

A role for liposomes in genetic vaccination

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Abstract

Genetic immunization by the use of plasmid DNA encoding antigens from bacteria, viruses, protozoa and cancers has often led to protective humoral and cell-mediated immunity, and has some practical advantages over conventional vaccines. However, naked DNA vaccines can be degraded by nucleases in situ, are unable to target antigen presenting cells (APCs), and exhibit poor performance when administered by routes other than the intramuscular, all of which have reduced the value of the approach. We have been able to avoid DNA degradation and also target DNA to APCs by the use of liposomes as DNA vaccine carriers. Entrapment of plasmid DNA within the aqueous spaces of cationic liposomes is effected by a one step procedure which results in most of the DNA being incorporated into a freeze dried, ready to use preparation. Animal experiments have shown that immunization by the intramuscular or the subcutaneous route with liposome-entrapped plasmid DNA encoding the hepatitis B surface antigen leads to much greater humoral (IgG subclasses) and cell mediated (splenic IFN- γ) immune responses than with naked DNA. In other experiments with a plasmid DNA encoding a model antigen (ovalbumin), a cytotoxic T lymphocyte (CTL) response was also observed. These results could be explained by the ability of liposomes to protect their DNA content from local nucleases and direct it to APCs in the lymph nodes draining the injected site.

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1. Introduction

Use of vaccines for the prevention of microbial infections is a preferred alternative to treatment. It has been applied successfully, for instance, in the eradication of smallpox as well as against tetanus, diphtheria, whooping cough, polio and measles, thus preventing millions of deaths each year. However, vaccines made of attenuated organisms, although efficacious in producing diverse and persistent immune responses by mimicking natural infections usually without the disease, can have certain disadvantages. Thus, there is a risk of reversion during replication of live viruses or even mutation to a more pathogenic state and with immunocompromised individuals some of the attenuated viruses may still provoke disease. On the other hand, although the extracellular localization of killed virus vaccines and their subsequent phagocytosis by professional antigen presenting cells (APCs) or antigen-specific B cells lead to MHC class II restricted presentation and to T helper cell and humoral immunity, they do not elicit significant cytotoxic T lympho-

cyte (CTL) responses. Moreover, subunit vaccines produced from biological fluids may not be entirely free of infectious agents. Unfortunately, subunit and peptide vaccines produced recombinantly or synthetically (and thus considered safe), are weak immunogens and often unable to induce appropriate immune responses. A great variety of experimental immunological adjuvants [1,2] now available go a long way in rendering such vaccines stronger and more efficient. However, 70 odd years after the introduction of aluminum salts as an adjuvant, only two other adjuvants, liposomes [3] and MF59 [1], have been approved for use in humans [4]. Thus, in spite of considerable progress, the road to the ideal vaccine appears as elusive as ever, that is until recently.

A novel and exciting concept now developed, namely de novo production of the required vaccine antigen by the host's cells in vivo, promises to revolutionize vaccination especially where vaccines are either ineffective or unavailable. The concept entails the direct injection of antigen-encoding plasmid DNA which, following its uptake by cells, finds its way into the nucleus where it transfects the cells episomally. Produced antigen, recognized as foreign by the host, is then subjected to pathways similar to those undergone by the antigens of internalized viruses (but without their disadvantages) leading to protective humoral and

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cell mediated immunity [5–9]. A succession of publications from 1992 onwards established first the ability of plasmid DNA to induce an immune (antibody) response to the encoded foreign protein [10] and then, in experiments with DNA encoding influenza nucleoprotein, that immunity was both humoral and cell-mediated and also protective in mice challenged with the virus [11,12]. This was the first demonstration of an experimental DNA vaccine. At about the same time, humoral and cell-mediated immunity against HIV-1 using plasmids encoding the HIV rev and env proteins was reported [13] and similar results were obtained a little later with a gene for the hepatitis B surface antigen (HBsAg) [14]. It also appeared that DNA immunization could be applied in cancer treatment: injection of plasmids encoding tumor antigens resulted in the induction of immune responses [15,16] which were protective in an animal model [6]. Following these pioneering studies, the concept of DNA immunization has now been adopted by vaccinologists worldwide using an ever increasing number of plasmids encoding immunogens from bacterial, viral and parasitic pathogens and a variety of tumors [8,9]. In many of these studies genetic immunization led to the protection of animals from infection [5–9]. Significantly, clinical trials for the therapy of or prophylaxis against a variety of infections are already in progress [8,9].

2. The plasmid vaccine

A successful DNA vaccine, usually [6] supercoiled, consists of the gene encoding the antigen of interest (normally the section of the target pathogen which elicits protective immunity), a promoter sequence (often derived from cytomegalovirus (CMV) or Rous sarcoma virus (RSV) to drive the transcription of the antigen gene insert, an mRNA stability polyadenylation region at the 3' end of the insert to ensure translation, the plasminogen activator gene which controls the secretion of the recombinant product, and ancillary signals. There are in addition an origin of replication for the amplification of the plasmid in bacteria and a gene for antibiotic resistance to select the transformed bacteria. As only one or two representative viral genes are selected for insertion in the DNA plasmid and not the full length viral genetic information, there is no danger of genetic recombination with superinfection by natural viral isolates.

DNA vaccines are easy and inexpensive to produce as plasmid vectors can be constructed and tested rapidly. Also, plasmids are more temperature stable than live vaccines and their storage in a lyophilized form is straightforward. Recent developments in genetic vaccination technology have led to a common set of operational procedures [7]. In brief, after its construction, the plasmid is amplified in *Escherichia coli*. A large set of different *E. coli* host strains have been studied to identify those producing large amounts of DNA per cell of the highest quality. The produced plasmid is then

purified from the lysed cells by gel chromatography or density gradient centrifugation, followed by phenol extraction. It is essential that plasmids made for in vivo use are highly homogeneous, sterile and free of all contamination, particularly endotoxins, RNA, protein and genomic DNA. To that end, the QIAGEN procedure has been approved by European countries and the USA [18].

The majority of the immunization procedures carried out so far have opted for the intramuscular and, to a lesser extent, the intraepidermal route. Other routes such as the oral, nasal, vaginal, intravenous, intraperitoneal and subcutaneous have also been used with various degrees of success [8,9]. For intramuscular injections, it is not uncommon to pretreat the tissue with cardiotoxin and other drugs that cause muscle damage followed by regeneration [5], or the anaesthetic bupivacaine which dilates local vessels thus enhancing DNA uptake by myocytes [13]. There are a number of other variables in the way vaccines are administered that need full evaluation before consensus is reached as to their significance in terms of optimal immune response [17].

It was initially observed [11,12] and subsequently confirmed [5–9] that intramuscular injection of DNA vaccines leads to such types of immunity as CTL. This was unexpected because antigen presentation requires the function of professional APCs [19]. However, myocytes which were shown [5] to take up the plasmid, albeit only to a small extent and with only a fraction of cells participating in the uptake, are not professional APCs. Although myocytes carry MHC class I molecules and can present endogenously produced viral peptides to the CD8⁺ cells to induce CTLs, they do so inefficiently [17] as they lack vital co-stimulatory molecules such as the B7-1 molecule. It has thus been difficult to accept that antigen presentation leading to a CTL response occurs via myocytes. Instead, it was reported [17] that CTL responses are, at least in part, the result of transfer of antigenic material between the muscle cells and professional APCs. It is also likely that plasmid secreted by the myocytes or as such is taken up directly by APCs infiltrating the injected site. Such cells would include dendritic cells which will express and present peptides to CD8⁺ cells following transport to the lymph nodes or spleen. On the other hand, CD4⁺ cells may be activated by APCs via MHC class II presentation of antigen secreted by the myocytes (or released from them after their destruction via a Tc response) and captured by the cells. Such events would lead to both cellular (Th 1) and humoral (Th 2) immunity. According to a recent report [6], dendritic cells are the essential APCs involved in immune responses elicited by intramuscularly given DNA vaccines.

3. Liposome-mediated DNA vaccination

As already mentioned, vaccination with naked DNA by the intramuscular route relies on the ability of myocytes to engulf the plasmid. Some of the DNA may also be en-

docytosed by APCs infiltrating the site of injection or in the lymph nodes following its migration to the lymphatics. The extent of DNA degradation by extracellular deoxyribonucleases is unknown but, depending on the time of its residence interstitially, degradation could be considerable. In the gut milieu, naked DNA has failed to elicit an immune response, probably because of its anticipated complete degradation [6]. It follows that approaches to protect DNA from the extracellular biological milieu, introduce it into cells more efficiently or target it to immunologically relevant cells should contribute to optimal DNA vaccine design.

It has been proposed [20] that, as APCs are a preferred alternative to muscle cells as targets for DNA vaccine uptake and expression, liposomes would be a suitable means of delivery of entrapped DNA to such cells. Locally injected liposomes are known [3] to be taken up avidly by APCs infiltrating the site of injection or in the lymphatics, an event that has been implicated [3] in their immunoadjuvant activity. Liposomes would also protect [21] their DNA content from deoxyribonuclease attack. Because of the structural versatility [22] of the system, its transfection efficiency could be further improved by the judicious choice of vesicle surface charge, size and lipid composition or by the co-entrapment of cytokine genes and other adjuvants (e.g. immunostimulatory sequences), together with the plasmid vaccine. Moreover, as a number of injectable liposome-based drug formulations including vaccines against hepatitis A and influenza have been already licensed in the USA and Europe for clinical use [22], acceptance of the system clinically would be less problematic.

3.1. Entrapment of plasmid DNA into liposomes

We have shown [8,23,24] that a variety of plasmid DNAs can be quantitatively entrapped into the aqueous phase of multilamellar liposomes by a mild dehydration–rehydration procedure [21,23,24]. This consists of mixing preformed small unilamellar vesicles (SUVs) with a solution of the DNA destined for entrapment, freeze–drying of the mixture and controlled rehydration of the formed powder followed by centrifugation to remove non-entrapped material. Incorporation values shown in Table 1 were, as expected, higher (57–90% of the amount used) when a cationic lipid was present in the bilayers. No apparent relationship was observed between amount of DNA used (10–500 µg) and values of incorporation for the compositions and lipid mass shown. The possibility that DNA was not entrapped within the bilayers of cationic liposomes but was rather complexed with their surface (as suggested by the similarly high “incorporation” values obtained on mixing; Table 1) was examined by exposing liposome-entrapped and liposome-complexed DNA to deoxyribonuclease: substantially more liposome-entrapped DNA remained intact than when it was complexed [21], presumably because of the inability of the enzyme to reach its substrate. The significant resistance of complexed DNA to the enzyme (despite its accessibility) could be attributed to its condensed state [25]. Further evidence of DNA actual entrapment within liposomes rather than surface-complexed, was obtained by gel electrophoresis of a mixture of cationic SUVs and plasmid DNA before (complexed) and after the dehydration–rehydration process (entrapped DNA). In the

Table 1
Incorporation of plasmid DNA into liposomes by the dehydration–rehydration method

Liposomes	Incorporated plasmid DNA (percentage of used)					
	pGL2	pRc/CMV HBS	pRSVGH	pCMV4.65	pCMV4.EGFP	VR1020
PC, DOPE (a)	44.2		45.6	28.6		
PC, DOPE (b)	12.1		11.3			
PC, DOPE, PS (a)	57.3					
PC, DOPE, PS (b)	12.6					
PC, DOPE, PG (a)			53.5			
PC, DOPE, PG (b)			10.2			
PC, DOPE, SA (a)	74.8					
PC, DOPE, SA (b)	48.3					
PC, DOPE, DC-Chol (a)		87.1	76.9			
PC, DOPE, DC-Chol (b)			77.2			
PC, DOPE, DOTAP (a)		80.1	79.8	52.7	71.9	89.6
PC, DOPE, DOTAP (b)		88.6	80.6	67.7		81.6
PC, DOPE, DODAP (a)			57.4			
PC, DOPE, DODAP (b)			64.8			

³⁵S-labeled plasmid DNA (10–500 µg) was incorporated (a) into or mixed (b) with neutral (PC, DOPE), anionic (PC, DOPE, PS or PG) or cationic (PC, DOPE, SA, DC-Chol, DOTAP or DODAP) dehydration–rehydration vesicles (DRVs). Incorporation values for the different amounts of DNA used for each of the liposomal formulations did not differ significantly and were therefore pooled (values shown are means of values obtained from three–five experiments). PC (16 µmol) was used in molar ratios of 1:0.5 (neutral) and 1:0.5:0:25 (anionic and cationic liposomes). PC, egg phosphatidylcholine; DOPE, dioleoyl phosphatidyl ethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; SA, stearylamine; DC-Chol, 3-(*N,N*-dimethylaminoethane) carbonyl cholesterol; DOTAP, 1,2-dioleoyl-3(trimethylammonium) propane; DODAP, 1,2-dioleoyl-3-dimethylammonium propane. Plasmid DNAs used encoded luciferase (pGL2), hepatitis B surface antigen (S region) (pRc/CMV HBS), human growth hormone (pRSVGH), mycobacterium leprosy protein (pCMV4.65), enhanced green fluorescent green protein (pCMV4.EGFP) and schistosome protein (VR1020).

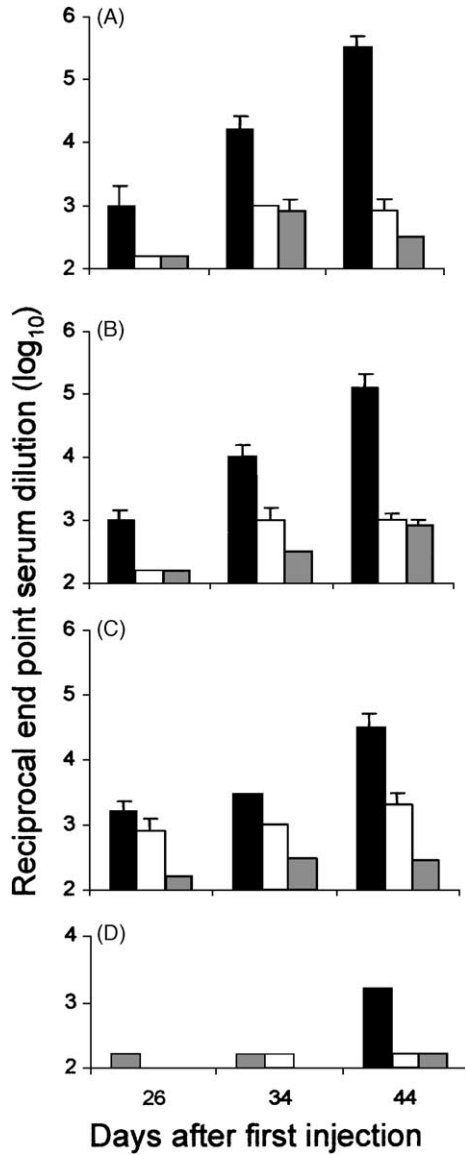
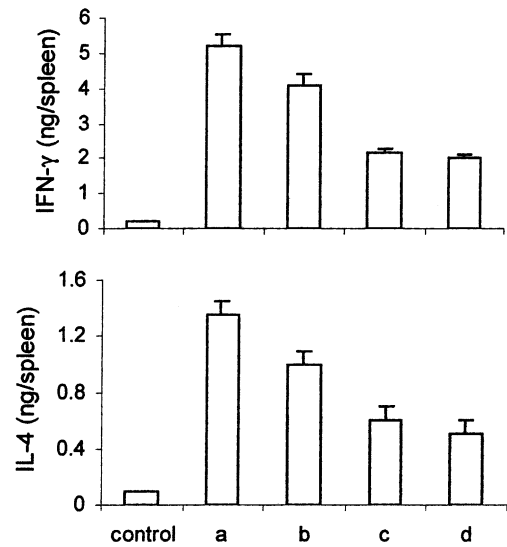


Fig. 1. Immune responses in mice injected with naked, or liposome-entrapped pRc/CMV HBS. BALB/c mice were injected intramuscularly on days 0, 10, 20, 27 and 37 with 5 µg of DNA entrapped in cationic liposomes composed of PC, DOPE and DOTAP (A); DC-Chol (B); or SA (C) (molar ratios 1:0.5:0.25); or in the naked form (D). Animals were bled 7, 15, 26, 34 and 44 days after the first injection and sera tested by ELISA for IgG₁ (black bars), IgG_{2a} (white bars) or IgG_{2b} (gray bars) responses against the encoded hepatitis B surface antigen (HBsAg; S region, ayw subtype). Values are means ± S.D. of log₁₀ of reciprocal end point serum dilutions required for OD to reach readings of about 0.2. Similar values (all groups) were obtained in mice injected as above with 10 µg DNA in a separate experiment (results not shown). Sera from untreated mice gave log₁₀ values of less than 2.0. IgG₁ responses were mounted by all mice injected with liposomal DNA but became measurable only at 26 days. Differences in log₁₀ 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). Reproduced with permission from [20].

presence of the anionic sodium dodecylsulfate (SDS) (incorporated in the gel), complexed DNA was dissociated from the SUVs presumably because of ionic competition for the cationic charges. In contrast, “entrapped” DNA retained its association with the liposomes, suggesting its unavailability to the competing SDS anion [26].

3.2. Immunization studies

Plasmid-containing liposomes, previously [21] found to transfect cells in vitro regardless of the vesicle surface charge, were tested in immunization experiments [20,27] using a plasmid (pRc/CMV HBS) encoding the S region of the hepatitis B surface antigen (HBsAg; subtype ayw). Fig. 1 and legend show that mice (BALB/c) injected repeatedly by the intramuscular route with 5 or 10 µg plasmid entrapped in cationic liposomes, elicited at all times tested much greater (up to 100-fold) antibody (IgG₁) responses against the encoded antigen than animals immunized with the naked DNA. Responses for other subclasses (IgG_{2a} and



- A: PC:DOPE:DOTAP(DNA)
- B: PC:DOPE(DNA)
- C: PC:DOPE:DOTAP-DNA
- D: Naked DNA

Fig. 2. Cytokine levels in the spleens of mice injected with naked, entrapped or complexed pRc/CMV HBS. BALB/c mice were immunized as in Fig. 1 with DNA entrapped into either cationic (DOTAP) (a) or uncharged liposomes (b), mixed with cationic (DOTAP) liposomes (c), or in the naked form (d). “Control” denotes cytokine levels in normal unimmunized mice. Three weeks after the final injection, mice were killed and their spleens subjected to IL-4 and IFN-γ analysis. Each bar represents the mean ± S.E. of a group of four mice. Cytokine values in mice immunized with cationic liposomes were significantly higher than those in the other groups ($P < 0.001$ – 0.05). Modified from [20].

IgG_{2b}) for the liposomal DNA were also greater albeit to a lesser extent (up to 10-fold) (Fig. 1). Significantly, IgG₁ responses for the liposome-entrapped DNA were also higher (up to 10-fold) than those obtained for DNA complexed with similar cationic liposomes [20]. This was also true for IFN- γ and IL-4 levels in the spleens of immunized mice (Fig. 2). In another study, the role of the route of injection of the pRc/CMV HBS plasmid was examined for both humoral and cell-mediated immunity, using BALB/c mice and an outbred strain (T.O.) of the same species. Results (Fig. 3) comparing responses for liposome-entrapped and naked DNA indicate greater antibody (IgG₁) responses for the former not only by the intramuscular route but also the subcutaneous and the intravenous routes. Interestingly, there was not much difference in the titers between the two strains (Fig. 3) suggesting that immunization with liposomal pRc/CMV HBS is not MHC restricted. A similar pattern of results was obtained with IFN- γ and IL-4 in the spleens (not shown).

The mechanism by which liposomes promote greater immune responses to the encoded antigen than seen with the naked plasmid is not likely to involve muscle cells. Although cationic liposomes could in theory bind to the negatively charged myocytes and be taken up by them, protein in the interstitial fluid would neutralize [22] the liposomal surface and thus interfere with such binding. Moreover, vesicle size (about 600–700 nm average diameter; [26]) would render access to the cells difficult if not impossible. It is then more likely that cationic liposomes are endocytosed by APCs, possibly including dendritic cells, in the lymphatics where liposomes eventually end up [28]. This is strongly supported by experiments in which mice were injected intramuscularly or subcutaneously with liposomes entrapping the plasmid (pCMV4.EFGP) encoding the enhanced fluorescent green protein or with the naked plasmid. Sections of the lymph nodes draining the injected site revealed (Fig. 4) much more green fluorescence when

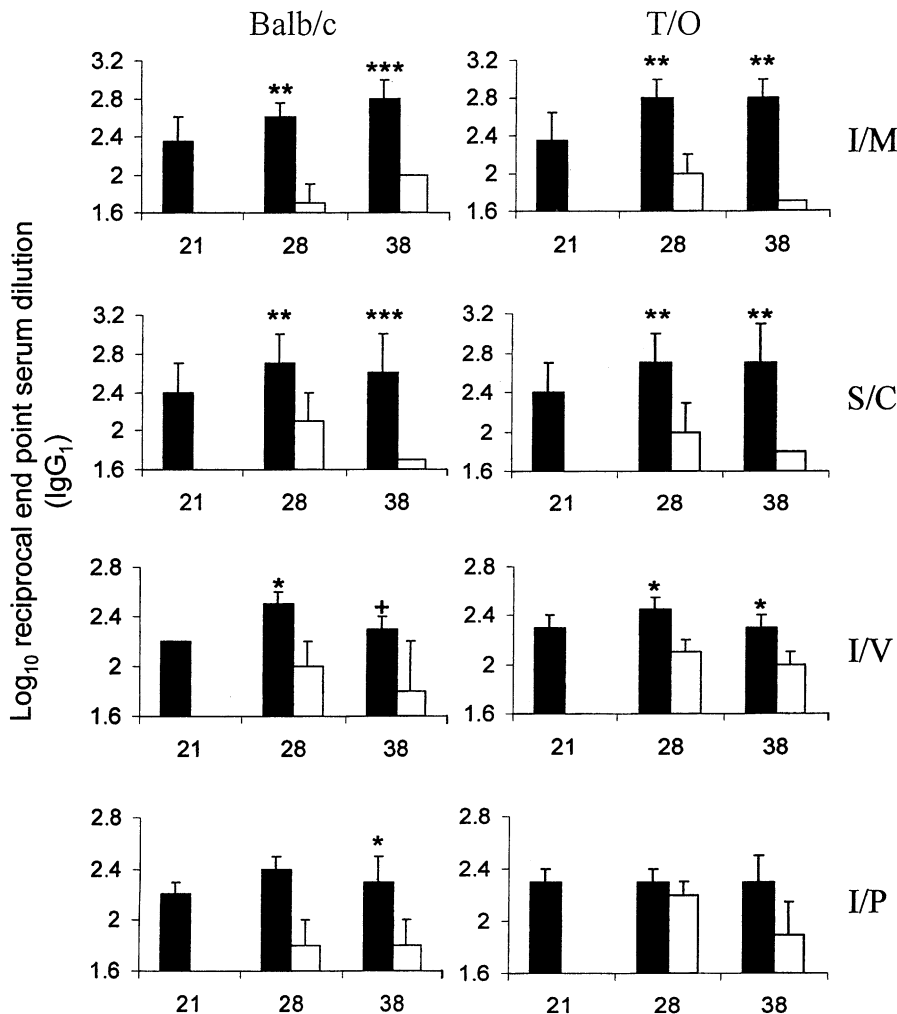


Fig. 3. The effect of route immunization with pRc/CMV HBS on IgG₁ immune responses in inbred (BALB/c) and outbred (T.O.) mice. Mice in groups of four were injected intramuscularly (I/M), subcutaneously (S/C), intravenously (I/V) or intraperitoneally (I/P) twice on days 1 and 21 with 10 μ g of liposome-entrapped (black bars) or naked (white bars) pRc/CMV HBS and bled on days 21, 28 and 38. Liposomes were composed of PC, DOPE and DOTAP (1:0.5:0.25 molar ratio). $^+P < 0.05$; $^*P < 0.01$; $^{**}P < 0.005$; $^{***}P < 0.008$. Reproduced with permission from [8].

the plasmid was administered in the entrapped form [27]. As discussed elsewhere [8,20], it appears that the key ingredient of the DNA liposomal formulations as used in Fig. 1 in enhancing immune responses is the cationic lipid. It is conceivable that some of the endocytosed DNA escapes the endocytic vacuoles prior to their fusion with lysosomes (by a mechanism similar to that proposed [29] for vesicle–DNA complexes) to enter the cytosol for eventual episomal transfection and presentation of the encoded antigen. It is perhaps at this stage of intracellular trafficking of DNA, spanning its putative escape from endosomes and access to their nucleus, that the cationic lipid, possibly together within the fusogenic phosphatidylethanolamine (PE) component, plays a significant role.

3.3. Induction of a cytotoxic T lymphocyte (CTL) response by liposome-entrapped plasmid DNA

Studies on the immune responses induced by liposome entrapped DNA vaccines have so far focused on the humoral response and used surrogate measurements (IgG_{2a} subclass and splenic IFN- γ to indicate a cell-mediated component. In a subsequent study we expanded [30] the analysis profile in order to include the CTL component of the immune response, as measured by the specific killing of syngeneic target cells pulsed with a recognized CTL epitope peptide derived from the antigen tested. To that end, we analyzed the type and degree of immune response induced following sub-

cutaneous injection of DNA in liposomes and compared it to that achieved by DNA alone delivered by the same route.

For this purpose 6–8-week old, female C57/BL6 (H-2^d) mice received either one or two doses of 2.5 or 10 μ g of ovalbumin (OVA)-encoding plasmid DNA (pCI-OVA) either alone or in liposomes. As positive control, animals were immunized subcutaneously with 100 μ g of OVA protein complexed with 1 μ g of cholera toxin (CT). One week after the last immunization, blood samples and spleens were collected from all animals and tested for anti-OVA serum total IgG antibody levels, CTL activity and cytokine release. After a single dose of antigen, only animals immunized with either protein or 10 μ g of liposomal DNA showed significant anti-OVA serum antibody titers by ELISA. After two doses of antigen only animals immunized with either protein or liposomal DNA (both 2.5 and 10 μ g) showed significant levels of seroconversion and serum antibody titers against OVA by ELISA [30]. Similarly, no anti-OVA CTL activity was detected in animals immunized with DNA alone. However, animals immunized with two doses of 10 μ g of liposome-entrapped DNA (LipodineTM) displayed a CTL response higher (60% cell killing versus 50%) than that obtained in the positive control group, immunized with OVA protein + adjuvant (CT) (Fig. 5). Thus, delivery of a small dose of liposomal plasmid DNA subcutaneously, a route of immunization not normally inducing significant plasmid DNA mediated immune activation [9], results in a strong antigen specific cellular response which is greater than that

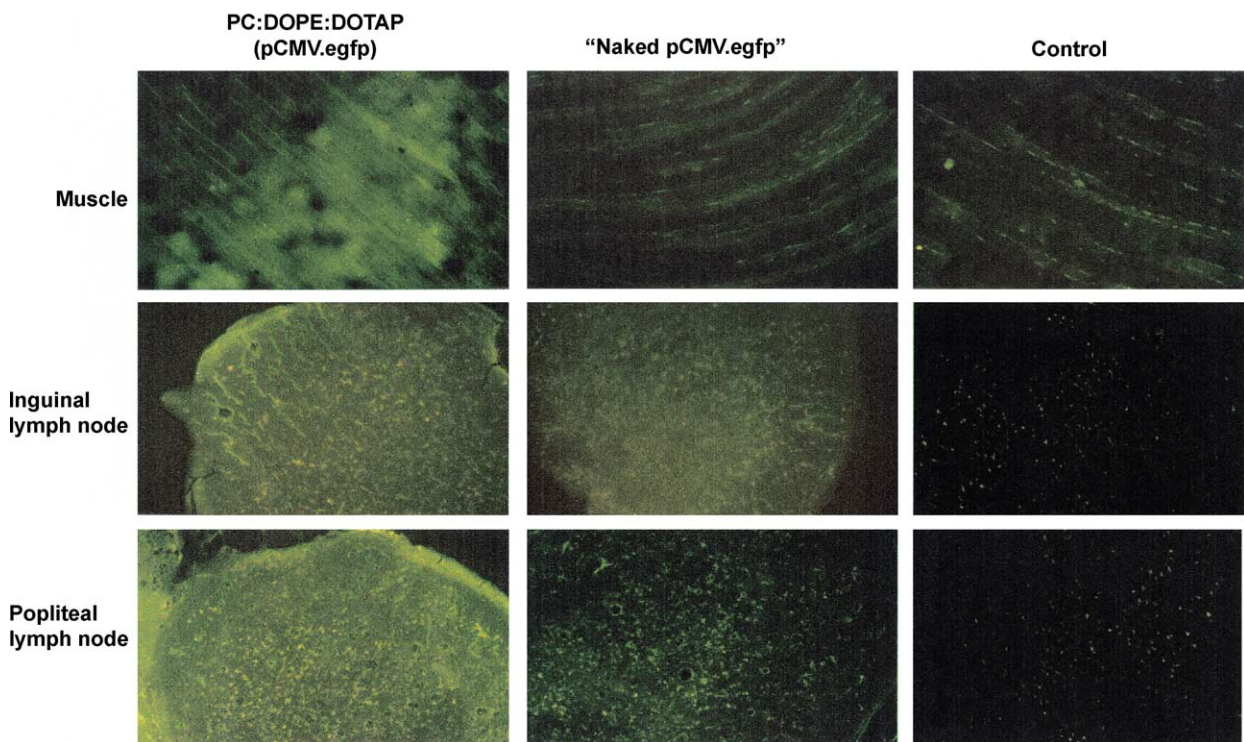


Fig. 4. Fluorescence images of muscle and lymph node sections from mice injected intramuscularly with 10 μ g liposome-entrapped or naked pCMV4.EGFP and killed 48h later. Sections from untreated animals were used as controls. Reproduced with permission from [27].

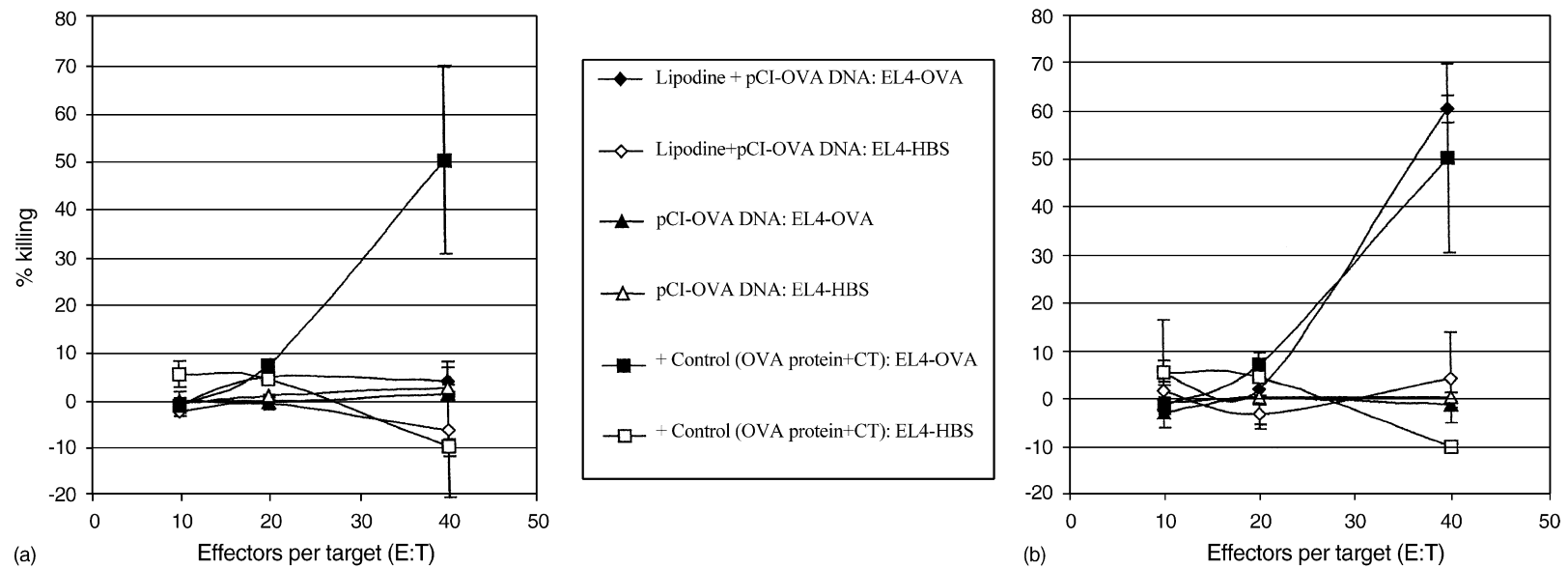


Fig. 5. CTL response to EL4 pulsed with an OVA CTL epitope peptide in animals immunized with 2.5 µg (a) and 10 µg (b) of pCI-OVA either alone or entrapped in cationic DOTAP liposomes (for vesicle composition see Table 1). (a) CTL activity (two times 2.5 µg pCI-OVA dose); splenocytes + OVA (peptide) + EL4 + IL-2 stimulation; (b) CTL activity (two times 10 µg pCI-OVA dose); splenocytes + OVA (peptide) + EL4 + IL-2 stimulation.

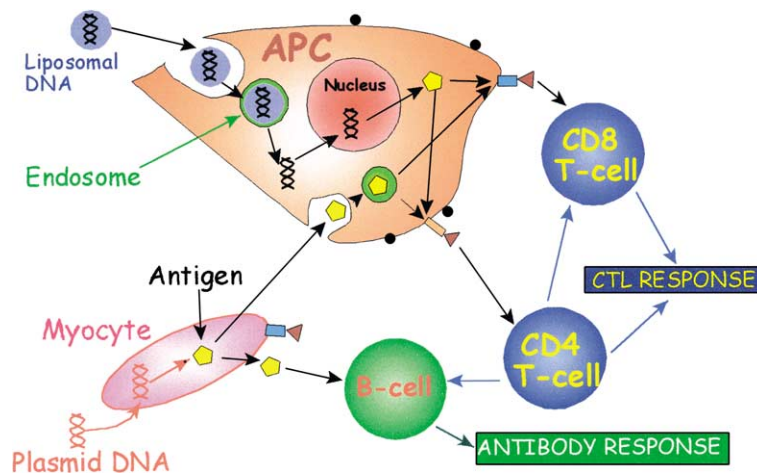


Fig. 6. Following vaccination with naked plasmid DNA, the plasmid is taken up by the myocytes which are transfected. The cells then release the antigen which is captured by antigen presenting cells (APCs) and B cells. Stimulation of CD4⁺ and CD8⁺ T cells by antigenic peptide includes cytotoxic T lymphocyte (CTL) responses and also induces B cells to produce antibodies. MHC class I (blue rectangular on the surface of myocyte) expression may occur on the myocytes, and MHC class I (blue rectangular) and II (orange rectangular) are expressed on APCs. Co-stimulatory molecules on the APCs are shown as black circles. Liposome-entrapped plasmid DNA may be taken up directly by APCs such as dendritic cells, which results in transfection and MHC classes I and II expression which stimulates the CD4⁺ and CD8⁺ T cells by antigenic peptide and induces CTL responses and also B cells to produce antibodies.

achieved by higher doses of a conventional protein antigen together with a powerful adjuvant (CT).

4. Conclusions

DNA immunization is a promising approach to the design of vaccines for situations where antigens are either ineffective or unavailable. However, plasmid DNA vaccines used as such are vulnerable to attack by deoxyribonuclease following their administration and do not normally target antigen presenting cells. Such problems can be circumvented by entrapping the DNA within cationic liposomes. The technique of entrapment is simple (one step), easy to use and generates a freeze-dried preparation that can be used on rehydration. Entrapped plasmid is not accessible to nuclease nor can it be replaced by other competing anionic molecules. Immunization studies have shown that cationic liposomes promote much greater humoral and cytotoxic T lymphocyte immune responses against the antigen encoded by the entrapped DNA vaccine. A possible mechanism by which liposomes augment immune responses following DNA immunization is illustrated in Fig. 6.

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