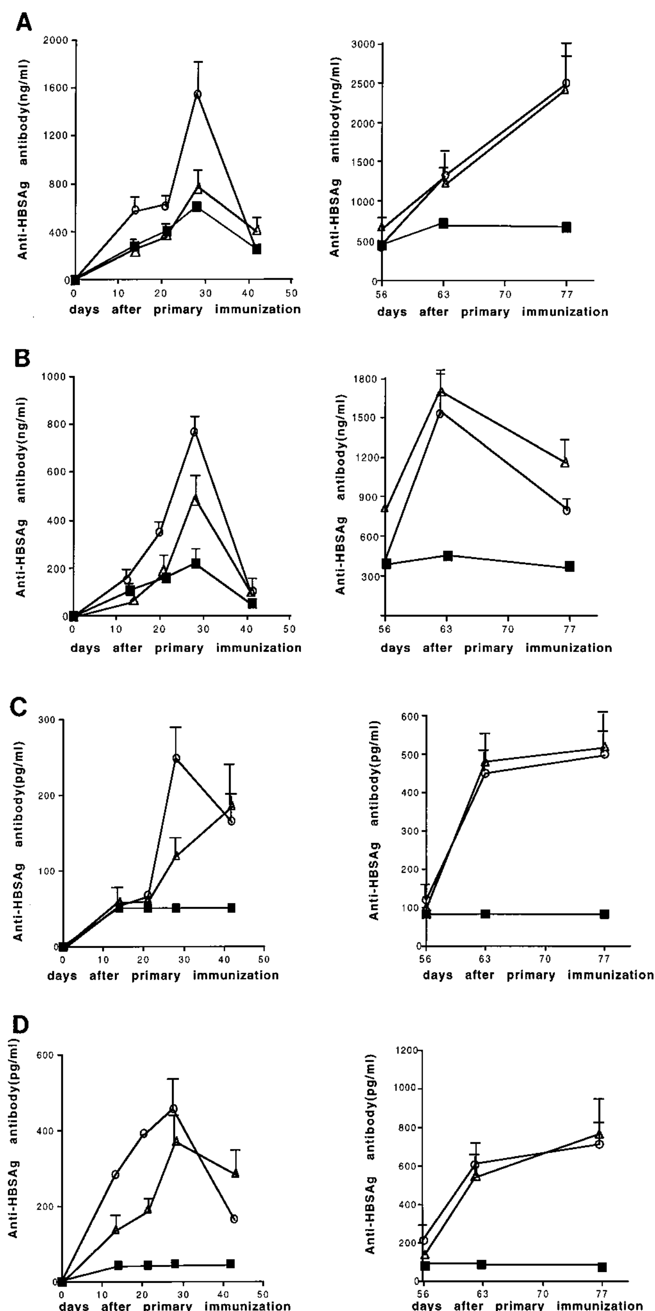

FIG. 2. Serum and mucosal antibody responses by normal mice following immunization with HBsAg in the presence or absence of $1,25(OH)_2D_3$ immunomodulation. Serum IgG (A) and IgA (B) as well as mucosal IgA (C) and IgG (D) from vaginal secretions were quantitatively assessed at various time points following immunization. Groups of animals were vaccinated with HBsAg alone (■) or with HBsAg and $1,25(OH)_2D_3$ administered topically (○) or directly incorporated into the vaccine formulation (△).

in addition to, and without any apparent compromise to, the normal systemic humoral immune response elicited by this standard subcutaneous vaccination procedure.

Experiments were next conducted to determine whether the immunization of normal animals with HBsAg incorporated directly into the vaccine formulation was able to influence antigen-inducible T-cell cytokine production. Normal female strain CBA mice were vaccinated subcutaneously with 1 μg of

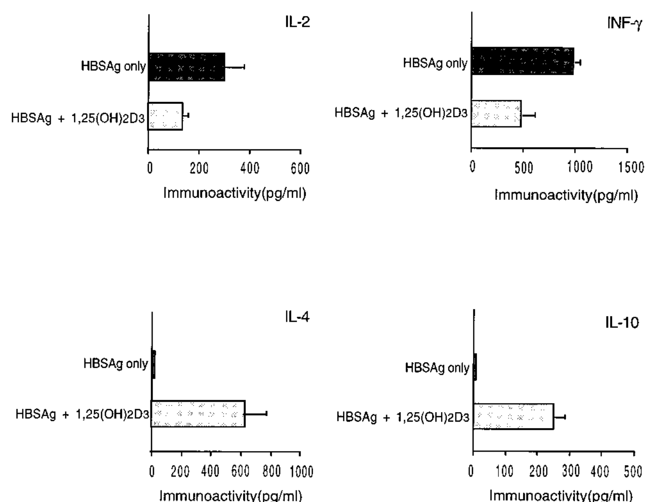

FIG. 3. Modulatory influence on antigen-specific T-cell cytokine production by direct incorporation of $1,25(OH)_2D_3$ into a subcutaneously administered vaccine formulation.

HBsAg or HBsAg containing 0.1 μg of $1,25(OH)_2D_3$. Fourteen days later, the animals were sacrificed and the lymph nodes draining the site of immunization as well as the spleens were excised and individually prepared for tissue culture. Cytokine production by antigen-specific T cells ($CD4^+$) was induced by adding soluble HBsAg (10 μg/ml) to parallel sets of cell cultures. The results (Fig. 3) demonstrate that 24 h after antigen stimulation in vitro, splenocytes and draining lymph node cells (data not shown) from animals vaccinated in the presence of $1,25(OH)_2D_3$ produced a dominant type 2 response while the same population of lymphoid cells from conventionally immunized mice produced primarily type 1 cytokines in response to antigen stimulation.

Finally, to confirm that $1,25(OH)_2D_3$ hormone immunomodulation coupled to vaccination was able to induce a common type of mucosal immunity, a microdot blot ELISA protocol was used to investigate the presence of anti-HBsAg antibodies in a number of mucosal secretions from conventionally immunized animals and animals subcutaneously immu-

TABLE 1. Microdot ELISA of swabs taken from mucosal surfaces of hormone-immunomodulated and conventionally vaccinated animals[a]

Source of mucosal secretion	Presence of anti-HBSAg IgG[b]		Presence of anti-HBSAg IgA	
	Vaccine only	Vaccine with $1,25(OH)_2D_3$	Vaccine only	Vaccine with $1,25(OH)_2D_3$
Lachrymal	−	+	−	+
Oral	−	+	−	+
Vaginal[c]	Trace[d]	+	Trace	+
Colorectal	−	+	−	+

[a] Samples were collected for analysis from immunized C3H mice on day 35 following primary immunization with 1.0 μg of HBsAg in alum.

[b] The presence of antibody response in the various secretions was determined qualitatively by the development of color around the sites of application of secretion samples as explained in Materials and Methods.

[c] The vaginal secretions from conventionally immunized mice possessed a very low level (<10%) of antibody when compared with the vaginal secretions from animals immunized with $1,25(OH)_2D_3$.

[d] One microgram of $1,25(OH)_2D_3$ was applied to the vaccination site 5 days after immunization.

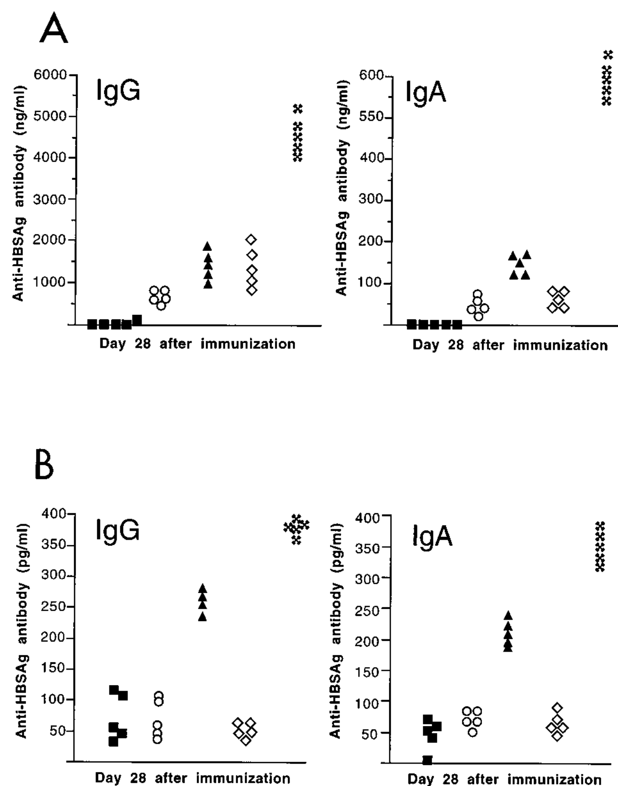

FIG. 4. A comparison of adjuvant properties of HBsAg vaccines formulated with alum alone (○) or with $1,25(OH)_2D_3$ (▲), DHEA (◇), or $1,25(OH)_2D_3$ and DHEA (⌘) added to the vaccine preparations relative to unimmunized controls (■). Serum IgG and IgA (A) and vaginal secretion IgG and IgA (B) antibody titers were analyzed 28 days following vaccination. Similar differences in antibody titers were also observed 21 days after vaccination (data not shown).

nized under conditions where the draining lymph nodes were immunomodulated by $1,25(OH)_2D_3$. The data presented in Table 1 represent the results obtained from cotton swab samples collected on day 35 following HBsAg immunization. This assay system was able to detect both IgA and IgG antibodies specific for the immunizing antigen in lachrymal, oral, vaginal, and colorectal secretions of the animals immunized in conjunction with the $1,25(OH)_2D_3$ immunomodulation. Virtually no antibody could be detected in the mucosal secretion samples taken from the animals given only a conventional subcutaneous immunization.

Vaccine preparations containing $1,25(OH)_2D_3$ plus DHEA elicit potent mucosal immune responses. We have reported previously that the addition of small quantities of the steroid DHEA to HBsAg vaccine formulations greatly enhances the titers of antibodies produced subsequent to immunization of aged mice (5). Experiments were therefore conducted to evaluate whether the addition of DHEA to vaccine preparations also containing $1,25(OH)_2D_3$ might influence the immune responses generated. Four groups of normal strain C3H mature adult mice were immunized with either (i) 1.0 μg of HBsAg in alum alone, (ii) the antigen plus 0.1 μg of $1,25(OH)_2D_3$, (iii) the antigen plus 2.0 μg of DHEA, or (iv) an antigen formulation combined with both $1,25(OH)_2D_3$ and DHEA. Serum and vaginal lavage samples were collected on days 21 and 28, and the amounts of IgG and IgA antibody specific for HBsAg were determined. Figure 4 presents the results obtained from individual animals 28 days after the primary immunization. Analysis of the serum samples demonstrated that both $1,25(OH)_2D_3$ and DHEA alone were able to augment the serum antibody responses (both IgG and IgA) to HBsAg immunization. A further enhancement in serum antibody titers was observed for the group of animals immunized with the vaccine preparation containing a combination of both immunomodulators. A more detailed analysis of serum samples collected 28 days after HBsAg immunization additionally revealed that incorporation of the various combinations of immunomodulators into the vaccine preparation was able to markedly influence both the IgG subclass distribution and the amount of serum IgA antibodies produced (Table 2). HBsAg vaccine formulated with $1,25(OH)_2D_3$ alone enhanced the amount of IgG1 (approximately twofold) and IgA (approximately fourfold) while reducing the amount of IgG2a antibody. The addition of DHEA alone to the vaccine preparation increased both IgG1 and IgG2a antibody levels, while vaccine preparations containing DHEA and $1,25(OH)_2D_3$ were found to show fourfold elevations of IgG1, 20-fold increases in IgA, and about a 50% reduction in IgG2a antibodies. These findings are quite consistent with predicted changes in immunoglobulin production by antigen-stimulated B cells under various cytokine microenvironments.

An evaluation of antibodies to HBsAg in the vaginal lavage fluids established that, unlike $1,25(OH)_2D_3$, DHEA alone was incapable of eliciting any detectable secretory antibody following vaccination (Fig. 4). However, when DHEA and $1,25(OH)_2D_3$ were combined with HBsAg into a single vaccine formulation, the DHEA was able to provide an enhancing influence to the mucosal antibody response elicited by the $1,25(OH)_2D_3$ addition to the vaccine formulation. In most experiments, the combined use of $1,25(OH)_2D_3$ and DHEA has been able to augment the amount of antibody found in the

TABLE 2. Ability of vaccination in the presence of various steroid hormone modulators to influence the IgG subclass and IgA distribution of serum antibodies

Vaccine component(s)	Anti-HBsAg serum antibody (ng/ml)[a]				
	IgG1	IgG2a	IgG2b	IgG3	IgA
Vaccine only[b]	2,280 ± 12.7	390 ± 26	5 ± 0.6	Und[c]	36 ± 4.0
Vaccine plus $1,25(OH)_2D_3$[d]	4,350 ± 19.8	213 ± 23	7.2 ± 0.8	Und	163 ± 3.0
Vaccine plus DHEA[e]	3,785 ± 366	590 ± 67	5.5 ± 0.33	Und	41.5 ± 6.3
Vaccine plus $1,25(OH)_2D_3$ and DHEA	8,250 ± 110	200 ± 28	14.3 ± 2.8	Und	781 ± 50

[a] Values are mean antibody concentrations ± standard deviations for five animals per group.
[b] One microgram of recombinant HBsAg in a 25-μl volume.
[c] Und, undetectable.
[d] One-tenth microgram of $1,25(OH)_2D_3$ was incorporated into the vaccine.
[e] Two micrograms of DHEA was incorporated into the vaccine.

mucosal secretions by two to five times the amount induced by $1,25(OH)_2D_3$ immunomodulation alone. Similar immunization protocols employing other protein vaccines (e.g., influenza virus or diphtheria toxoid) have yielded equivalent types of results (data not shown).

Experiments were next conducted to compare the $1,25(OH)_2D_3$-plus-DHEA-immunomodulated immunization strategy with a standard experimental approach known to induce a common mucosal immune response (18, 40, 53). Stool samples were collected on day 28 following the subcutaneous immunization of normal strain C3H mice with HBsAg alone or in combination with $1,25(OH)_2D_3$ and DHEA. A third group of mice were given an oral vaccination containing 10 μg of HBsAg plus 5 μg of CT (List Biologicals, Campbell, Calif.) by previously described protocols (20). Soluble antibodies were liberated from individual fecal pellets by incubating them in a small volume of PBS (125 μl) for 30 min and then subjecting them to vigorous vortexing and centrifugation. Antigen-specific and total IgAs were quantitated in the diluted samples, and the results are presented in picograms per milliliter (Fig. 5A) or as the percentage of the total feces-extracted IgA antibody calculated to be antigen specific (Fig. 5B). The results established that high titers of IgA coproantibodies were present in the fecal pellets from the groups of animals vaccinated with $1,25(OH)_2D_3$ and DHEA as well as from the animals orally immunized with HBsAg plus CT. Quantitative analysis of the relationship between anti-HBsAg IgA and total IgA in the stool samples determined that nearly 3% of the antibody from the $1,25(OH)_2D_3$ treatment group and nearly 1.0% of the coproantibody from orally immunized animals were antigen specific. Minimal HBsAg-specific IgA was found in fecal samples from animals vaccinated with HBsAg alone.

By day 35 postvaccination, the titers of coproantibodies in both treatment groups had declined substantially (350 to 450 pg/ml; data not shown). Animals from all three groups were orally challenged with 10 μg of HBsAg in sodium bicarbonate buffer on day 43. Seven and 14 days later, stool samples were collected and evaluated for memory responses. Elevated titers of anti-HBsAg antibody (IgA) could be detected by day 7 postchallenge, and optimum titers were achieved by day 14 (Fig. 5C). Comparable memory responses to challenge were found in the $1,25(OH)_2D_3$-plus-DHEA and the CT treatment groups. Antigen-specific IgG coproantibodies were also evaluated in this study but were undetectable (data not shown).

Kinetic analysis of specific antibody-producing cells in various lymphoid organs and mucosal tissues following hormone-modulated vaccination. A series of kinetic experiments was conducted to ascertain the lymphoid organ distribution of antibody-producing cells following hormone-modulated and conventional immunization protocols. Two groups of strain C3H animals were immunized with either HBsAg alone or HBsAg plus $1,25(OH)_2D_3$ and DHEA. At 0, 3, 6, and 12 days postimmunization, animals from each group were sacrificed and IgA and IgG spot-forming cells within various lymphoid organs were quantitated by ELISPOT assay. The results of the analysis of IgA-producing cells is presented in Table 3. Although some specific antibody-producing cells could be detected in the spleen as early as day 3 postimmunization and by day 6 in the draining lymph nodes, the vast majority of cells producing the specific antibody were detected in the mesenteric lymph nodes and Peyer's patches. This finding indicates that the hormone-modulated vaccination protocol, while fully capable of affecting T-cell function in the draining lymph nodes, does not appear to be promoting the actual differentiation of antibody-producing cells in these peripheral lymphoid organs. Instead, it appears that the primary lymphoid organ sites of terminal

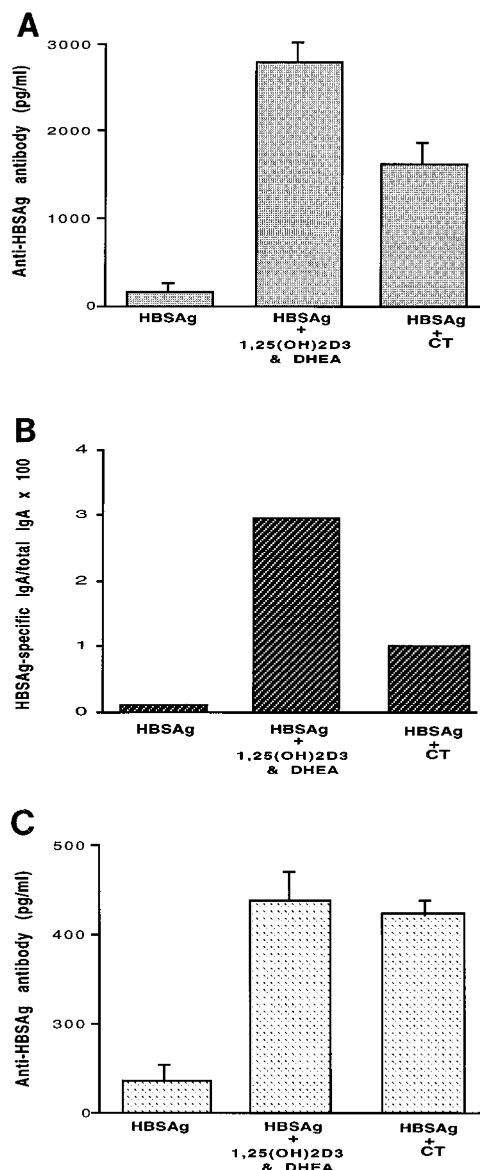

FIG. 5. A comparison of systemic hormone-immunomodulated peripheral immunization, employing DHEA and $1,25(OH)_2D_3$ containing HBsAg vaccine formulations, with oral HBsAg vaccine formulations administered with CT as the mucosal adjuvant. (A) Coproantibody IgA titers established on a day 28 postimmunization stool sample; (B) presentation of the day 28 data as a percentage of total IgA detected in the fecal secretions; (C) day 57 coproantibody titers elicited by an oral challenge of all experimental groups with HBsAg 14 days earlier (on day 43 postimmunization).

B-cell differentiation to secretory antibody-producing cells under hormone-modulated conditions are retained in the Peyer's patches and mesenteric lymph nodes. Analysis of the sites promoting development of IgG-producing cells determined that the spleen and draining lymph nodes contained the greatest numbers by day 6 (100 to $400/10^6$ cells), with the mesenteric lymph nodes and Peyer's patches demonstrating a lesser, but significant, number of antibody-producing cells at the optimum (6- to 12-day) time period postimmunization (Table 4).

The data presented in Fig. 6 and 7 represent the results of a series of experiments designed to question the kinetics associated with the emergence of antigen-specific antibody-produc-

TABLE 3. ELISPOT analysis of IgA antibody-forming cells isolated from lymphoid tissues of C3H mice at various times postimmunization with HBsAg in conjunction with vitamin D_3 and DHEA

Source of HBsAg-specific B cells[a]	Frequency of anti-HBsAg IgA spot-forming cells on[b]:			
	Day 0	Day 3	Day 6	Day 12
Draining lymph node				
(−)	0	0	0	0
(+)	0	0	54 ± 12	22 ± 5
Spleen				
(−)	0	0	13 ± 3	0
(+)	0	29 ± 7	25 ± 4	19 ± 5
Mesenteric lymph node				
(−)	32 ± 10	35 ± 12	74 ± 18	44 ± 12
(+)	32 ± 10	93 ± 17	350 ± 85	210 ± 55
Peyer's patch				
(−)	0	0	14 ± 4	18 ± 5
(+)	0	17 ± 7	125 ± 25	62 ± 20

[a] C3H strain mice were given a subcutaneous immunization of 1.0 μg of HBsAg with 0.1 μg of $1,25(OH)_2D_3$ and 2.0 μg of DHEA added directly to the vaccine formulation. (−), mice received HBsAg only; (+), mice were immunized with the vaccine formulation plus immunomodulators.

[b] The values represent the calculated numbers of antibody-secreting cells per 10^6 cells ± standard deviations.

ing cells into mucosal tissue sites following a hormone-modulated vaccination with HBsAg. Groups of animals were immunized with HBsAg as specified in the legends to Fig. 6 and 7, and subgroups were sacrificed for ELISPOT analysis of antibody-producing cells after 7, 14, and 21 days. Antigen-specific IgA-producing cells (Fig. 6) were easily detected in the lamina propria of both the intestines and lungs of both groups of mice vaccinated with $1,25(OH)_2D_3$. The generation of antigen-specific IgG-producing cells (Fig. 7) exhibited characteristics quite dissimilar from the IgA producers in that no cells committed to IgG production could be detected in the lamina propria of the intestine. ELISPOT-positive cells producing IgG antibody specific for HBsAg could easily be detected in the lung after day 21 postvaccination. The inclusion of $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$ plus DHEA in the vaccine formulation had only a minimal enhancing influence on the numbers of anti HBsAg IgG-producing cells found in the lungs of HBsAg-immunized animals.

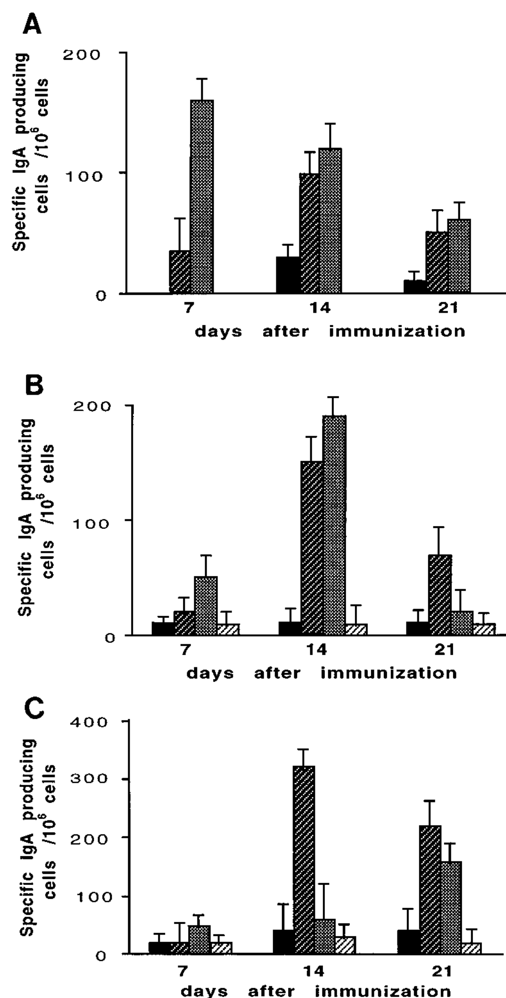

FIG. 6. Kinetic analysis of HBsAg-specific IgA antibody-producing cells present in the Peyer's patches (A) and in the lamina propria of the small intestine (B) and lungs (C) of conventionally HBsAg-immunized mice and mice immunized with vaccine formulations containing $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$ and DHEA. Two to three individuals within each treatment group were sacrificed at the specified time points, and specific antibody-producing cells were quantitated by ELISPOT analysis. Symbols: ■, HBsAg control; ▨, HBsAg + $1,25(OH)_2D_3$; ▩, HBsAg + $1,25(OH)_2D_3$ + DHEA; ▧, naive control.

TABLE 4. ELISPOT analysis of IgG antibody-forming cells isolated from lymphoid tissues of C3H mice at various times postimmunization with HBsAg in conjunction with $1,25(OH)_2D_3$ and DHEA

Source of HBsAg-specific B cells[a]	Frequency of anti-HBsAg IgG spot-forming cells on[b]:			
	Day 0	Day 3	Day 6	Day 12
Draining lymph node				
(−)	0	0	13 ± 5	0
(+)	0	22 ± 7[c]	107 ± 23	24 ± 5
Spleen				
(−)	0	58 ± 14	153 ± 16	105 ± 12
(+)	0	148 ± 11	377 ± 35	164 ± 21
Mesenteric lymph node				
(−)	0	18 ± 5	55 ± 17	10 ± 5
(+)	0	42 ± 9	148 ± 24	82 ± 20
Peyer's patch				
(−)	0	0	37 ± 12	42 ± 15
(+)	0	20 ± 7	162 ± 25	72 ± 20

[a] C3H strain mice were given a subcutaneous immunization of 1.0 μg of HBsAg with 0.1 μg of $1,25(OH)_2D_3$ and 2.0 μg of DHEA added directly to the vaccine formulation. (−), mice received HBsAg only; (+), mice were immunized with the vaccine formulation plus immunomodulators.

[b] The values represent the calculated numbers of antibody-secreting cells per 10^6 cells ± standard deviations.

DISCUSSION

The experimental findings presented herein indicate that it is technically feasible to manipulate the development of particular types of immune effector responses by formulating vaccine preparations with agents having specific and defined T-cell-influencing activities. Methods to manipulate the common mucosal immune system are particularly attractive, since such a high percentage of human pathogens gain access to their hosts across mucosal membranes. Mucosal tissues can also serve as direct targets of the pathogenic process. It is not surprising, therefore, that mucosal tissues possess a variety of innate and adaptive immune mechanisms to aid in the protec-

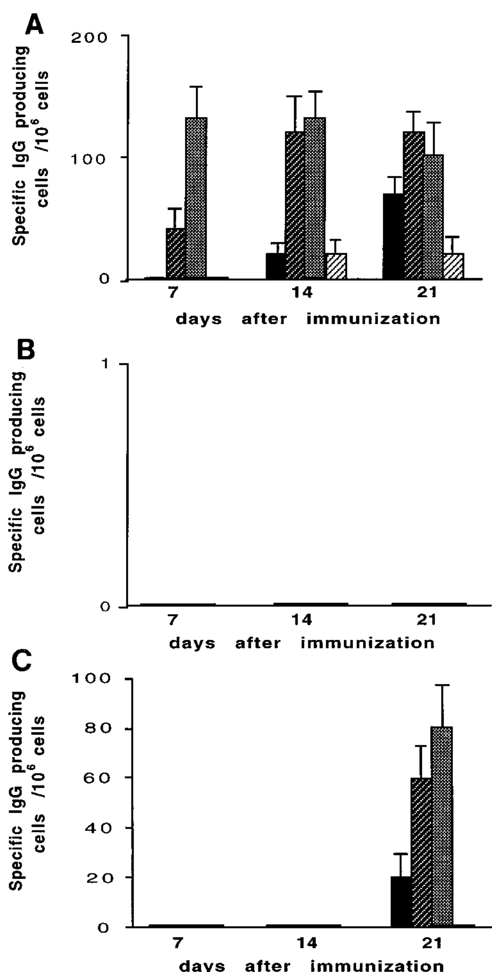

FIG. 7. Kinetic analysis of HBsAg-specific IgG antibody-producing cells present in the Peyer's patches (A) and in the lamina propria of the small intestine (B) and lungs (C) of conventionally HBsAg-immunized mice and mice immunized with vaccine formulations containing $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$ and DHEA. Two to three individuals within each treatment group were sacrificed at the specified time points, and specific antibody-producing cells were quantitated by ELISPOT analysis. Symbols: ■, HBsAg control; ▨, HBsAg + $1,25(OH)_2D_3$; ▩, HBsAg + $1,25(OH)_2D_3$ + DHEA; ▨, naive control.

tion of this barrier from invasion. The common mucosal immune system, particularly through the activities of secretory antibodies, is believed to represent an integral component in the repertoire of host defense mechanisms protecting mucosal surfaces (50). Antigen-specific secretory antibodies are predominantly of the IgA class and appear to function in protection through their capacity to reduce colonization of infectious agents at mucosal surfaces (25, 50). Lowering the capacity of a microorganism to colonize mucosal tissues reduces the chances of infection, resulting in less chance of disease. It is also possible, however, that secretory antibodies can exhibit toxin-neutralizing or enzyme-neutralizing activities (25, 34). It has also been postulated that locally produced IgA might exert some of its protective functions intracellularly, inhibiting virus replication by its capacity to interfere with viral assembly (34).

Although specific common mucosal immune responses are easily observed following recovery from an active mucosal infection or disease, the development of effective vaccines capable of eliciting protective mucosal immune responses has met with a number of significant challenges. The cellular elements and tissues associated with the common mucosal immune system evolved to respond defensively to challenge with a replicating infectious agent, and the mucosal immunity induction sites are clearly anatomically developed and positioned to best accommodate those forms of antigen presentation associated with active infection. Modified live virus or attenuated bacterial vaccines are not presently favored, since there is a possibility of the vaccine causing disease, especially in immunosuppressed individuals. Unfortunately, mucosally administered nonreplicating antigens, including recombinant proteins or purified polysaccharides, are not efficiently presented to the appropriate mucosal immunity induction sites. This is due, in part, to the tremendous surface area associated with the total mucosal surfaces compared with the limited surface area of the mucosal immunity induction sites. Therefore, the successful experimental induction of common mucosal immune responses to antigens presented to a mucosal surface generally requires high doses of antigen, repeated exposures, and the facilitating activities of potent mucosal adjuvants such as CT or heat-labile enterotoxin from *Escherichia coli* (20, 22, 40, 44, 53).

We demonstrated a number of years ago that T cells residing in mucosa-associated secondary lymphoid organs are committed to producing cytokines conducive to the induction of mucosal immune responses (5, 14). We therefore speculated that it might be possible to generate, within any secondary lymphoid organ, the types of effector immune responses which are normally initiated in the Peyer's patch microenvironment if the activated T-cell repertoire of cytokines is manipulated to conform to those normally present within mucosal lymphoid tissues. Exposure of splenic or lymph node T cells to the influences of $1,25(OH)_2D_3$ in vitro or in vivo was found to facilitate a switch from a predominant type 1 response to a type 2-like response, evidenced by an enhanced level of IL-4 and IL-10 and lower production of IL-2 and IFN-γ following T-cell activation. Additional studies in vitro determined that both polyclonally activated $CD4^+$ and $CD8^+$ T cells were similarly affected by $1,25(OH)_2D_3$ exposure, although the modulatory effects were more pronounced with enriched populations of $CD4^+$ T cells (unpublished observations). Our in vitro studies, when coupled with the fact that $1,25(OH)_2D_3$ appears to also promote an enhanced production of active TGF-β (42), suggest that this steroid might be capable of promoting a secretory immune response to antigens administered afferently to a lymphoid organ under its immunomodulatory influence. This was indeed the case, as evidenced by the ability to stimulate the generation of specific secretory immune responses to antigens injected subcutaneously or intramuscularly in combination with the $1,25(OH)_2D_3$. Common mucosal immunity was then verified to exist by our ability to detect simultaneously antigen-specific IgA in a number of distinct mucosal secretions. These responses were restricted only to those animals vaccinated under the regulatory influence of $1,25(OH)_2D_3$.

Numerous preliminary studies employing a variety of protein antigens were conducted during the course of these studies to optimize the dose, route of administration, and timing of $1,25(OH)_2D_3$ administration. At the present time, we have no scientific explanation for the findings that topical administration of $1,25(OH)_2D_3$ (1 to 2 μg) for up to 5 days postimmunization and the direct incorporation of $1,25(OH)_2D_3$ (0.5 to 1.0 μg) into the vaccine formulation have proven to be equally effective at promoting the development of common mucosal immunity. It might be possible that an early IL-2-dominated T-cell response to antigen is beneficial during the afferent phase of a mucosal immune response and that later switching of T-cell activities to responses dominated by IL-4 and IL-10

are then instituted to appropriately guide B-cell differentiation. This might partially explain the effectiveness of topical 1,25-$(OH)_2D_3$ administration a few days after vaccination.

It has been demonstrated that common mucosal immune responses can be augmented in intranasally vaccinated animals administered liposomes containing a recombinant source of IL-2 (1). The mechanisms underlying this enhancement are unknown but may relate to the synergistic activities between IL-2 and TGF-β on increases in IgA production (29). These findings suggested that the addition of DHEA to a vaccine formulation already containing 1,25$(OH)_2D_3$ might enhance secretory antibody titers, since DHEA is able to increase activated T-cell production of IL-2 in both mice and humans (16). This possibility was supported by experiments with freshly isolated murine T cells in vitro which determined that the addition of DHEA (10^{-7} M) to cultures containing modulatory doses of 1,25$(OH)_2D_3$ (10^{-8} M) was able to abrogate the ability of this hormone to depress production of IL-2 without affecting the enhanced production of IL-4 and IL-10 (unpublished results). Results of the experiments with HBsAg immunization in the presence of 1,25$(OH)_2D_3$ and DHEA supported our working hypothesis (5) and provided us with a novel hormone-modulated immunization strategy which is now proving to be comparable to the use of orally administered CT as a mucosal adjuvant in some experimental systems. The hormone-modulated immunization protocol has an advantage of requiring only small amounts ($<$10%) of antigen compared with that needed to elicit a comparable secretory immune response to vaccination with CT as the adjuvant. We have experimentally tested the possibility that 1,25$(OH)_2D_3$ administered transmucosally (intranasally or orally) with a vaccine preparation might be able to enhance or facilitate the development of a common mucosal immune response. Unfortunately, the results of these studies have not been encouraging, indicating that transmucosal vaccination is apparently not influenced by the simultaneous mucosal administration of various steroid immunomodulators.

Our highly reproducible findings by ELISPOT analysis, i.e., that mucosal lymphoid organs were the major sites where early-antigen-specific IgA-secreting cells reside, were unexpected. This indicates that unknown factors concerning the roles played by the mucosal lymphoid organs in B-cell differentiation into secretory antibody-producing cells still remain to be addressed. Clearly, additional work is necessary before we will fully understand the mechanisms operating to facilitate mucosal immunity following immunization under 1,25$(OH)_2D_3$ influences.

Oral and intranasally administered vaccine formulations are being actively pursued by numerous investigators because their use is preferred over that of hypodermic injections and because of their ability to promote, under appropriate conditions, the induction of mucosal immunity. However, for some of the newly emerging vaccination strategies, it may ultimately prove difficult to use transmucosal immunization routes for effective mucosal vaccination. This may represent the case with nucleic acid vaccines, in which effective cellular transfection is required for protein antigen generation in vivo. Hormonally immunomodulated vaccination strategies could prove to be beneficial with this new form of immunization, as evidenced by our recent ability to establish a level of protection in mice against herpes simplex virus type 2 following their immunization with a nucleic acid vaccine encoding glycoprotein D2 when administered intramuscularly in the presence of 1,25$(OH)_2D_3$ (28).

In summary, the present investigation describes a novel systemic immunization strategy which is capable of promoting the generation of both humoral and common mucosal immune responses to antigens administered by subcutaneous or intramuscular injection. The procedure employs a simple subcutaneous immunization with an antigen preparation supplemented with small amounts (0.1 μg) of 1,25$(OH)_2D_3$. The protocol appears to be cost effective and for the present test antigens is comparable in efficiency to oral vaccine preparations containing 10 times the amount of antigen plus CT. The protocol also appears to be safe, although additional experimentation is clearly warranted to determine whether the use of this hormone modulation technique influences the development of cellular immune responses. This becomes especially relevant under those vaccination conditions in which cell-mediated immune effector mechanisms would be beneficial for protection.

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