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Original Articles

Route and Method of Delivery of DNA Vaccine Influence Immune Responses in Mice and Non-Human Primates

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Abstract

Background: In spite of the large number of studies that have evaluated DNA-based immunization, few have directly compared the immune responses generated by different routes of immunization, particularly in non-human primates. Here we examine the ability of a hepatitis B surface antigen (HBsAg)-encoding plasmid to induce immune responses in mice and non-human primates (rhesus monkeys: *Macaca mulatta*) after delivery by a number of routes.

Materials and Methods: Eight different injected [intraperitoneal (IP), intradermal (ID), intravenous (IV), intramuscular (IM), intraperineal (IPER), subcutaneous (SC), sublingual (SL), vaginal wall (VW)] and six non-injected [intranasal inhalation (INH), intranasal instillation (INS), intrarectal (IR), intravaginal (IVAG), ocular (Oc), oral feeding (oral)] routes and the gene gun (GG) were used to deliver HBsAg-expressing plasmid DNA to BALB/c mice. Sera were assessed for HBsAg-specific antibodies (anti-HBs, IgG, IgG1, IgG2a) and cytotoxic T lymphocyte (CTL) activity measured. Three of the most commonly used routes (IM, ID, GG) were compared in rhesus monkeys, also using HBsAg-expressing vectors. Monkeys were immunized with short (0-, 4- and 8-week) or long (0-, 12- and 24-week) intervals between boosts, and in the case of GG, also with different doses, and their sera were assessed for anti-HBs.

Results: In one study, anti-HBs were detected in plasma of mice treated by five of eight of the in-

jected and none of the six noninjected routes. The highest levels of anti-HBs were induced by IM and IV injections, although significant titers were also obtained with SL and ID. Each of these routes also induced CTL, as did IPER and VW and one noninjected route (INH) that failed to induce antibodies. In a second study, GG (1.6 μg) was compared to ID and IM (100 μg) delivery. Significant titers were obtained by all routes after only one boost, with the highest levels detected by IM. Delivery to the skin by GG induced exclusively IgG1 antibodies (Th2-like) at 4 weeks and only very low IgG2a levels at later times; ID-immunized mice had predominantly IgG1 at 4 weeks and this changed to mixed IgG1/IgG2a over time. Responses with IM injection (in the leg or tongue) were predominantly IgG2a (Th1-like) at all times. IV injection gave mixed IgG1/IgG2a responses. In monkeys, in the first experiment, 1 mg DNA IM or ID at 0, 4, and 8 weeks gave equivalent anti-HB titers and 0.4 μg at the same times by GG induced lower titers. In the second experiment, 1 mg DNA IM or ID, or 3.2 μg by GG, at 0, 12, and 24 weeks, gave anti-HB values in the hierarchy of GG > IM > ID. Furthermore, high titers were retained after a single immunization in mice but fell off over time in the monkeys, even after boost.

Conclusions: Route of administration of plasmid DNA vaccines influences the strength and nature of immune responses in mice and non-human primates. However, the results in mice were not always predictive of those in monkeys and this is likely true for humans as well. Optimal dose and immunization schedule will most likely vary between species. It is not clear whether results in non-human primates will be predictive of results in humans, thus additional studies are required.

Introduction

DNA-based immunization is a recently developed method of immunization that, in principle, could overcome many of the deficits of traditional antigen-based vaccines. Strong cellular and humoral responses have been demonstrated in a number of different animal models, subsequent to the in vivo expression of antigen from directly introduced plasmid DNA (1,2), and several human clinical trials have been completed or are underway (3–6). Unfortunately, the promising results in animal models have not been realized in human trials and considerable effort is now being focused at understanding this difference and developing ways of improving the efficacy of DNA vaccines.

The induction of strong immune responses in animal models following introduction of DNA appears to be due to a combination of (*i*) the prolonged in vivo synthesis of antigen (7), (*ii*) in vivo antigen expression that results in major histocompatability complex (MHC) presentation (8,9), and (*iii*) the adjuvant effect of unmethylated immunostimulatory CpG motifs present in the DNA backbone (10,11). Thus, efforts to improve the efficacy of DNA vaccines include vector modifications, facilitated or targeted DNA delivery, and the use of adjuvants in an attempt to increase the amount of antigen expressed, to have the antigen expressed in the most appropriate cells and/or to improve the immune responses against the expressed antigen.

The strength and nature of immune responses in mice with DNA vaccines appear to be influenced by a number of factors (1,2); however, these variables may not be of similar importance in larger animals including humans. As such, optimization methods developed in mice may not necessarily be applicable to humans. Factors that have significant impact on subsequent immune responses in mice include the route and method of DNA delivery (12–14), likely because of differences in the efficiency of gene transfer and the type of cell(s) transfected. DNA vaccines have been delivered to a wide variety of tissues by both injected and noninjected DNA delivery methods, including (*i*) into the muscle by gene gun (GG) or intramuscular (IM) injection (13,15–20); (*ii*) into the skin by epidermal GG delivery (9,12,21) or intradermal (ID) (12,22,23) or subcutaneous (SC) (12,24) injection; (*iii*) into the circulatory system by intravenous (IV) injection (12,25,26); (*iv*) into the respiratory system by intranasal (IN) (12,27–29) or intratracheal (12) delivery; (*v*) into the digestive system by oral feeding of microencapsulated

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(30,31) or lipid-formulated DNA (32), injection into the oral mucosa (32), tongue (33), or jejunum (32), and GG delivery into the Peyer's patches of the bowel (34), tongue (35), or oral mucosa (36); (*vi*) into the genitourinary tract by intravaginal (IVAG) GG delivery (34) or instillation (37,38), and (*vii*) by topical ocular (Oc) administration (39).

In spite of the large number of routes previously reported, few studies have directly compared the immune responses generated by different routes of immunization, particularly in non-human primates. We have previously described a model for DNA-based immunization against the hepatitis B virus (HBV) whereby strong systemic immunity is induced by IM or ID injection of DNA encoding HBV surface antigen (HBsAg). Here we examine the ability of a HBsAg-encoding plasmid to induce immune responses in mice after delivery by a large number of routes and compare three of the most frequently used delivery methods, namely IM and ID injection, and GG delivery to skin, for immunization of mice and non-human primates (rhesus monkeys).

Materials and Methods

Plasmid DNA

Gene-transfer studies were carried out with plasmid DNA encoding the middle (M = preS2 + S) or major (S) surface proteins of the HBV envelope. These plasmids, which were each under the control of the cytomegalovirus (CMV) immediate-early promoter, with or without inclusion of the CMV intron A (A), were designated pCMV-S2.S (40), pCMV-S (16), and pCMV(A)-S (provided by PowderJect Vaccines, #WRG 7072). We have previously shown no difference in anti-HB titers against the major (S) envelope protein after immunization with pCMV-S2S or pCMV-S (40) or with pCMV-S or pCMV(A)-S (H. Davis et al., unpublished results). The DNA was purified on Qiagen anion-exchange chromatography columns (Qiagen, Chatsworth, CA) and resuspended in sterile saline (0.15 M NaCl, Sigma, St. Louis, MO) to a final concentration of 1 to 5 mg/ml or coated onto 1 to 3 μm gold beads for GG delivery. DNA prepared in this way has only low levels of endotoxin (18). DNA in saline or coated onto gold beads was stored at −20°C or 4°C (after desiccation) respectively, until required for administration.

Animals

MICE. All experiments were carried out using female BALB/c mice (Charles River, Montreal, Quebec) aged 6 to 8 weeks with 3 to 12 mice per experimental or control group. Mice were shaved over the administration site for most injected routes and GG. For most injected routes as well as IVAG instillation, mice were deeply anesthetized with Somnotol® (75 mg/kg IP; MTC Pharmaceuticals, Cambridge, Ontario) anesthesia. For other noninjected routes, GG and IM injection, mice were lightly anesthetized by inhalation of Halothane® (Halocarbon Laboratories, River Edge, NJ).

MONKEYS. All experiments were carried out with adult male and female rhesus monkeys (2.5 to 6.3 kg body weight) with four animals randomly assigned per experimental or control group. Animals were anesthetized with Ketamine HCl (10 mg/kg body weight; Phoenix Pharmaceutical, St. Joseph, MO) prior to immunization. Monkeys were maintained in the animal facility of Bioqual (Rockville, MD) or Primedica (formerly TSI Mason, Worcester, MA) and monitored daily by animal care specialists. No symptoms of general ill health or local adverse reactions at the sites of injection were noted.

DNA-Based Immunization of Mice

Each animal received a total of 100 μg pCMV-S, pCMV(A)-S, or pCMV-S2.S in 0.15 M NaCl, with the exception of GG delivery where each animal received a total of 1.6 μg DNA coated onto gold particles as per our standard protocol (41). Gold particles (Degussa, Theodore, AL) used in mice and monkeys were 1 to 3 μm in diameter. DNA loading rate was 1.6 μg DNA/mg gold, 0.5 mg gold per cartridge in mice. The number and volume of administrations varied with the route of administration and had been determined in preliminary studies as optimal for that particular route. Some of the mice immunized by IM or ID injection or GG delivery to the epidermis were boosted at different time points after priming.

INJECTED ROUTES OF ADMINISTRATION AND GG. DNA was delivered to two separate sites in the lower abdomen using the hand-held, helium-powered PowderJect XR particle acceleration device (formerly known as Accell® gene gun). For all injected routes, DNA was injected using an

insulin syringe with a 29-gauge needle attached (Becton Dickinson, Franklin Lakes, NJ) by one of the following routes: intraperitoneal (IP): 150 μl directly into the peritoneal cavity; ID: 50 μl at each of three separate sites in the skin of the lower back; IM: 50 μl bilaterally into the tibialis anterior (TA) muscles; IV: 150 μl into the tail vein; intraperineal (IPER): 50 μl into the perineal region; SC: 150 μl under the skin of the lower back; sublingual (SL): 50 μl in the lower surface of the tongue; vaginal wall (VW): 50 μl into the submucosa deep to the vaginal epithelium.

NONINJECTED ROUTES OF ADMINISTRATION. DNA solution was administered by one of the following routes: IN inhalation (INH): 3 × 50 μl at 15-min intervals, applied as droplets directly over both external nares of mice; IN instillation (INS): 3 × 25 μl at 15-min intervals, instilled directly into the nasal cavity by insertion of a gel-loading tip attached to a Gilson pipette a few mm into the nostril; intrarectal (IR): 100 μl DNA instilled via the anus using a 200-μl pipette tip, following which a small quantity of petroleum jelly was applied to prevent leakage; IVAG: 100 μl DNA slowly instilled into the mouse vaginal cavity using a 200-μl pipette tip, following which a small quantity of petroleum jelly was applied to prevent leakage (mice were anesthetized with Somnotol to prevent them from licking themselves and ingesting some of the DNA); Oc: 10 μl applied to each eyeball, following which the eyelids were gently massaged; oral feeding (oral): 100 μl administered directly into the stomach using a 1-c.c. tuberculin syringe (Becton Dickinson) attached to a 20-gauge olive tip steel feeding tube (Fine Science Tools, North Vancouver, British Columbia), which was passed through the oral cavity and into the esophagus.

DNA-based Immunization of Monkeys

In one experiment (carried out at Bioqual), monkeys were immunized at 0, 4, and 8 weeks with pCMV-S2.S DNA by IM or ID injection (1 mg) or GG delivery to the skin (0.4 μg). In a separate experiment (carried out at Primedica), monkeys were treated similarly except pCMV-S DNA was given at 0, 12, and 24 weeks and the GG dose was increased to 3.2 μg. IM injections were performed with the Biojector® needle-free jet injection system (Bioject, Portland, OR) using a barrel size previously shown to deliver almost all radioopaque injectate into the muscle belly (data not shown) and ID injections using an insulin syringe with a 29-gauge needle attached (Becton Dickinson). We have previously shown that the Biojector is superior to IM injection for DNA-based immunization of rabbits (42) and Aotus monkeys (23). The Biojector is not suitable for IM injection of mice since the force of the liquid jet stream is too powerful for such a small animal. GG immunization in monkeys was also with the PowderJect XR system (DNA loading rates: 0.2 and 0.8 μg DNA/mg gold, in the first and second studies, respectively, 0.5 mg gold per cartridge). DNA for each of these routes (4 monkeys per group) was delivered as follows: ID: 100 μl at each of four separate sites in the shaved skin of the lower back; IM: 250 μl at each of four sites by bilateral injection into the triceps and hamstring (Bioqual) or the triceps and quadriceps (Primedica); GG: 0.4 μg divided into four sites (Bioqual) or 3.2 μg divided into eight sites (Primedica) over the midabdomen. All sites were shaved and swabbed with alcohol prior to DNA administration.

Evaluation of HBsAg-specific Immune Responses in Mice

ANTI-HBs. Plasma was obtained by retro-orbital puncture at various time points after immunization. HBsAg-specific antibodies (anti-HBs) in plasma were detected by ELISA assay on individual samples using HBsAg-coated plates as previously described (43). End-point dilution titers for total immunoglobulin G (IgG) as well as IgG1 and IgG2a isotypes were defined as the highest plasma dilution that resulted in an absorbance value (OD 450) two times greater than that of nonimmune plasma, with a cut-off value of 0.05. In our laboratory we have previously determined that the relationship between end-point titers in mice and those in milli-international units (mIU), as defined by the World Health Organization (WHO), is close to 1:1 (44). A value of 10 mIU/ml is protective in humans. In addition, a panel of mouse standards was identified by comparing various mouse sera against human-derived standards defined by the WHO (Monolisa Anti-HBs Standards, Sanofi Diagnostics Pasteur, Montreal, Quebec) using a commercial kit (Monolisa Anti-HBs, Sanofi Diagnostics Pasteur). These mouse standards were then used to quantify anti-HB titers in some mouse groups in mIU/ml. Anti-HB titers were expressed as means (mIU/ml) or geometric means (end-point dilution) ± SEM of individual animal values, which

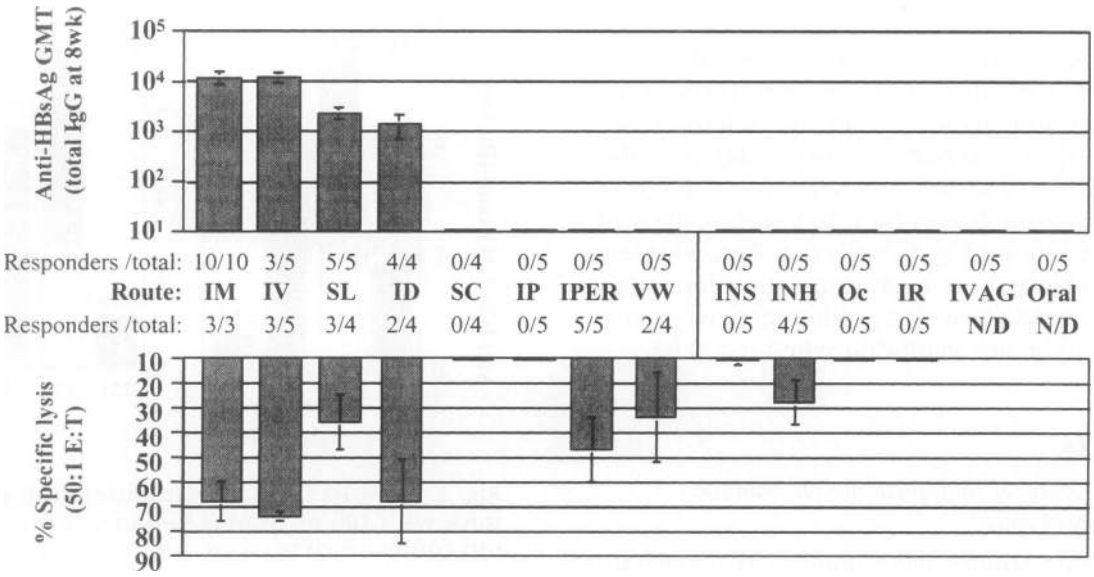

Fig. 1. Results from immunization of BALB/c mice with 100 μg pCMV(A)-S in saline by various routes. Upper panel: Each bar represents the group geometric mean titer (GMT) (±SEM) determined by end-point dilution ELISA assay for HBsAg-specific antibodies (anti-HBs, total IgG) in plasma of mice that responded (end-point titer >10) taken 8 weeks after immunization. Number of responders per group total is indicated below graph. End-point dilution titers were determined as the highest sample dilution resulting in an absorbance value two times that of nonimmune plasma, with a cut-off value of 0.05. Lower panel: Each bar represents the mean HBsAg-specific lysis (±SEM) as a percentage of the total possible lysis (% specific lysis) at effector-to-target ratio (E:T) of 50:1 in splenocytes from mice that responded (% specific lysis >10) 8 weeks after immunization. Number of responders per group total is indicated above graph. ND, not determined.

were themselves the average of triplicate assays. Nonimmune plasma was used as a negative control.

HBS AG-SPECIFIC CYTOTOXIC T-LYMPHOCYTE (CTL) ACTIVITY. Splenocytes taken at 4, 6, or 8 weeks after immunization were specifically restimulated for 5 days in vitro with an irradiated HBsAg-expressing cell line (P815S) and then used in a chromium release assay with the same HBsAg-expressing target cells, or a nonexpressing control cell line (P815), as previously described (45). Effector:target (E:T) ratios of 6.25:1, 25:1, 50:1, and 100:1 were used for all assays, although only data with the 50:1 ratio are shown (Figs. 1 and 4) as relative differences were similar with all ratios tested. Data were expressed as % specific lysis = % lysis with P815S cells − % lysis P815 cells. The percent lysis was calculated as [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100. Spontaneous release was determined by incubating target cells without effector cells and total release was determined by adding 100 μl of 2% Triton-X 100 to target cells. The percent specific lysis was calculated. Cytotoxic T lymphocyte (CTL) activity was expressed as group means ± SEM of individual animal values, which were themselves the average of values from triplicate assays.

Evaluation of Anti-HB Responses in Monkeys

Monkeys were bled by IV puncture prior to and at various time points after immunization and anti-HB antibodies were detected and quantified by ELISA on individual serum samples as follows. A solid phase of plasma-derived HBsAg particles (*ay* subtype, 100 μl of 1 μg/ml per well, overnight at room temperature; International Enzymes, Fallbrook, CA) was used to capture anti-HB antibodies in the serum (1 hr at 37°C), which were then detected using a non-species-specific conjugate and *o*-Phenylenediamine (OPD) as chromogen supplied as part of a commercial kit (Monolisa Anti-HBs). Anti-HB titers were expressed in mIU/ml based on comparison with WHO-defined standards (Monolisa Anti-HBs Standards).

Statistical Analysis

Data were analyzed using the GraphPAD InStat program (GraphPAD Software, San Diego, CA). The statistical significance of the difference between values obtained from two groups was determined by Student's two-tailed *t*-test and from three or more groups by one-factor analysis of variance followed by Tukey's test. Anti-HB titers were subjected to logarithmic transformation prior to statistical analysis. Differences were considered to be not significant when $p > 0.05$.

Results

Effect of Route of Immunization on Immune Responses in Mice

The mouse studies were done in two separate experiments. The first compared 14 different injected or noninjected routes. The second study compared IM and ID injection and GG.

HUMORAL RESPONSE. Anti-HBs were detected in plasma of mice immunized with 100 μg pCMV(A)-S by some of the injected, but none of the noninjected routes (Fig. 1, upper panel). The IM, SL, and ID routes resulted in 100% seroconversion compared to 60% with IV injection. Equivalent levels of anti-HBs were attained by 8 weeks postimmunization in responding mice in the IM and IV groups ($p = 0.56$), and in the SL and ID routes ($p = 0.91$), but the former pair had significantly greater titers than the latter pair ($p < 0.05$). Both IM and SL immunization induced mixed Th1/Th2 responses, but with considerably more IgG2a and IgG1. IgG2a antibodies are considered indicative of a Th1 response, although it is not possible to directly compare levels of antibodies because of different avidities for the two antibodies. IV and ID immunization also induced a mixed IgG1/IgG2a response at early time points, and over time these shifted slightly toward Th1-like for IV and slightly toward Th2-like for ID routes (Fig. 2). A humoral response was not detected with the other injected routes (IP, IPER, SC, and VW) or with any of the noninjected routes (INH, INS, IR, IVAG, Oc, and oral) (Fig. 1).

CELL-MEDIATED RESPONSES. HBsAg-specific CTL were detected following immunization by six injected routes (IM, IV, SL, ID, IPER, and VW) as well as by one noninjected route (INH) (Fig. 1, lower panel). The IM and IV routes resulted in significantly stronger cellular responses than INH

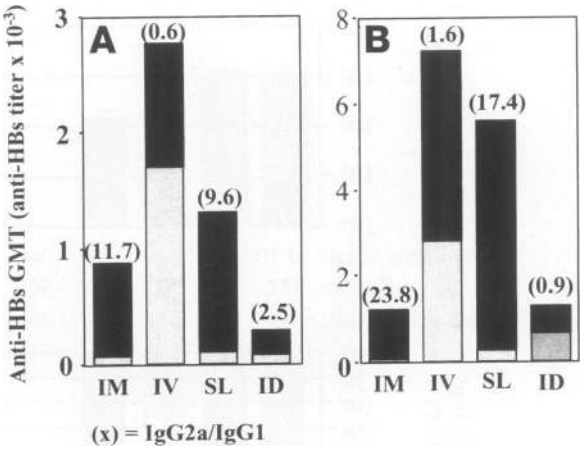

Fig. 2. Results from immunization of BALB/c mice with 100 μg pCMV(A)-S in saline by various routes. Each bar represents the group geometric mean titer (GMT) determined by end-point dilution ELISA assay for HBsAg-specific antibodies (anti-HBs) of IgG1 (gray bars) or IgG2a (black bars) isotypes in plasma taken 4 weeks (A) and 8 weeks (B) after immunization. Titers were defined as the highest plasma dilution resulting in an absorbance value two times that of nonimmune plasma, with a cut-off value of 0.05. Numbers above each bar indicate the IgG2a-to-IgG1 ratio, with a value >1 indicating a more Th-1 like response.

($p = 0.01$ and 0.006, respectively), and IV was stronger than SL ($p = 0.03$), but there were no other significant differences between routes ($p > 0.05$). Not all groups with CTL had anti-HBs (e.g., IPER, VW) and not all animals with humoral responses had cellular responses (e.g., for ID immunization, 4/4 had anti-HBs and 2/4 had CTL). Furthermore, CTL responses did not always correlate with anti-HB isotypes. For example, there were low levels of CTL with predominantly IgG2a (Th1-like) for SL yet strong CTL activity with both IgG1 and IgG2a (mixed Th1/Th2) for ID. Only with IM injection did 100% of mice both seroconvert and have CTL activity (Fig. 1).

Immune Responses in Mice with IM, ID, and GG Delivery

HUMORAL RESPONSES. There was 100% seroconversion (anti-HBs > 10 mIU/ml) by 2 weeks in mice immunized with 100 μg pCMV-S2.S by IM or ID injection (10/10). In mice immunized with >60-fold less (1.6 μg) DNA by GG there was 80% seroconversion at 2 weeks (8/10) and

100% by 4 weeks. Anti-HB total IgG titers at 4 weeks after priming were higher after IM than either ID or GG immunizations ($p = 0.0005$ and $p < 0.0001$, respectively), and ID gave higher titers than GG ($p = 0.015$). Boosting at 4 weeks raised anti-HB titers approximately 10-fold for IM and ID groups and 20-fold for GG-immunized mice ($p < 0.001$ relative to pre-boost titers for all routes). Post-boost titers remained the highest with IM ($p < 0.05$), but there was no longer a difference between the ID and GG groups ($p = 0.59$). Animals immunized by IM and ID, but not GG, received a second boost at 8 weeks. This did not increase anti-HB titers further ($p = 0.16$ and 0.35 for IM and ID, respectively compared to pre-boost levels) (Fig. 3A). Near-peak levels of anti-HBs were maintained until the end of the experiment in all groups (5 to 6 weeks after the last boost, $p > 0.05$).

Evaluation of plasma for IgG1 and IgG2a antibody isotypes, as indicators of Th2- and Th1-like responses, respectively, showed, 4 weeks after priming, only IgG1 antibodies following GG, predominantly IgG1 following ID, and mixed IgG1/IgG2a following IM delivery. These all shifted toward a Th1 response over time such that ID became mixed Th1/Th2 and IM became Th1-like, although they remained Th2-like for GG. Subsequent boosting did not change the ratio of antibody isotypes in any groups (Table 1). These findings indicate that, at least for the time points evaluated here, route and method of immunization can influence the kinetics, strength, and type of humoral response after DNA-based immunization.

CTL. HBsAg-specific CTL were detected after each of IM, ID, and GG immunizations. There was no difference in mean CTL activity 4 weeks after IM or ID delivery of 100 μg pCMV-S ($p > 0.05$), although there was lower variability with IM than ID (Fig. 4A). There was no significant effect on CTL activity in IM or ID groups by boosting mice in an identical manner at 2 weeks (not shown). CTL activity was weaker after two doses by GG administration than one dose by IM or ID injection ($p < 0.002$) (Fig. 4B). It should be noted that the dose was >60-fold lower with GG than the injected routes, and we do not have single-dose GG data to know whether there was a boost effect by the second dose. Nevertheless, results are comparable because all CTL assays were carried out simultaneously. There was no difference in CTL activity in mice boosted by GG at 2 or 4 weeks ($p > 0.05$), nor was there a difference in mice immunized IM with 100 μg pCMV-S or pCMV-S2.S ($p > 0.05$) (Fig. 4).

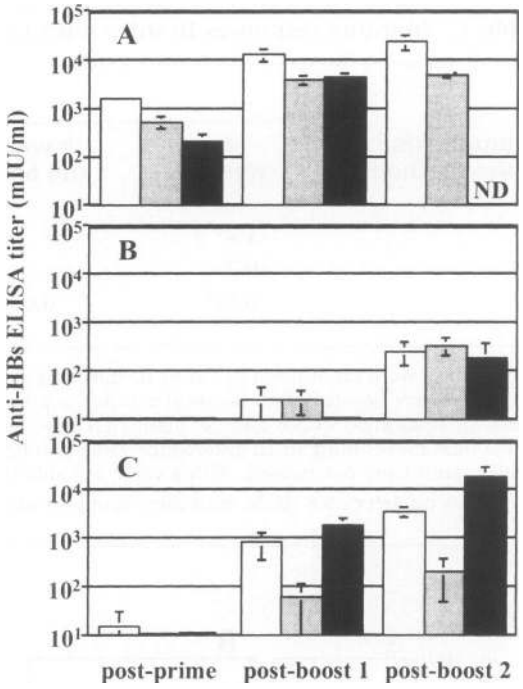

Fig. 3. Results from immunization of BALB/c mice and rhesus monkeys with pCMV-S2.S for various time periods. (A) BALB/c mice were immunized at 0, 4, and 8 weeks with 100 μg pCMV-S2.S by IM (white bars) or ID injection (gray bars) or with 1.6 μg by GG delivery (black bars). Each bar represents the group mean (±SEM) of anti-HB titer (mIU/ml) in plasma collected 4 weeks after priming or 2 weeks after each boost. End-point dilution titers were determined as the highest sample dilution resulting in an absorbance value two times that of nonimmune plasma, with a cut-off value of 0.05. Values in mIU/ml were determined from mouse standards identified by comparison with human-derived standards and the Monolisa Anti-HBs Detection Kit (Sanofi Diagnostics Pasteur). ND, not determined. (B) Rhesus monkeys were immunized at 0, 4, and 8 weeks with 1 mg pCMV-S2.S by IM (white bars) or ID injection (gray bars) or with 0.4 μg by GG delivery (black bars). Each bar represents the group mean (±SEM) of anti-HB titer (mIU/ml) in plasma collected 4 weeks after priming or 2 weeks after each boost. Values in mIU/ml were determined with human-derived standards and the Monolisa Anti-HBs Detection Kit (Sanofi Diagnostics Pasteur). (C) Rhesus monkeys were immunized at 0, 12, and 24 weeks with 1 mg pCMV-S by IM (white bars) or ID injection (gray bars) or with 3.2 μg by GG delivery (black bars). Each bar represents the group mean (±SEM) of anti-HB titer (mIU/ml) in plasma collected 4 weeks after priming or 2 weeks after each boost. Values in mIU/ml were determined with human-derived standards and the Monolisa Anti-HBs Detection Kit (Sanofi Diagnostics Pasteur).

Table 1. Immune responses in mice with IM, ID, and GG delivery

Immunization Route/Method	IgG2a/IgG1				
	4 weeks	9 weeks (no boost)	9 weeks (1 boost)	12 weeks (no boost)	12 weeks (2 boosts)
IM	1.2	—	—	2.7	3.2
ID	0.2	—	—	0.9	1.3
GG	0.01[a]	0.003[a]	0.002	—	—

BALB/c mice were immunized by IM or ID injection with 100 μg pCMV-S2.S or by GG delivery with 1.6 μg. At 4 and 6 weeks some mice were boosted in an identical manner to prime. HBsAg-specific antibodies of IgG1 or IgG2a isotypes were determined by end-point dilution ELISA assay in plasma taken at various time points after immunization. Titers were defined as the highest plasma dilution resulting in an absorbance value two times that of nonimmune plasma, with a cut-off value of 0.05. The IgG2a-to-IgG1 ratios were determined, with a value >1 indicating a predominantly Th-1 like response.

[a]There was no detectable IgG2a with these groups, thus a value of 1 was assigned for IgG2a to determine IgG2a/IgG1.

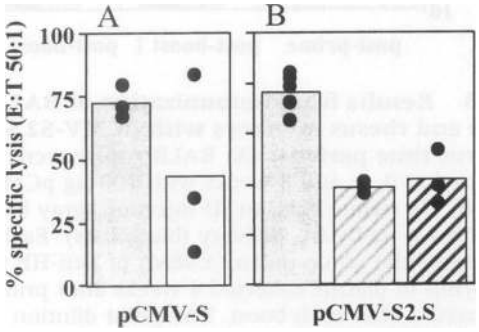

Fig. 4. Results from immunization of BALB/c mice with pCMV-S and pCMV-S2.S. (A) BALB/c mice were immunized with 100 μg pCMV-S by IM (gray bars) or ID (white bars) injection. Spleens were removed 4 weeks later and CTL activity was determined. Bars represent the mean HBsAg-specific lysis as a percentage of the total possible lysis (% specific lysis) at an effector-to-target ratio (E:T) of 50:1, and black dots represent % specific lysis for individual mice. (B) BALB/c mice were immunized with 100 μg pCMV-S2.S by IM (gray bars) or 1.6 μg by GG (diagonally striped bars) delivery. Mice immunized by GG were boosted in identical manner 2 weeks (light gray striped bars) or 4 weeks (black striped bars) later. CTL activity was determined on spleens removed 6 weeks after priming for IM and ID groups, or at 2 or 4 weeks after boost (which was carried out 4 weeks post-prime) for GG groups. Bars represent the mean HBsAg-specific lysis as a percentage of the total possible lysis (% specific lysis) at an effector-to-target ratio (E:T) of 50:1, and black dots represent % specific lysis for individual mice.

Humoral Response in Monkeys

RESPONSE AFTER PRIMING. After administration of pCMV-S2.S or pCMV-S DNA, anti-HBs were not detected by 4 weeks in the ID or GG groups (0/8) and detected in only one of eight (60 mIU/ml) of the IM groups. By 8 weeks after priming, two monkeys in the GG group (2/4) had seroconverted, as had one monkey in the ID group (1/4) by 12 weeks.

EXPERIMENT 1 (BOOSTING AT 4 AND 8 WEEKS). Although no monkeys in this experiment had seroconverted by the time of the first boost, by 2 weeks post-boost, low levels of anti-HBs (10–100 mIU/ml, $p = 0.69$) were detected in plasma of some monkeys immunized by IM (2/4) or ID (3/4), but not by GG (0/4). However, by 4 weeks after the first boost, one of the monkeys immunized by GG had seroconverted (16 mIU/ml). By 2 weeks after the second boost the number of responders increased for the IM and ID groups (3/4 and 4/4, respectively) and by 4 weeks post-boost for the GG group (2/4), with all animals attaining low to moderate levels (<1000 mIU/ml) and no significant differences among groups ($p = 0.3$) (Fig. 3B).

EXPERIMENT 2 (BOOSTING AT 12 AND 24 WEEKS). At the time of the first boost, only a few monkeys had seroconverted (IM 1/4, ID 1/4, GG 2/4). By 2 weeks after a 12-week boost, most monkeys had seroconverted (IM 4/4, ID 2/4, GG 3/4) and anti-HB titers had peaked at 62 ± 54, 816 ± 461, and 1827 ± 742 mIU/ml for the ID, IM, and GG groups, respectively. However, over the following 6 weeks, anti-HB levels decreased 2- to 6-fold in all groups. A second boost given at 24 weeks raised anti-HB levels ~20-fold by 2 weeks later for all groups and brought about seroconversion

in one more monkey of the ID group. Anti-HB titers decreased by 2- to 6-fold over the subsequent 6 weeks (data not shown). Overall, peak anti-HB titers attained with GG immunization were 5- and 100-fold higher than those with IM and ID delivery, respectively.

EFFECT OF TIMING OF BOOSTS. It is possible to compare timing of boosts for the IM and ID groups where dose was kept constant. The anti-HB response with IM-immunized monkeys was improved by lengthening the rest period between immunizations. Anti-HB titers attained for monkeys immunized IM were greater when boosted at 12 and 24 weeks than those at 4 and 8 weeks ($p = 0.008$, $p = 0.019$ for 2 weeks post-boost 1 and 2, respectively). In contrast, an increase in anti-HB levels was not seen with the increased rest period for ID-immunized animals ($p > 0.05$) (Fig. 3B,C). It is not possible to evaluate this effect with GG since the dose was also changed.

Discussion

DNA vaccines have been shown to induce strong humoral and cellular immune responses against numerous bacterial, viral, and parasite antigens in animal models. This, combined with the many potential economical and practical advantages that they offer, has led to intense interest in the development of DNA vaccines for use in humans. Unfortunately, early human clinical trials have not proven to be as successful as was predicted from preclinical studies, which were mostly carried out in mice. Considerable effort has been expended toward improving the efficacy of DNA vaccines through (*i*) improved efficiency of transfection with facilitated delivery methods such as liposomes (46), or DNA-compacting agents (47), and gene gun for direct intracellular delivery (48); (*ii*) improved vector design for high levels of expression and longer mRNA stability (1); (*iii*) addition of immunostimulatory CpG motifs to plasmid vectors (49); (*iv*) co-expression of co-stimulatory molecules or cytokines to augment immune responses (50,51); (*v*) optimization of immunization schedule (52,53); and (*vi*) combination with other vaccine approaches such as protein or attenuated viral vaccines (54,55). The route of delivery of the DNA vaccine can have an impact on the efficiency of transfection as well as the types and location of cells transfected, and thus potentially on the nature of the immune response (12–14,56,57).

Many different routes have been shown to be effective for DNA delivery in mice, as has been confirmed in the present study; however, few studies have compared responses obtained with different routes using the same antigen-expressing DNA, dose, and immunization schedule. There have been even fewer studies to compare routes of administration in non-human primates. We have attempted to bridge this gap by comparing the delivery of HBsAg-expressing vectors by 15 different routes in mice and 3 of the most common routes in rhesus monkeys.

When a large number of injected and noninjected routes were used to administer the DNA vaccine, anti-HBs were detected only in serum of mice treated by five of eight of the injected routes and by GG but none of the six noninjected routes. The highest levels of anti-HBs were induced by IM and IV injections, although reasonable titers were also obtained with sublingual and ID injections and GG delivery of DNA-coated gold particles to the epidermis. Each of these routes also induced CTL activity, but notably, CTL were also found with two other injected routes (perineum and vaginal wall injection) as well as one noninjected route (IN inhalation) that failed to induce antibodies. At one time it was thought that muscle was a preferred route for DNA vaccine delivery because early studies showed it to be more efficient than other tissues for the uptake and expression of plasmid DNA encoding reporter genes (58) and its postmitotic nature allowed expression from the episomally located plasmids to continue for many months (59). However, more recent studies with antigen-encoding plasmids have shown that antigen expression does not continue indefinitely, but rather is lost by some immune-mediated mechanism around 2–3 weeks after DNA injection (7). Furthermore, even though immune responses are possible following transplantation of antigen-expressing myoblasts (60), only professional antigen-presenting cells (APC) are actually capable of priming immune responses (60–63). Nevertheless, on the basis of findings that immune responses were unaffected when the injected muscle was removed within seconds of injection (64), it is uncertain what role the relatively few antigen-expressing muscle fibers play after direct injection of DNA vaccines into muscle. Indeed, it is possible that direct transfection of APC may be necessary in the case of DNA vaccines, and following IM injection, such transfected APC are

found in regional lymph nodes (65). However, it is not clear whether transfection occurs only at the site of DNA administration or whether the DNA can travel to lymphoid tissues and transfect cells there. Successful delivery routes appear to deliver the DNA to regions of concentrated lymphoid tissue or organs: IV and IP to the spleen, ID to Langerhan cells, IN and SL or VW injections to the extensive, diffuse interstitial lymphoid tissues of the naso-oropharynx and genitourinary systems, respectively. Why some approaches induced CTL without antibodies is difficult to say, although this may be largely a question of efficiency since these routes tended to have the weakest CTL responses. The generally absent responses with the noninjected routes were not unexpected, as the mucosal surfaces are protective barriers, physiologically designed to limit uptake of bacteria, viruses, and antigens, and, unless the mucosal surface has been broken or damaged, transfection efficiency using naked DNA is low. Efforts to circumvent this have included formulation of plasmid DNA with cationic lipids (24,29,32), the use of mucosal adjuvants (27,28,32), microencapsulation for oral delivery (30,31,66), or physical penetration of naked DNA into mucosal tissue using a GG (34,36).

A number of factors appear to influence the Th bias of the response, including (*i*) the antigen (20); (*ii*) the dose of antigen (67); (*iii*) whether the antigen is secreted, cytoplasmic, or membrane bound (68,69); (*iv*) the route and method of DNA administration (13,14,63); (*v*) the number of immunizations (14,70,71); (*vi*) the presence of CpG motifs (10,72); (*vii*) the haplotype of the mouse immunized (73); (*viii*) the presence of adjuvant (27,28,32); (*ix*) co-expression of cytokines (74,75); (*x*) whether DNA is formulated (e.g., with cationic liposomes) (24,29,32); and (*xi*) rest period between immunizations (51,52). Previous studies have reported Th2-like or mixed Th1/Th2 responses with DNA delivery to the skin and more Th1-like responses with IM injection (13,14,19,76,77), and this has been corroborated in the present study. However, antibody isotype did not change as a result of booster immunizations in the present study, which is in contrast with findings of other studies (14,70). These differences may be due to the nature of the encoded antigen, which can affect the antibody isotypes induced (20). While it is not clear exactly what factors determine the Th bias of responses with DNA vaccines, comparisons between studies should take into account the time at which antibody isotypes are determined and whether it is a primary or secondary response.

Anti-HB isotypes did not always correlate with CTL responses. For example, low CTL were found with SL administration despite predominantly IgG2a antibodies (Th1-like), and strong CTL were found with ID concomitant with a Th2-like antibody response. Although IgG2a and CTL are both considered indicative of Th1 responses, we have also noted such disassociation of antibody isotype and CTL activity with mucosal administration of antigen (45) or with DNA-based immunization of neonatal mice (44). Thus precise evaluation of the Th response may also require analysis of cytokine secretion from antigen-stimulated Th cells in vitro.

The realization that results in mice often do not predict the situation in humans has also led to a large number of DNA vaccine studies in non-human primates, including Aotus monkeys (23), rhesus monkeys (51,78–80), and chimpanzees (17,38,81–84). IM injection of plasmid DNA vaccines, while highly immunogenic in mice (see refs. 1,2) was found to be only relatively so in chimpanzees (17,38,79,83), and essentially not all in Aotus monkeys (23). Furthermore, although early human studies have demonstrated the safety and potential of DNA vaccines, results obtained have not been as good as predicted from animal models (3–6). Collectively, these results indicate that no animal model may be ideal for prediction of efficacy in humans. The relatively greater efficacy of IM in mice than primates may be related to morphological differences. Alternatively, it may be more related to dosage. The 10- to 100-μg doses of DNA vaccine typically used in a 20-g mouse would be equivalent to 35 to 350 mg in a 70-kg human, a dose range greatly in excess of what has been used to date in human clinical trials.

In summary, mice may have limited value for choosing the best route of DNA vaccine delivery for humans. While efficacy in murine models has preceeded the successful development of many human vaccines, it is probably safe to say that any vaccine that works in a human will work in a mouse, but not necessarily vice versa. Therefore, it is difficult to predict from mouse studies the potential of a new vaccine for humans. In fact, in those human trials that have been carried out, none of the DNA vaccines induced the strong immune responses that had been seen in mice with the same vectors. Furthermore, although non-human primate models are frequently used for development and testing

of human vaccines, it is not clear how predictive they will be in the case of DNA vaccines where efficacy, by virtue of the requirement first to transfect cells and express the antigen, relies on many factors other than immunological responses to the antigen. We will not know the answer to this until after greater experience has been achieved in non-human primates and human clinical trials.

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