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Intranasal Immunization of a DNA Vaccine with IL-12- and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)-Expressing Plasmids in Liposomes Induces Strong Mucosal and Cell-Mediated Immune Responses Against HIV-1 Antigens[1]

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A DNA vaccine constructed with the CMV promoter conjugated to *env* gp160 and *rev* genes has been shown to induce an effective Th1-type immune response when inoculated via an intramuscular route. In the present study, we obtained high levels of both humoral and cell-mediated immune activity by intranasal administration of this DNA vaccine. The production of mucosal IgA Ab in feces and vaginal fluid was stimulated significantly by intranasal DNA administration. This route of administration resulted in a significant level of HIV-1-neutralizing Abs in feces and serum. Cytokine assays revealed that intranasal administration of this DNA vaccine induces a Th2-type immune response. Interestingly, cationic liposomes greatly enhanced these activities. Abs against HIV-1 were present for at least 10 mo. Coadministration of the DNA vaccine with IL-12- and granulocyte/macrophage-CSF-expressing plasmids induced high levels of HIV-specific CTLs and an increase in delayed type hypersensitivity when administered by the intranasal route. These results clearly demonstrate that intranasal administration of this DNA vaccine with liposomes, together with IL-12- and/or granulocyte/macrophage-CSF-expressing plasmids, induces a strong level of anti-HIV-1 immune response. *The Journal of Immunology,* **1997, 159: 3638–3647.**

The induction of strong mucosal and cell-mediated immunity (CMI)[3] are important elements to consider in constructing an effective HIV-1 vaccine. We have designed a plasmid DNA vaccine (1) to achieve these goals. Our first objective was to stimulate HIV-specific mucosal immunity. Immunologically competent lymphocytes and Abs in mucosal sites form a critical first line of host defense against local HIV-1 infection. Our previous results suggested that mucosal IgA Ab inhibits HIV-1 replication (2). Although a variety of immunization protocols using HIV-1 vaccines have been shown to induce the production of serum neutralizing Abs after systemic vaccination, immunization via a systemic route rather poorly induces a secretory IgA immune response (3–6). Since circulating IgG and IgM only poorly penetrate mucosal surfaces, there is usually little relationship between resistance to mucosally associated infections and levels of circulating IgG or IgM Ab. Tissues of the female reproductive tract, urethra, and several mucosa have cells characteristic of mucosal effector tissues, including cells that have been shown to produce mucosal IgA Abs (7). There are some reports that intranasal (i.n.) administration of protein or peptide Ags induces both IgA and IgE Abs (5, 8, 9), which correlates with the activation of the Th2-type immune response (5). However, there have been only a few studies concerning i.n. administration of DNA plasmids (10). The main purpose of the present study was to determine whether i.n. DNA vaccination is capable of inducing a high level of HIV-1-specific mucosal immunities.

Work leading up to the present study involved the construction of a DNA vaccine from HIV-1 *env* and *rev* genes. Immunization with this vaccine resulted in a strong CMI response to HIV-1 gp160 Ag (1). This DNA vaccine induces Env and a minute amount of Rev proteins and facilitates CTL production (1, 11, 12). Use of cationic liposomes was thought to be advantageous for vaccine delivery as they have been reported to be effective adjuvants in immunization (14–16). Therefore, we tested whether i.n. administration of DNA vaccine with cationic liposomes could activate both mucosal immunity and a strong CMI.

To achieve protective immunity against HIV-1 infection, the induction of high CTL levels has been shown to be important (17–19). IL-12 enhances various types of CMI by Th1 cell activation (20–27). In addition, GM-CSF has been reported to enhance Ab responses (28). In this regard, we studied whether i.n. DNA vaccination with IL-12 and/or GM-CSF expression plasmids in liposomes could activate HIV-specific immune responses. Coadministration of IL-12 and HIV-1 expression plasmids by i.n. vaccination significantly enhanced HIV-1-specific CTL production. Coadministration of GM-CSF and HIV-1 expression plasmids enhanced both Th1- and Th2-type responses. Additional

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[3] Abbreviations used in this paper: CMI, cell-mediated immunity; GM-CSF, granulocyte/macrophage colony-stimulating factor; CT, cholera toxin; DTH, delayed-type hypersensitivity; i.n., intranasal or intranasally; V3 region, third hypervariable region.

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activation of HIV-specific Th1 and Th2 cells was observed by mixing these two cytokines. We conclude that i.n. vaccination using plasmid DNA vaccines encoding HIV Ags plus appropriate cytokine plasmids may be a useful approach for inducing protective immunity against HIV.

Materials and Methods

HIV viruses

HIV-1 strains of III_B were kindly donated by the National Institutes of Health AIDS Research and Reference Reagent Program.

Animals

All BALB/c (H-2^d) mice, 6 to 10 wk old, were purchased from Japan SLC, Inc., Hamamatsu, Japan.

DNA vaccine

DNA encoding the *env* region of the HIV-1 III_B strain was excised from clone pSMTE7 (29) by cleavage with *Sal*I and *Bpu*1102I and cloned into the expression plasmid pBC12/CMV at the *Hin*dIII and *Bam*HI sites to produce pCMV160IIIB, as reported previously (1, 30). The pCMV160IIIB plasmid, therefore, expresses the precursor gp160 envelope region of the HIV-1 provirus under the control of the immediate early promotor of CMV. pcREV contains the full-length HIV-1 regulatory *rev* gene linked to the CMV promoter sequence and has been shown to efficiently express HIV-1 Rev (31).

Mouse IL-12 expression plasmid, designated pCAGGSIL-12, was constructed as follows. Both mouse IL-12 p35 and p40 cDNAs, kindly provided by Dr. S. Wolf, Genetic Institute, Cambridge, MA, were inserted into the *Eco*RI site of the pCAGGS expression vector (32), resulting in pCAGGS-p35 and pCAGGS-p40, respectively. The expression unit for IL-12 p35, including the CMV immediate early enhancer-chicken β-actin hybrid promoter, IL-12 p35 cDNA, and a rabbit β-globin poly(A) signal, was excised from pCAGGS-p35 and was inserted downstream of the IL-12 p40 expression unit of pCAGGS-p40. The pCAGGSIL-12 is described in Figure 1*A*. The expression plasmid of mouse IL-4, which was a conjugate of the pCAGGS expression vector (Fig. 1*B*). The expression plasmid of mouse GM-CSF (28, 33) was provided by Dr. H. L. Davis, Loeb Medical Research Institute, Ottawa, Canada.

Preparation of liposomes

The detailed method for cationic liposome preparation has been reported previously (34). A mixture of 3β(*N*(*N'*,*N'*-dimethylaminoethane) carbamoyl) cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) in chloroform was dried, vacuum desiccated, and resuspended in sterile HEPES buffer (pH 7.8). After hydration, the dispersion was sonicated to form liposomes with an average diameter of 150 to 200 nm. Before injection, an appropriate amount of DNA plasmids in 0.15 mol PBS, pH 7.2, was mixed with the above liposome solution at a volume ratio of 17:3. These mixtures were vortexed and injected.

Immunization and DNA inoculation

Direct inoculation of the DNA vaccine into the gastrocnemius or quadriceps muscles of mice was performed as previously described (1, 35). Most of the data described in this paper were obtained by immunizing mice in the gastrocnemius muscles. In some cases, however, we inoculated into the quadriceps or biceps muscles to compare the immunogenicity of this DNA vaccine at different sites. No differences in immune activities have been reported between these muscles (34). Mice were immunized with a total of 1 to 10 μg of DNA vaccine in PBS mixed with 15% cationic liposomes before inoculation. The HIV-1 DNA vaccine was a mixture of pcREV DNA and pCMV160IIIB DNA used at a ratio of 1:5. An appropriate amount of cytokine plasmids was mixed in the same PBS. Instead of the liposomes, in some cases, we used 10 μg of cholera toxin (CT; List Biologic Laboratories, Campbell, CA) as adjuvant.

The i.n. route has been described in detail elsewhere (36). Mice were anesthetized with ethyl ether, and after about 20 s, 30 μl of the mixture of DNA vaccine and other reagents were dropped into the nasal cavity. The mice inhaled these preparations simply by breathing. The same procedure employing the same dose of immunogen was used for repeat immunizations on days 7 and 21. Five to seven days after the last boost, immune responses were studied.

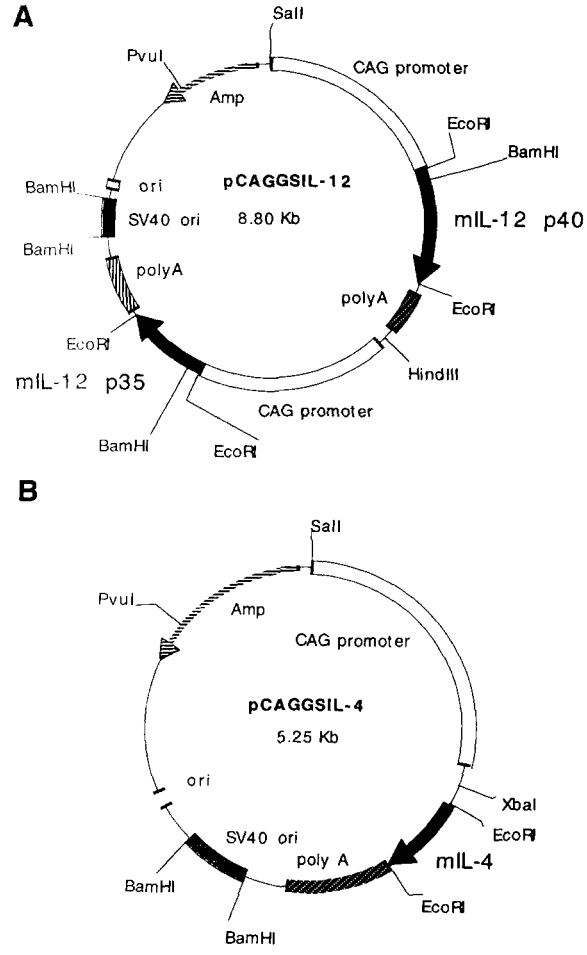

FIGURE 1. Genetic maps of pCAGGSIL-12 (*A*) and pCAGGSIL-4 (*B*).

ELISA

ELISA was performed as described previously (37, 38). First, 96-well microtiter plates were coated with 10 μg/ml of IIIB V3 (TRKSIRIQRG PGRAFVTIGKIGN) long peptide (2) or 1 μg/ml of gp160 protein, which were donated by the National Institutes of Health AIDS Research and Reference Reagent Program. The wells were rinsed with 0.15 M PBS and then blocked with 3% BSA in PBS for 1 h. Mouse sera were added and incubated for 1 h at 37°C. Following washing, the bound Ig was characterized using affinity-purified horseradish peroxidase-coupled goat anti-mouse IgG + A + M (H + L) + E (Pierce Chemicals), or horseradish peroxidase-coupled goat anti-mouse isotype-specific Abs for IgG1, IgG2a, and IgM (Southern Biotechnology Associates). For the estimation of secretory IgA (s-IgA) Ab against the V3 peptide, rabbit anti-rat secretory component (SC) Ab (kindly provided by Dr. B. Underdown, McMaster University Medical Center, Ontario, Canada) was also used. The titers were reported as the reciprocal number of the final detectable dilution, which gave an OD_{490} of $\geq$0.2 OD units.

Sample collection

Fecal extract samples were prepared as described elsewhere (2). Briefly, after 100 mg of fecal pellets were mixed with 1 ml of PBS, samples were spun on a vortex mixer, left to settle for 15 min, respun until all materials were resuspended, and centrifuged at 12,000 rpm. The supernatants were then collected and tested. Vaginal fluid samples were obtained by the method described by Staats et al. (5). Vaginal wash samples were collected by flushing the vaginal cavity with 100 μl of sterile PBS while mice were under isoflurane anesthesia. Estimations were made by comparing cpm values of the vaginal wash samples of preimmunized and immunized mice. Each vaginal sample was centrifuged in a microcentrifuge to remove particulate matter. All samples were stored at −20°C until the Ab assay. This procedure resulted in an approximate 10-fold dilution of vaginal samples, as determined by the group referred to above (5), and we were able to

confirm this. In this study, we described the value of Ab titers of undiluted original samples.

Antifusion assay

A previously described antifusion assay was used for measuring the neutralizing activity of the fecal solution (2). CEM cells were infected with HIV-III_B and then cultured for approximately 14 days. Stably infected CEM cells were first incubated for 2 h with several concentrations of immune sera or with fecal extracts. Uninfected MOLT-1 cells were then added to this culture system (infected cells:uninfected cells = 1:10). After the cells were incubated for 24 h at 37°C, giant cells were counted. The fusion assay evaluation was done in a blinded fashion. The neutralizing activity was expressed as the percentage of the reduction in syncytium formation by the fecal extract solution from immunized mice compared with that from preimmunized mice. The same experiments were performed several times, and we have provided representative data only.

Neutralization assay using p24 protein detection method

The p24 protein assay was performed by a method described elsewhere (2). A 4% solution of immune antisera or fecal extracts, as well as column-adsorbed fecal samples from each immunized or preimmunized mouse, was added to medium containing HIV-1 and incubated for 1 h as previously described (2). Uninfected CEM were then added to the medium containing the virus. After 4 h, the cells were washed and cultured for 5 days. The concentrations of p24 protein in the filtered cell-free supernatants were measured using an HIV-1-specific enzyme immunoassay (Abbott Laboratories, North Chicago, IL) according to the manufacturer's instructions. The concentration of p24 (ng/ml) was determined from a standard curve derived from dilutions of the known standard.

Cytokine assays

Two million regional lymphoid cells and two million spleen cells were vaccinated and restimulated in vitro in the presence of the same number of irradiated (30 Gy) syngeneic spleen cells pulsed with V3 (HIV-$1III_B$, RG PGRAFVTI) (2, 5) peptide in a 24-well plate. In other groups, 5 μg/ml of Con A or 1 μM of ionomycin and 10 ng/ml of PMA were added to the culture systems to examine cytokine synthesis. After 24 h, IL-4 in the culture supernatants was quantitated by an IL-4-specific ELISA. For the IL-4 ELISA, supernatants were collected and mAb BVD6-1011 (anti-IL-4, PharMingen, San Diego, CA) was used as the capturing agent. Then, BVD6-24G2-biotin (PharMingen) was used as biotinylated developing Ab. For the IFN-γ ELISA, supernatants of the 48-h cultures were collected and mAb R4-6A2 (anti-IFN-γ) was used as the capturing agent. XMG 1.2-biotin (PharMingen) was also used. Avidin-conjugated alkaline phosphatase was used in a colorimetric assay (39) to detect the presence of bound second Ab and human cytokine. The concentration of lymphokines was determined using a standard curve prepared for recombinant murine IFN-γ or IL-4 (both from PharMingen).

Footpad swelling response

The footpad swelling response was measured as previously described (40). The appropriate amount of DNA vaccine with or without liposomes was administered i.n. to mice on days 0, 7, and 21. As a control, we immunized with 5 μg of sperm whale myoglobin. For the first 15 days, 300 μg of purified IgG fraction of sheep anti-mouse IL-12 Ab or 100 μg of XMG1.2 (a rat IgG1 mAb to mouse IFN-γ provided by Dr. J. Miller DNAX research Institute, Palo Alto, CA) was i.v. injected every 2 days. The amount of Ab injected into mice was almost the same as other previous reports (41–43). Six days after the last vaccination, 25 μg of III_B V3 peptide RIQRG PGRAFVTIGK (44, 45), 10 μg of gp160 or 25 μg of sperm whale myoglobin peptide (ISEAIIHVLHSRHP, Gorai et al. (46)) were injected into mouse footpads. We also used V3 peptide in the tests of cell-mediated immune responses, because the sequence of V3 peptide (RGPGRAFVTI) was reported to overlap for the CTL and helper cell epitopes of HIV-1 IIIB (44, 45). After 24 h, the extent of footpad swelling was estimated as the difference in thickness in units of 10^{-2} mm between the preinjected and postinjected footpads.

HIV-specific cytotoxic test

Two micrograms of DNA vaccine with various adjuvants or liposomes were administered i.n. or i.m. to mice on days 0, 7, and 21. As above, for the first 15 days 300 μg of anti-IL-12 Ab or 100 μg of anti-IFN-γ Ab was i.v. injected every 2 days.

Five to seven days after the last DNA vaccination, spleen and regional immune lymph node cells were collected. Then, 1×10^6 lymphoid cells from the immunized mice were restimulated in vitro in the presence of irradiated (30 Gy) syngeneic spleen cells pulsed with 5 μg/ml of V3 peptide (RGPGRAFVTI) (2, 5). After culturing for 5 days, the cytotoxic activity of these spleen cells was measured by a 6-h ^{51}Cr release assay using V3 peptide-pulsed target cells (47). The target cells were prepared using the same III_B V3 peptide pulsed onto Con A blasts or P815 ($H\text{-}2^d$) cells. These treated cells were labeled with [^{51}Cr]Na_2O_4 (sodium chromate; Amersham Japan, Tokyo, Japan), washed thoroughly, and used as targets. In some cases, effector cells were incubated with purified anti-CD8 mAb (Accurate, Westbury, NY) for 1 h at 4°C. After washing, cells were incubated with a 1:10 dilution of low-toxicity rabbit complement (Accurate) for 1 h at 37°C. Cells were then washed and used for the cytotoxicity assay. E:T ratios ranged from 5:1 to 80:1. The percentage of specific ^{51}Cr release was calculated as 100 × (experimental release − spontaneous release)/(maximun release − spontaneous release). Target cells incubated in medium alone and with 5% Triton X-100 were used to determine the spontaneous and the maximum release of chromium, respectively.

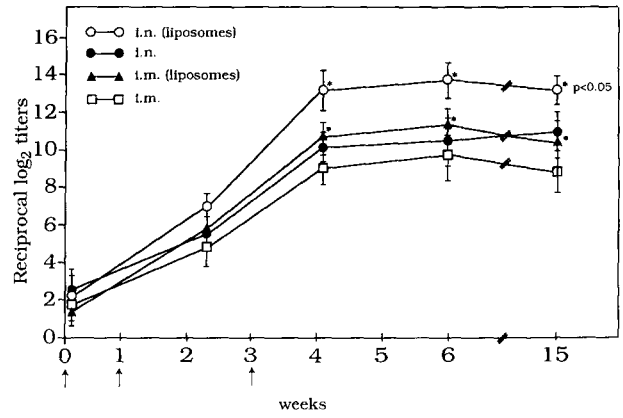

FIGURE 2. Time course of anti-gp160 IgG titers in immunized mice. Two micrograms of DNA vaccine were administered i.n. or i.m. on days 0, 7, and 21, and serum Ab titers were assayed by gp160-coated ELISA. Arrows indicate the day of immunization. Data are mean titers ± SE of five to eight independent mice. This is a representative result of three separate experiments. *, Signifies a statistically significant difference ($p < 0.05$) between the group i.m. administered DNA plus cationic liposomes (▲) and i.n. administered DNA plus liposomes (○).

Data analysis

All values were expressed as means ± SE. Statistical analysis of the experimental data and controls was performed using the two-tailed Student's *t* test.

Results

Serum Ab responses to i.n. immunization

The HIV DNA vaccine was administered i.n. on days 0, 7, and 21, and antisera collected 5 days after the third immunization. We initially studied the dose-response curve induced by i.n. DNA vaccination. When we used V3 IIIB long peptide as coating Ag for the ELISA assay, vaccine doses of 0.1, 2, 5, and 10 μg led to mean $\log_2$ Ab titers of 2.5 ± 1.2 (SE), 8.2 ± 1.8, 9.3 ± 2.1, and 10.0 ± 1.6, respectively. But when we used gp160 as a coating Ag, Ab titers of sera obtained by 0.1, 2, 5, and 10 μg of vaccine were 3.6 ± 1.4, 9.0 ± 2.4, 10.1 ± 2.4, and 11.3 ± 2.5, respectively. Therefore, we generally used a dose of 2 μg of vaccine, and serum Ab titers were determined using gp160 as a coating Ag. The serum Ab titers were compared for mice immunized with 2 μg of DNA injected into three kinds of muscles. The mean Ab titers of sera obtained by immunization into the gastrocnemius, quadricep, and bicep muscles were 8.7 ± 2.0, 8.6 ± 1.7, and 8.8 ± 1.6, respectively. From these results and considering the previous reports (33), we used both quadriceps and gastrocnemius muscles for i.m. DNA vaccination. We next studied the time course over which serum Abs were induced following i.n. or i.m. immunization (Fig. 2). Two micrograms

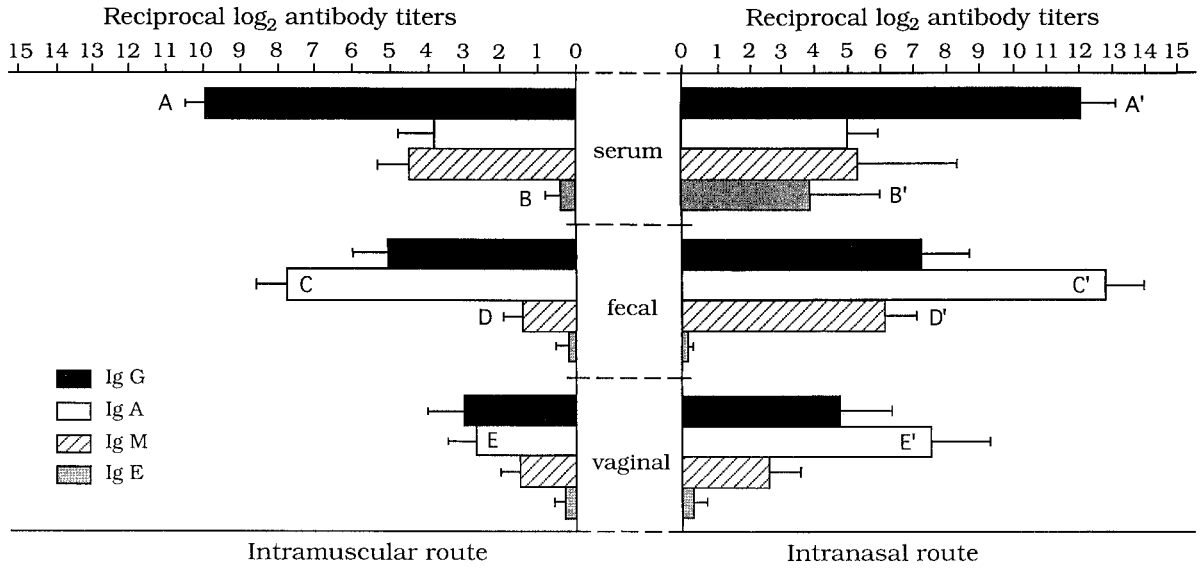

FIGURE 3. Comparison of the Ig isotypes and titers of serum, fecal extract, and vaginal fluid samples from mice immunized with DNA vaccine plus liposomes into gastrocnemius muscles or through the i.n. route. On days 0, 7, and 28, the vaccine was administered. Five days after the third immunization, fecal extract and vaginal fluid samples were collected, and Ig isotypes were measured by ELISA using long V3 peptides as a coating Ag. The data represent the mean ± SE of four to six mice. All experiments were repeated three times. There exists a statistically significant difference between A vs A′, B vs B′, C vs C′, D vs D′, and E vs E′ at $p < 0.05$.

of DNA vaccine, with or without cationic liposomes, were used to immunize mice, and Ab titers of each group of animals were compared. Intranasal administration of the vaccine combined with cationic liposomes induced the strongest and longest-lasting Ab response (Fig. 2). The i.n. route of immunization induced higher titers of HIV-specific IgG Abs than did the i.m. route. Moreover, serum IgG Ab titers after 10 mo by i.n. and i.m. immunization were 9.6 ± 1.1 and 7.3 ± 0.9, respectively.

Comparison of Ig isotype of serum and mucosal Abs

The Ig isotype and titers raised by DNA vaccination of serum, fecal extracts, and vaginal fluid were then examined. In our experiments, the Ab titer of fecal extracts and vaginal fluid by ELISA was clearly detected using the long V3 peptide as a coating Ag (2). We therefore used this peptide in the mucosal Ab assay. gp160 protein was used when the levels of serum Ab were determined. The serum IgG titer was the highest, whereas IgM and IgA serum levels were considerably lower (Fig. 3). IgE Abs were detectable in serum with i.n. immunization only. Fecal extract samples contained more IgA than IgG following i.n. and i.m. vaccination. However, IgA Ab levels were greater than IgG levels in vaginal fluids. The titer of mucosal IgA Ab was rather enhanced when DNA vaccine was i.n. administered. The titers of anti-SC Ab in fecal and vaginal samples were 5.3 ± 1.2 and 5.5 ± 0, respectively, whereas the level of this Ab in serum was undetectable. Compared with the i.m. route, i.n. vaccination elicited higher levels of mucosal IgA. Seventeen weeks after the last i.n. administration of vaccine, the mean IgA Ab titers of serum, fecal, and vaginal samples were 4.0 ± 0.6, 8.8 ± 1.7, and 4.6 ± 0.6, respectively, whereas those obtained by i.m. DNA vaccination were 3.5 ± 0.2, 5.3 ± 1.1, and 3.6 ± 0.8, respectively. These results suggest that i.n. administration of the DNA vaccine induced higher levels of secretory IgA Ab in the mucosas of both gut and vagina than i.m. administration.

Changes in Ab levels using cytokine expression plasmids or various adjuvants

The next experiments evaluated adjuvants and cytokines for their potential to enhance the production of HIV-specific serum Abs following i.n. vaccination. As shown in Figure 4, cationic liposomes enhanced serum Ab levels when used in combination with the vaccine. When the IL-4 or GM-CSF expression plasmid was included in the vaccine, a significant increase in serum Ab levels occurred. A CT exhibited no adjuvant activity under this condition. Coinjection of vaccine plus an IL-12 expressing plasmid also failed to modify HIV-specific serum Ab titers. Coadministered GM-CSF and IL-12 expression plasmid resulted in Ab titers comparable to that of mice given GM-CSF plasmid as adjuvant.

We then analyzed fecal IgA levels and the immunomodulatory effects of adjuvants and cytokine plasmids following i.n. administration of the vaccine (Fig. 5). Fecal IgA Ab titers were significantly greater after i.n. vs i.m. immunization in mice immunized with vaccine alone in the absence or presence of liposome. The coadministration of IL-4 or GM-CSF expression plasmid further increased the level of fecal IgA. Coadministration of IL-12 expression plasmids did not modify fecal IgA Ab levels. Again, however, a strong level of anti-V3 Ab was observed when we coadministered IL-12 plus GM-CSF expression plasmids. In the absence of cytokines, fecal Ab titers of i.n. vaccinated groups were significantly higher than the i.m. vaccinated groups. As mentioned above, these mucosal IgA patterns persisted for 15 wk.

Antifusion activity

The capacity of immune antisera to inhibit HIV-mediated fusion was then examined. Two groups of 6 to 7 mice were immunized i.n. or i.m. (gastrocnemius muscles) three times with the DNA vaccine. The antifusion activity of the resulting antisera were compared. The maximum dilution that inhibited giant cell formation by >50% was taken as the antifusion titer. The mean antifusion titer of the i.n. DNA administered group was 4.2 ± 0.7, whereas that of the i.m. group was 4.0 ± 1.2. The mean titer of the control sera

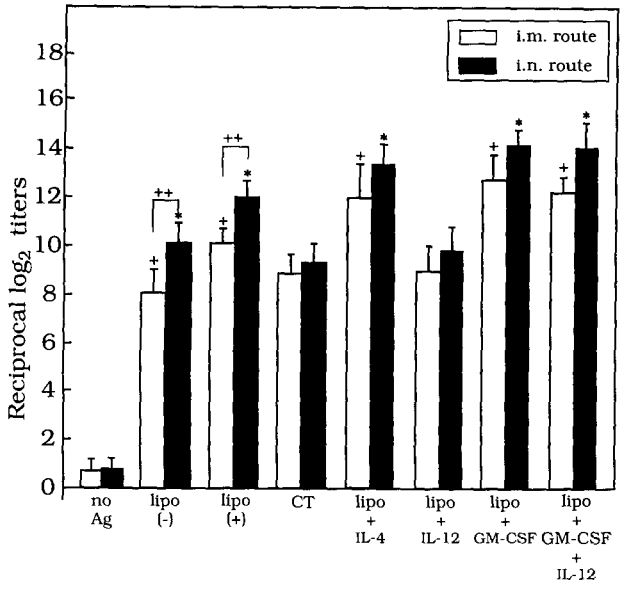

FIGURE 4. Changes in serum Ab response titers using various adjuvants and cytokine expression plasmids. On days 0, 7, and 21, 2 μg of DNA vaccine were administered with various adjuvants (liposomes (lipo), 10 μg of CT, and various cytokine expression plasmids (2 μg of IL-4, IL-12, and/or GM-CSF). On day 28, each serum was collected, and Ab titers were measured. Data are mean titers ± SE of seven to nine mice. Identical experiments were performed on two other groups of mice and produced almost the same results. * and $^{+}$, Signify statistically significant differences ($p < 0.05$) between the marked group and the DNA vaccine plus liposomes group; $^{++}$ signifies a statistically significant difference ($p < 0.05$) between two adjacent groups.

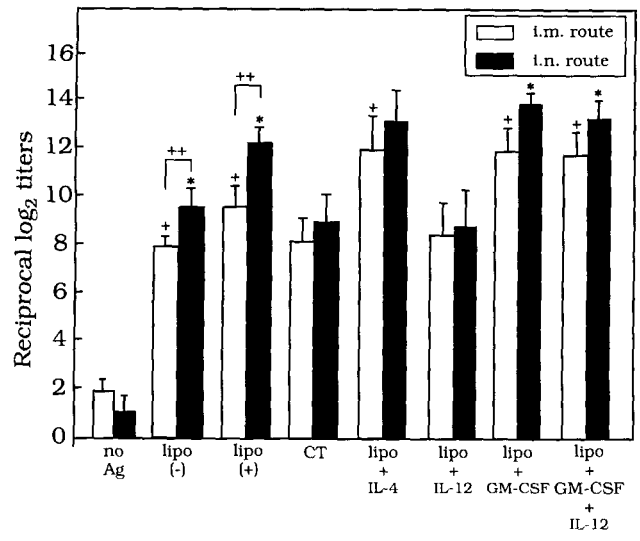

FIGURE 5. Changes in fecal IgA Ab titers using various adjuvants and cytokine expression plasmids. Samples were collected on day 28, and fecal IgA Abs were assayed. We used long V3 peptide as a coating Ag. Data represent mean titers ± SE of six to eight mice. This is a representative result of four separate experiments. Data analysis are the same as in Figure 4.

from the preimmunized group was 0.8 ± 1.2. These results suggest that the antisera obtained by i.n. or i.m. DNA vaccination were equally capable of inhibiting HIV-1-specific fusion.

The antifusion activity of immune fecal extracts was also studied. Using groups of five mice each, the mean antifusion titers of fecal extracts of mice immunized i.n. or i.m. with the DNA vaccine were 4.3 ± 1.8 and 3.4 ± 1.0, respectively. The antifusion titer ± SE of the preimmunized control group was 1.1 ± 1.3. These results again suggested that there was no significant difference in the antifusion activity of i.n. and i.m. immunized animals, although the titers of antifusion activity were generally low.

Inhibition of p24 protein production by Ab from immunized mice

We next determined whether these high-titered antisera or fecal extracts inhibited the growth of HIV-1. HIV-III_B was incubated with Ab preparations for 2 h. Virus replication was assayed by the p24 protein binding method. We then compared the inhibition of p24 production using sera or fecal extracts obtained from i.n. or i.m. DNA vaccinated mice (Table I). Both groups showed a substantial level of p24 inhibition. The mean inhibition value of preimmune fecal extracts was almost the same as those of control samples in Table I. There was no statistically significant difference between the i.n. and i.m. groups. Fecal Ab of animals given the combination of DNA vaccine with IL-4 expression plasmids slightly inhibited p24 protein production. This inhibition disappeared by adsorption of samples on both IgA and V3 peptide binding columns; however, the inhibition by Abs was weak. Similarly, coadministration of IL-12 and liposome with the i.m. vaccine did not modify the fecal response.

Table I. *HIV-1 neutralizing activity of Ab from immunized mice using a p24 protein assay*[a]

Serum or Fecal Samples	p24 Protein Production (ng/ml) HIV-1 III_B (mean ± SE)
I. i.n. administration	
Serum sample IIIB/REV	15.0 ± 0.8^{+}
Fecal sample IIIB/REV	15.3 ± 0.7*
IIIB/REV + IL-4 + liposomes	14.6 ± 1.7*
IIIB/REV + IL-4 + liposomes (adsorbed on a HIV-1 V3 peptide Ag column)	25.7 ± 3.5
IIIB/REV + IL-4 + liposomes (adsorbed on an anti-IgA column)	27.9 ± 2.2
II. i.m. administration	
Serum sample IIIB/REV	17.3 ± 1.1^{+}
Fecal sample IIIB/REV	16.2 ± 2.0*
IIIB/REV + IL-12 + liposomes	16.3 ± 3.1*
III. Nonimmune control	
Serum sample	28.3 ± 2.9†
Fecal sample	29.8 ± 5.2‡

[a] Five microliters of fecal extracts from five to seven BALB/c mice immunized i.n. or i.m. with DNA vaccine or DNA vaccine plus IL-4 expression plasmid. The same inclination was observed in three other independent experiments. * and + show statistically significant differences (*$p < 0.05$; +, < 0.05) against nonimmune control groups of serum (†) and fecal (‡) samples.

CMI responses

The next study examined whether i.n. administration of vaccine stimulated an enhanced CMI response. The DTH levels of the i.n. immunized groups were about the same as those of the i.m. immunized groups (Table II). Administration of IL-12 expression plasmid enhanced the degree of HIV *env*-specific swelling, whereas coinjection with IL-4 expression plasmid suppressed it. When IL-12 and GM-CSF expression plasmids were coadministered, the DTH response was greater than that with GM-CSF and comparable to that with IL-12. Injection of anti-IL-12 Ab strongly inhibited the DTH response. Neither plasmid vector alone (without

Table II. *Footpad swelling response of mice immunized by i.n. administration of HIV-1 DNA vaccine*[a]

Immunogens (μg/mouse)		Swelling Response (Mean ± SE × 10^{-2} mm)	
		III_B-V3 peptide	Myoglobin peptide
I. i.n. administration			
pCMV160IIIB/REV	0.2	14.3 ± 1.7†	NT
	0.2 + IL-12	18.9 ± 1.5*	NT
	0.2 + IL-12 + anti-IL-12 Ab	6.9 ± 2.0**,+	NT
	0.2 + IL-4	9.0 ± 0.8*	NT
	0.2 + GM-CSF	15.8 ± 1.9	NT
	0.2 + GM-CSF + IL-12	19.3 ± 3.1*	NT
	2.0	14.4 ± 1.6	1.6 ± 1.2
	20.0	13.6 ± 2.3	1.9 ± 1.0
II. i.m. administration			
pCMV160IIIB/REV	0.2	13.9 ± 0.7‡	NT
	0.2 + IL-12	17.3 ± 1.8§	NT
	0.2 + GM-CSF	15.9 ± 2.0	NT
	0.2 + IL-12 + GM-CSF	18.6 ± 3.6§	1.2 ± 0.7
	2.0	14.2 ± 1.2	1.5 ± 0.7
	20.0	15.7 ± 2.3	NT
III. Control			
i.n. pCMV empty	10.0	1.7 ± 0.1	1.4 ± 0.5
i.n. myoglobin	10.0	2.0 ± 0.3	14.0 ± 2.2

[a] BALB/c mice were i.n. or i.m. (*Musculus gastrocnemius*) immunized with each dose of DNA vaccine or myoglobin. Seven days after the second boosting, footpad swelling responses directed to III_B-V3 peptide (RGPGRAFVTI) were examined. For the first 15 days, 0.3 mg of anti-IL-12 Ab (anti-IL-12 Ab) was injected every 2 days. The data represent the mean ± SE of four to six mice. All experiments were repeated three times. NT, not tested. * and **, Show a statistically significant difference against the † and other groups (*, $p < 0.05$; **, $p < 0.01$). +, Indicates statistically significant differences at $p < 0.05$ between these two groups. §, Indicates statistically significant difference beween ‡ and other groups.

insert DNA) nor sperm whale myoglobin-immunized mice exhibited a swelling response when IIIB peptide was injected.

Experiments were performed to determine whether i.n. administration of the DNA vaccine induced a CTL response. BALB/c mice were treated with cationic liposomes and DNA vaccine together with cytokine plasmids. After the last immunization, spleen cells were collected and cocultured with V3 peptide-pulsed syngenic spleen cells. As shown in Figure 6*A*, both i.n. and i.m. immunization with the DNA vaccine alone induced a CTL response. However, coadministration of vaccine with liposomes, IL-12 plasmid, or GM-CSF and IL-12 expression plasmids induced greater CTL responses than vaccine alone (Fig. 6*B*). But GM-CSF expression plasmids did not show such a strong level of CTL (not shown). In contrast, immunization with liposomes, vaccine, and the IL-4 expression plasmid resulted in a lower level of CTL induction (Fig. 6*B*). These CTL responses were significantly suppressed by the in vivo injection of anti-IL-12 or anti-IFN-γ Ab (Fig. 6*C*), suggesting that IFN-γ as well as IL-12 play important roles in these plasmid-mediated immune responses. These results demonstrate that i.n. administration of DNA vaccine with IL-12 and GM-CSF expression plasmids induced the highest level of CTL activity of combinations evaluated. Our present results also demonstrate that i.n. administration of DNA also induced a significant CTL response.

Determination of IgG and subclasses of helper T cells

The Ig subclasses of the i.n. or i.m. immune antisera were determined using ELISA. Figure 7 shows that the mean Ig IgG1/IgG2a ratios of antisera obtained by i.n. administration of vaccine and liposomes with IL-4 expression plasmid, of vaccine and liposomes with IL-12 plasmid, or vaccine and liposomes were 5.1, 0.8, and 2.9, respectively. The mean IgG1/IgG2a ratios by i.m. administration of the vaccine with and without liposomes were 1.3 and 2.1, respectively. In addition, when DNA vaccination by the two routes was compared, i.n. administration induced higher IgG1 production than did the i.m. route, regardless of whether liposomes were employed. The IL-4 expression plasmids help to induce mainly IgG1 Ab, and the IL-12 expression plasmid helps to increase production of IgG2a. The coadministration of IL-12 and GM-CSF expression plasmids enhanced both IgG1 and IgG2a Abs.

The last experiment was designed to assay cytokine levels in culture fluids obtained by 24 or 48 h cocultures of spleen cells from animals immunized with vaccine and the IL-12 plasmid with V3 peptide-pulsed syngeneic spleen cells or with nonspecific reagents (Fig. 8). There was no great difference in cytokine production between groups subjected to i.n. or i.m. immunization when we cultured cells in medium only. However, lymphocytes from i.n. immunized mice showed elevated levels of IL-4 production when stimulated with Con A or PMA and ionomycin relative to i.m. immunized mice. In contrast, the level of IFN-γ increased in cultures of Con A or PMA and ionomycin to stimulated spleen to cells from mice i.m. immunized with IL-12 expression plasmid and DNA vaccine. A significant level of IFN-γ production was observed in the group when lymphoid cells from i.m. immunized mice were cultured with V3 peptide-pulsed syngeneic spleen cells. In contrast, only lymphoid cells from mice immunized i.n. with DNA vaccine plus the IL-12 expression plasmid expressed significant levels of IL-4 when these cells were cocultured with V3 peptide-pulsed syngeneic spleen cells. Lymphoid cells from nonimmune mice expressed undetectable levels of both IL-4 and IFN-γ. These results suggest that i.n. administration of DNA vaccine with the IL-12 plasmid induces both Th1- and Th2-type responses.

Discussion

Several studies have shown that i.n. administration of protein Ags induce strong immune responses (5, 8, 9, 48). However, there have been only a few reports of a systematic analysis of i.n. DNA vaccination. The present study shows that i.n. administration of an HIV-1 DNA vaccine induces strong Ab production, particularly production of mucosal IgA (Fig. 5). We also observed high titers and persistent serum IgG and fecal IgA Ab responses (Fig. 3).

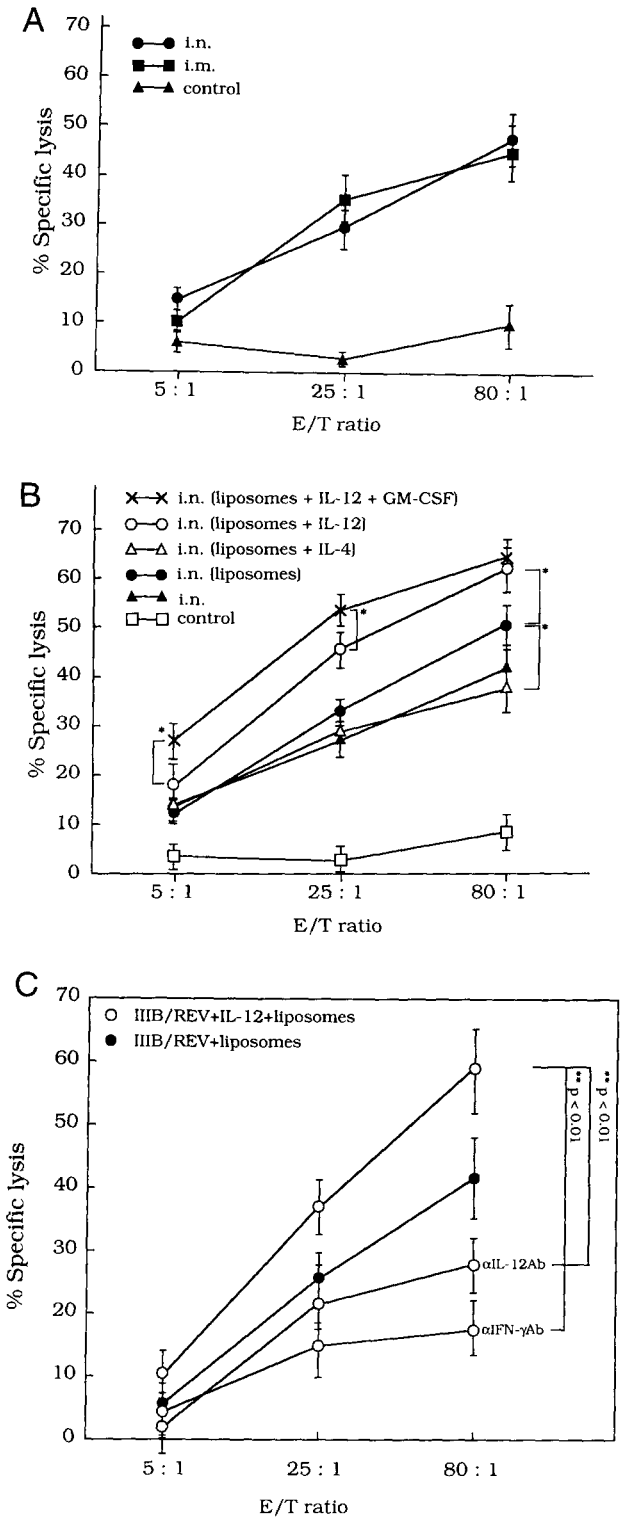

FIGURE 6. The CTL response of BALB/c mice to V3 peptide-pulsed target cells. Lymphoid cells from each immunized group were restimulated for 5 days. V3 peptide-pulsed syngeneic spleen cells were used as target cells, and restimulated lymphocytes were used as a source of CTL. Data are mean titers of four to five independent groups of three to four mice. Control, lymphoid cells from nonimmunized mice. *A*, Comparison of the CTL response to i.n. vs i.m. vaccination, *B*, Adjuvant effect of liposomes or cytokine on the DNA vaccine-mediated CTL response. *C*, In vivo inhibition studies by using anti-cytokine Abs. For the first 15 days, 0.3 mg of Abs was injected i.p. every 2 days. * and **, Signify statistically significant differences between the i.n. DNA vaccinated group and other groups (*, $p < 0.05$; **, $p < 0.01$).

There have been reports showing that i.n. immunization with influenza hemagglutinin (49), *Chlamydia trachomatis* (50), *Streptococcus mutans* Ag I/II (51), or recombinant *S. mutans* Pac (52) induces Ag-specific serum IgA as well as IgG production. Our previous studies revealed that oral administration is also effective at raising IgA Abs, but that this response was short lasting (2). The reason for these short periods of high Ab production or oral tolerance (53) is not yet clear; however, turnover of intestinal epithelium is faster than that of upper respiratory or pulmonary tissue. There is retention of Ag or DNA in the pulmonary tissues. The pulmonary tissues contain vast areas of epithelial cells that are directly exposed to the Ag. Since many lymphoid organs lie adjacent to these epithelial cells, this might explain the development of a good Ag-specific immune response. However, detailed analyses are necessary to explain the long-lasting high titer Abs induced by i.n. immunization.

HIV-specific IgA Ab titers in both fecal and vaginal fluid were highest when the vaccine was administered i.n. (Fig. 5), consistent with other reports (49–52). As shown in Table I, fecal extract inhibited HIV-1 replication, indicating that the IgA was functionally active. But the inhibition by fecal extract was weak. This type of HIV-1 inhibition was also observed using a peptide vaccine as the immunogen, as has been previously described in detail (2). Intranasal immunization induced IgA production in vaginal fluid as well as feces, suggesting this DNA plasmid immunization strategy may be useful in the development of HIV-1 vaccines. The present finding that IgA inhibited HIV-1 growth is particularly important since two of the most common points of entry for the virus are the anal and vaginal regions. However, it might be still questioned whether the method we used for the intramuscular immunization is just the best way or not. We did not use bupivacaine for preimmune procedures (11, 12). There are several groups using methods similar to our procedure (DNA in saline). There are several methods of DNA vaccination, such as 50-μg injection into the muscles of mice in PBS or 5 to 20-μg injection by gene gun. We could not accurately compare these conditions in this study. From our present data we can at least say that the i.n. route of DNA plasmid immunization induces high levels of HIV-specific immunity.

The present study also demonstrates that cationic liposomes significantly enhance immunity when administered i.n. together with the DNA vaccine (Figs. 2, 4, 5, and 6). Used as adjuvants, cationic liposomes have been shown to increase the effect of protein immunization (14–16). Systemic effects with these liposomes have also been observed with intramuscular DNA vaccination (Okuda et al., manuscript in preparation). Wheeler et al. (10) have reported that a novel cationic lipid enhances plasmid DNA delivery and expression in mouse lung. However, to our knowledge, this is the first report showing that liposomes enhance immune responses when coadministered i.n. with a DNA vaccine.

Generally speaking, the i.n. method of administration is useful for inducing both IgE and IgA Abs, which are typical indicators of Th2 cell activation (8, 10, 52, 54). Others have also shown that mucosal immunization with soluble proteins plus CT as a mucosal adjuvant induces Th2-like responses (5). The predominance of Th2-type induction was also observed by our cytokine assay data (Fig. 8), which showed a rather high IL-4 production and a moderate IFN-γ production when i.n. immunization was employed. The IgG1/IgG2a ratio with the i.n. route was higher than with i.m. administration (Fig. 7), which again suggests an activation of Th2-type cells. The characteristics of the strong induction of a Th1-type response by DNA vaccination (1) was also confirmed by present studies, and this predominance was activated by the administration of IL-12 expression plasmid.

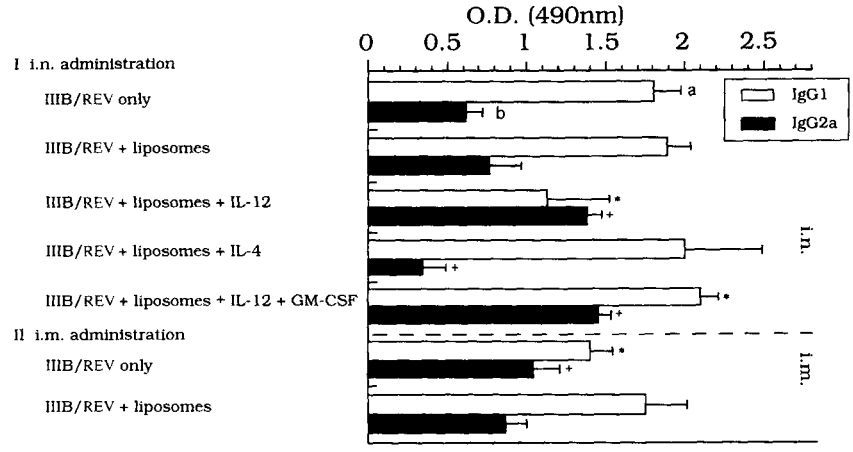

FIGURE 7. Ig subclasses of HIV-specific Ab. On days 0, 7, and 21, 2 μg of DNA vaccine and equal amounts of expression plasmid were administered by the i.n. or i.m. route. After 7 days, antisera from each group of five to seven mice were collected, and Ig subclasses were assayed by ELISA using a gp160 as the coating Ag. Data represent mean OD ± SE. *, Signifies a statistically significant difference ($p < 0.05$) between *a* and other groups. $^+$, Signifies a statistical significant difference ($p < 0.05$) between *b* and other groups.

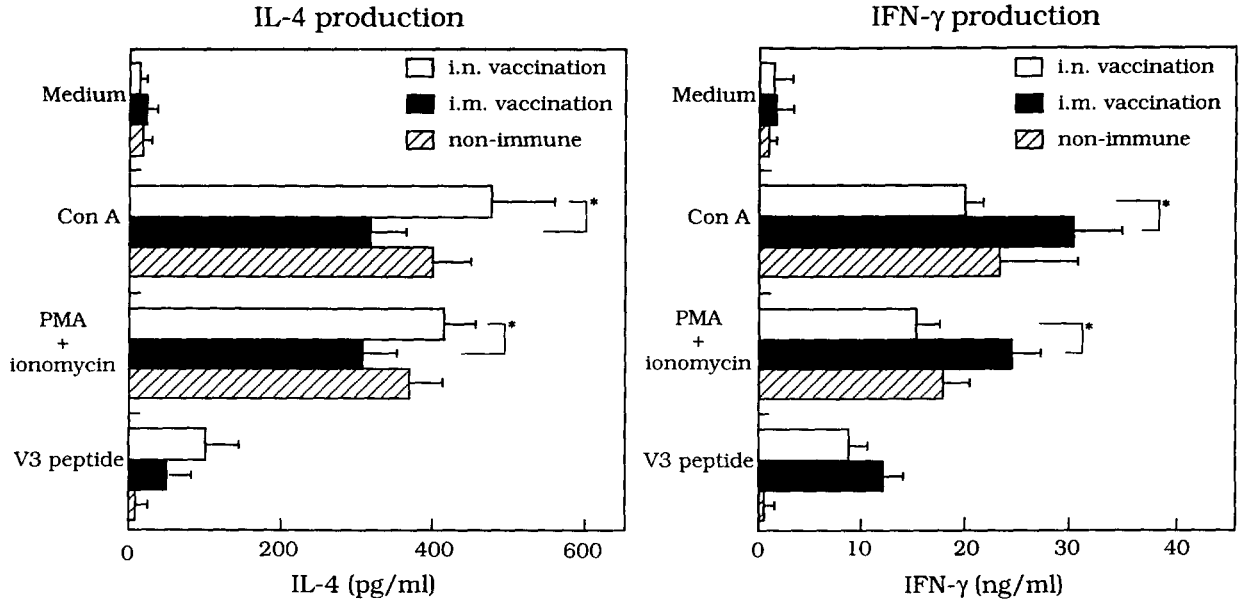

FIGURE 8. Influence of the route of DNA vaccination on cytokine production. Two micrograms each of IIIB/REV and IL-12 expression plasmids were immunized i.m. or i.n. into mice on days 0, 7, and 21. The immune lymphoid cells were collected and cocultured with each reagent. Mean levels ± SE of cytokines were assayed. Data represent mean cytokine levels ± SE of five to seven mice. These data are representative of three other individual experiments.

We detected more IgE Ab in the sera of mice immunized by the i.n. vs i.m. routes (Fig. 2). IgE production by i.n. or perioral administration has been reported by others (5), which is a sign of Th2-type response. It is uncertain at this time whether the serum IgE Ab seen with i.n. DNA vaccination is sufficiently high to cause anaphylaxis or allergy. There is a report that anti-DNA Abs induced by DNA vaccination do not cause autoimmune syndromes in any autoimmune model mice (55). Our mice demonstrated no clinical disease. We also used CT as an adjuvant but did not observe any significant increases in Ab titers under our experimental conditions. Many studies (2, 5, 8) have shown that CT helps to increase the immunogenicity of various peptide or protein-containing vaccines. In the future, we might find conditions under which these adjuvants would be more effective. IL-12, an important factor in the differentiation of Th1 cells (19), has been reported to increase 1) NK cell and CTL activities (20, 26); 2) T cell proliferation (56); and 3) Th1 cell maturation and production of IFN-γ (21). In addition, IL-12 inhibits the progression of various intracellular pathogens in mice (22, 23) and protects against the development of murine AIDS (24). These reports were relevant to the development of our DNA vaccine for HIV-1.

We predicted that the Th2-type response might even be suppressed by coadministration of the IL-12 expression plasmid, since it has been reported that IL-12 inhibits IgE response (23). The tendency for IL-12 expression plasmids to enhance IFN-γ production and somewhat suppress IL-4 production was also observed in cytokine assays (Fig. 8). These observations are very common in the course of Th1 or Th2 maturation.

Our results revealed that coadministration of an IL-12 expression plasmid with an HIV DNA vaccine did not reduce IgA Ab production or levels of serum Ig (Figs. 4 and 5). There are several reports that IL-12 does not suppress Ab production (25, 26). It did in fact strongly enhance both DTH and CTL responses. This is quite similar to the sequence of events in CTL activation by live attenuated vaccines, and the intracellular steps involved are essential for inducing a strong CTL production (25). Our present study demonstrated that administration of IL-12 expression plasmid induces IFN-γ production (Fig. 8), which also plays an important

role in CTL induction, because in vivo injection of anti-IFN-γ Ab strongly suppressed CTL responses (Fig. 6*C*). The results of cytokine assays (Fig. 8) and Ig subclass determinations (Fig. 7) also revealed that the coadministration of DNA vaccine with IL-12 expression plasmid preferentially activated Th1-type cells. In another set of experiments, we injected mice i.v. with the IL-12 protein to consider whether this protein was able to increase DTH or CTL responses. The Th1-type responses were weak in these studies compared with the present coinjection of IL-12 expression plasmid (Okuda et al., manuscript in preparation). One explanation for this is that protein decay in the mouse is fast, and the effect of IL-12 is only transient (20); therefore, the continuous production of or stimulation by the IL-12 expression plasmid might be important for activation of Th1-type cells in this animal (20). In addition, coadministration of GM-CSF also activated CMI. This is the first observation that this type of strong enhancement was induced by coadministration of both GM-CSF and the IL-12 plasmid. This coactivation of GM-CSF and IL-12 expression plasmids is very important for not only the HIV-1 vaccine but also for many other vaccine. The activation of both Th1 and Th2 is one of the ideal adjuvant. The mechanism of this type of coactivation is still unclear. However, we conjecture that GM-CSF activates or helps to grow APC, that IL-12 activates Th0 to Th1 maturation, and that Th1 and Th2 do not interfere with each other.

Our present study clearly demonstrates that DNA vaccine with an IL-12 expression plasmid induces good levels of both HIV-specific mucosal IgA Ab and CTL activity. Good enhanced HIV-specific immunities were also observed by coadministration of IL-12 and GM-CSF expression plasmids. Intranasal administration of DNA preparations appears to be an effective method for delivery, because it is simple, easy, and safe, and involves no infectious microbes along with their associated side effects.

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