An immune response to ovalbumin covalently coupled to liposomes is prevented when the liposomes used contain doxorubicin

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Abstract

It is now well established that liposomes with surface associated proteins are immunogenic. Repeated administration of protein coated liposomes elicits the generation of antibodies and the elimination of proteoliposome increases markedly in animals 'immunized' with such liposomes. This immune response compromises the therapeutic potential of liposomal formulations that rely on the use of protein- or peptide-based targeting ligands to enhance cell specificity. Strategies to suppress or inhibit such immune responses must be developed if this technology is going to prove therapeutically viable. This study evaluates whether an immune response to a protein, covalently attached to liposomes by a thioether bond between *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP)-modified-protein and *N*-(4-(*P*-maleimidophenyl)butyryl) (MPB)-activated lipids, can be suppressed when the liposomes used contain the anti-cancer drug doxorubicin. To assess this, the highly immunogenic protein ovalbumin was conjugated onto liposomes composed of distearoylphosphatidylcholine/cholesterol (DSPC/Chol) with sufficient poly(ethylene glycol)-modified distearoyl phosphatidylethanolamine (PEG-DSPE) (2 mol%) to prevent liposome aggregation during protein coupling and to engender increased circulation lifetimes. The immune response to these liposomes with and without encapsulated doxorubicin was measured by: (1) monitoring liposome elimination after 3 weekly i.v. injections in C3H/HeJ mice and (2) measuring the anti-ovalbumin antibody levels by an ELISA assay. One week after a single dose of ovalbumin-coated PEG liposomes (50 μg protein/mouse) the immune response resulted in rapid elimination of a second dose of ovalbumin-coated PEG liposomes. Rapid liposome elimination was correlated to generation of high levels (> 9 μg/ml plasma) of circulating anti-ovalbumin IgG. In contrast, anti-ovalbumin antibodies were not detected when the liposomes used contained doxorubicin. Plasma elimination of these drug loaded protein coated liposomes decreased following repeated weekly i.v. doses, an

effect that is consistent with liposomal doxorubicin mediated suppression of phagocytic cells in the liver. © 1997 Elsevier Science B.V.

1. Introduction

Although it is unlikely that monoclonal antibody based anti-cancer pharmaceuticals will become useful for first-line treatment of solid tumors, it is evident that the specificity achieved through the use of antibodies will be of therapeutic value (Shockley et al., 1992; Vitetta and Uhr, 1994). Advancement of this technology is still hindered, however, by the fact that following initial treatment immune responses to administered antibodies restricts further applications. Specifically, generation of heterologous antibodies leads to rapid elimination of the therapeutic antibody and significantly reduces in vivo targeting efficiency. HAMA, or human anti-mouse antibody, responses can be minimized through use of humanized murine antibodies, however production of anti-idiotypic antibodies is still observed (Khazaeli et al., 1994). Alternatively, immunosuppressive agents may be used to prevent an immune response against a therapeutic monoclonal antibody (Ledermann et al., 1988).

Targeted liposomal carriers must also contend with the potential for generation of an immune response against surface associated proteins. As shown by Shek et al. (1986) and Phillips et al. (1994), protein coated liposomes are highly immunogenic when injected into immune competent animals. Immunogenicity, as measured by formation of protein specific plaque forming cells and circulating mouse IgG, causes a reduction in the circulation lifetime of the antibody coated liposome and decreases the potential for these systems to interact with target cells. Surprisingly this immune response is not effected by use of surface grafted poly (ethylene glycol), a hydrophilic polymer known to inhibit liposome-cell interactions (Phillips et al., 1994). Since our applications are focused on development of targeted formulations of anticancer drugs, drugs that are known to suppress immune responses, we chose to address a simple question. Is the immune response to a protein-coated liposome inhibited if the liposome contains the anticancer drug doxorubicin? In this regard it is well known that tissue macrophages play an important role in the generation of an immune response to foreign proteins (Vidal et al., 1993; Brewer et al., 1994) and that macrophages play a dominant role in removing liposomes from the circulation. It is also known that the presence of encapsulated doxorubicin inhibits the activity of phagocytic cells in the liver (Bally et al., 1990b; Parr et al., 1993; Daemen et al., 1995) and this may inhibit generation of a humoral response to antigens associated with the liposome surface. The results presented demonstrate that encapsulated doxorubicin inhibits the immune response to a protein, ovalbumin, that was covalently attached to the liposome's surface.

2. Materials and methods

2.1. Materials

1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL). *N*-(4-(*p*-maleimidophenyl) butyryl)-dipalmitoyl phosphatidylethanolamine (MPB-DPPE) was obtained from Northern Lipids (Vancouver, BC). MePEG$_{2000}$-S-DSPE (PEG-DSPE) was obtained from Dr. Ansell and prepared as described elsewhere (Parr et al., 1994). Imject® ovalbumin and *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) were obtained from Pierce. Cholesterol, *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulphonic acid) (HEPES), Sepharose CL-4B, Sephadex G-25, Sephadex G-50 and dithiothreitol (DTT) were obtained from Sigma. [^{3}H] cholesteryl hexadecyl ether ([^{3}H]-CHE) was obtained from Amersham. Doxorubicin was obtained from Adria Laboratories. Freund's complete and incomplete adjuvant was obtained from GIBCO (Grand Island, NY). Female C3H/HeJ mice were obtained from the B.C. Cancer Agency Joint Animal Facility breeding colony who obtain parental stock from Jackson Laboratories (CA). It should be noted that all animal studies were done in accordance to the guidelines of the Canadian Council of Animal Care.

2.2. Preparation of liposomes

Large unilamellar vesicles were prepared according to the method of Hope et al. (1985). Lipid mixtures consisting of 52% DSPC/45% cholesterol/2% PEG-DSPE/1% MPB-DPPE (mol ratio) and trace amounts of [^{3}H] cholesteryl hexadecyl ether (1–5 μCi/100 μmol total lipid) were dissolved in chloroform. Previous studies have shown cholesteryl hexadecyl ether is a non-exchangeable and non-metabolizable lipid that can be employed as a liposomal lipid marker (Derksen et al., 1987; Bally et al., 1993). The solution was subsequently concentrated under a stream of nitrogen gas to produce a homogenous lipid film. The film was then placed under high vacuum for at least 4 h before hydration at 65°C with 300 mM citric acid (pH 4.0). The resulting lipid preparation (50 mM) was frozen and thawed 5 times (Mayer et al., 1985) prior to extrusion 10 times through stacked 100 nm polycarbonate filters (Poretic, Mississauga, Ont.) using an extrusion apparatus set to 65°C (Lipex Biomembranes, Vancouver, BC). Liposome particle size was determined by quasielastic light scattering (QELS) measurements (using a Nicomp 370 particle sizer operating at a wavelength of 632.8 nm). The external buffer of the liposome suspension (citrate pH 4.0), was exchanged into HBS (25 mM HEPES, 150 mM NaCl, pH 7.5) by gel filtration on Sephadex G-25.

2.3. Coupling of thiolated ovalbumin to liposomes

Imject® ovalbumin was modified with the amine reactive crosslinker SPDP according to procedures described previously (Loughrey et al., 1990). An ovalbumin solution was prepared in 0.9% NaCl at 8 mg/ml. SPDP was dissolved in ethanol and diluted in HBS prior to ovalbumin modification. The reaction was carried out with a 5-fold molar excess of SPDP to ovalbumin at room temperature for 30 min and subsequently passed down a Sephadex G-50 column equilibrated with HBS to remove any unreacted SPDP. The modified ovalbumin was then reduced with DTT (25 mM for 30 min) and the thiolated product isolated by gel filtration on Sephadex G-50 equilibrated with HBS. Modified ovalbumin was coupled with liposomes at a ratio of 90 μg of protein per μmol lipid (15 mM final lipid

concentration). The coupling reaction was carried out in HBS and stirred at room temperature for 18 h. Ovalbumin conjugated liposomes were then passed down a Sepharose CL-4B column equilibrated with HBS to remove any free ovalbumin. The amount of ovalbumin coupled to the liposomes was determined using the Pierce Micro BCA assay in the presence of 1% Triton X-100 to disrupt the liposomes. The reaction typically results in 30–40 μg of ovalbumin coupled per μmol lipid, consistent with results obtained for other proteins in this laboratory (Loughrey et al., 1990; Longman et al., 1995; Harasym et al., 1995).

2.4. Doxorubicin encapsulation

Doxorubicin was encapsulated in selected preparations using the transmembrane pH gradient driven loading procedure as previously described (Mayer et al., 1986). Briefly, ovalbumin-coated liposomes (pH$_{in}$ 4.0/pH$_{out}$ 7.5) and doxorubicin (solubilized in 0.9% NaCl) were pre-heated to 65°C for 5 min prior to mixing. The liposomes were added to doxorubicin to achieve a drug-to-lipid ratio of 0.2:1 (mol/mol). This mixture was incubated for 10 min at 65°C with intermittent mixing. Drug encapsulation efficiency was determined as described elsewhere (Mayer et al., 1990) and in entrapment efficiencies of > 98% were obtained. In experiments where empty liposomes were used, the same procedure was followed with the exception that a solution of 0.9% NaCl was used in place of doxorubicin.

2.5. In vivo liposome elimination studies

Groups of four C3H/HeJ mice per experimental point were given the specified treatment as a single i.v. dose via a lateral tail vein. The injection, given in a volume of 200 μl, contained 50 μg of ovalbumin. In animals receiving control (protein free) liposomes, the dose was chosen based on the lipid dose given for the ovalbumin-coated liposome group. This lipid dose was approximately 45 mg/kg. In the groups receiving doxorubicin, the drug dose was approximately 8 mg/kg. It is important to note that the maximum tolerated dose of doxorubicin encapsulated in DSPC/Chol liposomes is greater than 60 mg doxorubicin/kg (Parr et al., 1997). Therefore, the 8

mg/kg drug dose is well below the maximum tolerated dose and was well tolerated by the animals. Previous studies have demonstrated that a single 2 mg/kg drug dose is sufficient to inhibit accumulation of liposomes in the liver of mice (Bally et al., 1990b; Parr et al., 1993)

Plasma levels of injected liposomal lipid was determined 4 h after i.v. injection. Liposomal lipid was measured by using the lipid marker [3H]-cholesteryl hexadecyl ether. Blood (25 μl) was obtained via a nick of a lateral tail vein and collected with pre-rinsed (200 mM EDTA) microcapillary tubes. Subsequently, the capillary tube was rinsed several times with 200 mM EDTA, adjusting the final volume of blood and EDTA solution to 225 μl with 200 mM EDTA. The sample was placed on ice until it was centrifuged (500 × g). The pelleted blood cells were washed once with 200 μl of 200 mM EDTA and the supernatants (original plus wash) were collected and mixed with 5 ml of Pico-Fluor 40 scintillation cocktail (Packard). The amount of radioactivity in each sample was determined using a Beckman LS3801 scintillation counter. It was assumed that the hematocrit of the mice was 48% (Bally et al., 1993), therefore, each 25 μl of whole blood contained 13 μl of plasma.

One week after the third injection (week four) all four groups of mice were injected with freshly prepared ovalbumin-coated liposomes (without doxorubicin). All mice were injected at an ovalbumin dose of 50 μg per mouse. Whole blood was collected at 1 and 4 h by the tail nick procedure described above. At 24 h animals were asphyxiated by CO_2 and blood was collected via cardiac puncture and placed into EDTA coated microtainers (Becton Dickenson). For the latter sample, plasma was prepared by centrifuging at 500 × g for 10 min. Lipid levels in the plasma were determined as described above where the amount of radioactivity in 50 μl of plasma was measured.

2.6. Doxorubicin mediated inhibition of immune response to free ovalbumin

Additional groups (4 mice per group) of animals were immunized with free Imject® ovalbumin mixed with Freund's adjuvant. The ovalbumin was dissolved in 0.9% NaCl to achieve a concentration of 0.5 mg/ml. For the first immunization, this solution was emulsified with Freund's complete adjuvant. Subsequent immunizations used ovalbumin emulsified with Freund's incomplete adjuvant. Sterile emulsions were made either in saline (control) or from a 4 mg/ml solution of ovalbumin. This protein solution was drawn into a 6 cc sterile glass syringe while an equivalent volume of Freund's adjuvant was drawn into a second syringe. The two syringes were connected and mixed until a thick stable homogeneous solution was obtained (~20 min). Saline was substituted for ovalbumin for control preparations. Mice were injected with 50 μg of ovalbumin intraperitoneally (200 μl) for three consecutive weeks (fresh preparations weekly). The animals were immunized every week for three weeks and either terminated on the fourth week for assessment of circulating anti-ovalbumin IgG levels or were used to evaluate the elimination of ovalbumin doxorubicin loaded liposomes following i.v. administration.

To determine whether liposomal doxorubicin effected the ability of animals immunized with ovalbumin to generate an immune response, 4 groups were established: (i) Freund's adjuvant mixed with saline (no ovalbumin); (ii) Freund's adjuvant with ovalbumin; (iii) Freund's adjuvant with ovalbumin and an i.v. dose of DSPC/Chol liposomes and (iv) Freund's adjuvant with ovalbumin and an i.v. dose of doxorubicin encapsulated in DSPC/Chol liposomes. For the latter two groups, liposomes (without surface associated ovalbumin) were administered i.v at a lipid dose of 45 mg/kg, 4 h prior to administration of the ovalbumin Freund's mixture. When the liposomes contained doxorubicin, the drug dose was approximately 8 mg/kg.

2.7. Protein analysis

The stability of ovalbumin when incubated in the presence of doxorubicin at 65°C was assessed by SDS–PAGE according to standard procedures. Ovalbumin (M_r 45 kDa; 4 mg/ml) in HBS pH 7.5 was heated in a water bath to 65°C in the absence and presence of doxorubicin (14.8 mg/ml) for 10 and 20 min, respectively. The samples (16 μg) were subjected to electrophoresis through a 12% denaturing polyacrylamide gel (Bio-Rad Laboratories) and stained with Coomassie blue.

2.8. ELISA assay for anti-ovalbumin immunoglobulin

Microtitre plates (Flow Laboratories) were coated with 50 μl of 40 μg/ml ovalbumin and incubated at 4°C overnight. The plates were washed twice (phosphate buffered saline containing 0.1% (vol/vol) Tween 20) and then blocked with 2% bovine serum albumin (BSA) in Hank's balanced salt solution (HBSS) for 1 h at 37°C. The plates were rinsed prior to addition of mouse plasma in a final volume of 100 μl (the extent to which the plasma was diluted depended on treatment group). After a 1 h incubation the plates were washed three times and 100 μl of 0.5 μg/ml biotin labeled goat anti-mouse IgG (diluted in Hank's solution with 1% BSA) was added. The plates were incubated at room temperature for 30 min and washed 3 times prior to addition of 100 μl of 500 mU/ml of streptavidin-β-galactosidase (diluted in Hank's solution with 1% BSA). Plates were incubated at room temperature for 30 min, washed, and the amount of plate associated β-galactosidase was determined. Chlorophenol red-β-D-galactopyranoside (100 μl of a 2 mg/ml) in HBSS, plus 1% BSA was added to the plates and incubated at room temperature for 1 h. The amount of chlorophenol red produced was determined by measuring absorbance at 570 nm on a Titertek Multiscan plate reader. Antibody levels were estimated on the basis of standard curves consisting of known quantities of a rabbit polyclonal anti-ovalbumin IgG (Cortex Biochemicals, Ca). Controls consisted of undiluted plasma samples obtained from untreated C3H/HeJ mice.

3. Results

3.1. Elimination behavior of ovalbumin-coated liposomes

Plasma elimination of liposomes with and without surface associated ovalbumin is shown in Fig. 1. These results were obtained following i.v. administration of liposomes at a lipid dose of approximately 45 mg/kg and show that liposomes with surface associated ovalbumin are eliminated from the circulation at a rate faster than that observed for control
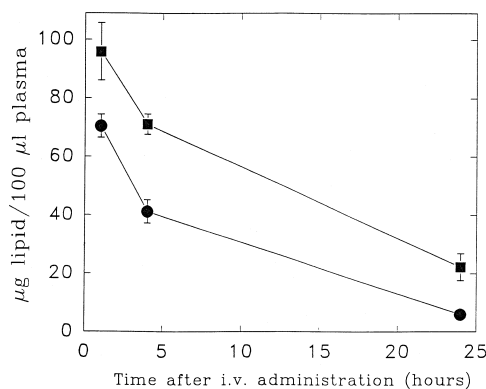

Fig. 1. Elimination of ovalbumin-coated PEG liposomes. Mice were injected i.v. with either 2% PEG liposomes (■) or ovalbumin-coated 2% PEG liposomes (●). Blood was collected at various time points and the amount of lipid remaining in the blood determined. The results are plotted as an average obtained from four animals $\pm$ standard deviation.

liposomes. One day following administration, for example, significantly higher levels of control liposomes (DSPC/Chol/MPB-PE/PEG-PE; 52:45:1:2 mol ratio, approximately 20% of the injected lipid dose) can be found in the plasma compartment in comparison to identical liposomes with surface associated ovalbumin (less than 5% of the injected lipid dose). This result is consistent with previous studies showing that liposomes with surface associated proteins are cleared more rapidly than control liposomes following i.v. administration and that the clearance rate is dependent on the protein used (Harasym et al., 1995; Longman et al., 1995). Protein mediated decreases in circulation lifetime are observed under conditions where the size of the liposomes used are comparable (100–150 nm) as measured by QELS.

3.2. Effect of encapsulated doxorubicin on immune response to ovalbumin-coated liposomes

Studies by Phillips et al. (1994) showed that repeated i.v. administration of IgG coated anionic DPPC/DMPG liposomes elicited an immune response that decreased subsequent plasma circulation lifetimes. In addition, Shek et al. (1986) demonstrated an immune response to BSA coupled liposomes after repeated i.p. administration. It was important to demonstrate that our neutral liposomal formulations (prepared with saturated phosphatidyl-
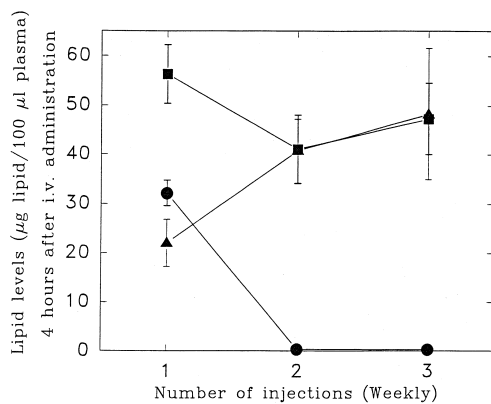
Fig. 2. Elimination of ovalbumin-coated PEG liposomes as a function of injection number. Mice were injected (i.v.) for three consecutive weeks with either 2% PEG liposomes (■), ovalbumin-coated 2% PEG liposomes (●) or ovalbumin-coated doxorubicin loaded 2% PEG liposomes (▲). Four hours after injection blood was collected and the concentration of lipid remaining in the plasma determined. The results are plotted as an average obtained from four animals ± standard deviation.

choline, cholesterol and PEG-modified lipids) could engender an immune response to an attached protein following i.v. administration. These small liposomes (100–150 nm) were designed for serum stability and enhanced circulation characteristics. As shown in Figs. 2 and 3, these ovalbumin-coated liposomes appear to be highly immunogenic. Weekly injections of control liposomes did not result in significant changes in circulating blood levels, i.e. 56 μg lipid/100 μl plasma remained after the first injection (corresponding to approximately 50% of the injected dose) in comparison to lipid levels of 41 and 48 μg/ 100 μl plasma which were observed at 4 h after the second and third injections, respectively. When ovalbumin-coated PEG liposomes were administered, approximately two fold less lipid (in comparison with protein free liposomes) was recovered 4 h after the first injection, corresponding to 25% of the injected dose. After the second injection this value decreased significantly ($p < 0.01$), to less than 0.5% of the injected dose (< 0.7 μg/100 μl plasma) in the blood compartment. Rapid elimination was also observed after the third injection. It should be noted that immediately after the third injection of ovalbumin-coated liposomes mice showed signs of acute stress (convulsions, labored breathing and hunched posture), but after 5 min the animals recov-

ered, remaining inactive for periods in excess of one hour after administration. Animals receiving control (protein free) liposomes exhibited no changes in behavior after repeated liposome administration.

Remarkably different elimination behavior was observed when ovalbumin liposomes contained doxorubicin (Fig. 2) were injected. The circulating lipid levels observed 4 h after i.v. administration of drug loaded liposomes increased with each subsequent injection. After the third administration, the levels of doxorubicin-loaded ovalbumin-coated PEG liposomes measured in the blood compartment (approximately 50% of the injected dose) were comparable to those obtained with control liposomes (no protein, no doxorubicin). In comparison, less than 0.5% of the injected dose was observed for ovalbumin-coated PEG liposomes in the absence of encapsulated drug. Entrapped doxorubicin mediated increases in liposome circulation lifetimes have been reported in previous studies (Bally et al., 1990b). The increased circulation lifetimes have been attributed to the cytotoxic activity of encapsulated doxorubicin on the phagocytic cells of the RES and, in particular, Kupffer cells residing in the liver (Parr et al., 1993; Daemen et al., 1995). Administration of free doxorubicin at levels of 20 mg/kg (single dose) has no impact on the elimination behavior of liposomes (Bally et al., 1990b).
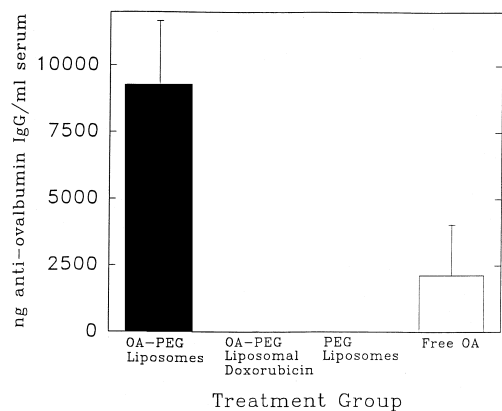

Fig. 3. The level of anti-ovalbumin antibodies in serum was determined by ELISA (see Section 2) for samples obtained one week after three consecutive weekly i.v. injections of the samples described in Fig. 2. The results are plotted as an average obtained from four animals ± standard deviation, with IgG levels determined in triplicate for each animal.

Since it has been demonstrated that Kupffer cells are required for the generation of IgG antibodies (Brewer et al., 1994), it is reasonable to suggest that entrapped doxorubicin may inhibit generation of anti-ovalbumin antibodies. Therefore, IgG levels were measured in plasma obtained from C3H/HeJ mice given 3 weekly i.v. injections of control liposomes, free ovalbumin, ovalbumin-coated PEG liposomes or doxorubicin-loaded ovalbumin-coated PEG liposomes (Fig. 3). Anti-ovalbumin IgG was not detected in the plasma of mice receiving control liposomes or doxorubicin-loaded ovalbumin-coated PEG liposomes. In contrast, anti-ovalbumin IgG levels of 2.1 and 9.3 μg/ml plasma were obtained in animals injected i.v. with free ovalbumin and ovalbumin-coated PEG liposomes, respectively. These results clearly demonstrated that generation of antibodies against liposome surface associated proteins can be inhibited by the presence of encapsulated doxorubicin.

It is known that under certain conditions (heating to $> 56°C$, vigorous mixing and/or interactions with chemicals) that ovalbumin can be denatured and it is necessary to establish that the conditions used to prepare doxorubicin-loaded ovalbumin-coated lipo-somes did not result in protein denaturation. As shown in Fig. 4, PAGE analysis of ovalbumin following a 10 min incubation at 65°C (lane 3) or a 20 min incubation at 65°C in the presence of doxorubicin (lane 4) demonstrated that the protein was stable under the conditions required for doxorubicin loading. An additional test of whether ovalbumin covalently coupled onto liposomes with entrapped doxorubicin could be recognized in vivo by antibodies generated against free (unmodified) ovalbumin is shown in Fig. 5. Animals were injected i.p. for three consecutive weeks with Freund's adjuvant mixed with saline (filled circles) or ovalbumin (open circles) and one week after the last injection the animals were given a single i.v. injection of doxorubicin-loaded ovalbumin-coated liposomes. In animals given the Freund's/saline mixture the elimination behavior of the doxorubicin-loaded ovalbumin-coated liposomes was comparable to that observed in animals not pre-treated with the adjuvant. In contrast, doxorubicin-loaded ovalbumin-coated liposomes were rapidly removed (less than 15 min) from the blood compartment of animals immunized with free ovalbumin. This suggests that antibodies generated against ovalbumin were capable of increasing the
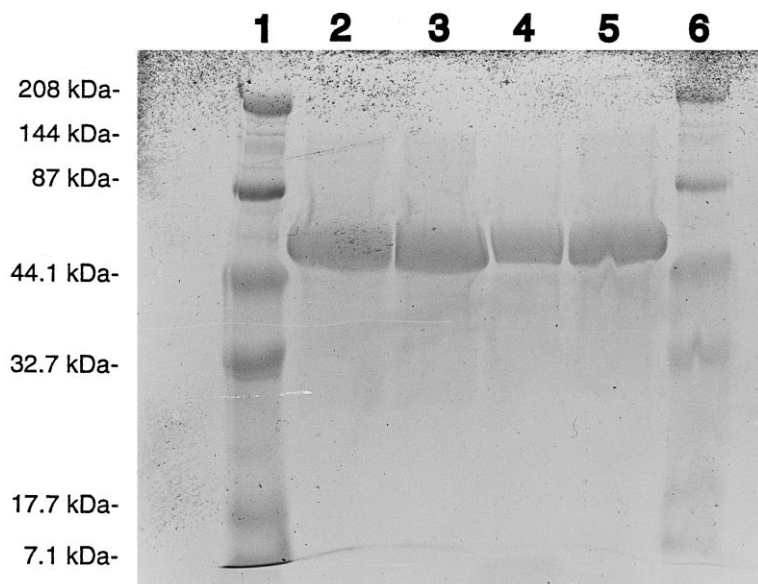

Fig. 4. Stability of ovalbumin under conditions required for doxorubicin loading. Ovalbumin (M_r 45 kDa; 4 mg/ml) samples (16 μg) were separated by 12% SDS–PAGE and stained with Coomassie blue. Lanes 1 and 6, pre-stained standards; lanes 2 and 5, non-heated ovalbumin controls; lane 3, ovalbumin heated to 65°C for 10 min; lane 4, ovalbumin + doxorubicin heated for 20 min.
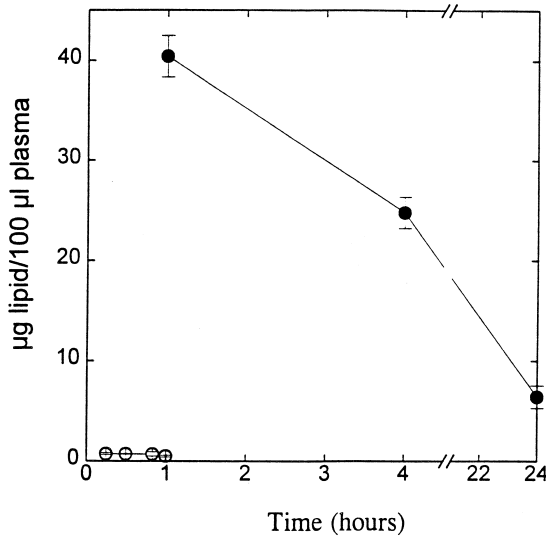
Fig. 5. Elimination of ovalbumin-coated doxorubicin-loaded PEG liposomes in the presence or absence of prior immunization with free ovalbumin. Two groups of mice (12 mice/group) were injected (i.p.) for three consecutive weeks with either an emulsion of Freund's adjuvant containing saline (●) or Freund's adjuvant containing ovalbumin (○), as outlined in Section 2. On the fourth week mice from both groups were injected (i.v.) with 50 μg of ovalbumin-coated doxorubicin loaded PEG liposomes (200 μl). At the indicated time points mice were terminated and plasma collected and assayed for liposomal lipid. The results are plotted as an average obtained from at least three animals $\pm$ standard deviation.

elimination rate of ovalbumin liposome that were exposed to 65°C as required for doxorubicin loading. It is important to note that the ELISA used to measure circulating IgG levels relied on coating plates with ovalbumin at 4°C and we can not discount the possibility that antibodies generated against ovalbumin heated at 65°C would efficiently recognize this bound ovalbumin.

The effect of prior i.v. injection of ovalbumin-coated liposomes on the elimination of ovalbumin-coated liposomes at a single time point (4 h) was shown in Fig. 2. More extensive time course studies are shown in Fig. 6, which include data obtained from four experimental groups that were all given (i.v.) ovalbumin-coated PEG liposomes (no encapsulated doxorubicin) at a lipid dose of 45 mg/kg. Four groups of animals were used, each differing in terms of the type of pre-treatment. Animals given weekly i.v. injections of free ovalbumin and ovalbumin-

coated PEG liposomes had measurable levels of anti-ovalbumin antibodies in the plasma (see Fig. 3). As shown in Fig. 6, plasma elimination of ovalbumin-coated PEG liposomes was most rapid in these animals. One hour after injection less than 10% and 30% (respectively) of the injected ovalbumin-coated PEG liposome dose was recovered in the plasma compartment. Plasma elimination of ovalbumin-coated PEG liposomes was unaffected by pre-treatment with control liposomes. The circulation half-life observed for these liposomes was approximately 1.5 h, similar to that observed in animals that received no prior treatment. It is important to note that the control liposomes used contained 2% PEG-modified lipid as well as 1% MPB-PE, suggesting that the immune response elicited against ovalbumin-coated PEG liposomes was likely not due to antigenicity of these lipid components. The circulation half-life of the ovalbumin-coated PEG liposomes was significantly enhanced in animals that had received three prior injections of doxorubicin-loaded ovalbumin-coated PEG liposomes. The absence of anti-ovalbumin IgG in the plasma in combination with the RES blockade, both of which are mediated by pre-treatment with doxorubicin containing lipo-
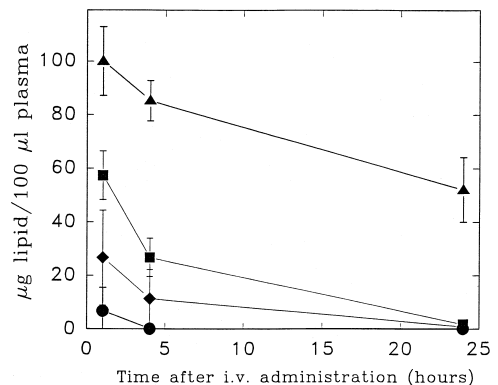

Fig. 6. Elimination of ovalbumin-coated PEG liposomes. Mice were injected (i.v.) for three consecutive weeks with either free ovalbumin (♦), 2% PEG liposomes (■), ovalbumin-coated 2% PEG liposomes (●) or ovalbumin-coated, doxorubicin loaded 2% PEG liposomes (▲). On the fourth week, all mice were injected i.v. with ovalbumin-coated 2% PEG liposomes. Elimination of the fourth dose was monitored by collecting blood at various time points and the amount of lipid remaining in the blood determined. The results are plotted as an average obtained from four animals $\pm$ standard deviation.

somes, resulted in a circulation half-life increase from 1.5 h to greater than 24 h for the ovalbumin-coated PEG liposomes.

3.3. Liposomal doxorubicin inhibition of anti-ovalbumin antibody production

The results presented thus far clearly demonstrate that the presence of encapsulated doxorubicin can inhibit the generation of an antibody response to a surface associated protein present on doxorubicin loaded liposomes. This may be a consequence of toxicity to cells responsible for antigen processing, a mechanism involving RES blockade rather then RES saturation. RES blockade is used here to describe conditions where specific drugs adversely effect phagocytic cell function (Bally et al., 1990b; Parr et al., 1993; Daemen et al., 1995). It is also possible that this effect may be due to the non-specific inhibition of proliferating immune cells generated in response to an antigen and that i.v. administration of

ovalbumin-coated PEG liposomes is not optimal in terms of generation of an immune response. Subsequent studies were designed to determine whether liposomal doxorubicin (without surface associated protein) was capable of inhibiting the generation of anti-ovalbumin antibodies under more stringent conditions. Animals were given three weekly i.p. injections of Freund's adjuvant plus ovalbumin (50 μg/mouse) and the level of circulating anti-ovalbumin IgG was determined one week after the last injection. Four hours prior to each i.p. injection of Freund's adjuvant/ovalbumin mixture, animals were given an i.v. injection of saline, control liposomes or liposomal doxorubicin (see Section 2). The results, shown in Fig. 7, clearly demonstrate that production of anti-ovalbumin IgG is prevented when the animals are pre-treated with liposomal doxorubicin. Those animals pre-injected (prior to each i.p. injection) with liposomes without encapsulated drug had anti-ovalbumin IgG levels of > 30 μg/ml serum. This was not significantly different from that observed in animals given 3 weekly i.p. injections of Freund's adjuvant/ovalbumin mixtures alone (> 40 μg/ml serum of anti-ovalbumin IgG). In contrast, antibody levels were not detectable in animals pre-injected with liposomal doxorubicin.
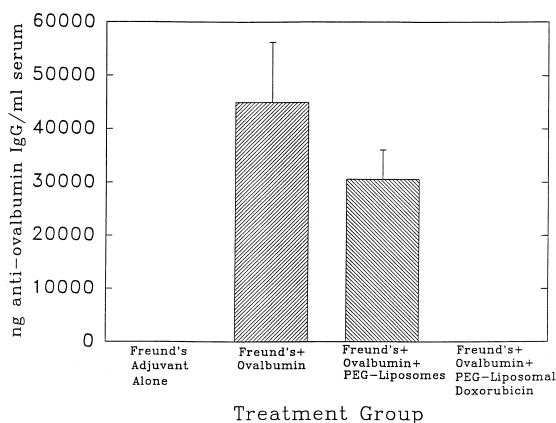

Fig. 7. The level of anti-ovalbumin antibodies in serum was determined by ELISA (see Section 2) for samples obtained one week after the three consecutive weekly i.p. injections of: (i) Freund's adjuvant (mixed with saline), (ii) Freund's adjuvant mixed with free ovalbumin, (iii) Freund's adjuvant mixed with free ovalbumin and given 'empty' PEG-liposomes four hours prior to each i.p. injection, and (iv) Freund's adjuvant mixed with free ovalbumin and given doxorubicin loaded PEG-liposomes four hours prior to each i.p. injection (see Section 2). The results are plotted as an average obtained from four animals ± standard deviation.

4. Discussion

It has recently been shown that antibody coated liposomes containing the anti-cancer drug doxorubicin can be used to effectively treat murine lung tumors (Ahmad et al., 1993). One of the primary limitations regarding further development of this technology, however, concerns the generation of immune responses to surface associated proteins (Phillips and Dahman, 1995) and/or the heterobifunctional linking reagents used for protein coupling (Boeckler et al., 1996). This has been confirmed in recent studies from Phillips et al. (1994) indicating that liposomes, even when prepared with polyethylene glycol modified lipids, significantly enhance immunogenicity of surface associated IgG (Phillips and Dahman, 1995). These data were consistent with earlier studies by Shek et al. (1986) and

Shek and Heath (1983) for liposomes prepared in the absence of PEG-modified lipids.

The focus of this report was to determine if an immune response generated by a protein coated liposome could be blocked when those liposomes contained doxorubicin, a cytotoxic and immunosuppressive drug. In this regard the objectives are similar to those developed by Shek et al. (1986) who demonstrated that encapsulated methotrexate could modulate (increase or decrease) the immune response to BSA (bovine serum albumin) covalently coupled to liposomes. There are several important distinctions that should be made when comparing previous studies to those reported here. First, Phillips et al. (1994) used liposomes prepared of DMPC/DMPG. These liposomes lack cholesterol and contain significant levels of the anionic lipid phosphatidylglycerol (PG) both of which are known to promote liposome elimination from blood and increase removal by phagocytic cells of the RES. Second, the studies of Shek et al. (1986) used liposomes prepared by reverse phase evaporation techniques which likely produced large liposomes (> 400 nm) that were subsequently given by repetitive i.p. administration. The liposomes used in this report have been selected on the basis of physical (size) and chemical (lipid composition) characteristics that are known to be optimal for systemic use. In this regard, optimized systems refer to the development of carriers that are retained in the blood compartment for extended time periods, retain encapsulated drug well after systemic administration, exhibit a reduced tendency to interact with phagocytic cells of the RES systems and have a propensity to accumulate (non-specifically) in regions of tumor growth. We initiated these studies in hopes of demonstrating that such optimized systems would be less immunogenic, in comparison to anionic liposomes or large liposomes, when given intravenously. As indicated in this report, these hopes were not realized.

The results, however, clearly indicate that the humoral response to proteins attached to liposomes can be prevented when the liposomes contain doxorubicin and it is of interest to speculate on the mechanism(s) that may be responsible for generation and prevention of this immune response. Since the specific immune cells affected following administration of the ovalbumin-coated liposomal doxorubicin

formulations have not be identified, it is difficult to ascribe a mechanism responsible for suppression of the immune response observed here. A number of cell types have been identified as antigen presenting cells; including fixed tissue macrophages (Shek and Lukovich, 1982; Su and van Rooijen, 1989), dendritic cells (Nair et al., 1993) as well as B lymphocytes (Dal Monte and Szoka, 1989); all of which could be directly or indirectly affected by treatment with liposomal formulations of doxorubicin. We believe that B lymphocytes are an unlikely target because the liposomal drug dose used was below that required to engender effective suppression of peripheral leukocytes or spleen cells (Bally et al., 1990a). Further, administration of free doxorubicin at the maximum tolerated dose, where significant immune suppression is observed, was not able to inhibit the immune response measured here. Finally, lymphocytes are not actively phagocytic and it would be improbable that these cells could directly accumulate the ovalbumin-coated liposomal drug.

In contrast, tissue macrophages as well as dendritic cells are known to be phagocytic and it is well established that these cells are responsible for the elimination of liposome based drug carrier systems. The results presented are consistent with reports demonstrating inhibition of adjuvant elicited IgG production in animals pre-treated with agents known to block or eliminate macrophages (Brewer et al., 1994). Further, we and others have shown that a pre-dose (i.v.) of liposomal doxorubicin can interfere with phagocytic cells responsible for removing liposomes from the circulation (Bally et al., 1990b; Parr et al., 1993; Daemen et al., 1995). Efficient inhibition of these cells in vivo engenders increases in liposome circulation lifetimes. Since the phagocytic cells play an important role in antigen presentation (Alving, 1991), suppression of these cells may inhibit the generation of an immune response. Further studies identifying which phagocytic cell populations are influenced by administration of liposomal doxorubicin are ongoing.

Concerning potential clinical development of protein based targeted liposomal drugs, it is reasonable to conclude that the generation of anti-proteoliposome antibodies will not be a significant problem for certain targeted drug formulations. The choice of drug, however, will be critical. Multiple injections of

protein-coated liposomes may only be practical if the liposomes contain drugs, such as anti-cancer drugs that exhibit specific toxicities towards antigen presenting cells. In contrast, targeted formulations of anti-microbial or anti-fungal agents may still elicit major immune responses generated against liposome associated targeting ligands.

Acknowledgements

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