Modulation of Cellular Immune Response Against Hepatitis C Virus Nonstructural Protein 3 by Cationic Liposome Encapsulated DNA Immunization

Xuanmao Jiao, Richard Y.-H. Wang, Zhiming Feng, Harvey J. Alter, and James Wai-Kuo Shih

A vaccine strategy directed to increase Th1 cellular immune responses, particularly to hepatitis C virus (HCV) nonstructural protein 3 (NS3), has considerable potential to overcome the infection with HCV. DNA vaccination can induce both humoral and cellular immune responses, but it became apparent that the cellular uptake of naked DNA injected into muscle was not very efficient, as much of the DNA is degraded by interstitial nucleases before it reaches the nucleus for transcription. In this paper, cationic liposomes composed of different cationic lipids, such as dimethyl-dioctadecylammonium bromide (DDAB), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), or 1,2-dioleoyl-sn-glycerol-3-ethylphosphocholine (DOEPC), were used to improve DNA immunization in mice, and their efficiencies were compared. It was found that cationic liposome-mediated DNA immunization induced stronger HCV NS3-specific immune responses than immunization with naked DNA alone. Cationic liposomes composed of DDAB and equimolar of a neutral lipid, egg yolk phosphatidylcholine (EPC), induced the strongest antigen-specific Th1 type immune responses among the cationic liposome investigated, whereas the liposomes composed of 2 cationic lipids, DDAB and DOEPC, induced an antigen-specific Th2 type immune response. All cationic liposomes used in this study triggered high-level, nonspecific IL-12 production in mice, a feature important for the development of maximum Th1 immune responses. In conclusion, the cationic liposome-mediated gene delivery is a viable HCV vaccine strategy that should be further tested in the chimpanzee model. (HEPATOLOGY 2003;37:452-460.)

It is estimated that more than 170 million people worldwide have been infected by the hepatitis C virus (HCV), more than 4 times as many as with the human immunodeficiency virus.[1] Despite the near eradication of transfusion-associated HCV infection, it is presumed that 36,000 to 150,000 new HCV infections and 8,000 to 10,000 deaths occur in the United States each year.[1,2] Approximately 20% of those chronically infected with HCV develop cirrhosis, and 1% to 5% of patients with cirrhosis progress to hepatocellular carcinoma.[1] Currently, the most effective treatment for chronic HCV infection is the combination of interferon alfa with ribavirin, but only 38% to 43% of treated patients have sustained benefit from antiviral therapy.[3,4] The development of a therapeutic as well as a preventive vaccine is therefore a goal of high priority and vast potential impact.

HCV-specific immune responses in patients with acute self-limited HCV infection or patients recovered from chronic HCV infection following treatment suggest that T-cell-mediated immune responses play an important role in determining the outcome of HCV infection.[5] HCV-specific CD4$^+$ T cells directed against recombinant HCV core, NS3, and NS4 antigens have been detected during the first few weeks of acute hepatitis C[5]; NS3-specific CD4$^+$ T-cell immune responses are strong in patients who resolve acute hepatitis and may be necessary for viral clearance.[6] The immune response associated with recovery frequently displays a Th1 or Th0 cytokine pro-

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Abbreviations: HCV, hepatitis C virus; NS3, nonstructural protein 3; DDAB, dimethyl-dioctadecylammonium bromide; EPC, egg yolk phosphatidyl-choline; DOEPC, 1,2-dioleoyl-sn-glycerol-3-ethylphosphocholine; IL, interleukin; PBS, phosphate-buffered saline; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; SUV, small unilamellar vesicle; IFN-γ, interferon gamma; FSFC, frequency of spot-forming cells.

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Received March 18, 2002; accepted November 9, 2002.

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0270-9139/03/3702-0028$35.00/0
doi:10.1053/jhep.2003.50051

file, whereas the activation of Th2 responses seems to be involved in the development of chronic hepatitis.[7] The immunodominant epitope recognized by nonstructural protein 3 (NS3)-specific Th cells is at amino acid position 1,251-1,259 within HCV NS3[8] and is completely conserved within HCV genotypes 1a, 1b, 1c, 2a, and 2b.[5] Accordingly, a vaccine strategy directed to increase Th1 cellular immune responses, particularly to HCV NS3, has considerable potential.

DNA vaccination can induce both humoral and cellular immune responses, and its potency against HCV has been evaluated by a variety of immunization strategies.[9-20] In most of these studies, naked plasmid DNA encoding HCV proteins or HCV derived epitopes were injected intramuscularly. However, it became apparent that the cellular uptake of naked DNA injected into muscle was not very efficient, presumably because much of the DNA is degraded by interstitial nucleases before it reaches the nucleus for transcription.[21-23] Thus, many alternate approaches to enhance cellular uptake of the injected DNA have been used, including particle-mediated DNA delivery (gene gun),[16,24] intramuscular electric gene transfer,[25] biodegradable aliginate microspheres,[26] attenuated intracellular bacteria,[27] cationic lipid/DNA complex or cationic liposome encapsulated DNA,[28-31] and virosome encapsulated DNA.[32,33] Among these, cationic liposome and cationic lipid/DNA complexes have demonstrated their ability to enhance DNA transfer[34] and to modulate immune responses against tumor[29,30,35] and HBV.[36] In this paper, cationic liposomes composed of different cationic lipids were used to mediate DNA immunization in mice, and their efficiencies were compared. It was found that cationic liposomes composed of dimethyl-dioctadecylammonium bromide (DDAB) and equimolar egg yolk phosphatidylcholine (EPC) were effective in generating high-level Th1 type immune responses against HCV NS3, whereas liposomes composed of DDAB and 1,2-dioleoyl-sn-glycerol-3-ethylphosphocholine (DOEPC) induced Th2 type immune responses. It was also found that all cationic liposomes used in this study triggered high-level, nonspecific interleukin 12 (IL-12) production in mice, a feature important for the development of maximum Th1 immune responses.

Materials and Methods

Construction of HCV NS3 Expression Vector, Expression, and Purification of Recombinant HCV NS3 Protein. Recombinant HCV NS3 (rNS3) was expressed and purified with the QIAexpress System (Qiagen Inc., Valencia, CA) according to the manufacturer's handbook. Briefly, plasmid pQE-HCV/NS3 was constructed by inserting the HCV NS3 gene encoding aa 1,027-1,657 (nt 3,420-5,312, cloned from HCV strain H of genotype 1a) into the *Bam*HI restricted enzyme site of pQE-11 plasmid (Qiagen Inc) pretreated with Klenow fragment of DNA polymerase I and bacterial alkaline phosphatase. *Escherichia coli* DH5aF′IQ was transformed by the DNA ligation mixture. In addition to the insert, the new construct encoded a 6-histidine tag at the N-terminus that provided for nickel-affinity purification of the expressed protein. HCV NS3-producing clones were screened by Western blot, and human HCV positive serum was used as first antibody.[9]

Selected clone (pQE-HCV/NS3) was grown in LB-B media, and the expression of recombinant HCV NS3 was induced by IPTG. The cells were collected and washed once with washing buffer (50 mmol/L Tris-HCl, pH 8.0, 5 mmol/L EDTA, 0.15 mol/L NaCl, with protease inhibitor cocktail tablets from Boehringer-Mannheim GmbH, Mannheim, Germany). The cells were lysed by lysozyme digestion followed with sonication. The insoluble inclusion-body proteins were pelleted and resuspended in lysis buffer (0.1 mol/L NaH_2PO_4, 10 mmol/L Tris-HCl, 0.3 mol/L NaCl, 1% Triton X-100). The residual insoluble proteins were pelleted and dissolved in loading buffer (8 mol/L urea, 0.1 mol/L NaH_2PO_4, 10 mmol/L Tris-HCl, 10 mmol/L 2-ME, pH 8.0). The supernatant was collected after an additional centrifugation.

To affinity purify rNS3, the supernatant was loaded onto a nickel-NTA resin (Qiagen Inc.) column preequilibrated with loading buffer. Unbound protein was washed thoroughly with column washing buffer (8 mol/L urea, 0.1 mol/L NaH_2PO_4, 0.01 mol/L Tris-HCl, pH 6.3). Bound rNS3 was eluted with eluting buffer (8 mol/L urea, 0.1 mol/L NaH_2PO_4, 0.01 mol/L Tris-HCl, pH 4.5). The fractions containing rNS3 were determined by Western blot assay and could be used directly in an ELISA assay. For use as stimulator in the enzyme-linked immunospot assay (ELISPOT) assay, the elution containing rNS3 protein was concentrated by Centricon-10 (Amecon, Bedford, MA) and then diluted with 10 to 20 volumes of phosphate buffered saline (PBS). The rNS3 protein precipitate was formed then pelleted by centrifugation and resuspended in PBS by sonication. The concentration was determined by Bio-Rad DC (Bio-Rad Corp., Hercules, CA) protein assay. Endotoxin was determined to be free by limulus amebocyte lysate Pyrogent-5000 method. Recombinant HCV NS5b protein was prepared by the same procedure.

Construction and Preparation of HCV NS3 Expression Vector Used in Immunization. pCI-HCV/NS3 was constructed by inserting HCV NS3 gene encoding aa 1,027-1,657 (nt 3,420-5,312 cloned from

HCV strain H of genotype 1a) between *Mlu*I and *XbaI* restriction enzyme sites of the pCI plasmid treated with Klenow DNA polymerase. *Escherichia coli* Top 10F′ was transformed by the construct. The plasmid DNA from selected clones was purified using QIAGEN Mega Plasmid Kit (QIAGEN Inc.) according to the manufacturer's instructions. Endotoxin was determined to be free by limulus amebocyte lysate Pyrogent-5000 method. The blank pCI was purified with the same procedure. The purified endotoxin-free plasmid was dissolved in 0.2× Tris-EDTA buffer and then used in the experiments below.

Western Blot and Immunofluorescence Assays to Detect In Vitro Expression in Mammalian Cells. The expression of HCV NS3 in CHO-K1 (CCL61, ATCC, Rockville, MD) cells was detected by Western blot assay or immunofluorescence staining assay. CHO-K1 cells were seeded 1 day prior to transfection in a 6-well plate for Western blot assay or in a 2-well chamber slide for immunofluorescence staining assay. Transfection of CHO-K1 cells or Balb/3T3 cells with pCI-HCV/NS3 or pCI was mediated by Lipofectamine Plus (Life Science, Gaithersburg, MD) according to the manufacturer's instructions. Western blot or immunofluorescence staining was performed as described previously.[9]

Preparation of Liposome-Encapsulated pCI-HCV/NS3. pCI-HCV/NS3 and pCI-blank were entrapped into cationic liposome by a dehydration and rehydration procedure.[37] Cationic liposomes used in this experiment were composed with equimolar EPC and a cationic lipid, either DDAB, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), or DOEPC. Liposomes composed of equimolar DDAB and DOEPC were also used. All lipids were purchased from Avanti Polar Lipids, Alabaster, AL. Briefly, 3.96 μmol of each lipid was mixed in chloroform solution and dried under a gentle stream of nitrogen. The mixture of lipids was hydrated with 600 μL of injection grade water and sonicated subsequently in a bath-type sonicator until the suspension was translucent; thus, a small unilamellar vesicle (SUV) suspension was obtained, and the endotoxin was determined to be free by limulus amebocyte lysate Pyrogent-5000 method. Six hundred micrograms of the plasmids in 600 μL 0.2× TE solution was added to the SUV suspension, and the mixture was frozen in dry ice and dried in a freezing drier. Sixty microliters of 1/10× PBS was added to rehydrate the lipid-DNA mixture; thus, a dehydration-rehydration vesicle containing plasmid DNA was obtained. The liposome-DNA was diluted with 1× PBS to a final volume of 600 μL. To determine the entrapment efficiency, the liposome-DNA suspension was centrifuged at 100,000*g* × 2 hours; the absorbance of the supernatant was measured at 260 nm, and the concentration of unentrapped DNA was calculated. The optimal DNA/lipid ratio was determined by observing the change in turbidity upon titration of DNA into SUV suspension or titration of SUV into DNA solution. The turbidity was determined by reading at 400 nm. The initial concentration of liposome and DNA were 13.2 μmol/mL and 1,000 μg/mL, respectively.

Plasmid DNA Immunization. Female BALB/c mice were housed in approved animal care facilities during the experimental period and were handled following the international guidelines required for experimentation with animals. All animal study protocols were approved by NIH Clinical Center Animal Care and Use Committee. Six- to 8-week-old mice were primed by direct intramuscular injection of 100 μg liposome encapsulated or free plasmid DNA (pCI-HCV/NS3 or pCI-blank control) into the tibialis anterior muscle of both legs following anesthesia. The mice were boosted twice by injection of the respective plasmid constructs into the same region 4 and 6 weeks later.

ELISPOT Assay. The ELISPOT assay was used to determine IL-2, IL-4, IL-12, and interferon gamma (IFN-γ) secreting cells among the spleen cells from mice immunized with liposome-encapsulating or free pCI-HCV/NS3 and pCI-blank, under the stimulation of recombinant HCV NS3 protein. Spleen cells from immunized mice were separated by Ficoll-Paque, adjusted to 4×10^6 cells/mL, and then cultured with recombinant HCV NS3 proteins at 3 μg/mL. Ninety-six-well filtration plates with 0.45 μm surfactant-free mixed cellulose ester membrane, type MAHA S45 (Millipore Co., Bedford, MA), were coated with purified anticytokine antibody (IL-2, IL-4, IL-12, or IFN-γ) at the concentration of 10 μg/mL in 20 mmol/L borate buffer (pH 8.4) and incubated overnight at room temperature. The plates were overcoated with 5% bovine serum albumin in PBS. At day 2 or day 4, 4×10^5 spleen cells in 100 μL volume were added to each well and incubated with antigen for another 24 hours at 37°C in the presence of 5% CO_2, allowing for production and capture of released cytokines. All determinations were run in duplicate. After incubation, cells were removed by washing 6 times with PBS containing 0.05% NP-40 and 2 times with distilled water. One μg/mL biotin-labeled anticytokine monoclonal antibody was used as the detective antibody, and 1:4,000 diluted streptavidin-horseradish peroxidase was used for antibody detection. Finally, optimal 4CN peroxidase substrate (Bio-Rad) was added and incubated for 20 to 30 minutes at room temperature to develop the spots. The reaction was stopped by washing with distilled water. The spots were counted automatically by ELISPOT reader (Carl Zeis Vision, Hallbergmoos, Germany). The fre-

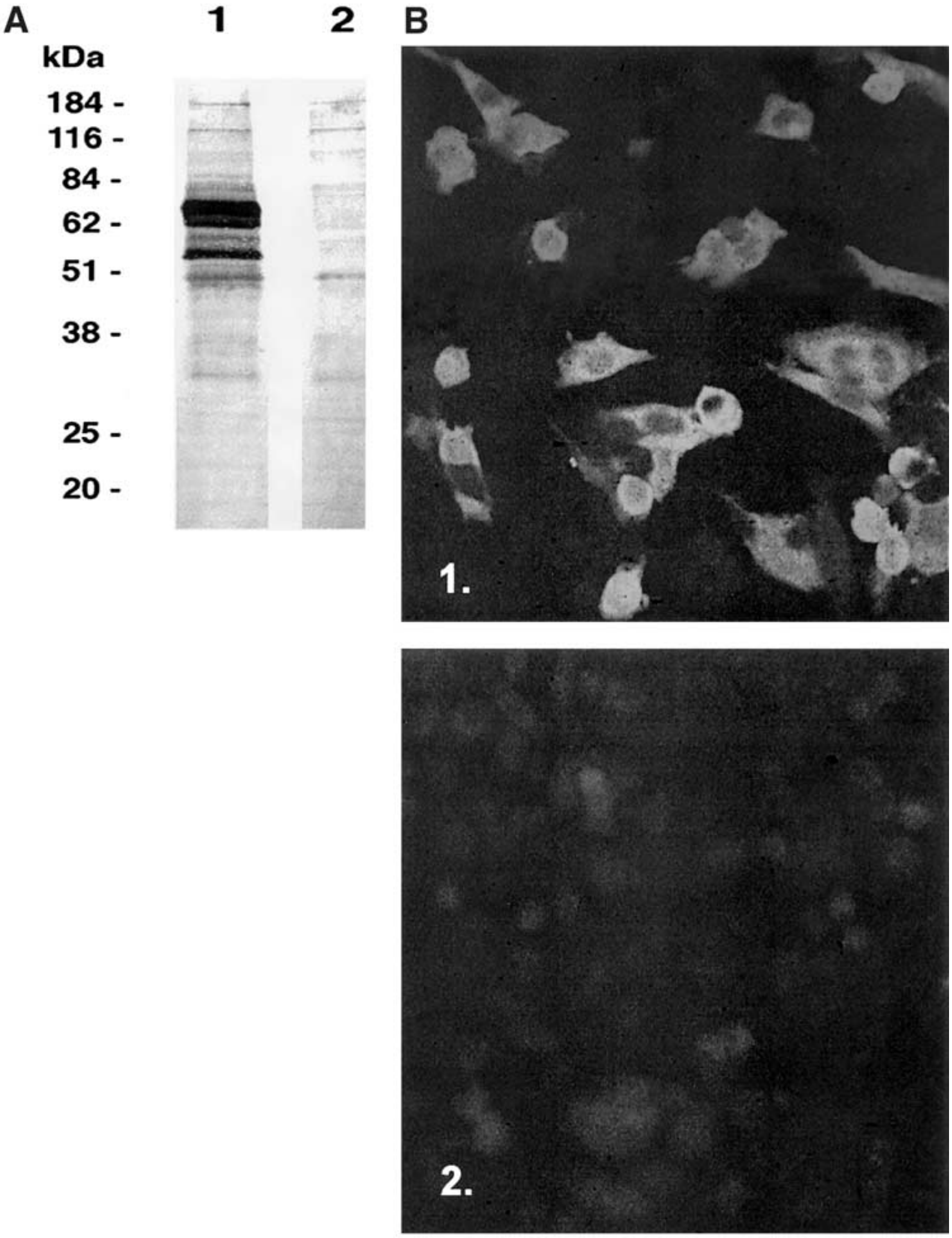

Fig. 1. Expression of HCV NS3 protein in CHO-K1 cells transfected with pCI-HCV/NS3 or its blank control mediated by Lipofectamine Plus. (A) HCV NS3 protein in cell lysates was detected by Western blot 24 hours after the cells were transfected with plasmids. (B) Twenty-four hours after transfection of CHO-K1 cells with pCI-HCV/NS3 or its control plasmid, the cells were examined by indirect fluorescence after staining with human HCV positive patient serum. Label 1 is pCI-HCV/NS3 and Label 2 is blank pCI plasmid.

quency of antigen-specific cytokine-secreted cells was defined as the frequency of the cytokine-secreted cells in the mice immunized with plasmids encoding antigen minus the frequency in the mice immunized with control plasmids.

Results

Expression of HCV NS3 Protein in pCI-HCV/NS3-4748 Transfected Mammalian Cells. Expressed HCV NS3 produced in pCI-HCV/NS3 transfected CHO-K1 cells was detected by Western blot (Fig. 1A). The molecular mass of HCV NS3 expressed by pCI-HCV/NS3-transfected cells was 69 kd, identical to the protein purified from pQE-HCV/NS3-transformed *E. coli* cells. The transient expression of HCV NS3 was also observed through immunofluorescence staining in pCI-HCV/NS3-transfected BALB/3T3 cells (Fig. 1B). Label 1 is pCI-HCV/NS3 and Label 2 is blank pCI plasmid.

The DNA/Lipid Ratio and Entrapment Efficiency of Liposome-Encapsulating Plasmids. The optimal DNA/lipid ratio is a key factor for cationic liposome-mediated gene transfer. At the ideal DNA/lipid ratio, the cationic lipid/DNA complex should be homogeneous and carry net positive charges. To find a suitable DNA/lipid ratio, the aggregation of liposomes induced by the titration of plasmids or the aggregation of DNA induced by the titration of cationic liposomes was observed

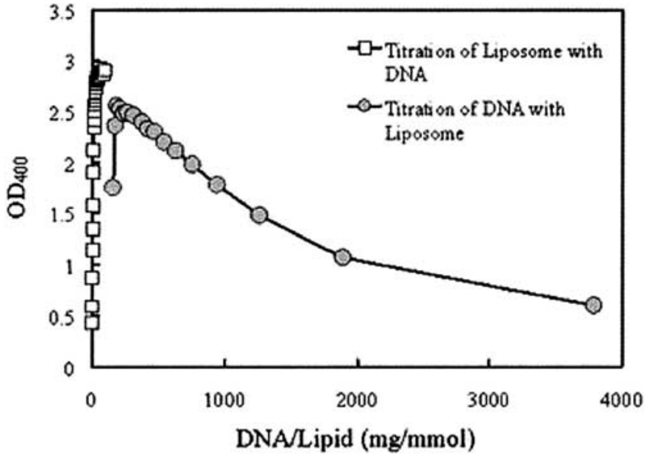

Fig. 2. The aggregation of liposome induced by the titration of plasmids or the aggregation of DNA induced by the titration of cationic liposome. Sediments were formed at 172 μg/μmol of the ratio of DNA/lipid. At this point, the net electric charge of liposome/DNA complex was assumed to be zero, according to DLVO theory.[38] The liposome/DNA complex with a ratio of DNA/lipid below this point should carry positive electric charges and above this point should carry negative electric charges.

(Fig. 2). Liposome/DNA complex was formed after adding the plasmids into SUV suspension; the formation of sediments was not found up to 90 μg/μmol of the ratio of DNA/lipid. In this situation, the complex should carry net positive electric charges. As the DNA solution was titrated with SUV, the sediments were formed at 172 μg/μmol of the ratio of DNA/lipid. At this point, the net electric charge of liposome/DNA complex is assumed to be zero according to DLVO theory.[38] The liposome/DNA complex with a ratio of DNA/lipid above this point should carry net negative electric charges. The DNA/lipid ratio used in the experiments was determined to be 75 μg/μmol. The entrapment efficiency of the plasmids into cationic liposomes was above 90% with all the cationic liposomes used in this experiment.

Induction of Specific CD4$^+$ Th1 Immune Response After DNA Immunization With Naked or Cationic Liposome Encapsulating Plasmids Encoding HCV NS3. Using the ELISPOT technique, cells secreting either Th1 cytokines, IFN γ and IL-2, or Th2 type cytokines, IL-4, were detected 6 days after stimulation with recombinant HCV/NS3 proteins. The frequency of spot-forming cells (FSFC) was expressed as the spot number per 400,000 cells. The number of mice in each group is shown in Table 1.

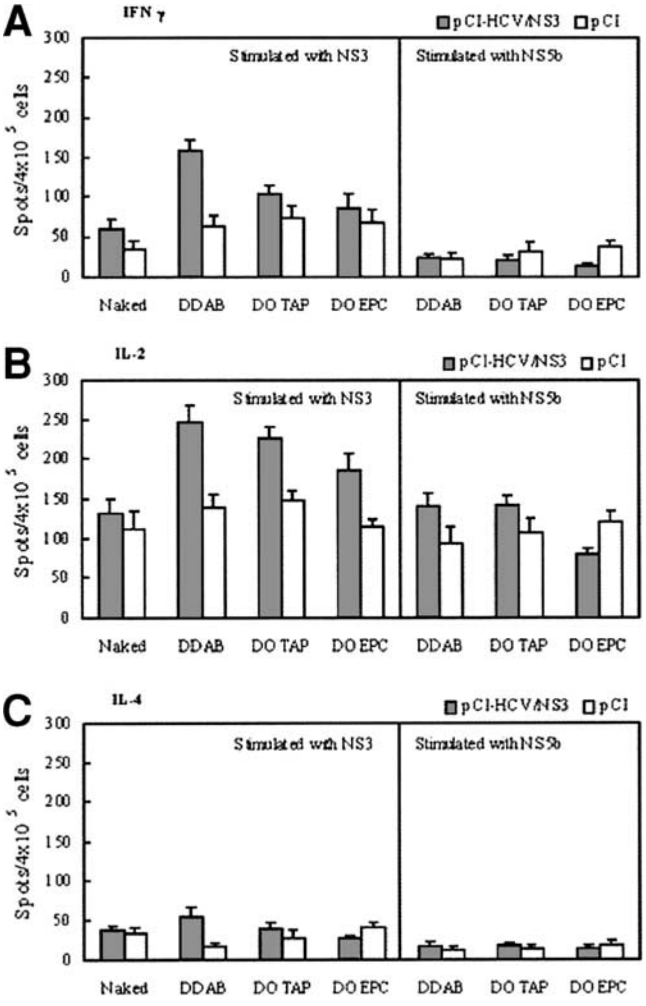

Fig. 3. Numbers of (A) interferon γ (IFN-γ), (B) interleukin-2 (IL-2), and (C) interleukin-4 (IL-4) spot-forming cells (SFC) in response to 5 days stimulation with recombinant HCV NS3 protein (3 μg/mL) in the mice immunized with naked or cationic liposome encapsulating plasmids encoding HCV NS3 or their blank controls. Recombinant HCV NS5b protein (3 μg/mL) was used as control. The mice were immunized by direct IM injections and boosted identically 4 and 6 weeks later. The numbers of SFC were obtained from 4 × 10^5 cells. As a conclusion, the cationic liposome-mediated DNA immunization induced stronger antigen-specific immune response than immunization with naked DNA alone. DDAB/EPC liposome-pCI-HCV/NS3 induced strongest antigen-specific Th1 type immune responses.

As shown in Fig. 3A, FSFC secreting IFN γ in the mice immunized with DDAB/EPC liposme-pCI-HCV/NS3

Table 1. Number of Mice in Each Experimental Group

Group	pCI-HCV/NS3	EPC/DDAB-pCI-HCV/NS3	EPC/DOTAP-pCI-HCV/NS3	EPC/DOEPC-pCI-HCV/NS3	DDAB/DOEPC-pCI-HCV/NS3
No. of mice	10	10	10	10	5
Group	pCI	EPC/DDAB-pCI	EPC/DOTAP-pCI	EPC/DOEPC-pCI	DDAB/DOEPC-pCI
No. of mice	10	8	10	10	5

was significantly higher than the mice immunized with DDAB/EPC liposome encapsulating blank plasmid ($P < .001$). The difference of FSFC secreting IFN-γ between the mice immunized with naked pCI-HCV/NS3 and blank pCI was not statistically significant ($P = .164$). The FSFC secreting IFN-γ in the mice immunized with DDAB/EPC liposme-pCI-HCV/NS3 was significantly higher than naked pCI-HCV/NS3 immunized mice ($P < .001$).

FSFC of IL-2 (Fig. 3B) was much higher than FSFC of IFN-γ in all immunized mice. As in FSFC of IFN-γ, FSFC secreting IL-2 in the mice immunized with DDAB/EPC liposme-pCI-HCV/NS3 was significantly higher than its control ($P = .002$). There was no statistically significant difference of FSFC of IL-2 between naked pCI-HCV/NS3 immunized mice and its control ($P = .48$). The FSFC secreting IL-2 in the mice immunized with DDAB/EPC liposme-pCI-HCV/NS3 was significantly higher than naked pCI-HCV/NS3 immunized mice ($P < .001$).

In the mice immunized with DDAB/EPC liposome-pCI-HCV/NS3, FSCS of IL-4 (Fig. 3C) was significantly higher than its control ($P = .012$). FSCS of IL-4 in naked pCI-HCV/NS3 immunized mice was not different from its control. There was no significant difference of FSFC of IL-4 between the mice immunized with naked and DDAB/EPC liposome-pCI-HCV/NS3. In the mice immunized with DDAB/EPC liposome-pCI-HCV/NS3, FSFC of IL-4 was significantly lower than FSFC of IFN-γ or IL-2.

The ability of cationic liposomes composed with DOTAP/EPC or DOEPC/EPC to enhance DNA immunization was also tested (Fig. 3). Only FSFC of IL-2 in these cationic liposome-pCI-HCV/NS3 immunized mice was found significantly different from their control (DOTAP/EPC: $P < .001$; DOEPC/EPC: $P = .005$). FSFC of IFN-γ in the mice immunized with DOTAP/EPC or DOEPC/EPC liposome encapsulating pCI-HCV/NS3 was higher than their control but not significantly different (DOTAP/EPC: $P = .11$; DOEPC/EPC: $P = .49$) and was lower than in mice immunized with DDAB/EPC liposome encapsulated pCI-HCV/NS3 ($P = .004$ for DOTAP/EPC and $P = .005$ for DOEPC/EPC). FSFC of IL-4 did not differ according to the cationic liposome used and was not significantly different from blank plasmid controls.

These results demonstrated that DDAB/EPC liposome has the best ability to promote DNA immunization. This liposome was composed with a cationic lipid, DDAB, and a neutral lipid, EPC. The ability of another liposome, made with a cationic lipid DOEPC substituting for the neutral lipid EPC, was investigated (Fig. 4). Neither FSFC of IFN-γ nor IL-2 in the mice immunized with DDAB/DOEPC liposome-pCI-HCV/NS3 was significantly different from their controls ($P = .078$ for IFN-γ and $P = .11$ for IL-2). FSFC of IL-4 in the mice immunized with DDAB/EPC liposome-pCI-HCV/NS3 was significantly higher than its control ($P = .003$). Compared with mice immunized with DDAB/EPC liposome, mice immunized with DDAB/DOEPC liposome showed considerably lower IFN-γ and IL-2 responses and much higher IL-4 responses. When the cells from cationic liposome-pCI-HCV/NS3 immunized mice were stimulated with recombinant NS5b proteins, only a few spot forming cells were detected (data not shown), illustrating that the induced Th-cell immune responses were antigen specific.

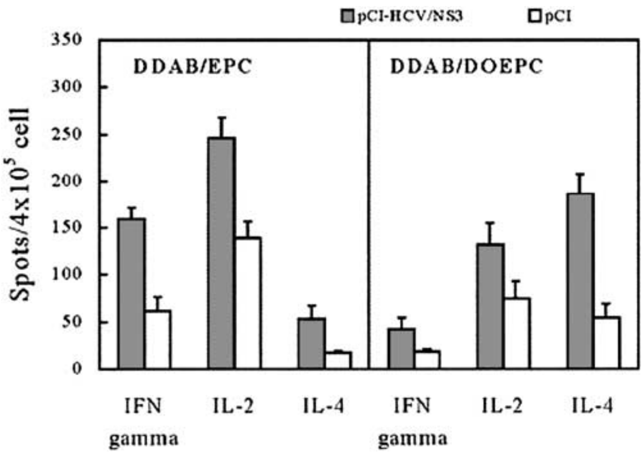

Fig. 4. The cytokine profiles obtained from the mice immunized with pCI-HCV/NS3 encapsulated in different type cationic liposomes. In contrast to DDAB/EPC liposome, DNA immunization mediated with DDAB/DOEPC liposome, composed with 2 cationic lipids, induced Th2 type immune responses.

From the above results, it can be concluded that cationic liposome-mediated DNA immunization induced stronger antigen-specific immune responses than immunization with naked DNA alone. DDAB/EPC liposome-pCI-HCV/NS3 induced the strongest antigen-specific Th1 type immune responses among the cationic liposome investigated, whereas DDAB/DOEPC liposome-pCI-HCV/NS3 induced an antigen-specific Th2 type immune response.

Nonspecific IL-12 Secretion Induced by Cationic Liposome Encapsulated Plasmids Immunization. FSFC of IL-12 in the mice immunized with naked or cationic liposome encapsulating plasmids was measured 5 days after stimulation with recombinant HCV/NS3 proteins (Fig. 5). It was found that FSFC of IL-12 in the mice immunized with cationic liposomes, including DDAB/EPC, DOTAP/EPC, DOEPC/EPC, and DDAB/DOEPC, encapsulating pCI-HCV/NS3 or its blank plasmid pCI was significantly higher than in naked pCI-HCV/

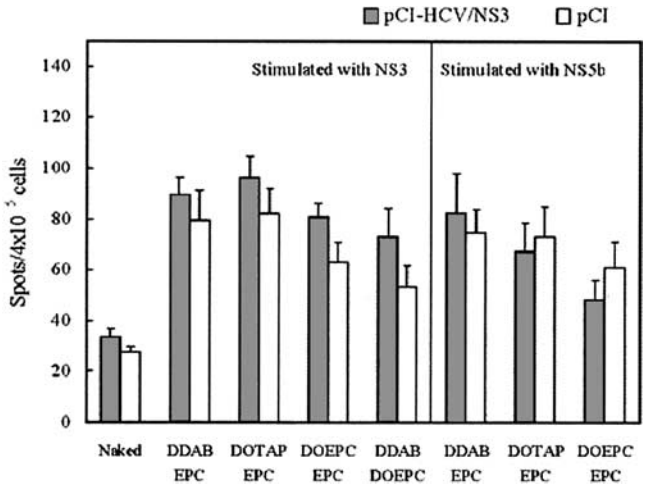

Fig. 5. IL-12 related spot-forming cells in response to the stimulation with recombinant HCV NS3 protein in the mice immunized with naked or cationic liposome encapsulating plasmids encoding HCV NS3 or their blank controls. Recombinant HCV NS5b protein (3 μg/mL) was used as control. It can be concluded that the immunization with cationic liposome encapsulating plasmids induced strong nonspecific IL-12 secretion.

NS3 or blank pCI immunized mice. There was no significant difference in the FSFC for IL-12 between the mice immunized with plasmids encoding HCV/NS3 and their blank control. Spot forming cells of IL-12 in the mice immunized with cationic liposome encapsulating plasmids were also detected when the cells were stimulated with recombinant HCV/NS5b proteins. It can be concluded that the immunization with cationic liposome-encapsulating plasmids induced strong nonspecific IL-12 secretion.

Discussion

Naked DNA immunization is less efficient than expected for 2 primary reasons. First, injected DNA is degraded by interstitial nucleases before it reaches the nucleus for transcription.[21-23] Two studies showed that the titer of anti-NS3 IgG induced by naked DNA immunization was only 20 to 100.[39,40] Our data also showed that the titer of anti-NS3 IgG induced either by pCI-HCV NS3 or pcDNA-HCV NS3 was not exceeded by 100 (unpublished data). Th1 cytokine such as IL-2 and IFN γ responded to NS3 stimulation induced by naked DNA immunization was almost in same level with recombinant protein immunization. We also showed that the cell number secreted IL-2 and IFN-γ responded to HCV NS3 stimulation in pCI-HCV NS3 or pcDNA-HCV NS3 immunization was little different from recombinant HCV NS3 protein immunization (unpublished data). There is an electrostatic obstacle to the uptake of naked DNA by the cells; in aqueous solution, both DNA and cellular membranes are negatively charged, so that they will repel each other.[41] Cationic liposomes can associate with the negatively charged DNA through the positively charged polar head of cationic lipids and subsequently enhance the interaction with the negatively charged cellular membrane. To improve the efficiency of DNA immunization against HCV NS3 in this study, the cationic lipid DDAB was used to form the cationic liposome together with a neutral lipid, EPC. Compared with naked DNA immunization, a 4-fold increase in antigen-specific IFN-γ-secreting cells, a 5-fold increase in antigen-specific IL-2-secreting cells, and a 9-fold increase in antigen-specific IL-4-secreting cells were found in DDAB/EPC liposome-pCI-HCV/NS3 immunized mice. It is thus clear that DDAB/EPC liposomes can enhance the cellular immune responses induced by DNA immunization. A Th1 type immune response appears to be essential for the clearance of HCV infection.[5-8] Th1 or Th2 type immune responses can be determined by the ratio of the cells secreting the Th1 cytokine, IFN γ, and the Th2 cytokine, IL-4. In DDAB/EPC liposome-pCI-HCV immunized mice, antigen-specific IFN-γ-secreting cells were 3-fold as many as the antigen-specific IL-4-secreting cells, indicating that the immune response was of Th1 type.

In an attempt to optimize the formulation of the cationic liposome, DDAB was replaced with DOTAP and DOEPC, 2 commonly used cationic lipids in gene transfection and gene therapy, but their ability to mediate DNA immunization was found inferior to DDAB. Delivery of therapeutic agents to the cytoplasm of target cells by liposomes requires liposome-cellular membrane fusion. Fusion can occur either between the liposomal membrane and the plasma membrane directly or between the liposomal membrane and the endosomal membrane following liposome endocytosis.[42] The ability mediating membrane fusion of the 3 kinds of cationic lipid used in this experiment was DDAB > DOTAP > DOEPC according to the analysis of their head group structure. It appears that the more fusogenic the liposome, the better the ability of the liposome to mediate DNA immunization, but the results were totally different when another more fusogenic liposome was used.

In contrast to DDAB/EPC, which is composed of equal-molar quantities of cationic lipid and neutral lipid, DDAB/DOEPC liposome is composed of equal-molar quantities of 2 cationic lipids. Because DDAB/DOEPC has a higher net positive charge than DDAB/EPC, it was anticipated that it would fuse more efficiently to negatively charged cell membranes and thus enhance immunogenicity. However, our experiments showed that DDAB/DOEPC liposome-pCI-HCV/NS3 presented an altered cytokine profile as compared with the less highly charged DDAB/EPC, resulting in a Th2 response compared with the Th1 response induced by DDAB/EPC. This disparity between the observed and the expected outcome might be explained by the findings of Gregoriadis et al.,[28,31] who demonstrated that the binding of positively charged DNA-containing liposomes to negatively charged muscle cells was inhibited by proteins in the interstitial fluid that confer to a net negative charge to the liposomal surface. We assumed that the moderately positive charged DDAB/EPC-liposome-plasmid might have been up-taken by circulating APC while highly positive charged DDAB/DOEPC-liposom-plasmid might have failed to be neutralized and, thus, internalized at site of injection through binding to the muscle surface membrane. Our ELISPOT cytokine analysis would suggest that plasmids adsorbed by muscle cells will induce a Th2 type immune responses, whereas those captured directly by APC would induce a Th1 type immune response.

Of interest in this study, all of the cationic liposome encapsulating plasmids induced strong IL-12 secretion in immunized mice. IL-12 is very important in the differentiation of Th0 cells to Th1 cells at the site of antigen encounter[43]; Th1 cells, in turn, are induced to secret IFN-γ. It is well known that bacterial DNA triggers innate immune responses and activation of B cells, macrophages, and NK cells.[44-47] Under bacterial DNA stimulation, macrophages, one form of APCs, can be directly activated and release IL-12.[46,48] The immunogenicity of bacterial DNA is due in part to its increased purine content, which is enriched in CpG motifs relative to eukaryotic DNA.[44-47,49,50] By inference, the enhanced immunogenicity of liposome complexes relative to naked DNA, as found in this study, is most likely the result of lipid-facilitated intracellular entry and nuclear translocation of DNA.[48,51] Furthermore, cationic liposomes protect DNA from degradation and prolong circulation time, which may in turn facilitate binding to monocytes and macrophages.[52] DDAB/EPC-liposome-plasmid could be captured directly by APC, and the antigen could be presented on the surface of the same cell. When naïve T cells were primed by APC, it could bind with IL-12 secreted by the same cell immediately, thus differentiated to Th1 cells. Otherwise, DDAB/DOEPC-liposome-plasmid was suggested to be absorbed by muscle cells, and the antigen would be presented by the cells that did not secrete IL-12. When naïve T cells are primed by these cells, they have many opportunities of binding with other kinds of cytokines such as IL-4, thus different to Th2 cells. This could be the reason that even DDAB/DOEPC-liposome-plasmid induced high level of IL-12 secretion, but, because they lack the ability to deliver the plasmids directly to APC, the immune responses favored Th2.

In summary, we clearly demonstrate that cationic liposomes have the ability to enhance and modulate immune responses against HCV NS3 induced by DNA immunization. We propose that the cationic liposome-plasmid complex is captured by APCs and the DNA then released the cytoplasm through fusion with the endosomal membranes and that this pathway induces a Th1 type immune response. We also found that cationic liposome-encapsulating plasmids induced strong IL-12 secretion, which is necessary in the differentiation Th0 cells to Th1 cells. We conclude that the cationic liposome-mediated gene delivery is a viable HCV vaccine strategy that should be tested further in the chimpanzee model.

Acknowledgment: The authors thank Virginia Besch for reviewing the manuscript.

References

1. Cohen J. The scientific challenge of hepatitis C. Science 1999;285:26-30.
2. Stratton KR, Durch JS, Lawrence RS, Hepatitis C. Vaccines for the 21st century. Washington, DC: National Academy Press, 2000.
3. Poynard T, Marcellin P, Lee SS, Niederan C, Minuk G, Bain V, Heathcote J, et al. Randomised trial of interferon a2b plus ribavirin for 48 weeks or for 24 weeks versus interferon a2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. Lancet 1998;352:1426-1432.
4. MaHutchison JG, Gorden SC, Schiff ER, Lee WM, Rustgi VK, Goodman ZD, Ling MH, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. New Engl J Med 1998;339:1485-1492.
5. Rehemann B, Chisary FV. Cell mediated immune response to the hepatitis C virus. Curr Top Microbiol Immunol 2000;242:299-325.
6. Diepolder HM, Zachoval RM, Hoffmann RM, Wierenga EA, Santantonia T, Jung MC, Eichenlaub D, et al. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 1995;346:1006-1007.
7. Tsai S-L, Liaw Y-F, Chen M-H, Huang C-Y, Guo GC. Detection of type-2 like T-helper cells in hepatitis C virus infection: implication for hepatitis C chronicity. HEPATOLOGY 1997;25:449-458.
8. Diepolder HM, Gerlach J-T, Zachoval RM, Hoffmann RM, Jung MC, Wierenga EA, Scholz S, et al. Immunodominant CD4$^+$ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. J Virol 1997;71:6011-6019.
9. Chen Z, Wang RY-H, Alter HJ, Shih JW-K. Genetic immunization of mice with plasmids containing hepatitis C virus core protein-encoding DNA. Vaccine Res 1995;4:135-144.
10. Major ME, Vitvitski L, Mink MA, Schleef M, Whalen RG, Trepo C, Inchauspe G. DNA-based immunization with chimeric vector for the induction of immune response against the hepatitis C virus nucleocapsid. J Virol 1995;69:5798-5805.
11. Hu G-J, Wang RY-H, Han D-S, Alter HJ, Shih JW-K. Characterization of the humoral and cellular immune response against hepatitis C virus core induced by DNA-based immunization. Vaccine 1999;17:3160-3170.
12. Vadalin O, Tanaka E, Spengler U, Trepo C, Inchauspe G. Targeting of hepatitis C virus core protein for MHC I or MHC II presentation does not enhance induction of immune response to DNA vaccination. DNA Cell Biol 1999;18:611-621.
13. Tedeshi V, Akatsuka T, Shih JW-K, Ballegay M, Feinstone SM. A specific antibody response to HCV E2 elicited in mice by intramuscle inoculation of plasmid DNA containing coding sequence for E2. HEPATOLOGY 1997; 25:459-462.
14. Saito T, Sherman GJ, Kurokohchi K, Guo Z-P, Donets M, Yu M-YW, Berzofsky JA, et al. Plasmid DNA-based immunization for hepatitis C

virus structural proteins, immune response in mice. Gastroenterology 1997;112:1321-1320.

15. Nakino I, Maertens G, Major ME, Vitvitski L, Dubuisson J, Fournillier A, De Martynoff G, et al. Immunization with plasmid DNA encoding hepatitis C virus envelop E2 antigenic domains induces antibodies whose immune reactivity is linked to the injection mode. J Virol 1997;71:7101-7109.
16. Forns X, Emerson SU, Tobin GJ, Mushahwar IK, Purcell RH, Bukh J. DNA immunization of mice and macaques with plasmids encoding hepatitis C virus envelop E2 protein expressed intracellularly and on the cell surface. Vaccine 1999;17:1992-2002.
17. Cho JH, Lee SW, Sung YC. Enhanced cellular immunity to hepatitis C virus nonstructural proteins by codelivery of granulocyte macrophage-colony stimulating factor gene in intramuscular DNA immunization. Vaccine 1999;17:1136-1144.
18. Pancholi P, Liu Q, Tricoche N, Zhang P, Perkus ME, Prince AM. DNA prime-canarypox boost with polycistronic hepatitis C virus (HCV) genes generates potent immune responses to HCV structural and nonstructural proteins. J Infect Dis 2000;182:18-27.
19. Zucchelli S, Capone S, Fattori E, Folgori A, Di Marco A, Casimiro D, Simon AJ, et al. Enhancing B- and T-cell immune response to a hepatitis C virus E2 DNA vaccine by intramuscular electrical gene transfer. J Virol 2000;74:11598-11607.
20. Lazdina U, Hultgren C, Frelin L, Chen M, Lodin K, Weiland O, Leroux-Roels G, et al. Humoral and CD4(+) T helper (Th) cell responses to the hepatitis C virus non-structural 3 (NS3) protein: NS3 primes Th1-like responses more effectively as a DNA-based immunogen than as a recombinant protein. J Gen Virol 2001;82:1299-1308.
21. Lewis PJ, Babiuk LA, DNA vaccines: a review. Adv Virus Res 1999;54: 129-188.
22. Gregoriadis G. Genetic vaccines: strategies for optimisation. Pharm Res 1998;15:661-670.
23. Davis HL, Whalen RG, Demeneix B. Direct gene transfer in skeletal muscle in vivo: factors influencing efficiency of transfer and stability of expression. Hum Gene Ther 1993;4:151-156.
24. Creusot RJ, Thomsen LL, van Wely CA, Topley P, Tite JP, Chain BM. Early commitment of adoptive transferred CD4$^+$ T cells following particle mediated DNA vaccination: implications for the study of immunomodulation. Vaccine 2001;19:1678-1687.
25. Zucchelli S, Capone S, Fattori E, Folgori A, Marco AD, Casimiro D, Simon AJ, et al. Enhancing B- and T-cell immune response to a hepatitis C virus E2 DNA vaccine by intramuscular electrical gene transfer. J Virol 2000;74:11598-11607.
26. Mittal SK, Aggarwal N, Sailaja G, van Olphen A, HogenEsch H, North A, Hays J, et al. Immunization with DNA, adenovirus or both in biodegradable alginate microspheres: effect of route of inoculation on immune response. Vaccine 2001;19:253-263.
27. Dietrich G, Gentschev I, Hess J, Ulmers JB, Kaufmann SHE, Goebel W. Delivery of DNA vaccine by attenuated intracellular bacteria. Immunol Today 1999;20:251-253.
28. Gregoriadis G, Saffie R, de Souza JB. Liposome-mediated DNA vaccination. FEBS Lett 1997;402:107-110.
29. Dow SW, Fradkin LG, Liggitt DH, Willson AP, Heath TD, Potter TA. Lipid-DNA complex induce potent activation of innate immune responses and antitumor activity when administered intravenously. J Immunol 1999;163:1552-1561.
30. Lanuti M, Rudginsky S, Force SD, Lambright ES, Siders WM, Chang MY, Amin KM, et al. Cationic lipid: bacterial DNA complexes elicit adaptive cellular immunity in murine intraperitoneal tumor models. Cancer Res 2000;60:2955-2963.
31. Perrie Y, Frederick PM, Gregoriadis G. Liposome mediated DNA vaccination: the effect of vesicle composition. Vaccine 2001;19:3301-3310.
32. Zhou W-Z, Kaneda Y, Huang SKS, Morishita R, Hoon DSB. Protective immunization against melonoma by gp-100 DNA-HVJ-liposome vaccine. Gene Ther 1999;6:1768-1773.
33. Cusi MG, Zurbriggen R, Valassina M, Bianchi S, Durrer P, Valensin PE, Donity M, et al. Intranasal immunization with mumps virus DNA vaccine delivered by influenza virosomes elicits mucosal and systemic immunity. Virology 2000;277:111-118.
34. Crystal RG. Transfer of genes to humans: early lessons and obstacles to success. Science 1995;270:404-410.
35. Whitmore M, Li S, Huang L, LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth. Gene Ther 1999;6:1867-1875.
36. Brunel F, Darbouret A, Ronco J. Cationic lipid DC-Chol induces an improved and balanced immunity able to overcome the unresponsiveness to the hepatitis B vaccine. Vaccine 1999;17:2192-2203.
37. Kirby C, Gregoriadis G. Dehydration-rehydration vesicles: a simple method for high yield drug entrapment in liposomes. Biotechnology 1984; 11:979-984.
38. Vold MJ, Vold RD. Colloid chemistry: The science of large molecules, small particles, and surfaces. New York: Reinhold Publishing Corp., 1964.
39. Encke J, zu Putlitz J, Geissler M, Wands JR. Genetic immunization generates cellular and humoral immune responses against the nonstructural proteins of the hepatitis C virus in a murine model. J Immunol 1998;161: 4917-4923.
40. Gorden EJ, Bhat R, Liu Q, Wang Y-F, Tackney C, Prince AM. Immune responses to hepatitis C virus structural and nonstructral proteins induced by plasmid DNA immunizations. J Infect Dis 2000;181:42-50.
41. Bennet DE, O'Brien DF. Photoactivated enhancement of liposome fusion. Biochemistry 1995;34:3102-3113.
42. Leikins S, Parsegian VA, Rau DC, Hydration forces. Annu Rev Phys Chem 1993;44:369-395.
43. Kourilsky P, Truffa-Bachi P. Cytokine fields and the polarization of the immune response. Trends Immunol 2001;22:502-509.
44. Pisetsky DS. Immune activation by bacterial DNA: a new genetic code. Immunity 1996;5:303-310.
45. Krieg AM. Lymphocyte activation by CpG dinucleotide motifs in prokaryotic DNA. Trends Microbiol 1996;4:73-76.
46. Stacey KJ, Sweet MJ, Hume DA. Macrophages ingest and are activated by bacterial DNA. J Immunol 1996;157:2116-2122.
47. Klinman DM, Yi A, Beaucage SJ, Conover J, Krieg AM. CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin-6, interleukin-12, and interferon-γ. Proc Natl Acad Sci U S A 1996; 93:2879-2883.
48. Chace JH, Hooker NA, Mildenstein KL, Krieg AM, Cowdery JS. Bacterial DNA-induced NK cell IFN-γ production is dependent on macrophage secretion of IL-12. Clin Immunol Immunopathol 1997;84: 185-193.
49. Yi A, Klinman DM, Martin TL, Matson S, Krieg AM. Rapid immune activation by CpG motifs in bacterial DNA. J Immunol 1996;157:5394-5402.
50. Ballas ZK, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J Immunol 1996;157:1840-1845.
51. Friend DS, Papahadjopoulos D, Debs RJ. Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. Biochim Biophys Acta 1996;1278:41.
52. McLean JW, Fox EA, Baluk P, Bolton PB, Haskell A, Pearlman R, Thurston G, et al. Organ-specific endothelial cell uptake of cationic liposome-DNA complexes. Am J Physiol 1997;273:H387-H404.