Liposome-mediated DNA vaccination

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Abstract Numerous reports have indicated that intramuscular injection of antigen-coding naked plasmid DNA can trigger humoral and cell-mediated protective immunity against infection. This follows DNA uptake by muscle fibres, leading to the expression and extracellular release of the antigen. Here it is shown for the first time that intramuscular immunization of mice with pRc/CMV HBS (encoding the S region of hepatitis B antigen; HBsAg) entrapped into positively charged (cationic) liposomes leads to greatly improved humoral and cell-mediated immunity. These cationic liposome-entrapped DNA vaccines generate titres of anti-HBsAg IgG_1 antibody isotype in excess of 100-fold higher and increased levels of both IFN-γ and IL-4 when compared with naked DNA or DNA complexed with preformed similar (cationic) liposomes. It is likely that immunization with liposome-entrapped plasmid DNA involves antigen-presenting cells locally or in the regional draining lymph nodes.

Key words: Liposome; Plasmid DNA; DNA vaccination

1. Introduction

Intramuscular injection of antigen-encoding naked plasmid DNA can trigger humoral and cell-mediated protective immunity against infection [1–4]. Immunity follows DNA uptake by muscle fibres, leading to the expression and extracellular release of the antigen [5,6]. Potential disadvantages with naked DNA vaccination include uptake of DNA by only a minor fraction of muscle cells [5], exposure of DNA to nucleases in the interstitial fluid, the use of relatively large quantities of DNA (e.g. 50–200 μg per mouse) [5] and, often, the need to inject into regenerating muscle in order to enhance immunity [5,7,8].

Administration of antigen-encoding plasmid DNA via liposome could, on the other hand, circumvent the need of muscle involvement and facilitate [9] instead its uptake by antigen presenting cells (APC), for instance those infiltrating the site of injection or in the lymphatics, at the same time protecting DNA from nuclease attack [10]. Moreover, transfection of APC with liposome-entrapped DNA could be promoted by the judicial choice of vesicle surface charge and lipid composition [1,10]. To that end, we have recently developed a method [10] which allows for the quantitative entrapment of DNA into neutral, anionic and cationic liposomes all of which are capable of transfecting cells in vitro with varying efficiency [10]. Here, it is shown for the first time that intramuscular immunization of mice with pRc/CMV HBS (encoding the S region of hepatitis B surface antigen; ayw subtype) entrapped into such liposomes leads to improved humoral and cell-mediated immunity. Cationic liposome-entrapped DNA vaccines, for instance, generate titres of IgG_1 antibody isotype in excess of 100-fold higher and increased levels of both IFN-γ and IL-4 when compared with naked DNA or DNA complexed with preformed similar (cationic) liposomes. It is likely that immunization with liposome-entrapped plasmid DNA involves antigen-presenting cells (APC) locally or in the regional draining lymph nodes.

2. Materials and methods

2.1. Materials

Plasmid pRc/CMV HBS, cloned by Dr. Robert Whalen using pRc/CMV (InVitrogen) as vector backbone, expresses sequences coding for the S (small) protein of hepatitis B virus (HBsAg, subtype ayw). Recombinant hepatitis B surface antigen (HBsAg) (S region; subtype ayw) was purchased from Genzyme, West Malling, Kent, UK. The sources and grades of egg phosphatidylcholine (PC), dioleoyl phosphatidylcholine (DOPE), stearylamine (SA), and phosphatidylserine (PS) have been described elsewhere [10]. 1,2-Dioleoyloxy-3-(trimethylammonium)propane (DOTAP) was from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and 3β-(*N,N*-dimethylaminoethane)carbamyl cholesterol (DC-Chol) was a gift from Dr. C. Kirby.

2.2. Entrapment of plasmid DNA into liposomes

pRc/CMV HBS mixed with ^{35}S-labelled tracer, was entrapped in liposomes as previously described for a luciferase encoding plasmid DNA [10]. Briefly, small unilamellar vesicles (SUV) prepared [10,11] from 16 μmol PC and 8 μmol DOPE in the absence (uncharged) or presence of either 4 μmol PS (anionic) or 4 μmol of SA, DOTAP or DC-Chol (cationic liposomes), were mixed with 10–100 μg DNA and freeze-dried overnight. Following rehydration under controlled conditions [10,11], the generated dehydrated-rehydrated vesicles (DRV liposomes) were washed by centrifugation and suspended in 0.15 M sodium phosphate buffer supplemented with 0.9% NaCl, pH 7.4 (PBS). DNA entrapment values were, as expected high for instance [10], 53–58% (neutral and anionic) and 77–83% (cationic liposomes) of the amount used (results not shown). In some experiments, preformed cationic (DOTAP) DRV were mixed with 10–100 μg DNA, incubated for 30 min at 20°C and then washed by centrifugation as above. Values of complexed DNA were 83–94% of the amount used (results not shown).

2.3. DNA immunisation

Male Balb/c mice 4–8 weeks old were bled from the tail vein 1–2 days before a single or several intramuscular (hind leg) injections of 50 μl PBS containing 1–10 μg naked, complexed or liposome-entrapped (about 0.5 mg PC) pRc/CMV HBS (see legends to Figs. 1–4 for details). Animals were bled again at time intervals after the first injection and sera (diluted 20-fold in PBS) kept at −40°C. Sera were tested for anti-HBsAg (S region; ayw subtype) IgG_1, IgG_{2a} and IgG_{2b} by the enzyme-linked immunoadsorbent assay (ELISA) (using the same antigen to coat the plates) as applied previously [12] for the same antigen. Immune response was expressed as the $\log_{10}$ of the reciprocal of serial 3-fold serum dilution required for OD to reach a reading of about 0.200 (end point dilution). Sera from untreated mice gave $\log_{10}$ values of less than 2.0.

2.4. Cytokine extractions

Endogenous levels of IFN-γ and IL-4 in the spleen were determined using the method of Nakane et al [13] as previously modified [14]. Individual spleens were weighed, homogenized in ice-cold RPMI con-

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taining 1% 3-[(cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS; Sigma) in a Dounce tissue homogenizer and 10% (w/v) homogenates were prepared. Homogenates were left on ice for 1 h and insoluble debris were then removed by centrifugation at 2000×*g* for 20 min. The clear supernatants were stored at −70°C.

2.5. Cytokine assays

Standard capture ELISAs were used with monoclonal antibody pairs and Maxisorp (NUNC, UK) plates. Primary monoclonal antibodies against IFN-γ (R46A2) and IL-4 (11B11) and secondary biotinylated anti-mouse IL-4 (BVD6-24G2) and anti-mouse IFN-γ (XMG1.2) monoclonal antibodies (Pharmingen, USA) were used with streptavidin peroxidase (Dako, Denmark) and *o*-phenylenediamine (Sigma) as substrate. Recombinant IFN-γ and IL-4 standards were from Pharmingen. Results (mean ± S.D.) are expressed as ng/spleen from at least 4 mice.

2.6. Statistical analysis

Significance levels were determined by the Student's *t*-test for unpaired observations.

3. Results and discussion

In a number of separate experiments, Balb/c mice were injected intramuscularly several times with pRc/CMV-HBS as naked DNA, entrapped into uncharged or charged liposomes, or complexed with similar preformed cationic liposomes. Fig. 1 and legend show that in mice injected repeatedly during a 37 day period with two different doses (5 and 10 μg) of DNA entrapped in positively charged liposomes incorporating a cationic lipid (DOTAP, DC-Chol or SA), titres of antibody (IgG_1) responses against the encoded antigen (HBsAg) were up to at least 100-fold greater at all times tested (26, 34 and 44 days after the first injection) than titres in mice immunized with naked DNA. IgG_{2a} and IgG_{2b} titres for the liposomal DNA were also greater, albeit to a lesser extent (about 10-fold).

Complexes of cationic vesicles with plasmid DNA are highly effective in transfection in vitro [1,15] and, in a recent study [10], one such complex of Lipofectin with DNA (pGL_2-control expressing the luciferase reporter gene from a SV40 promoter) was 10–100-fold more efficient in transfecting cultured COS-7 cells than the same DNA entrapped by the present technique in uncharged or charged liposomes. However, Lipofectin complexed with antigen-encoded plasmid DNA did not augment antibody responses to the antigen in another study [16] in vivo. We therefore compared entrapped (charged and uncharged liposomes) and complexed (with similar cationic liposomes) pRc/CMV-HBS in terms of immune responses to the encoded antigen using the same protocol of immunization as in Fig. 1. Results (Fig. 2) show that, contrary to the aforementioned [10] in vitro transfection efficiency data, DNA entrapped in cationic (DOTAP) liposomes produced greater (over 80-fold) IgG_1 titres than complexed DNA (10 μg dose; 28 days). Interestingly, uncharged liposomes were also found capable of transfecting entrapped DNA although, judging from the antibody titres (Fig. 2, 10 μg), with reduced efficiency. Animals from the experiment in Fig. 2 were also tested for T cell responses and to that end, levels of IFN-γ and IL-4 in their spleens, 3 weeks after the final immunization, were measured as indicators of Th1 and Th2 subset T cell activation. The data (Fig. 3) show that activation for both Th1 and Th2 subsets was greater with liposome-entrapped DNA when compared with naked or complexed DNA. It therefore appears that immunization

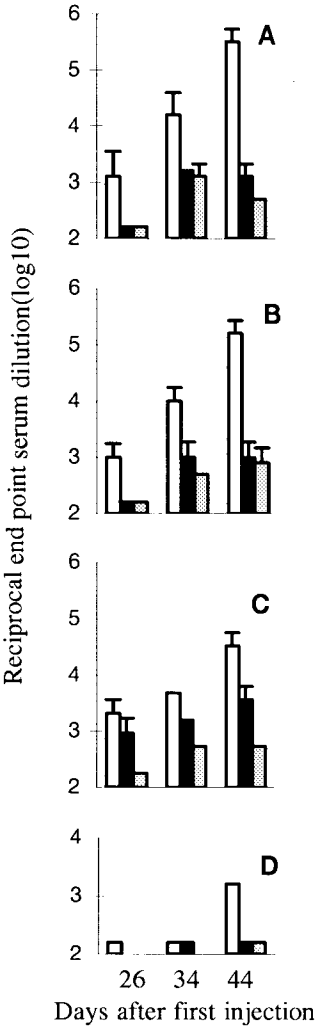

Fig. 1. Comparison of immune responses in mice injected with plasmid DNA as such or entrapped in different cationic liposomes. Balb/c mice in groups of four were injected intramuscularly on days 0, 10, 20, 27 and 37 with 5 μg of pRc/CMV HBS entrapped in positively charged liposomes composed of PC, DOPE and DOTAP (A), DC-Chol (B) or SA(C), or in the naked form (D). Animals were bled 7, 15, 26, 34 and 44 days after the first injection and sera tested for IgG_1 (white bars), IgG_{2a} (black bars) or IgG_{2b} (dotted bars) responses against the encoded antigen hepatitis B surface antigen (HBsAg; S region, ayw subtype). Values are means ± S.D. of $\log_{10}$ of reciprocal end point serum dilutions required for OD to reach readings of about 0.200. Similar values (all groups) were obtained in mice injected as above with 10 μg DNA in a separate experiment (results not shown). Immune responses were mounted by all mice injected with liposomal DNA but became measurable only at 26 days. Differences in $\log_{10}$ values (all IgG subclasses at all time intervals) in mice immunized with liposomal DNA and mice immunized with naked DNA were statistically significant ($P < 0.0001$–0.002).

with liposome-entrapped plasmid DNA induces both humoral and cell-mediated immunity.

Most reports [1,3,4,6,17–20] on naked DNA vaccination have employed protocols of multiple injections, but a single dose also produces a humoural response to the encoded antigen [5,21]. For instance, total IgG response for the naked pRc/CMV HBS (identical to the plasmid used here) was detectable 1–2 weeks after injection, to reach peak values by 4–8 weeks [5,22]. Under the present conditions of single immuni-

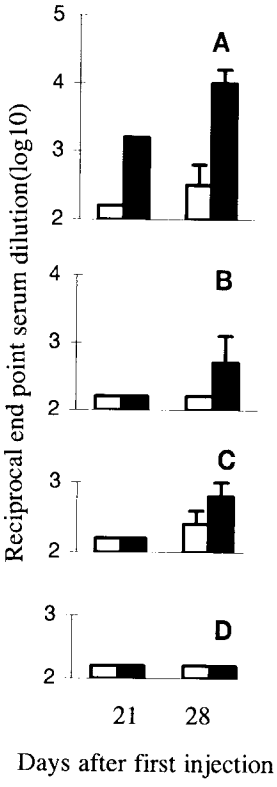

Fig. 2. Comparison of immune responses in mice injected with complexed or liposome-entrapped plasmid DNA. Balb/c mice in groups of four were injected intramuscularly on days 0. 7, 14, 21 and 28 with 1 (white bars) or 10 μg (black bars) of pRc/CMV HBS entrapped in positively charged liposomes composed of PC, DOPE and DOTAP (A), uncharged liposomes composed of PC and DOPE (B), complexed with similar preformed cationic DOTAP liposomes (C) or in naked form (D). Sera from animals bled at 7, 14, 21 and 28 days after the first injection were analysed for anti-HBsAg IgG_1 by ELISA. Immune responses were mounted by all mice injected with liposomal DNA but became measurable only at 21–28 days. For other details see legend to Fig. 1. Differences in $\log_{10}$ values (10 μg dose, 21 and 28 days) between mice immunized with cationic liposomal DNA and mice immunized with neutral liposomal, complexed and naked DNA as well as differences between neutral or liposomal or complexed and naked DNA were statistically significant ($P < 0.0001$–0.005).

zation (Fig. 4) with much lower doses of pRc/CMV HBS (2 and 10 μg), anti-HBsAg IgG_1 response for naked and complexed DNA was barely detectable even by 10 weeks. In contrast, there was an early and pronounced IgG_1 response for DNA entrapped in cationic liposomes (peaking at 5–7 weeks), and a delayed but significant response for DNA entrapped in neutral or negatively charged liposomes (Fig. 4 and legend).

The mechanism of liposome-mediated immunity to the antigen encoded by the plasmid DNA is unknown at present. With naked DNA vaccination, several, possibly concurrent, pathways leading to Th1 and Th2 immunity have been suggested [5,6]. They include secretion of the antigen by the DNA-transfected muscle cells and subsequent processing and presentation by resident Langerhans cells or infiltrating APC; destruction of transfected muscle cells via a Tc cell response resulting in release of the antigen; and transfection of both muscle cells and resident APC causing simultaneous activation of both T cell subsets. Work [23,24] on the fate of liposomes in vivo has produced no evidence of significant vesicle uptake by muscle cells after local injection. Instead, liposomes enter the lymphatic system to localize in the lymph nodes [9] and, indeed, such fate has been implicated in the action of liposomes as immunological adjuvants [25]. Although positively charged (DNA-containing) liposomes could conceivably bind to the negatively charged surface of muscle cells and be interiorized by them, this is probably prevented by proteins in the interstitial fluid which, on injection, are expected [23] to confer a net negative charge on the liposomal surface. It is more likely that positively charged liposomes (in which about 25% of total DNA content is exposed on the vesicle surface [10]) are phagocytosed by APC infiltrating the site of injection or in the lymphatics. Phagocytosis can apparently [26] be facilitated by the condensed state of the DNA (attained [27] on interaction with cationic lipids) and could explain the reduced levels of immune responses with DNA entrapped in uncharged or negatively charged liposomes. The fusogenic [27] DOPE component of liposomes, possibly in conjunction [27] with the cationic lipid, may then mediate DNA entry into the cytoplasm for eventual episomal transfection and presentation of the encoded antigen. Regardless of the mechanisms involved however, liposome-mediated DNA vaccination under the conditions described, appears

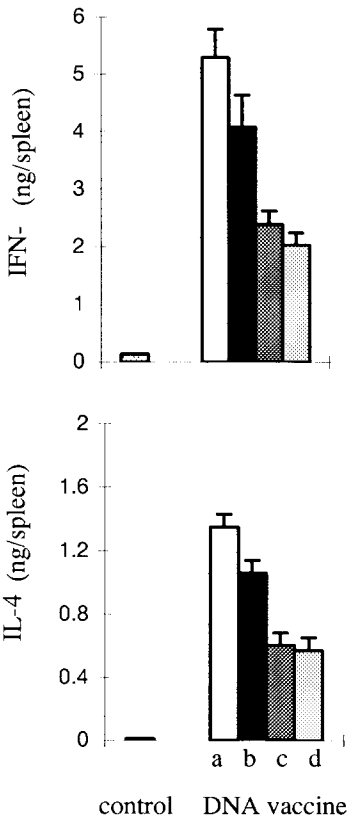

Fig. 3. Cytokine levels in the spleens of mice immunized with naked, complexed or liposome-entrapped plasmid DNA. Mice were immunized as in Fig. 2 with pRc/CMV HBS entrapped into either positively charged (a) or uncharged liposomes (b), complexed with positively charged liposomes (c), or in naked form (d). 'Control' represents cytokine levels in normal unimmunized mice. 3 weeks after the final injection, mice were killed and their spleens subjected to cytokine analysis. Each bar represents the mean ± S.D. of a group of 4 mice. Cytokine values in mice immunized with cationic liposomes were significantly higher than those in the other groups ($P < 0.001$–0.05).

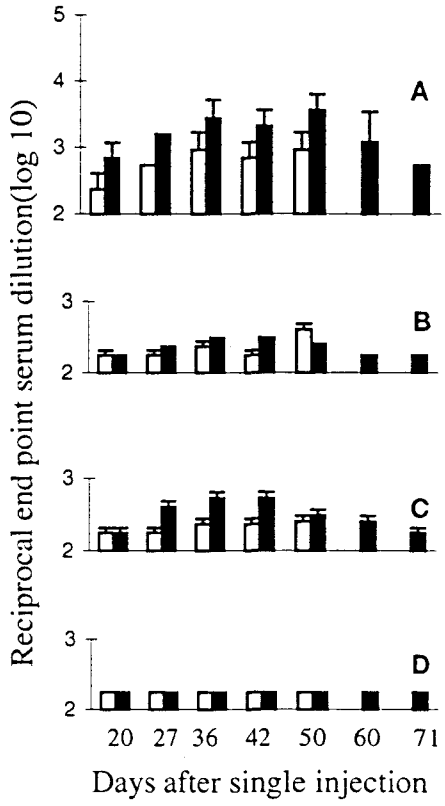

Fig. 4. Immune responses in mice after a single injection of plasmid DNA. Balb/c mice in groups of four were injected once intramuscularly with 2 (white bars) or 10 μg (black bars) of pRc/CMV HBS entrapped in positively charged liposomes composed of PC, DOPE and DOTAP (A), uncharged liposomes composed of PC and DOPE (B), complexed with preformed similar DOTAP liposomes (C) or in the naked form (D). Anti-HBsAg IgG_1 responses were analysed (ELISA) in sera obtained at time intervals after injection. Immune responses were mounted by all mice injected with liposomal DNA but became measurable only at 20–27 days. For other details see legend to Fig. 1. Differences in $\log_{10}$ values (both doses; all time intervals) between mice immunized with cationic liposomal DNA and mice immunized with naked DNA were statistically significant ($P < 0.0001$–0.002). In a fifth group of four mice immunized once as above with 10 μg pRc/CMV HBS entrapped in anionic liposomes composed of PC, DOPE and PS (see Section 2), IgG_1 immune responses ($\log_{10}$) were 2.25 ± 0.0 and 2.73 ± 0.0 at 21 and 29 days, respectively.

more effective than naked DNA in inducing both humoral and cell mediated immunity.

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