JOURNAL OF LIPOSOME RESEARCH, 12(1&2), 173–183 (2002)

INDUCTION OF A CYTOTOXIC T LYMPHOCYTE (CTL) RESPONSE TO PLASMID DNA DELIVERED VIA LIPODINE™ LIPOSOMES*

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ABSTRACT

We have previously shown[1–3] that liposome-mediated plasmid DNA immunisation may be a preferred alternative to the use of naked DNA. Lipodine™ DNA formulations consist of liposomes containing entrapped DNA plasmid by the dehydration–rehydration (DRV) method.[1] Such liposome formulations are distinct from liposomes with externally complexed DNA in that the majority of the DNA is "internal" to the liposome structure and hence protected from DNAase degradation.[3] Previous studies on the immune response induced by DNA vaccines entrapped in Lipodine™ have focused on the humoural response. In the present study, we have expanded the analysis profile in order to include the cytotoxic T lymphocyte (CTL) component of the immune response. We have analysed the immune response induced by DNA entrapped in Lipodine™ compared to that induced by DNA alone when delivered subcutaneously, a route of administration not normally inducing significant plasmid DNA mediated immune activation.

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Our results indicate that delivery of a small dose of plasmid DNA in Lipodine™ results in an improved antibody response to the plasmid encoded antigen and a strong antigen specific CTL response compared to that induced by DNA delivered alone.

Key Words: Plasmid DNA; Liposome (Lipodine™); Cytotoxic T lymphocyte (CTL) response

INTRODUCTION

A substantial number of studies[2,4,5] have shown that immunisation of experimental animals with naked plasmid DNA encoding antigens from a variety of viruses, bacteria, protozoa and cancers, induces humoural and cell-mediated protective immunity. This approach to immunisation, i.e., de novo production of vaccines by the host's cells in vivo, has the potential to transform vaccinology and clinical trials are already in progress.[4,6] Following DNA immunisation, the transgene encoded antigen is processed in pathways that are similar to those undergone by antigens produced by intracellular pathogens (e.g., viruses), leading to Th1 type immune responses, which are characterised by the induction of CTL and the production of such cytokines as IFN-γ.

Induction of optimal immune responses requires the antigen to be presented by professional antigen-presenting cells (APC). This is often cited as a weakness of DNA immunisation, particularly when carried out intramuscularly, the most common route of delivery. Myocytes, which have been shown to take up and express the DNA encoded antigen, possess MHC class I molecules, but lack the vital costimulatory molecules[4,5] which are present on professional APC. Therefore, it has been proposed[1,2,7] that APCs might be a preferred alternative to myocytes as targets for the uptake and expression of DNA vaccines.

Injected liposomes are endocytosed rapidly by APC infiltrating the site of injection or in the draining lymph nodes,[8,9] and hence appear to be an appropriate delivery system for DNA. Indeed, previous reports[1,2] involving the intramuscular or subcutaneous delivery of plasmid DNA coding for the S region of the hepatitis B surface antigen (HBsAg) entrapped in cationic liposomes elicited much greater humoural (IgG subclasses) and cell-mediated (IL-4 and interferon-γ) immune responses in mice than naked DNA or DNA complexed to similar, preformed liposomes.

Previous studies on the immune response induced by liposome-entrapped DNA vaccines have focused on the humoural response and used surrogate responses (IgG_{2a} subclass and splenic IFN-γ response) to indicate a cell-mediated component. In the present study, we have expanded the analysis profile in order to include the cytotoxic T lymphocyte (CTL) component of the

immune response, as measured by the specific killing of syngeneic target cells pulsed with a recognised CTL epitope peptide derived from the antigen tested.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC), dioleoyl phosphatidylethanolamine (DOPE) and 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP), ovalbumin (Grade VI) and cholera toxin were purchased from Sigma Chemical Co., UK. All lipids were stored (−20°C) dissolved in chloroform and purged with nitrogen. Plasmid pCI-OVA (a kind gift of Dr. T. Nagata, Hamamatsu University School of Medicine, Japan) contains the chicken egg albumin protein (ovalbumin, OVA)[10] cDNA cloned at the EcoR1 site of the pCI plasmid (Promega, Madison, WI) downstream from the CMV enhancer/promoter region. The plasmid for dosing was commercially produced by Aldevron (Fargo, USA) and contained <100 endotoxin units (EU)/mg of DNA with no residual protein detectable. Peptides for target cell (EL4) loading were ovalbumin MHC class I restricted ($H-2^b$)[11] epitope (SIINFEKL) and a hepatitis B surface (HBS) antigen MHC class I restricted ($H-2^b$)[12] epitope (ILSPFLPL). They were prepared by F-MOC chemistry and purified (> 90% purity) by reverse-phase HPLC. All other reagents were of appropriate analytical or tissue culture grade.

Methods

Preparation of Lipodine™ Formulation

Plasmid DNA pCI-OVA mixed with ^{35}S-labelled (pCI-OVA) tracer, was entrapped in liposomes as described.[13] Briefly, small unilamellar vesicles (SUV) prepared from 16 μmoles egg phosphatidylcholine (PC), 8 μmoles dioleoyl phosphatidylcholine (DOPE) and 42 μmoles 1,2-dioleoyloxy-3-(trimethylammonium) propane (DOTAP) were mixed with 100 μg of plasmid DNA and freeze-dried overnight. Following rehydration under controlled conditions,[13,14] the generated dehydrated–rehydrated vesicles (DRV liposomes) were centrifuged to remove non-incorporated DNA. The pellets were then resuspended in 0.1 M sodium phosphate buffer pH 7.2 supplemented with 0.9% NaCl (PBS) to the required dose volume. DNA incorporation into liposomes was estimated on the basis of ^{35}S radioactivity recovered in the suspended pellets. Liposomes with entrapped DNA were subjected to microelectrophoresis and photon correlation spectroscopy (PCS) at 25°C in a Malvern Zetasizer 3000 to determine their zeta potential (ZP) and z-average diameter respectively.[3]

Animal Procedures

Female C57BL/6 mice 6–12 weeks old (Harlan, UK) were immunised by subcutaneous injection. DNA doses of 10 µg and 2.5 µg (per mouse) as such (naked) or entrapped in liposomes (Lipodine™) were administered in 0.2 ml dose volume. Additional positive and negative controls received ovalbumin protein admixed with and cholera toxin,[15] and PBS respectively. Mice received two doses of antigen at days 0 and 14, with sample bleeds collected from the tail vein at day 13. On day 21 all animals were terminally bled, culled by cervical dislocation and their spleens harvested, pooled and processed.

Enzyme Linked Immunosorbent Assay

Sera obtained from sample bleeds were diluted 20-fold in PBS and kept at −20°C until assayed by the enzyme-linked immunoadsorbent assay (ELISA). Certified binding chemistry 96 well plates (Costar, EIA/RIA) were coated overnight at 4°C with 100 µl/well of 60 µg/ml ovalbumin in 0.1 M sodium carbonate buffer (pH 9.6). After removing the excess ovalbumin solution, wells were coated with 200 µl of 2% (w/v) BSA in PBS. After 2 h at room temperature, the blocking solution was removed and doubling dilutions (starting with 1/100 dilution) of the different experimental sera samples were added to the wells (50 µl sample/well). Following 1 h incubation at 37°C. The wells were washed four times with PBS/Tween 20 and overlaid with 50 µl/well of rabbit anti-mouse total Ig HRP-conjugated sera (Dako). After 1 h at 37°C, plates were washed four times with PBS/Tween 20 and overlaid with 50 µl/well of substrate solution *o*-phenylenediamine (Sigma, Fast OPD). The reaction was stopped by adding 50 µl/well of stopping solution (3 M sulphuric acid) and the adsorbance of each well at 490 nm was determined. The antibody response was expressed as the $\log_{10}$ of the reciprocal serum dilution required for OD to reach a reading of 0.200 (end point dilution). Log_{10} values for sera from negative control animals was always lower than 2.0.

Cell Culture

The EL4 (H-2^b) cell line is a chemically induced mouse thymoma line derived from C57BL/6 mice.[16] The cell line was maintained in RPMI-1640 (Sigma) supplemented with 50 IU/50 µg/ml of penicillin/streptomycin and 10% foetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2. Mouse splenocyte cultures were obtained from C57BL/6 mice and maintained in phenol red-free IMDM (Life Technologies) supplemented

with 0.02 mM β-mercaptoethanol, 50 IU/50 μg/ml of penicillin/streptomycin, 0.01 mg/ml of apo-transferrin, 1 mg/ml of bovine albumin, 0.015 mM linoleic acid and 0.015 mM oleic acid at 37°C in a humidified atmosphere of 5% CO_2.

Mitomycin C Treatment of EL4 Cells

EL4 cultures in exponential phase were harvested by centrifugation (250 g, 5 min) and resuspended in serum-free RPMI-1640 medium containing 50 μg/ml of mitomycin C (Sigma). After 45 min incubation at 37°C, the cell suspension was washed four times in serum-free RPMI-1640 medium (250 g, 5 min) and finally resuspended in complete medium.

Preparation of Splenocyte Cell Cultures and CTL Assay

Mouse spleens were gently pressed between two frosted slides and red blood cells removed from the resulting cell suspension by treatment with Red Cell Lysis Buffer (9 parts 0.16 M NH_4Cl and 1 part of 0.17 M *Tris*, pH 7.2). Splenocyte suspensions from each experimental group were seeded in an upright 25 cm^2 flask at a density of 1.5×10^6 cells/ml containing, as stimulators for the CTL population, 10^5 mitomycin C-treated EL4 cells/ml, 10 μM of the OVA CTL epitope peptide and 10 U/ml of recombinant mouse interleukin-2 (IL-2). After 6 days incubation at 37°C, the splenocyte suspensions were harvested, resuspended in complete IMDM and tested for CTL activity, as effectors (E), against EL4 targets (T) using the CytoTox96™ LDH (lactate dehydrogenase) release colorimetric assay (Promega) according to a modification of the manufacturer's recommendations. This technology has been demonstrated to provide identical results (within the experimental error) to those determined in a parallel ^{51}Cr release assay.[17,18] Briefly, EL4 targets were prepared from EL4 cultures harvested in exponential phase and resuspended at a density of 10^5 cells/ml in either IMDM or IMDM containing 10 μM of a CTL epitope peptide (OVA or HBS). Assays were set up by seeding, in triplicate, three sets of doubling dilutions of effector splenocytes (from 2×10^5 cells/well to 5×10^4 cells/well) in U bottom 96-well plates (Nunc) in a volume of 50 μl/well. Each set of wells was then overlaid with 50 μl of either EL4 cells (effector spontaneous), EL4 + OVA peptide (experimental) or EL4 + HBS peptide (experimental), resulting in E:T ratios of 40:1, 20:1 and 10:1. Duplicate sets of three wells containing only complete IMDM were also overlaid with 50 μl of one of the three EL4 target suspensions. These wells provided the minimum (target spontaneous) and, following complete cell lysis by a Triton X-100 solution, the maximum (target maximum) LDH release for each of the three EL4 target suspensions. After 4 h incubation at 37°C, 50 μl of supernatant was collected from each of the

experimental and control wells and the LDH activity in the samples measured by colormetric assay read at 490 nm. Cytotoxic activity at different E:T ratios was established from the optical density (OD) values at 490 nm and according to the following formula:

$$\%\text{Cytotoxicity} = \frac{OD_{\text{experimental}} - (OD_{\text{effector spontaneous}} + OD_{\text{target spontaneous}})}{OD_{\text{target maximum}} - OD_{\text{target minimum}}} \times 100$$

RESULTS AND DISCUSSION

Liposome Characterisation

Incorporation values of DNA into the liposomes employing the DRV method were high ($91 \pm 3\%$) (mean $\pm$ SD, $n=4$) and consistent with values previously found [3] with pRc/CMV HBS plasmid DNA. Physical characterisation of the liposome formulations yielded a cationic zeta potential of 25 ± 7 mV and a z-average diameter of 609 ± 80 nm (mean $\pm$ SD, $n=4$). Again, these values were not significantly different than those determined for liposomes containing pRc/CMV HBS plasmid DNA.[3]

Immunology

Ideally, an effective vaccine must be stable without the need for cold storage and capable of inducing an effective immune response using only a small dose of antigen and without the need for multiple immunisations. DNA vaccines do, in principle, fulfil the first requirement described above, but most efficient vaccination procedures described up to date require either multiple deliveries of large doses of DNA or delivery of the DNA complexed with expensive gold particles.[19–21] We have shown in several previous reports that plasmid DNA entrapped in Lipodine™ liposomes can induce an effective serum [1–3,7] and mucosal [22] antibody response. Nonetheless, the development of prophylactic and therapeutic immunity against viral infections and cancers requires the generation of a cytotoxic T cell (CTL) response in addition to the humoural response. To address the question of whether the use of Lipodine™ to deliver DNA vaccines results in the induction of both CTL and humoural responses, as well as in a reduction in the dose requirement, we have compared the immune response induced by two doses of DNA delivered either on their own or entrapped in liposomes (Lipodine™) by subcutaneous injection, a route not usually associated with the induction of immunity by DNA vaccines.[23]

Vaccination with Lipodine™ DNA Induces an Increased Antibody Response

As expected, all control animals immunised with 100 µg of OVA protein admixed with 1 µg of CT had seroconverted, developing a strong antibody responses against OVA ($\log_{10} > 5$) (not shown). This level of response is similar to that described elsewhere [15] and contrasts with the lack of immunogenicity of the OVA protein when used in the absence of CT. In the test groups, (Fig. 1a; upper frame) no antibody response against OVA could be detected in animals immunised with 2.5 µg of pCI-OVA after the first immunisation, independently of whether the DNA was delivered on its own or entrapped in Lipodine™. On the other hand, although no antibody response was detected in animals immunised with 10 µg of pCI-OVA alone (Fig. 1b; upper frame), 50% (4/8) of animals immunised with 10 µg of pCI-OVA entrapped in Lipodine™ developed an OVA specific antibody response. This was lower than that induced in the control group (OVA protein admixed with CT) but was significantly different (*t*-test $p < 0.05$) to that observed in the other experimental groups. After the second immunisation, there was a significant increase in the anti-OVA antibody response in the control animals ($\log_{10}$ titer > 6) (not shown), as expected for a secondary response. In all experimental groups (Fig. 1a&b , lower frame) significant differences were also observed. Over 60% (5/8) of the animals immunised with 2.5 µg of pCI-OVA entrapped in Lipodine™ had now seroconverted, whilst only 12% (1/8)

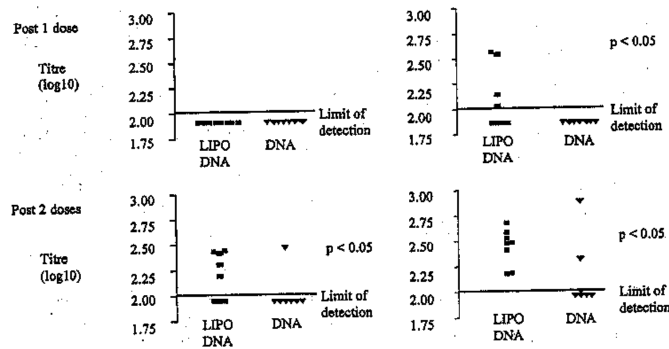

Figure 1. Total serum antibody response to OVA+CT protein at days 13 (after first dose; upper frame) and 21 (after second dose; lower frame) in animals immunised with 2.5 µg (a, left hand frames) and 10 µg (b, right hand frames) of pCI-OVA either alone or entrapped in Lipodine™. LIPO DNA and DNA denote Lipodine™ DNA and naked DNA respectively.

of the animals immunised with 2.5 μg of pCI-OVA alone had seroconverted. This difference was even more evident in animals immunised with 10 μg of pCI-OVA, since now only 25% of animals immunised with DNA alone had seroconverted whilst seroconversion was 100% (8/8) for the animals treated with DNA entrapped in Lipodine™.

These data clearly indicate that immunisation with DNA entrapped in Lipodine™ significantly increases the antibody response to the plasmid encoded antigen, allowing for a reduction in the DNA dose necessary to induce a specific level of response. This last observation is evident by the fact that the level of the response and the degree of seroconversion observed for animals receiving 2 doses of 2.5 μg of pCI-OVA entrapped in Lipodine™ is significantly higher than that observed in animals which received 2 doses of 10 μg of DNA alone.

Although our data show that seroconversion rates and antibody response levels are higher in the control (OVA + CT dosed) animals than in any of the experimental animals, this observation must be considered within the following context. Firstly, as indicated earlier, OVA protein alone has been reported to be a very poor immunogen, inducing little to no antibody responses. It is only when complexed to a very potent adjuvant like CT, which is certainly not licensed for human use, that the protein becomes highly immunogenic. Secondly, whilst in the control animals induction of the OVA specific immune response is immediate after injection, in the experimental groups there is a delay with regards to the time at which OVA protein first becomes available (following DNA expression) to the immune system. Finally, taking into account the reported levels of expression of plasmids in mammalian cells, the amount of protein produced even by the highest DNA dose used here (10 μg) will always be significantly lower than the amount of protein provided by any conventional immunisation protocol. These three factors together (lower immunogenicity of the non-complexed OVA protein produced by the plasmid, time delay in the availability of OVA protein to the immune system in animals immunised with DNA and the reduced levels of antigen available) are indeed those which define the differences in the level of the antibody response between control and experimental animals and can now be clearly understood.

Vaccination with Lipodine™ DNA Induces an Increased CTL Response

CTL responses are, as indicated earlier, essential for the resolution of viral infections and the treatment of established carcinomas. As shown in Figure 2, control animals immunised with 100 μg of OVA protein complexed with 1 μg of CT generated a strong OVA specific CTL response (50% lysis at E:T 40:1). Animals immunised with 2.5 μg of pCI-OVA, independently of

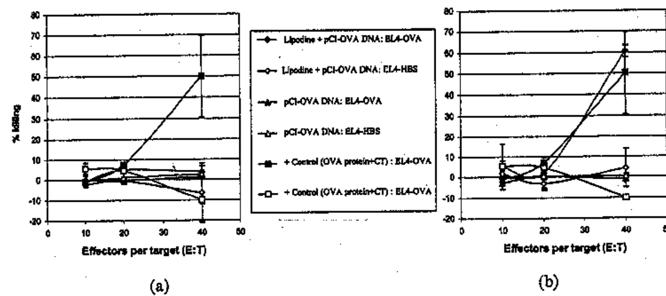

Figure 2. CTL response to EL4 cells pulsed with an OVA CTL epitope peptide in animals immunised with 2.5 µg (a, left hand frame) and 10 µg (b, right hand frame) of pCI-OVA either alone or entrapped in Lipodine™.

whether the DNA was delivered on its own or entrapped in Lipodine™, generated no detectable CTL responses against OVA (Fig. 2a). Similarly, immunisation with 10 µg of pCI-OVA alone failed to induce any detectable CTL response (Fig. 2b). In contrast, animals immunised with 10 µg of pCI-OVA entrapped in Lipodine™ generated an OVA specific CTL response (Fig. 2b) which was equal, if not higher, to that detected in the control animals.

Briefly, the induction of a CTL response depends on the effective presentation by professional antigen presenting cells of a large concentration of antigen-derived highly immunogenic CTL epitope peptides within an appropriate environment of cytokine and costimulatory molecules.[24] In our experiments, the CTL epitope peptides derived from the antigen used in the control and experimental immunisations are expected to be the same, independently of the method of delivery (protein, DNA or DNA entrapped in Lipodine™) and the presence of any adjuvant. In addition, the potential concentration of CTL epitope peptides derived from 100 µg of OVA protein would certainly be higher than that expected to be produced from a two 10 µg doses of pCI-OVA entrapped in Lipodine™. Considered together, these observations and our experimental results clearly indicate that delivery of DNA vaccines entrapped in Lipodine™ result in more effective antigen presentation and immune activation, at least at the CTL level, that immunisation with DNA alone or protein complexed with a strong adjuvant.

In conclusion, entrapment of plasmid DNA vaccines in Lipodine™ results in an increase in the antibody response to the plasmid encoded antigen compared to DNA alone, and in the induction of an antigen specific CTL

response which is equal to, if not higher than, that achieved by immunisation with protein admixed with a strong adjuvant.

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