

Brief Report

Chitin Micro-Particles (CMP): A Useful Adjuvant for Inducing Viral Specific Immunity when Delivered Intranasally with an HIV-DNA Vaccine

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INTRODUCTION

WE HAVE BEEN DEVELOPING an HIV-DNA vaccine based on an HIV-1 *env* and *rev* expression plasmid (20). Intranasal (i.n.) administration of this vaccine with an appropriate adjuvant induces high levels of antibody (Ab) production, especially secretory IgA (sIgA) Ab and HIV-specific cytotoxic T lymphocyte (CTL) activity in mice (7,23,31). HIV-specific sIgA Ab is an important aspect of vaccine design as it is effective at blocking HIV penetration of the mucosal membranes, which are the primary site for infection of several viruses including HIV. Our group and others have reported that mucosal IgA inhibits HIV-1 replication (3,8). A second component of a successful vaccine is the induction of strong CTL activity for the efficient elimination of virus-infected cells (6,30). An ideal HIV vaccine should be capable of inducing both strong mucosal and systemic CTL activity. Strong et al. reported that the intranasal application of chitin micro-particles (CMP) derived from shrimp or crab shells in mouse models of allergy markedly reduced allergic symptoms resulting from an up-regulation of IL-12, IFN- γ and TNF- α (26). Shibata et al. indicated that chitin has a unique Th1 adjuvant effect on the development of immunity against a mycobacterial antigen (25). Other natural Th1 potentiators, such as heat-killed *Brucella abortus* or CpG immunostimulatory DNA sequences have proved very effective as vaccine adjuvants (10,13,32). The current study was designed to examine whether CMP has an adjuvant effect and whether this might be useful for nasal vaccination with HIV-DNA vaccine.

MATERIALS AND METHODS

Experimental animals and HIV-DNA vaccine formulation. Six to eight-weeks-old female BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan). These animals were treated humanely and in full compliance with our Institutional Animal Care and Use Committee. The immunogenic plasmids, pCMV160IIIIB and pcREV, which encode the *env* and *rev* genes of HIV-1_{IIIIB}, respectively, were described in our previous report (20). A mixture of equal amounts of the two plasmids was used as the HIV-DNA

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vaccine (hereafter DNA vaccine) to elicit an env-specific immune response since a previous study showed that expression of env protein is dependent on rev co-expression (15). A volume of 30 μL of phosphate buffer saline (PBS) containing 5 μg DNA vaccine and 100 μg of CMP or a 50% solution of liposome, prepared as described previously (12) was administered to BALB/c mice via the intranasal (i.n.) route. As controls, 30 μL PBS or CMP alone was administered. Mice were anesthetized with diethyl ether, and 30 μL of the prepared vaccine formulation was dropped into both nostrils gradually to prevent suffocation. The mice were therefore able to inhale the vaccine preparation in a natural manner. Inoculations were performed once weekly for 3 weeks and humoral and cell-mediated immune responses were evaluated 7–10 days after the final immunization. Each group contained three to six mice.

Collection of samples. Mouse sera were collected by retro-orbital puncture and stored at 4°C. Fecal pellets (100 mg) were suspended in 1 mL of PBS and centrifuged at 15,000g for 5 min and the supernatants stored at –20°C until used.

Under anesthesia with diethyl ether, the lung and spleen were removed from mouse and homogenized. Red blood cells were lysed with 0.01 M Tris-HCl buffer solution (pH 8.3) containing 0.83% ammonium chloride. The cell suspension was passed through a wire mesh, centrifuged to remove cell debris and re-suspended in the complete culture medium, RPMI 1640 supplemented with antibiotics (100 $\mu\text{g}/\text{mL}$ streptomycin and 100 IU/mL penicillin), 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol and 10% heat-inactivated fetal calf serum.

Delayed-type hypersensitivity (DTH) response. For a DTH assay, the footpad swelling response was measured on the 7th day after the final treatment, as previously described (20). An injection of 4 μg of the HIV-1 V3 region peptide (NNTRKRIQRGPGRAFVTIGKIGN), which is both a T-cell and CTL epitope (19), or the sperm whale myoglobin peptide (ALVEADVA) (21) as a control was injected into the mouse footpads. After 24 h, the extent of footpad swelling was measured with a dial thickness gauge (Ozaki Seisakusyo, Tokyo, Japan) and expressed as the difference in footpad thickness in units of 10^{-2} mm between the pre-injected and post-injected footpads.

Enzymed-linked immunosorbent assay (ELISA). ELISA was performed to determine Ab responses against HIV-1III_B as previously described (7,23,31). Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with a 10 $\mu\text{g}/\text{mL}$ solution of V3 peptide and incubated at 4°C overnight. The wells were then blocked with PBS containing 1% BSA and incubated at RT for 2 h. For the evaluation of serum IgG Ab titer, affinity-purified HRP-labeled goat anti-mouse IgG (Sigma, St. Louis, MO) was used as secondary Ab. For the estimation of fecal IgA Ab against HIV-1, 100 mg of mouse fecal pellets were extracted in 1 mL of PBS. HRP-labeled anti-mouse IgA (Zymed Laboratories, San Francisco, CA) was used as secondary Ab. The plates were then colored with *o*-phenylenediamine dihydrochloride (Wako Chemical, Osaka, Japan) and absorbance read at 490 nm on an automated plate reader. Antibody titers were expressed as the reciprocal \log_2 numeral of the final detectable dilution, which gave an optical density at 490 nm (OD_{490}) of ≥ 0.2 OD units.

Enzyme-linked immunospot (ELISpot) assay. Ten days after final immunization, IFN- γ and IL-4 ELISpot assays were performed using a previously described method (17) with minor modification. Briefly, a MultiScreen-IP plate (Millipore Corp., Bedford, MA) was coated with 50 μL of purified rat anti-mouse IFN- γ mAb (AN18, 1 mg/mL; Mabtech AB, Nacko, Sweden) or anti-mouse IL-4 mAb (11B11, 1 mg/mL; Mabtech AB) per well and was incubated at 4°C over night. The plate was then washed with PBS and blocked using PBS containing 0.5% BSA at 37°C for 2 h. Freshly isolated immune lymphocytes (1×10^6) and 5 μg of V3 peptide were added to the each well and incubated in a 5% CO_2 atmosphere for 24 h, in triplicate. The control wells contained lymphocytes alone. After incubation, cells were removed and incubated with biotinylated anti-mouse IFN- γ mAb (R4-6A2, 1 mg/mL; Mabtech AB), or anti-mouse IL-4 mAb (BVD6-G2, 500 $\mu\text{g}/\text{mL}$; Mabtech AB) for 2 h at 37°, followed by washing and 0.1% alkaline phosphatase-streptavidin 100 $\mu\text{L}/\text{well}$ (Mabtech AB) for 1.5 h. Finally, the plate was treated with 5-bromo-4-chloro-3-indolyl-phosphatase/nitroblue tetrazolium BCIP/NBT-membrane phosphatase substrate (50 $\mu\text{L}/\text{well}$; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) at room temperature for 20 min and the reaction was stopped under running distilled water. The number of positively stained spots in the V3 peptide culture was counted using computer-assisted video image analysis and expressed as a proportion of 1×10^6 cells.

Intracellular cytokine staining (ICCS) assay. The ICCS assays were performed according to the instructions of the Cytofix/CytoPerm Plus GolgiPlug kit manufacturer (BD PharMingen, CA). Briefly, splenocytes were stimulated for 24 h with a 10 $\mu\text{g}/\text{mL}$ concentration of the HIV V3 peptide. GolgiPlug (1 $\mu\text{L}/\text{mL}$; BD PharMingen) was added for the last 2 h of stimulation. Cells were washed with staining buffer, blocked with 4% normal mouse serum, and stained with PE-labeled anti-mouse CD8a antibody (Ly-2; BD PharMingen). The cells were then suspended in 250 μL of Cytofix/CytoPerm solution at 4°C for 20 min, washed with Pem/Wash solution, and stained with FITC-labeled anti-mouse IFN- γ antibody (BD PharMingen) at 4°C for 30 min followed by flow cytometric analysis (Becton Dickinson FACScan).

Tetramer assay. The tetramer assay was carried out 10 days after final immunization as previously described (29). The H-2D^d/p18 tetramer (RGPGRAFVTI) labeled with phycoerythrin (PE) was prepared by the NIH AIDS Research and Reference Reagent Program. Briefly, lymphocytes were incubated at 4°C for 30 min with 4% normal mouse serum in PBS. Cells were stained with FITC-labeled anti-mouse CD8a (Ly-2; BD PharMingen) at 0.5 $\mu\text{g}/10^6$ cells and incubated at 4°C for 30 min. After two washes in PBS, the cells were incubated with the tetramer reagent at 37°C for 15 min followed by flow cytometric analysis.

Data analysis. Statistical analysis of the experimental data and controls was performed using the two-tailed Student's test.

RESULTS

Increased HIV-1-specific DTH and antibody responses. The antigen-specific DTH reaction was assayed by the footpad-swelling test. As shown in Table 1, coinoculation of CMP (CMP/DNA) gave a stronger DTH reaction to the V3 peptide (18.2 ± 4.6) than that obtained with immunogenic DNA alone (9.2 ± 1.3). The strength of the adjuvant effect was comparable to that of liposome (19.7 ± 5.0). There was no induction of DTH response by administration of CMP alone, and no substantial changes in responses to myoglobin peptide (negative control) demonstrate that the adjuvant activity of CMP was HIV antigen-specific.

Serum IgG and fecal IgA Abs titers to HIV-1 antigen were determined by ELISA (Table 1). Both administration of DNA vaccine together with CMP (CMP/DNA) or with Liposome (Lipo./DNA) strongly enhanced the HIV-specific serum IgG Ab, with 11.2 ± 1.5 and 10.8 ± 1.8 , respectively, when compared to

TABLE 1. ADJUVANT ACTIVITY OF CMP ON THE SPECIFIC IMMUNE RESPONSES

Administration	Swelling response (10^{-2} mm) ^a		Specific antibody titer ^b			
	V3 peptide	Myoglobin peptide	Serum IgG	Fecal IgA	IFN- γ -secreting cells ^c	IL-4-secreting cells ^d
PBS	4.5 ± 1.2	4.2 ± 1.0	1.5 ± 0.8	1.6 ± 0.6	21.2 ± 10.2	49.0 ± 12.1
CMP	4.7 ± 1.0	NT	2.1 ± 1.2	2.2 ± 0.2	28.3 ± 9.5	60.4 ± 9.6
DNA	$9.2 \pm 1.3^*$	NT	$8.3 \pm 1.4^*$	$7.2 \pm 1.0^*$	$65.3 \pm 12.6^*$	$204.5 \pm 22.3^*$
CMP/DNA	$18.2 \pm 4.6^{**}$	4.9 ± 1.2	$11.2 \pm 1.5^{**}$	$10.2 \pm 1.2^{**}$	$115.4 \pm 15.3^{**}$	$345.4 \pm 40.1^{**}$
Lipo./DNA	$19.7 \pm 5.0^{**}$	4.5 ± 1.4	$10.8 \pm 1.8^{**}$	$9.5 \pm 1.6^{**}$	$122.5 \pm 18.3^{**}$	$300.4 \pm 42.1^{**}$

^aHIV-1-specific V3 peptide or irrelevant sperm whale myoglobin peptide was injected into footpads. The extent of footpad swelling was expressed as the difference in footpad thickness between the pre- and post-injection measurements in units of 10^{-2} mm.

^bHIV-1-specific Ab titers were measured by ELISA using V3 peptide as Ag and 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.

^{c,d}IFN- γ and IL-4 ELISpot assays were performed as described in MATERIALS AND METHODS using freshly isolated immune spleen cells. The number of positive cells was expressed as a proportion of 1×10^6 cells.

***A mean value significantly different ($p < 0.05$) from that obtained with PBS alone group and DNA alone group, respectively.

Data are expressed as means \pm standard error of the mean from five to six mice per group.

DNA vaccine alone ($8. \pm 31.4$). Furthermore, these administration also strongly augmented the HIV-specific fecal IgA Ab (CMP/DNA; 10.2 ± 1.2 , Lipo./DNA; 9.5 ± 1.6 , DNA alone; 7.2 ± 1.0). These results demonstrated that CMP has a potent adjuvant effect on the DNA vaccine.

Augmented HIV-1-specific ELISpot Assay and ICCS assay. Measurement of IFN- γ - and IL-4-producing cells in the spleen cells re-stimulated with V3 peptide was performed by ELISpot assay and ICCS assay 10 days after the final immunization. ELISpot assay revealed a marked increase in the number of both IFN- γ -, and IL-4-secreting lymphocytes isolated from the spleens of mice immunized with CMP/DNA and Lipo./DNA compared with that of animals immunized with DNA vaccine alone (Table 1). Mice receiving CMP or PBS alone showed the same responses, which was insignificant. Similar results to the ELISpot assay also were obtained by ICCS assay (Fig. 1A). Co-inoculation with CMP enhanced the HIV-1-specific IFN- γ intracellular staining response in both lung (A-1) and spleen (A-2) stimulated *in vitro* with V3 peptide compared with inoculation with DNA alone. Positive lung cell stimulated *in vitro* was 0.2 % for DNA alone, compared to 0.35 % for CMP/DNA inoculation. In splenocytes, that of DNA alone and CMP/DNA group was 0.16 and 0.23 %, respectively. These results showed that co-inoculated with CMP increased the number of IFN- γ - and IL-4-producing cells.

Tetramer binding assay. A tetramer-binding assay was used to quantify MHC class I-restricted HIV-specific T cells in immunized mice (Fig. 1B). 0.94% CD8⁺ lymphocytes from lungs of mice immunized

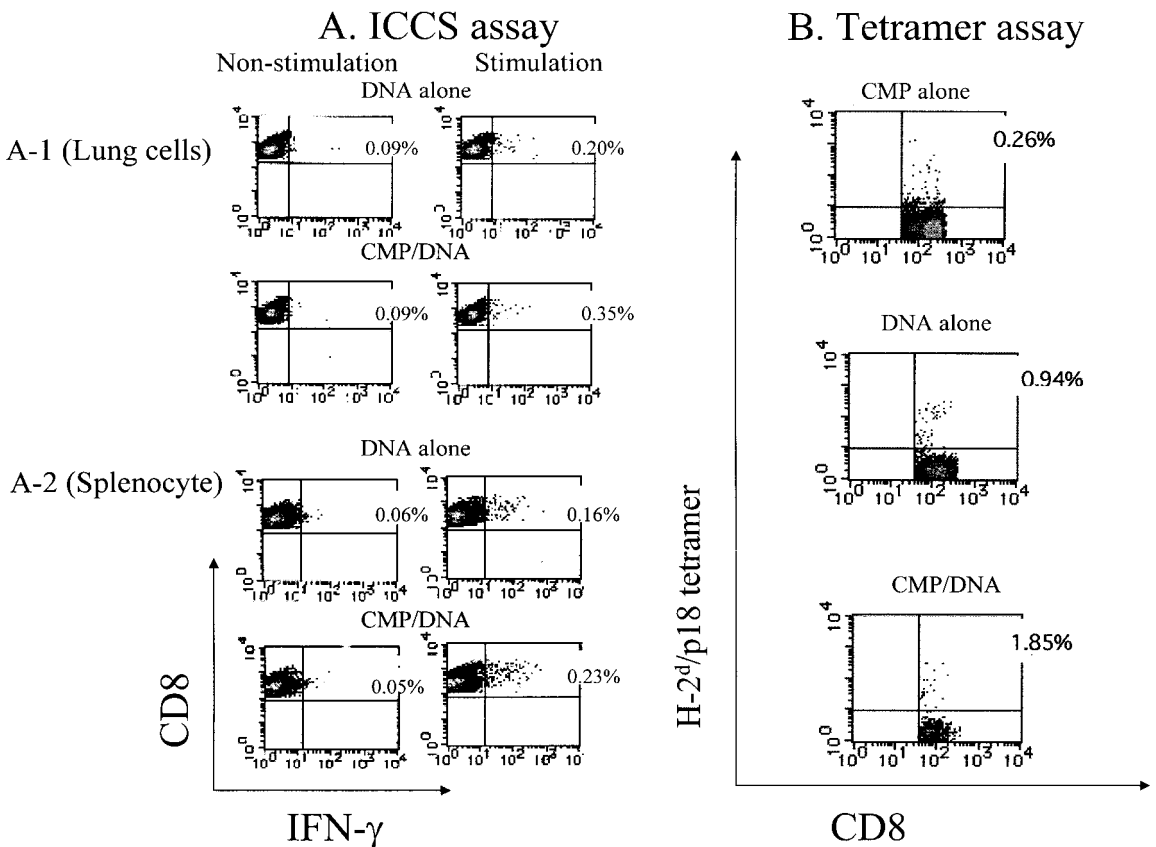


FIG. 1. ICCS assay and tetramer binding assay. The number of antigen-specific intracellular IFN-g-secreting lung cells (A-1) and spleen cells (A-2) were measured by ICCS and by the tetramer-binding (lung cells) assay (B). Assays were performed 10 days after the final immunization. The data shows the percentage of CD8⁺/IFN- γ positive T cells and CD8⁺/p18 positive T cells, respectively. Lung cells and spleen cells were prepared as in the Materials and Methods. Similar results were obtained in another independent experiment.

with DNA vaccine alone bound HIV peptide p18, whereas 1.85% CD8⁺ lymphocytes bound HIV peptide p18 in the mice immunized with CMP/DNA. Lung cells from mice administered with CMP alone gave a value of 0.26%. Similar results were obtained in another independent experiment.

DISCUSSION

We have developed an HIV-DNA vaccine that induces a variety of types and degrees of response, depending on the route of immunization and immunogenicity was augmented by the addition of appropriate immunomodulators or their expression plasmid (2,7,11,19,23,24,27,28,31). It was reported that HIV-1-specific fecal sIgA is capable of neutralizing HIV-1 *in vitro* (3,19,22). We have shown that the intranasal route of DNA vaccination induces a higher titer of antigen-specific serum IgG and fecal IgA than the intramuscular (i.m) route (23). Nasal or oral vaccination studies targeting mucosal immunity have commonly employed cholera toxin (CT) adjuvant (9,12,14,16). Although CT is reliable as a mucosal adjuvant, it is classified as a Th2-type adjuvant and it is not considered suitable for inducing systemic Th1-derived cell-mediated immune responses. In the present study we examined CMP, a natural micro-particulate carbohydrate, for adjuvant activity in enhancing the immune response to HIV-1 via the intranasal route of delivery of the DNA vaccine and compared the CMP adjuvant activity with that of liposome. There are many reports that cationic liposomes have a strong adjuvant effect in inducing humoral and cellular immunity (1,4,11,19).

DNA vaccine formulated with CMP and delivered intranasally enhanced HIV-specific Abs (Table 1). Generally speaking, intranasal administration has the advantage of inducing both IgE and IgA Abs, which are typical indicators of Th2 phenotype activation. The ELISpot assay showed that DNA/CMP vaccination significantly increased the numbers of IFN- γ - and IL-4-secreting cells when compared to DNA vaccination alone (Table 1).

A tetramer-binding assay demonstrated that MHC class I-restricted HIV-specific T cell response was increased in lungs from mice intranasally immunized with DNA/CMP when compared with DNA alone (Fig. 1B). Similar results were obtained in a separate experiment. However, we could not detect a significant increase in CD8⁺/p18 positive in spleen cells (data not shown). This difference may reflect a difference between the local immune response and systemic response induced by intranasal immunization. It may also reflect the immunization protocol used in this study and perhaps increasing the frequency of intranasal immunizations would show an enhanced effect. However, the results from this preliminary study suggest that HIV-DNA vaccine formulation with CMP and given intranasally can activate both Th1-type and Th2-type responses and enhances the humoral and cell-mediated immune responses to DNA vaccine in mice. Our results demonstrated that the adjuvant activities of CMP are comparable with liposome. Liposomes are phospholipid vesicles, which have been evaluated both as adjuvant and as delivery systems for antigens and drugs (4,5,11). The adjuvant activity of liposome may be related to an increase in the uptake of DNA into cells by fusion of liposome-DNA complexes with the cell membrane. The mechanism of action of CMP is unclear at the present time. Chitin in the form of micro-particles of 1–10 μm , can be efficiently phagocytosed by macrophages and this might facilitate entry of DNA and subsequent antigen presentation (24). CMP is a potent enhancer of IL-12 and IFN- γ , which strongly favors the development of Th1 and cell-mediated immune responses (24,26). Thus an adjuvant effect might be predicted when co-administered with DNA vaccine. It is also possible that CMP protects the DNA from degradation. Our previous study revealed that 30 μL of vaccine formulation containing 5 μg of DNA vaccine per mouse is sufficient to obtain a satisfactory specific immune responses and a 50% liposome solution shows a strong adjuvant activity. In the present study with CMP as adjuvant, we achieved a response using 30 μL of vaccine solution containing 5 μg of DNA and 100 μg of CMP per mouse. A more detailed examination of CMP and vaccine formulation and use of different intranasal vaccination protocols is required to fully evaluate the adjuvant effect of CMP. However, these results, taken together with our preliminary data suggest that CMP is comparable to liposome, one of the best adjuvant for DNA vaccination. CMP as an adjuvant is likely to be of clinical benefit for intranasal or mucosal vaccination because it is safe and easy to produce.

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