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Doxorubicin entrapped within liposome-associated antigens results in a selective inhibition of the antibody response to the linked antigen

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Abstract

The generation of an immune response can dramatically alter the circulation lifetime of a targeted liposome, particularly when the response is generated against the surface-coupled ligand. Following repeated administrations, rapid elimination of the carrier system is observed, thereby limiting potential applications for targeted liposomes in a therapeutic setting. In this study, we have investigated whether the encapsulation of a toxic drug within the carrier could prevent an immune response against a surface-bound protein. Liposome clearance and humoral immune response were monitored throughout multiple administrations of liposomes containing doxorubicin with surface-conjugated ovalbumin. The results show that low doses of encapsulated doxorubicin can prevent humoral immunity against repeated administration of liposomes conjugated with ovalbumin. The immunosuppressive effect was specific for the ovalbumin coupled to the liposome surface. This selective suppression of immunity against a surface conjugated protein could prove advantageous for safe repeated administration of protein containing liposomal systems. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The newest generation of liposomal drug-delivery systems has focused on delivery to specific target cells through the use of antibodies and other ligands coupled to the membrane surface. The earliest techniques used to couple antibodies to liposomes re-

sulted in rapidly cleared, unstable formulations [1,2]. The subsequent addition of PEG-polymers improved stability and reduced the RES uptake of targeted systems [3], unfortunately, the addition of PEG can also interfere with the binding of ligands to their target receptors [4,5]. Despite these difficulties, liposomes and their contents have been successfully delivered to the target cell population both in vitro and in vivo [6–9].

One of the major remaining obstacles in the application of targeted liposome-delivery systems concerns immune responses to the surface-coupled molecules. The natural adjuvant property of liposomes has been recognized since the 1970s [10], with the

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most significant humoral immune response generated against vesicles with protein covalently coupled to the surface [11,12]. The generation of a humoral immune response will result in the rapid elimination of these targeted drug-delivery systems after the first administration. Without the potential for multiple administrations, targeted drug-delivery systems will be of limited value in the treatment of disease. Encapsulation of cytotoxic drugs (such as the anti-cancer drug, doxorubicin), has been suggested as a possible solution to inhibit immune responses generated against surface-coupled molecules. These agents have been shown to interfere with antigen processing and presentation [13–15]. The role of the macrophage in the generation of an immune response to liposomal antigens has been well documented [16–18], and liposomal doxorubicin is known to be an inhibitor of liver Kupffer cell and splenic macrophage activity [19,20]. The administration of liposomal doxorubicin should therefore directly inhibit immune responses mediated by these cells. Additional evidence has documented direct interactions between liposomal surface proteins and antigen-specific lymphocyte subpopulations [13,17,21–24], providing a potentially more efficient route for the suppression of the immune response. The primary objective of these experiments was to characterize the dose limitations and possible antigen selectivity of the immune suppression induced by doxorubicin encapsulated within liposomes with surface-associated protein.

2. Materials and methods

2.1. Materials

DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine) and MPB-DSPE (*N*-(4-(*P*-maleimidophenyl) butyryl) distearoyl phosphatidylethanolamine) were purchased from Northern Lipids (Vancouver, BC). PEG-DSPE was generously provided by Dr. Steven Ansell of Inex Pharmaceuticals (Vancouver, BC). Lipid marker, [³H]cholesteryl hexadecyl ether (CHE) was purchased from NEN, Mississauga, ON. Doxorubicin was obtained from Adrian Laboratories. Imject Ovalbumin, biotinylated goat anti-mouse IgG, and streptavidin-linked β -galactosidase were purchased from Pierce (Rockford, IL). Female

CD-1 mice were obtained from Charles River (St. Constant, PQ). Cholesterol (Chol), SPDP (*N*-succinimidyl 2-(2-pyridyldithio) propionate), dithiothreitol (DTT), HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane-sulfonic acid), citrate, CL-4B Sepharose, G-50 Sephadex, hen egg lysozyme (HEL), bovine serum albumin and most other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Preparation of liposomes

Large unilamellar vesicles (LUVs) were prepared at various concentrations by extrusion of freeze-thawed multilamellar vesicles through two stacked 100-nm polycarbonate filters (Nucleopore, Pleasanton, CA) using an extrusion device (Lipex Biomembranes, Vancouver, BC) [25]. DSPC/Chol (55:45, mol%) and DSPC/Chol/DSPE-PEG₂₀₀₀/MPB-DSPE (52:45:2:1, mol%) liposomes were prepared in HEPES buffered saline solution (HBS; 20 mM HEPES, pH 7.4, 145 mM NaCl) at 2–200 mM total lipid concentrations. The average size of these liposomes was 100 ± 30 nm as determined by quasielastic light-scattering analysis (QLS) using a Nicomp Model 270 Submicron Particle Sizer (Nicomp Instruments, Santa Barbara, CA). Liposomes were radio-labelled using a lipid marker, [³H]cholesteryl hexadecyl ether (CHE) (NEN, Mississauga, ON). When used for biodistribution, liposomes contained 1 μ Ci [³H]CHE/3 μ mol of total lipid. For all other samples, a trace amount of this label (0.01 μ Ci/ μ mol) was used to monitor changes in lipid concentration. This label is non-exchangeable and non-metabolizable due to low levels of cholesteryl ester transfer protein activity [26–28], and this label has been shown to be similarly stable in CD-1 mice (P.R. Cullis et al., unpublished results). Specific activities of liposome preparations were determined by standard liquid scintillation counting methods using a Beckman LS 3801 Liquid Scintillation System (Beckman Instruments, Fullerton, CA), and a colorimetric phosphorous assay [29]. When a pH gradient was required for drug-loading, the liposomes were prepared in 300 mM citrate (pH 4.0) and the external buffer exchanged with HEPES-buffered saline solution (HBS; 20 mM HEPES, pH 7.5, 145 mM NaCl) by gel filtration on pre-packed Sephadex G-25 mini-columns (PD-10 columns) (Pierce, Rockford, IL).

2.3. Protein coupling to liposomes

Imject ovalbumin (10 mg/ml in 0.9% saline) was modified with the amine cross-linker SPDP as described previously by Loughrey et al. [30,31]. Ovalbumin was selected because of its immunogenicity, possesses well-studied antigenic epitopes and immune response behavior, and has been prepared in a purified, stable form ideal for the coupling procedure used. Briefly, SPDP was dissolved in ethanol and diluted with HBS just prior to addition to the ovalbumin solution in a 5-fold molar excess. After 30 min at room temperature, the unreacted SPDP was removed using a G-50 Sephadex column equilibrated with HBS (pH 7.5) and then reduced for 30 min with 25 mM DTT. The activated ovalbumin was isolated on a G-50 Sephadex column just prior to coupling to MPB-DSPE containing liposomes at a ratio of 90 μg protein/ μmol lipid (15 mM final lipid concentration), for 18 h at room temperature under nitrogen. Unconjugated ovalbumin was separated from protein-coupled liposomes by passage down a Sepharose CL-4B column (HBS, pH 7.5). Quantification of ovalbumin coupling was carried out using the Pierce Micro BCA protein assay in the presence of 1% Triton X-100 to disrupt the liposomes. The ovalbumin coupling procedure consistently resulted in 30–40 μg protein/ μmol lipid. Coupling of HEL followed the same procedures outlined above and consistently produced approximately 40 μg protein/ μmol lipid. HEL was chosen due to its highly antigenic nature and well-studied immunogenic behavior of the protein in animal systems.

2.4. pH gradient doxorubicin encapsulation

Doxorubicin was encapsulated in selected formulations using a transmembrane pH gradient as previously described by Mayer et al. [32–34]. Ovalbumin-coupled liposomes with an internal pH of 4.0 (citrate buffer) and an external pH of 7.5 (HBS) were heated to 65°C and then mixed with a doxorubicin solution for 10 min (10 mg/ml in 0.9% saline, 65°C). This procedure results in >95% trapping efficiencies, eliminating the need to separate free doxorubicin [33]. Drug-to-lipid molar ratios ranging from 0.03 to 0.2 were generated in these studies. For ovalbumin–liposome formulations not requiring

doxorubicin, a mock loading procedure was carried out using a drug-free 0.9% saline solution.

2.5. *In vivo* mouse biodistribution studies

Liposome formulations (200 μl) were administered via the lateral tail vein of female CD-1 mice. Lipid doses (except where specified in figure legends) contained 50 μg of coupled protein, equivalent to a lipid dose of approximately 45 mg lipid/kg body weight and a doxorubicin dose of 8 mg/kg. All time points were taken 4 h after the designated injections, with mice being sacrificed by exposure to carbon dioxide. Blood was removed by cardiac puncture, collected in EDTA-coated plasma collection tubes (Bectin Dickinson, Franklin Lakes, NJ) and centrifuged at 3000 rpm for 5 min to isolate the plasma fraction. Plasma liposome concentrations were determined from the specific activity of the injected liposomes, using standard liquid scintillation counting techniques. Plasma volume was assumed to be 5% the total body weight. All *in vivo* analyses used four mice per time point, and the standard error of the mean to represent variability of results.

2.6. Immunization studies with ovalbumin-coupled liposomes

All immunization experiments used liposome formulations as described above, with samples containing [^3H]CHE only when monitoring biodistributions. Unless otherwise designated in figure legends, mice were injected *i.v.* with specified formulations for 2 consecutive weeks (primary and secondary stimulation). Plasma elimination studies examined the biodistribution of the second weekly injection at the 4-h timepoint. Immune response to the coupled protein was determined by ELISA detection of antigen-specific IgG, 7 days after the second weekly injection. Additional experiments on the prevention, specificity, or elimination of immune response used procedures outlined above with the dosing and injection schedules described within the figure legends.

2.7. ELISA assay for antigen-specific immunoglobulin

Micro-assay plates (Bectin Dickinson, Franklin Lakes, NJ) were coated with ovalbumin (50 μl /well

of 40 $\mu\text{g/ml}$) and incubated at 4°C overnight (HEL replaced ovalbumin in the specificity study examining anti-HEL antibody production). The plates were washed twice with PBS–Tween 20 (phosphate-buffered saline, Ca^{2+} and Mg^{2+} free, 0.1% (v/v) Tween 20), before blocking for 1 h with 2% bovine serum albumin (in PBS) at 37°C. After rinsing with PBS–Tween 20 and then 1% BSA (in PBS), 100 μl of plasma diluted in PBS was allowed to incubate at room temperature for 1 h. Plates were subsequently rinsed three times with PBS–Tween 20, before addition of 100 μl aliquots of 0.5 $\mu\text{g/ml}$ biotinylated goat anti-mouse IgG (1% BSA, PBS) (30 min, room temperature). Plates were again rinsed three times with PBS–Tween 20, before addition of 100 μl of 500 mU/ml of streptavidin-linked β -galactosidase (1% BSA, PBS) (30 min, room temperature). Plate-associated β -galactosidase was quantified by the addition of 100 μl CPRG (chlorophenol red- β -D-galactopyranoside) at a concentration of 2 mg/ml. After 20 min, the production of chlorophenol red was monitored by determining the absorbance (relative to controls) at 570 nm on a Titertek Multiscan plate reader (Titertek Instruments, Huntsville, AL). Controls consisted of plasma from untreated female CD-1 mice.

3. Results

3.1. Entrapped doxorubicin eliminates the humoral immune response against liposomal ovalbumin

Investigations using liposomes with surface-bound molecules have revealed the generation of humoral immunity. In the experiments presented here, we use liposomes coupled to the highly immunogenic protein ovalbumin to study the effects of cytotoxic drugs, such as doxorubicin, on this immune response. This ‘proteoliposome’ model system will assist us in identifying potential immune-response problems associated with targeted liposomes containing similar cytotoxic drugs.

When empty ovalbumin proteoliposomes are administered i.v., a significant immune response is generated, resulting in the rapid elimination of subsequent injections (Fig. 1A). During the second and third administrations, the proteoliposomes were completely cleared from the blood, predominantly by the organs of the RES, before the 4-h timepoint. Rapid removal of the carrier was also observed for proteoliposome doses as low as 5 μg conjugated ovalbumin/900 μg lipid (results not shown). The pro-

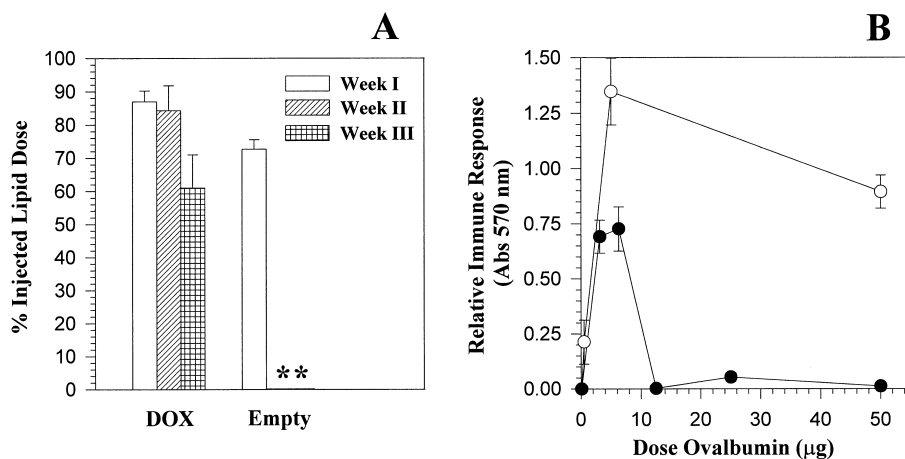


Fig. 1. Dose titration of ovalbumin–proteoliposomes with and without entrapped doxorubicin. Female CD-1 mice were injected i.v. for three consecutive weeks with ovalbumin-coupled liposomes (50 μg of protein/900 μg lipid). Formulations were either empty or contained doxorubicin encapsulated at a 0.2 drug-to-lipid ratio (mol/mol), producing a maximum lipid dose of 45 mg/kg containing 8 mg/kg of drug. (A) Plasma levels of injected lipid 4 h after weekly injections. Scintillation counting was used to detect the radiolabelled lipid marker (^3H)CHE incorporated in the lipid bilayer. (B) The relative production of ovalbumin-specific IgG (absorbance 570 nm) as determined by ELISA assays of plasma isolated 7 days after the second weekly injection. The ovalbumin dosing range was achieved by injecting various amounts of the same formulation (50 μg of protein/900 μg lipid). As such, as the ovalbumin dose decreases, the total doxorubicin dosage decreases. Solid symbols represent doxorubicin-loaded and open symbols indicate empty liposomes. All data points represent the mean and standard error of four mice. *No measurable plasma lipid.

duction of ovalbumin-specific IgG correlated with the rapid elimination of the vesicles from the blood (Fig. 1B). The results suggest an ‘all-or-none’ humoral immune response, with no detectable immune response seen only at the 0.5 μg proteoliposome dose. Importantly, when rapidly cleared 50 μg proteoliposome formulations contained doxorubicin, it prevented the formation of ovalbumin-specific IgG, and restored the long circulation lifetimes of subsequent injections (Fig. 1A). The total doxorubicin dosage used to achieve this effect was 8 mg/kg, which is well below the maximum tolerable dose of 50 mg/kg for DSPC/Chol-based carriers [33]. When the dose of doxorubicin loaded ovalbumin-coupled liposomes was decreased to 6 μg of ovalbumin, an immune response was observed. This corresponds to a total doxorubicin dose of less than 1 mg/kg, suggesting a threshold level of doxorubicin is required to prevent the generation of an anti-ovalbumin immune response.

3.2. Influence of drug concentration on the immune response against liposomal ovalbumin

To determine the role of drug concentration on the immune response, liposomes containing a range of drug concentrations were examined. This was achieved by altering the drug-to-lipid ratio of the formulation. Preliminary experiments demonstrated that two injections were sufficient to manifest changes in immune response and liposome behavior, therefore each subsequent study examined immune responses following two injections. To determine the lowest dose at which doxorubicin would eliminate the immune response, mice were injected with doxorubicin-encapsulated proteoliposomes containing various drug-to-lipid molar ratios for 2 consecutive weeks. The total lipid and protein dose was kept constant (45 mg lipid/kg body weight, 50 μg ovalbumin) so that the decreasing drug-to-lipid ratio resulted in a decreased total drug dose. The results in Fig. 2 show that lowering the drug-to-lipid ratio to 0.1 (equal to a 4 mg/kg total drug dose) still successfully suppressed the ovalbumin-specific immunity generated by the second injection (Fig. 2). This resulted in high plasma lipid concentrations for the second injection (results not shown). At drug-to-lipid ratios lower than 0.1, an immune response is gener-

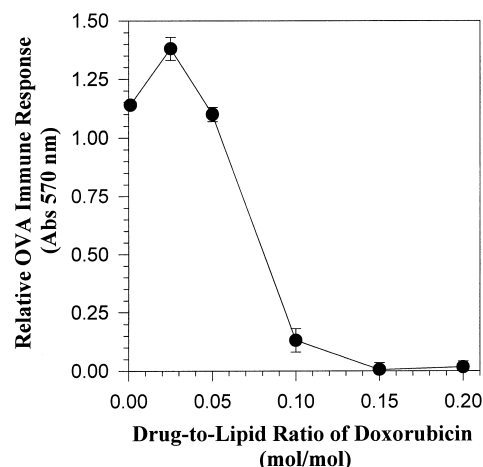


Fig. 2. Effect of drug-to-lipid ratio on the generation of an anti-ovalbumin immune response. Female CD-1 mice were injected i.v. for 2 consecutive weeks with doxorubicin entrapped in ovalbumin-coupled liposomes at a constant protein and lipid dose (50 μg ovalbumin; 45 mg lipid/kg b.wt.). Plasma samples were isolated 7 days after the second weekly injection for immune response determination. All data points represent the mean and standard error of four mice.

ated, resulting in rapid elimination of the carrier after the second injection.

3.3. Influence of total doxorubicin dose on the immune response against liposomal ovalbumin

Results of the previous section indicate that a high drug-to-lipid ratio results in more effective immune response suppression. In order to determine the lowest total drug dose that would result in suppression of the immune response, we administered two weekly injections of doxorubicin-proteoliposomes at a constant 0.2 drug-to-lipid ratio, but decreased the total drug dose by administering a lower lipid dose. Fig. 3 shows that high drug-to-lipid ratio preparations were more effective at eliminating immune responses than equivalent doses of doxorubicin encapsulated at lower drug-to-lipid ratios (Fig. 2). Only doxorubicin doses of 1 mg/kg or less failed to suppress the production of anti-ovalbumin IgG (Fig. 3). This indicates that delivery of highly concentrated doxorubicin within liposomes (high drug-to-lipid ratios) was twice as effective at inhibiting antibody production against the liposomal surface protein compared to lower drug-to-lipid ratio preparations. This is based

on the minimum drug dose required to suppress the immune response in the above experiments (4 and 2 mg/kg doxorubicin, respectively).

3.4. Influence of doxorubicin location on the immune response against liposomal ovalbumin

Liposomal doxorubicin has been shown to eliminate phagocytic cells, which act as antigen-presenting cells for the surface-coupled proteins [19,20]. This might result in the inhibition of immune response by inhibiting the antigen processing and presentation functions of these cells. The significant uptake of liposomes and proteoliposomes by the macrophages of the liver and spleen [16,17,35,36], suggests that liposomal doxorubicin could be just as effective as proteoliposomes containing the drug, as both are accumulated within these phagocytic cells of the RES. To test this possibility, mice were injected with a mixture of liposomal doxorubicin and empty ovalbumin proteoliposomes. The resulting plasma elimination and ELISA immune response data indicate that only the highest dose of liposomal doxorubicin (8 mg/kg) prevented the immune response and rapid elimination of co-administered ovalbumin proteoliposomes (Fig. 4A,B). This suggests that although high doses of liposomal doxorubicin could

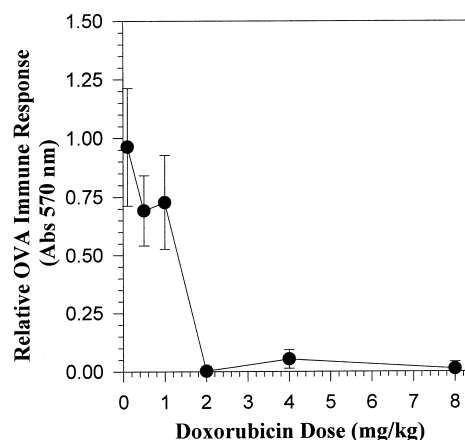


Fig. 3. Effect of total doxorubicin dose on the generation of an anti-ovalbumin immune response. Female CD-1 mice were injected i.v. for 2 consecutive weeks with various doses of doxorubicin entrapped in ovalbumin-coupled liposomes (50 μ g of protein/900 μ g lipid). Plasma samples were isolated 7 days after the second weekly injection for immune response determination. All data points represent the mean and standard error of four mice.

act generally against phagocytic cells to prevent an immune response, encapsulation of doxorubicin within the proteoliposomes was more effective in preventing an immune response against the proteoliposome.

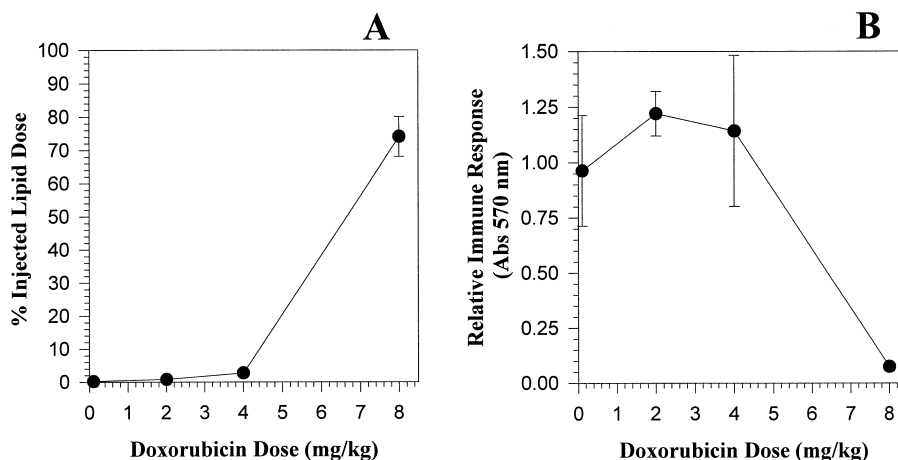


Fig. 4. Ovalbumin proteoliposome immune response and plasma elimination when co-injected with liposomal doxorubicin. Female CD-1 mice were injected i.v. for two consecutive weeks with 50 μ g of empty ovalbumin-liposomes and various doses of 0.2 drug-to-lipid ratio liposomal doxorubicin (DSPC/Chol/PEG-DSPE; 53:45:2, mol%), up to a maximum drug dose of 8 mg/kg. (A) Plasma levels of ovalbumin-liposomes 4 h after the second weekly injection as determined using standard scintillation counting. (B) The relative production of ovalbumin-specific IgG (absorbance 570 nm) as determined by ELISA assays of plasma isolated 7 days after the second weekly injection of each formulation. All data points represent the mean and standard error of four mice.

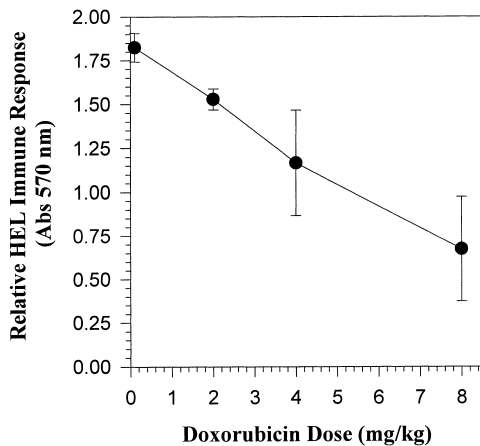


Fig. 5. Immune response to HEL proteoliposomes with simultaneous injection of doxorubicin encapsulated within ovalbumin-liposomes. Female CD-1 mice were co-injected with 50 μ g of empty HEL-liposomes and varying doses of 0.2 drug-to-lipid ratio doxorubicin in ovalbumin-coupled liposomes for 2 consecutive weeks. The figure shows the relative production of HEL-specific IgG (absorbance 570 nm) as determined by ELISA assays of plasma isolated 7 days after the second weekly injection of each formulation. All data points represent the mean and standard error of four mice.

3.5. The antibody response to antigen-associated liposomes is selectively inhibited by entrapment of doxorubicin within liposomes

To determine whether the doxorubicin-induced immune suppression was selective for a specific protein or involved a general blockade of the immune system, the humoral immune response to an unrelated liposomal protein was studied in drug-treated animals. The humoral response to liposomal HEL was monitored in animals co-administered with doxorubicin-loaded ovalbumin-liposomes encapsulated at a 0.2 drug-to-lipid ratio. The purpose of the study was to determine whether the drug-treated mice could mount an immune response to an unrelated liposomal antigen which did not contain encapsulated drug. As demonstrated in Fig. 5, doxorubicin doses as high as 8 mg/kg fail to block the majority of anti-HEL antibody production against the liposomal HEL. An inhibition of the humoral response against liposomal HEL is observed, but an inhibition which is only partial despite administration of ovalbumin-liposomes with encapsulated doxorubicin at doses which successfully blocked the antibody response (Fig. 2). Comparatively, the antibody response to

ovalbumin was completely blocked when ovalbumin-liposomes were co-injected with high dose of encapsulated doxorubicin (Fig. 4B). Altogether, these results indicate that the encapsulation of doxorubicin within a proteoliposome can impart a relatively selective inhibition of some components of the immune system for the specific protein associated with the liposome.

4. Discussion

In this study, we observed that the humoral response generated against an ovalbumin-coupled liposome could be blocked by the encapsulation of doxorubicin within the carrier. The extent of this immune regulation appears to be highly dependent on the drug-to-lipid ratio of the carrier. We also observed that the immune suppression is relatively specific to the protein coupled to the doxorubicin containing liposomes. This selective immune suppression could be useful for proteoliposome administrations where multiple dosing schedules are required.

Repeated injections of ovalbumin-liposomes induce production of antibodies against ovalbumin which cause subsequent injections to exhibit rapid elimination from the circulation. As shown in the dose titration experiments, low doxorubicin doses can prevent this antibody response. Complete suppression of the ovalbumin immune response could be achieved with 2 mg/kg doxorubicin administered at a drug-to-lipid ratio of 0.2:1 (mol:mol) (Fig. 3). This same dose of doxorubicin encapsulated at lower drug-to-lipid ratios did not block the production of ovalbumin antibodies, resulting in rapid elimination of subsequent injections. These results suggest that the concentration of doxorubicin within the carrier is crucial in blocking an immune response against a surface-coupled protein. When the doxorubicin was encapsulated in a separate carrier, a drug dose of 8 mg/kg was required to block the immune response. This is four times the dosage required when the drug is encapsulated within the same carrier. Macrophages of the liver and spleen are the predominant cells mediating liposome uptake. These cells are also involved in the processing and presentation of liposomal proteins and can be killed or inactivated in vivo

by liposomal doxorubicin [19,20]. Daemen et al. have shown that one i.v. injection of 5 mg/kg doxorubicin in PEG-containing liposomes reduced the phagocytic capacity of rat liver macrophages by 30% without a substantial change in the actual number of macrophages at 24 h after injection. By 72 h, uptake was reduced by 65% and a substantial decrease in the number of macrophages was observed [20]. This is in contrast to the initial studies of Daemen where doxorubicin liposomes lacking PEG only decrease the phagocytic activity of the macrophages. Doxorubicin encapsulated in PEG liposomes (long circulating liposomes) are taken up slowly compared to conventional liposomes, this explains the delay necessary to observe both a decrease in the phagocytic activity as well as numbers of phagocytic cells. According to these observations, the impaired macrophage uptake may lead to a diminution of the antibody response against the linked antigen due to the lack of antigen presentation. Comparatively, the suppression of the antibody response against an antigen conjugated to empty liposomes will require a higher dose of doxorubicin. When the phagocytic activity is partially reduced with Dox-liposomes at low doses, liposomes with covalently linked OVA will access the pool of macrophages not yet damaged and an immune response will occur. It is only at doses where the uptake is significantly reduced and the number of severely damaged macrophages is high that we observe a dramatic inhibition of the anti-OVA humoral response. However, the doxorubicin-induced unresponsiveness is not simply a consequence of the diminution of the number of phagocytic cells or reduced uptake itself; co-injection of HEL-linked liposomes with high doses of entrapped doxorubicin within OVA-linked liposomes did not abolish the anti-HEL antibody response. The decreased antibody production observed is not sufficient enough to prevent the rapid elimination of HEL-liposomes from the plasma upon the second injection (data not shown). We propose that the injection of Dox-liposomes-OVA, at doses of doxorubicin which are toxic for macrophages, will lead to the extensive death of the mature macrophages, the fraction of large size cells described by Daemen [19,20], and that T-cell cross-priming may occur. We believe that in the second round of injections, immature dendritic cells and macrophages may become involved and may effi-

ciently present OVA antigens derived from the processing of internalized apoptotic bodies to class II restricted T-cells. The T-cell activation resulting from the processing of phagocytosed apoptotic cells by immature phagocytic cells yields antigens which will be recognized by antigen-specific CD⁴⁺ T-cells. This process is termed 'T-cell cross-priming' [37]. Indeed, cells undergoing apoptosis may elicit an immune response initiated from the interaction among components of the immune response, CD⁸⁺ T-cells, CD⁴⁺ T- and B-cells, and requires the direct involvement of professional phagocytic cells. However, depending on the dose of Dox-liposomes-OVA, the number of apoptotic cells may be different and either T-cell activation or T-cell blockade can result. Following extensive killing of macrophages after injections of high dose of Dox-liposomes, immature dendritic cells may be overwhelmed by apoptotic cells which may consequently result in a functional and specific T-cell blockade that will affect the immune response against the associated antigen. Experiments looking at the effect of doxorubicin on specific T-cell activation in mice injected with high doses of doxorubicin entrapped in liposomes with conjugated OVA are currently ongoing to verify this hypothesis. Several considerations may explain the failure of Dox-liposomes-OVA to elicit a specific immune response. Processing of the dying cells by phagocytic cells may lead to the production and presentation of an altered epitope repertoire [38]. The engulfment of apoptotic cells by macrophages/dendritic cells does not trigger activation and up-regulation of MHC and co-stimulatory molecules. Finally, macrophages, upon engulfment of apoptotic cells may release immunomodulatory factors, such as anti-inflammatory IL-10 and TGF- β [39,40]. Indeed, in a recent investigation on the immunogenic potential of apoptotic cells in vivo, it was found that IL-10 was consistently released in the serum of animals immunized with apoptotic tumor cells [38]. Furthermore, injection of apoptotic cells significantly impaired cell-mediated immune responses in mice as measured by delayed-type hypersensitivity reactions. Our results suggest that a prevalence of professional phagocytes may favor antigen sequestration and paralysis of the immune response via release of soluble factors. Their ability to cross prime T-lymphocytes can be affected by the fact that apoptotic cell clearance by phagocytic cells is associ-

ated with the production of immunoregulatory factors such as IL-10.

In summary, the results presented here show that doses of liposome-encapsulated doxorubicin as low as 2 mg/kg can be repeatedly administered with surface-coupled ovalbumin without the generation of a humoral immune response. Low doses of doxorubicin selectively inhibit humoral immunity, while high drug doses induce general immune suppression. The results presented here suggest that encapsulation of doxorubicin, and therefore possibly other cytotoxic drugs, could be successfully used in targeted liposome-delivery systems due to their inhibitory effects on target molecule immunogenicity. Initial studies also suggest that similar systems could be potentially useful in the development of antigen selective immune suppression, a possibility that warrants further investigation.

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