

RAPID COMMUNICATION

Intranasal Administration of HIV-DNA Vaccine Formulated with a Polymer, Carboxymethylcellulose, Augments Mucosal Antibody Production and Cell-Mediated Immune Response

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We previously reported that intramuscular (i.m.) immunization of DNA vaccine encoding human immunodeficiency virus type 1 (HIV-1)_{IIIIB} *env* and *rev* genes alone or in combination with appropriate adjuvant induces substantial and enhanced immune response against HIV-1. In the present study, we examined whether a polymer, low-viscosity carboxymethylcellulose sodium salt (CMCS-L), has an adjuvant effect on immune response induced by DNA vaccination. BALB/c mice were immunized with HIV-DNA vaccine formulated with CMCS-L via the intranasal (i.n.) and i.m. routes. The combination with the polymer elicited higher levels of antigen-specific serum IgG and fecal IgA antibodies than DNA vaccine alone. For cell-mediated immunity, HIV-specific delayed-type hypersensitivity response and cytotoxic T lymphocyte activity were measured by the footpad-swelling test and the ⁵¹Cr-release assay, respectively. Both were enhanced by the combination with CMCS-L via i.n. and i.m. inoculation. Cytokine analysis in culture media of bulk splenocytes harvested from immunized animals showed higher levels of IL-4 production in i.n.-immunized mice compared with i.m.-immunized mice. Nevertheless, the increased IFN- γ production resulting from the combination with CMCS-L was observed only in i.n.-immunized mice. These data indicate that i.n. immunization of HIV-DNA vaccine formulated with CMCS-L enhances HIV-specific mucosal antibody (Ab) and systemic Ab and cell-mediated immune response. © 1998 Academic Press

Key Words: HIV-DNA vaccine; intranasal administration; mucosal immunity; carboxymethylcellulose sodium.

INTRODUCTION

The intestinal and urogenital mucosae are almost the first line of host defense against HIV-1. Our previous studies suggested that the mucosal IgA antibodies (Abs) could inhibit HIV-1 replication (1–3). Although a variety of protocols of systemic HIV-1 vaccination have been shown to induce the protection of serum-neutralizing Abs after systemic vaccination, the production of secretory IgA (s-IgA) has been rather poor (4, 5). There are some reports that intranasal (i.n.) administration of protein or peptide antigens induces both IgA and IgE Abs via activation of a Th2-type immune response (6, 7). Several studies also showed that direct DNA inoculation of a functional gene into animals can lead to significant expression of encoding proteins (8–10). Wang *et al.* (11) reported that inoculation of HIV-DNA plasmid could induce HIV-neutralizing Abs and that lymphocytes from DNA-immunized mice could proliferate to HIV antigen. In theory, direct DNA immunization may mimic certain aspects of an attenuated vaccine in that the viral protein may be synthesized in host cells rather than simply being present as injected materials in the circulation. We have developed an HIV-DNA vaccine incorporated with appropriate immunomodulators or their expression plasmids which could induce augmented HIV-specific humoral and cell-mediated immune (CMI) responses in mice, rabbits, and macaques via intramuscular (i.m.) immunization (12–16). However, there are only a few studies concerning i.n. administration of HIV-DNA vaccine.

On the other hand, it has been known that polymers or copolymers can be formulated with antigen as hydrophobic delivery vehicles (17–19). Low-viscosity carboxymethylcellulose sodium salt (CMCS-L) (a water-soluble polymer) has been utilized in detergents as a food stabilizer and as a soil-suspending agent. A vaccine delivery system which enhances both mucosal Ab

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TABLE 1
Serum Antibody Titer and Footpad Swelling Response of Mice Administered with DNA Vaccine

Immunogen	Reciprocal log ₂ antibody titer ^a (4 week)	Swelling response ^b (10 ⁻² mm)	
		HIV-specific peptide	Myoglobin peptide
i.n. administration			
DNA vaccine formulated			
With 0.5% CMCSL-L	12.0 ± 0.8*	15.5 ± 2.1*	NT
With 1.0% CMCSL-L	12.5 ± 1.8*	16.2 ± 2.5*	3.1 ± 1.5
With 2.5% CMCSL-L	9.2 ± 1.3	12.3 ± 2.3	NT
With 5.0% CMCSL-L	9.5 ± 1.7	11.6 ± 3.1	NT
DNA vaccine alone	9.5 ± 0.8	10.3 ± 2.1	1.2 ± 1.0
1% CMCSL-L	NT	6.3 ± 1.8	NT
i.m. administration			
DNA vaccine formulated			
With 0.5% CMCSL-L	9.2 ± 1.3	18.2 ± 4.1*	NT
With 1.0% CMCSL-L	9.2 ± 1.5	17.4 ± 3.9*	2.5 ± 1.1
With 2.5% CMCSL-L	8.5 ± 1.3	15.3 ± 3.3	NT
With 5.0% CMCSL-L	8.8 ± 1.7	12.0 ± 2.9	NT
DNA vaccine alone	8.9 ± 1.8	11.4 ± 3.0	1.8 ± 1.3
1% CMCSL-L	NT	7.2 ± 2.6	NT
None		3.2 ± 2.1	NT

Note. Data are expressed as the means ± standard error of the mean of five mice.

^a The mean Ab of sera 7 days after the third immunization.

^b Four micrograms of peptide was injected into the mouse footpad, and the extent of footpad swelling was assayed 24 h after peptide injection.

* Mean values significantly different from those of mice injected with DNA vaccine alone ($P < 0.05$).

and CMI response is important for achieving an effective vaccine. In this study, we examined whether CMCS-L has an adjuvant effect on immune response induced by i.n. or i.m. administration of HIV-DNA vaccine.

MATERIALS AND METHODS

Experimental animals. All 7-week-old BALB/c female mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). These animals were maintained in horizontal laminar-flow cabinets and provided with sterile food and water.

HIV-DNA vaccine. pCMV160IIIB encoding *env* of HIV-1_{IIIB} and pCREV encoding HIV-1 *rev* were previously described (12).

Synthetic peptide. HIV-1-specific peptides (TRPN-NTRKSIRIQRGPGRAFVTIGKIG (20) and RGPGR-AFVTI) (21), corresponding to the V3 loop of HIV-1_{IIIB} gp120, and sperm whale myoglobin peptide (ISEAIIHV-LHSRHP) (hereafter myoglobin) (22) were synthesized using the peptide synthesizer or in some cases were purchased from Sawady Technology (Tokyo, Japan).

Polymer. CMCS-L was purchased from Sigma (St. Louis, MO). CMCS-L was dissolved and diluted in sterile phosphate-buffered saline (PBS) and formulated into vaccine preparation.

Immunization of mice. Direct immunization of the DNA vaccine into the biceps muscles of mice was performed as previously described (12). Mice were immunized with 4 µg DNA vaccine dissolved in sterile PBS alone or formulated with 0.5–5.0% CMCS-L. The i.n. administration has been described in detail elsewhere (3). The same procedure employing the same dose of immunogen was used for immunization on days 0, 7, and 21. Five to seven days after the last boosting, humoral and cell-mediated immune responses were evaluated.

Enzyme-linked immunosorbent assay. ELISA was performed as described previously (1). HIV-1-specific peptide and myoglobin peptide were used as ELISA antigens. Affinity-purified horseradish peroxidase-coupled goat anti-mouse IgG, IgA, IgM, and IgE Abs (Organon Teknika Corporation, NC) or horseradish peroxidase-coupled goat anti-mouse isotype-specific IgG1 and IgG2a Abs (Southern Biotechnology Associates, Inc. AL) were used as secondary Abs. Antibody titers were expressed as the reciprocal log₂ numeral of the final detectable dilution which gave an optical density at 490 nm (OD₄₉₀) of ≥0.2 OD unit.

CMI response. The footpad-swelling response as delayed-type hypersensitivity (DTH) test was measured as described (23). Four micrograms of HIV-1-

specific peptide or myoglobin peptide as a control was injected into mouse footpads. 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 the postinjected footpads.

The HIV-specific cytotoxic test was performed as described (24). Spleen cells isolated from immunized or nontreated mice and, in some cases, spleen cells treated with anti-CD8⁺ monoclonal Ab and complement were cultured for 5 days in the presence of 30 μ g HIV-specific peptide in 10 ml RPMI complete medium supplemented with 10% fetal calf serum and used as effector cells. CTL activity was determined by effector cell-mediated lysis of P815 mastocytoma cells (H-2^d) pulsed with HIV-specific peptide in a 6-h chromium-release assay. The ratio of effector cells to target cells ranged from 5:1 to 80:1. The percentage of specific ⁵¹Cr release was calculated as $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$.

Cytokine assay. Spleen cells from immunized mice (5×10^6 cells/ml) were restimulated *in vitro* in the presence of the HIV-specific peptide (3 μ g/ml) or 5 μ g/ml of Con A to examine cytokine synthesis in a 96-well plate (200 μ l/well). IL-4 and IFN- γ were quantified by each specific ELISA using an immunoassay kit according to the manufacturer's protocol (Bio Source International, Inc., CA) in 24- and 48-h cultured supernatants, respectively.

Data analysis. Statistical analyses of the data were performed by one-way factorial analysis and Fisher's exact test.

RESULTS

Serum Ab responses to i.m. and i.n. immunization. The DNA vaccine, alone or formulated with 0.5–5.0% CMCS-L, was administered via the i.n. or the i.m. route on days 0, 7, and 21, and serum Ab titers of each group were determined by ELISA (Table 1). The means of 12.5 ± 1.8 and 9.2 ± 1.5 serum Ab titers were obtained on day 7 after the third immunization with the DNA vaccine formulated with 1% CMCS-L via the i.n. and the i.m. route, respectively. The i.n. immunization of DNA vaccine formulated with CMCS-L induced slightly higher titers of HIV-specific Abs than the i.m. immunization. The time course of antibody profile was also observed (Fig. 1). The vaccine formulated with 1% CMCS-L induced a strong and long-lasting Ab response.

Antibody titer and IgG subclasses. As shown in Table 1, i.n. administration with 1% CMCS-L strongly enhanced the HIV-specific serum IgG Ab titer. Both i.n. and i.m. administration with or without CMCS-L induced higher serum IgG titer compared to serum IgA titer (Table 2). Higher fecal IgA titer and similar levels of fecal

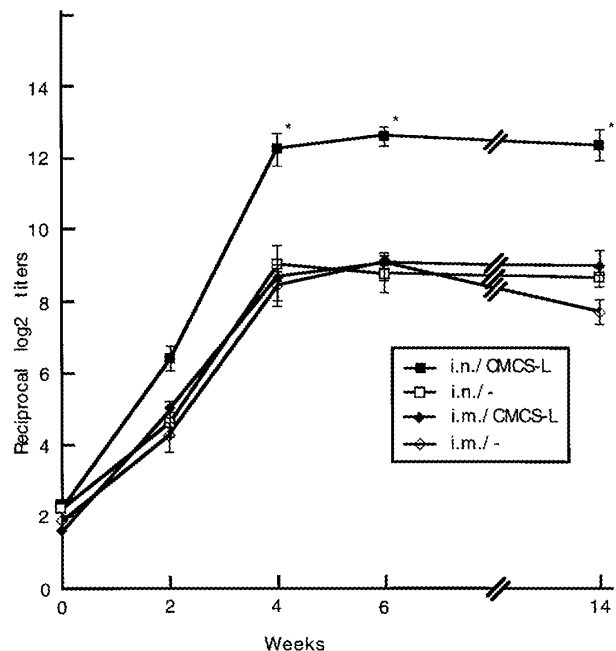


FIG. 1. Time course of anti-HIV-specific Ab titers in immunized mice. Four micrograms of DNA vaccine alone or formulated with 1% CMCS-L was administered via the i.n. or i.m. route on days 0, 7, and 21, and serum Ab titers were assayed by HIV-specific ELISA. Data are given as mean titers $\pm$ standard error of the mean from five mice. * Mean value significantly different from the other DNA vaccine-injected groups ($P < 0.05$).

IgA and IgG titers were observed in the mice i.n. and i.m. administered together with CMCS-L. Compared with the i.m. route, the i.n.-immunized group showed slightly higher IgA and IgG Ab levels. DNA vaccine formulated with CMCS-L and administered via the i.n. route induced significantly higher levels of fecal IgA and serum IgG than Abs via the i.m. route. Both i.n. and i.m. administration with CMCS-L decreased the IgG1/IgG2a ratio compared to DNA vaccine alone.

CMi response. DTH response was measured by the footpad-swelling test (Table 1). DTH response was significantly increased in the group administered 0.5–1% CMCS-L compared with the group administered DNA vaccine alone via i.n. and i.m. immunization. However, the increase was not observed in the complex with other concentrations of CMCS-L.

A series of CTL experiments was designed to determine whether inoculation of DNA vaccine formulated with CMCS-L via the i.n. route augments a CTL response. As shown in Fig. 2A, the i.n. administration of 0.5, 1.0, and 2.5% CMCS-L-formulated DNA vaccine demonstrated substantial adjuvant effects on CTL activities, with 61.3, 51.5, and 28.6% specific lysis at E:T ratio 80:1. Similar results were obtained also for i.m.

TABLE 2
Comparison of Serum and Fecal IgG and IgA Titers, Ig Subclass, and Cytokine Levels
in Mice Immunized with DNA Vaccine^a

Immunogen	Reciprocal log ₂ antibody titer				IgG ₁ /IgG _{2a} ^b	Cytokine ^c	
	Serum		Fecal			IL-4 (pg/ml)	IFN-γ (ng/ml)
	IgG	IgA	IgG	IgA			
i.n. administration							
DNA vaccine formulated with 1.0% CMCSL-L	12.1 ± 1.0*	4.6 ± 0.8	7.0 ± 1.8	12.5 ± 1.9***	2.1 ± 0.8*	453.0 ± 51.2*	18.4 ± 4.2**
DNA vaccine alone	9.0 ± 1.0	3.7 ± 0.8	6.8 ± 2.0	9.2 ± 1.8	3.0 ± 0.5*	423.1 ± 31.9*	10.0 ± 3.6
i.m. administration							
DNA vaccine formulated with 1.0% CMCSL-L	8.8 ± 1.0	4.4 ± 1.0	6.6 ± 1.0	7.0 ± 1.8	1.3 ± 0.4	321.2 ± 68.1	30.6 ± 5.8
DNA vaccine alone	8.2 ± 1.0	3.5 ± 1.0	5.8 ± 1.2	6.8 ± 1.4	1.9 ± 0.6	315.8 ± 61.0	20.0 ± 5.2

Note. Mean values significantly different ($P < 0.05$) from those of mice injected with *DNA vaccine formulation with 1.0% CMCS-L via the i.n. route and **DNA vaccine alone via the i.n. route.

^a Seven days after the third immunization, samples were collected and assayed by ELISA (see Materials and Methods). Data are expressed as the means ± standard error of five to six mice.

^b The IgG₁/IgG_{2a} ratio was calculated as the IgG₁ titer/IgG_{2a} titer ratio, which was determined by ELISA.

^c Spleen cells from immunized mice were cultured in the presence of Con A (5 μg/ml). After 24 h for IL-4 and 48 h for IFN-γ, each cytokine in the supernatant was quantified by the respective cytokine-specific ELISA.

administration (data not shown). CTL activities were not detectable when native P815 cells, myoglobin peptide-pulsed P815 cells, or EL4 cells were used as targets. CTL activity was also not detected when CD8⁺ T cell-depleted spleen cells harvested from an immunized mouse were used as effector (Fig. 2B).

Cytokine assay in culture supernatant of spleen cells from immunized animals (Table 2). Spleen cells from i.n.-immunized mice produced a significantly increased IL-4 production when stimulated with Con A compared to i.m. immunization. The i.n. administration of DNA vaccine formulated with CMCS-L induced significantly

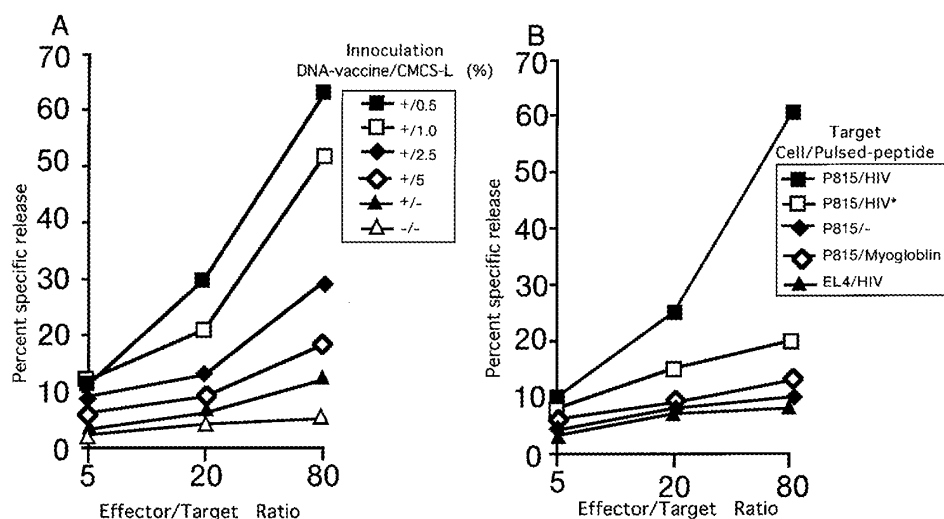


FIG. 2. (A) CTL responses induced by i.n. administration of DNA vaccine formulated with 0.5–5.0% CMCS-L. Spleen cells were restimulated *in vitro* with HIV-specific peptide (see Materials and Methods) for 5 days. One of three experiments is presented. HIV-specific peptide-pulsed P815 cells were used as targets. (B) Augmented CTL activity by 1.0% CMCS-L formulation DNA vaccine is antigen specific and is an MHC class I-restricted response. Immunization was performed in the same manner as in (A). P815 (H-2^d) and EL-4 (H-2^b) cells were used as targets, and HIV-specific peptide and myoglobin peptide were used as peptides for pulse labeling. Immunized spleen cells treated with anti-CD8⁺ mAb and complement were used as control. *CD8⁺ cell-depleted spleen cells were used as effector cells.

higher levels of IFN- γ production than DNA vaccine alone. When the spleen cells were cultured in the presence of the HIV-1-specific peptide, a similar increase was observed (data not shown). The cytokines were not detectable in the medium in the absence of antigen or mitogen.

DISCUSSION

Protective immunity against HIV may require humoral antibody response, CMI response, the presence of secretory immunoglobulin at mucosal surfaces, or a combination of these responses. An ideal HIV vaccine therefore should be capable of inducing both strong mucosal antibody production and CMI response. Our group and others have tried a number of approaches to enhance DNA-derived immunity, including particle bombardment (25, 26) and the use of expression vectors of certain cytokines (3, 14, 16, 27, 28) or other immunomodulatory molecules (13, 15). Previous studies have demonstrated that pathogen-specific s-IgA is able to protect a host against i.n. challenge with influenza virus (29). We and others also found that HIV-1-specific fecal s-IgA is capable of neutralizing HIV-1 *in vitro* (1–3). Nasal or oral vaccination studies targeting mucosal immunity have commonly employed cholera toxin (CT) adjuvant (30, 31). Although CT is reliable as a mucosal adjuvant, it is classified as a Th2-type adjuvant (32), and it is not considered suitable for inducing systemic Th1-derived CMI. Polymers have been evaluated in aqueous buffer, in oil–water, and in water-in-oil emulsion. Several of those formulations can induce protective antibodies in preerythrocytic or erythrocytic malaria vaccine models (17) or in pneumococcal vaccine models (18). In those models, protective immunity is associated with the development of IgG2a subclass antibodies (19). These studies showed that some polymer formulation could enhance the production of Th1-type responses. The goal of the present study was to examine whether CMCS-L, one kind of polymer, has potent adjuvant activity in enhancing the immune response to HIV-1 via i.n. and i.m. administration of DNA vaccine. Our results showed that the i.n. route of DNA vaccination induced a higher titer of serum antigen-specific IgG and fecal IgA Abs than the i.m. route, consistent with other reports (3, 33–36). DNA vaccine formulated with a low concentration of CMCS-L enhanced higher and substantial levels of HIV-specific Abs by both i.n. and i.m. administration (Fig. 1 and Table 1). The i.n. administration enhanced higher IgG1/IgG2a ratios than the i.m. administration (Table 2). Generally speaking, the i.n. administration has the advantage of inducing both IgE and IgA Abs, which are typical indicators of Th2 subset activation (6, 29). The CTL activity (Fig. 2), DTH response, and production of IFN- γ (Table 2) were strongly enhanced when DNA vaccine formulated with CMCS-L was com-

pared to DNA vaccine alone. These results indicate that the Th1-type response was stimulated. In summary, HIV-DNA vaccine formulation with a low concentration of CMCS-L via the i.n. route can activate both Th2-type and Th1-type responses and can induce stronger mucosal IgA and CTL responses in mice.

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