ELSEVIER

Journal of Immunological Methods 191 (1996) 1–10

JOURNAL OF IMMUNOLOGICAL METHODS

Immunogenicity of new heterobifunctional cross-linking reagents used in the conjugation of synthetic peptides to liposomes

Christophe Boeckler [a], Benoît Frisch [a], Sylviane Muller [b], Francis Schuber [a,*]

[a] *Laboratoire de Chimie Bioorganique, CNRS URA 1386, Faculté de Pharmacie, 74 Route du Rhin, 67400 Illkirch, France*
[b] *Institut de Biologie Moléculaire et Cellulaire, CNRS UPR 9021, 15 Rue Descartes, 67000 Strasbourg, France*

Received 7 August 1995; revised 30 October 1995; accepted 14 November 1995

Abstract

We have investigated the immunogenicity of six thiol-reactive heterobifunctional cross-linking reagents that permit the conjugation of cysteine carrying peptides to the surface of liposome containing monophosphoryl lipid A. Such constructs elicit an immune response against short synthetic peptides and our aim was to find the least immunogenic linkers to limit potential carrier-induced epitopic suppression. For that purpose the properties of three new polyoxyethylene linkers of different lengths and thiol-reactive moieties (maleimide, bromoacetyl, dithiopyridine) were compared to known derivatives obtained by reacting the classical reagents SMPB and SPDP or *N*-succinimidyl bromoacetate with phosphatidylethanolamine. The least immunogenic linkers were the bromoacetate derivatives whereas those containing a maleimide group evoked a significant anti-linker immune response. In addition, using IRGERA as a model peptide, we found that all six liposomal constructs strongly elicited the production of anti-peptide IgG antibodies. This immune response was therefore independent of the length of the linkers (ranging between 0.3 and 1.6 nm) and of the nature of the linkage between the peptide and the thiol-reactive moieties of the cross-linkers, i.e. stable thioether or bio-reducible disulfide bonds.

Keywords: Liposome; Spacer arm; Cross-linker; Heterobifunctional reagent; Immunogenicity; Peptide

1. Introduction

The use of synthetic peptides corresponding to the protective epitopes of pathogens has attracted considerable attention in the search for new approaches to vaccine design (Arnon and Horwitz, 1992). When short peptides are used for immunization their conjugation to carrier proteins (Katz, 1980), synthetic polymers or the employment of multiple antigen peptide (MAP) systems (Chai et al., 1992; Wang et al., 1995) are usually required to elicit a strong response. In many cases, however, the coupling of the peptide is not well characterized and therefore, the advantage of using structurally defined peptides

Abbreviations: Chol, cholesterol; DCC, *N,N'*-dicyclohexylcarbodiimide; DPPE, dipalmitoylphosphatidylethanolamine; FAB, fast atomic bombardment; HPLC, high performance liquid chromatography; MAP, multiple antigen peptide; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide; MCS, *N*-5-maleimidocaproyl-*N*-hydoxysuccinimide; MPB-PE, *N*-4-(*p*-maleimidophenyl)butyrylphosphatidylethanolamine; MPL, monophosphoryl lipid A; NHS, *N*-hydroxysuccinimidyl; PBS, phosphate buffered saline; PC, phosphatidylcholine; PDP-PE, *N*-3-(2-pyridyldithio)propionylphosphatidylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SMPB, 4-(*p*-maleimidophenyl)butyryl-*N*-hydroxysuccinimide; SPDP, 3-(2-pyridyldithio)propionyl-*N*-hydroxysuccinimide; SUV, small unilamellar vesicles.

* Corresponding author. Tel.: (+33) 88 67 68 40; Fax: (+33)88678891.

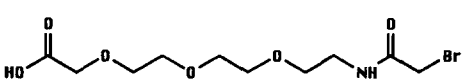
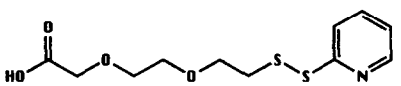
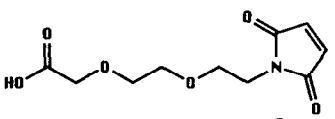
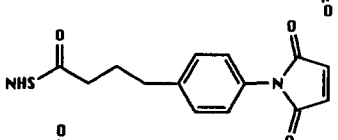
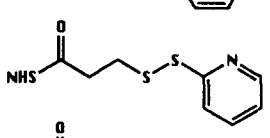
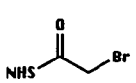
SSDI 0022-1759(95)00284-7

for the design of synthetic vaccines might be offset by the bioconjugation procedures.

Within this context liposomes, which are by themselves non-immunogenic, non-toxic and biodegradable, are particularly valuable. They have been found to significantly enhance the immune response against poorly immunogenic proteins, either entrapped or surface-bound (for reviews see Alving, 1991; Buiting et al., 1992; Gregoriadis, 1994). The intensity of the response can be further enhanced by the incorporation of adjuvants such as monophosphoryl lipid A (MPL) in the vesicles (Verma et al., 1992). In contrast to the many studies dealing with liposomal-associated protein antigens, little work has been performed with short synthetic peptides. In previous reports we have shown that small unilamellar vesicles, containing MPL, were able to induce a strong and long-lasting immune response against a model synthetic hexapeptide antigen of sequence IRGERA, corresponding to the C-terminal sequence of histone H3, that was coupled to their surface (Frisch et al., 1991; Friede et al., 1993). When applied to the cyclic 139–147 peptide mimicking site A of influenza hemagglutinin, this strategy permitted us to observe over 70% protection against viral infection in a murine vaccination model (Friede et al., 1994).

Peptides can be coupled to liposomes by chemically well defined procedures. Thus, in our previous studies, peptides extended at their N-terminal end by a CG spacer were conjugated to the surface of preformed vesicles containing MPB-PE (Fig. 1). This

Table 1
Structure of the heterobifunctional reagents used in this study

Compounds	Structure	Linker length (Å)[a]
1		16.1
2		11.3
3		13.1
SMPB		12.5
SPDP		6.9
4		2.7

[a] The length of the extended conformation of the linkers, between their carbonyl function and the sulfur atom of the conjugated peptide, has been estimated by use of a molecular modeling computer program (Personal CAChe).

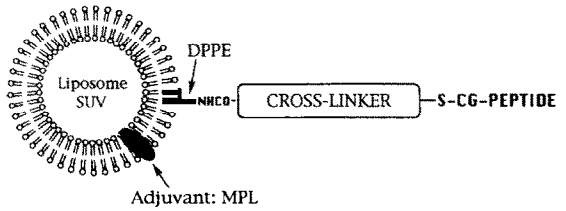


Fig. 1. Liposome-based construct for immunization with synthetic peptides. The peptide carrying a cysteine residue, e.g. at its N-terminus, is conjugated to the surface of preformed SUV containing a thiol-reactive phosphatidylethanolamine derivative and MPL as adjuvant.

thiol-reactive derivative (Martin and Papahadjopoulos, 1982) is obtained by reaction between PE and SMPB, a commercially available and widely used heterobifunctional reagent. Because it has been mentioned that MBS, a reagent structurally related to SMPB, when used for hapten-carrier conjugation elicited an immune response against the corresponding linker (Bernatowicz and Matsueda, 1986), we decided to investigate the immunogenicity of different cross-linkers used in immunoconjugation. We have also synthesized and studied the immunogenicity of new cross-linking reagents (Table 1) which, because of their polyoxyethylene structures, were hoped to be less immunogenic (see e.g. Zalipsky, 1995).

2. Materials and methods

Reagent-grade solvents were used without further purification. Cholesterol, recrystallized in methanol, egg yolk PC, and PG (transesterified from egg PC) were purchased from Sigma Chemical Co. (St-Quentin Fallavier, France). DPPE was a gift from D3F (Doullens, France). BSA was from Boehringer (Mannheim, Germany). *N*-hydroxysuccinimide, DCC and bromoacetic acid were obtained from Aldrich Chemical Co. and glutaraldehyde from Fluka Chemical Co. (St-Quentin Fallavier, France). Monophosphoryl lipid A was from Ribi Immunochem Research (Hamilton, MO) and SMPB from Pierce Chemical Co. (Rockford, IL). SPDP, MPB-DPPE and PDP-DPPE were prepared according to Carlsson et al. (1978), Martin and Papahadjopoulos (1982) and Martin et al. (1981) respectively. All the other heterobifunctional reagents (**1–3**; Table 1) and their DPPE derivatives were prepared in our laboratory as described by Frisch et al. (1996). *N*-succinimidyl bromoacetate (**4**; Table 1) was obtained as described by Bernatowicz and Matsueda (1986).

^{1}H-NMR spectra were recorded at 200 MHz on a Bruker WP-200 SY spectrometer (chemical shifts are given in ppm). High resolution mass spectra were obtained with a VG ZAB-HF mass spectrometer.

2.1. Synthesis of hexadecanoic acid 3-(2-(2-bromoacetylamino)ethoxy)hydroxyphosphoryloxy-2-hexadecanoyloxypropyl ester (PE-4) [1]

DPPE (50 mg, 72.2 μmol) was dissolved, at 40°C, in 15 ml anhydrous $CHCl_3$ containing diisopropylethylamine (11 mg, 86.8 μmol) and bromoacetic chloride (12 mg, 72.2 μmol). The reaction was then allowed to proceed for 2 h at 25°C under argon. Thin layer chromatography ($CHCl_3/CH_3OH/H_2O$, 65:25:4) revealed a quantitative conversion of DPPE to a less polar product. The solvent was evaporated under reduced pressure and the products redissolved in $CHCl_3$. This organic phase was extracted twice with 10 ml of 1% NaCl, twice with 10 ml of H_2O to remove water soluble by-products, then dried on $MgSO_4$ and finally evaporated. The reaction product was purified by chromatography on a 10 ml silica gel (BioSil,HA) column which had been activated (120°C overnight) and prewashed with 100 ml of $CHCl_3$. The column was eluted with an additional 20 ml volume of $CHCl_3$ followed by 20 ml $CHCl_3/CH_3OH$ mixtures (40:1, 30:1, 25:1, 20:1, 15:1) and finally with 60 ml of $CHCl_3/CH_3OH$ (10:1). Analysis by TLC indicated a single phosphate-positive and ninhydrin-negative spot for the fractions eluting with 15:1 and 10:1 $CHCl_3/CH_3OH$. These fractions were pooled and

[1] The following conventions, given for compound **1** as an example, are used throughout this manuscript: (i) PE-**1**, stands for the thiol-reactive DPPE derivative obtained by formation of an amide bond between the carboxylic group of the heterobifunctional reagent **1** and the phospholipid amine moiety; (ii) similarly BSA-**1** stands for **1** having reacted with amine functions of BSA; (iii) in PE-[**1**]-peptide, the squared brackets designate the linker, i.e. the structure of **1**, formed between the carbonyl of **1** and the sulfur atom (here a thioether) originating from the peptide.

evaporated under high vacuum to afford a white solid (47 mg, 80% yield); R_f 0.62 ($CH_2Cl_2/CH_3OH/HOAc$, 30 : 5 : 1). ^{1}H-NMR ($CDCl_3/CD_3OD$, 1 : 1) δ 5.30–5.20 (m, 1H, CH_2CHCH_2OP), 4.44–4.12 (m, 2H, CH_2CHCH_2OP), 4.03–3.96 (m, 4H, CH_2CHCH_2OP, $POCH_2CH_2O$), 3.87 (s, 2H, $COCH_2Br$), 3.54 (m, 2H, $POCH_2CH_2O$), 2.34–2.29 (m, 4H, $CH_2CH_2CO_2$), 1.71–1.50 (m, 4H, $CH_2CH_2CO_2$), 1.28 (m, 48H, $(CH_2)_{12}$), 0.89 (t, $J = 6.6$ Hz, 6H, CH_3). High resolution mass spectra (FAB +) m/z calculated for $C_{39}H_{75}O_9NPBrNa$ ($M + Na^+$) 834.4260, found 834.4249.

2.2. Mice

BALB/c (H-2^d) female mice of about 8 weeks old were purchased from IFFA Credo (L'Arbresle, France).

2.3. Peptides

The peptides used in this study were synthesized and purified as described previously (Benkirane et al., 1993). In order to enhance the accessibility of the IRGERA peptide when bound to carriers, two additional residues were added to its amino terminus to provide the peptides GGIRGERA and CGIRGERA. This permitted selective conjugation of the peptides to BSA and liposomes respectively. The degree of purity of the peptides, as assessed by HPLC, was at least 90%.

2.4. Preparation of the antigens

To avoid side reactions between the thiol groups of BSA, and the maleimide function (or alternatively bromoacetate and dithiopyridyl functions) of the heterobifunctional reagents, the carrier protein was first reacted with iodoacetic acid. To that end, BSA (400 mg, 6.7 μmol) in 20 ml 0.1 M PBS (pH 7.0) was mixed over a period of 1 h, at room temperature, with five times 65 μl of a 0.5 M solution of iodoacetic acid in 0.1 M PBS (pH 7.0). The protein was then dialyzed extensively against water and lyophilized.

To conjugate the heterobifunctional reagents to modified BSA, the free carboxylic moieties of **1**, **2** and **3** (Table 1) were first transformed into their corresponding *N*-succinimidyl esters according to the following procedure: DCC (4.3 mg, 20.7 μmol) was added to a stirred solution of 17.3 μmol of the heterobifunctional reagent and *N*-hydroxysuccinimide (2.4 mg, 20.7 μmol) in 500 μl dichloromethane, at 4°C. After 1 h, the mixture was allowed to return to room temperature and insoluble dicyclohexylurea was eliminated by centrifugation. The solvent was then removed by evaporation and the residue was dissolved in about 300 μl dimethylformamide.

Coupling of SPDP, SMPB, and the NHS esters of **1–4**, to modified BSA proceeded as follows: to 50 mg of the carrier protein dissolved in 1 ml 0.1 M PBS, pH 7.0 (4°C) containing 1 mM EDTA, were added 8.6 μmol of each heterobifunctional reagent in 150 μl of dimethylformamide and the solution was brought to room temperature. After 30 min of reaction, a similar quantity was added and, after an additional period of 30 min, the solutions were centrifuged to eliminate any precipitate and dialyzed extensively against 0.1 M PBS (pH 7.0) for BSA in which maleimide and dithiopyridyl functions have been introduced and against 50 mM ethanolamine buffer (pH 9.0) for BSA bearing bromoacetate functions. The extent of conjugation of the heterobifunctional reagents to BSA was estimated by quantifying the number of residual free amino groups with fluorescamine as previously described (Böhlen et al., 1973; Friede et al., 1993). Finally, the thiol-reactive functions were blocked with a peptide bearing a cysteinyl residue at its N-terminus, namely the peptide 290–304 of sequence CG-TLDPDTANPW-LILSE from the 52 kDa SSA/Ro ribonucleoprotein (Ricchiuti et al., 1994).

GGIRGERA peptide was conjugated to BSA with 1% glutaraldehyde and the yield of the coupling reaction was calculated by determining the amino acid composition of the conjugate (Briand et al., 1985).

2.5. Preparation of liposome-linked peptides

SUV were prepared from PC, PG, MPB-PE (or alternatively: PE-(**1–4**) or PDP-PE) and Chol (molar ratio of 65 : 20 : 15 : 50) as previously described (Frisch et al., 1991). Briefly, the lipids in chloroform solution with MPL (1 nmol per μmol lipid) were dried under high vacuum and resuspended to 7.5

μmol lipid/ml in 10 mM Hepes buffer (pH 7.4) containing 145 mM NaCl by 2 min vortexing and sonication (20 min) under nitrogen at 25°C using a bath-type sonicator (Laboratory Supply Co., Hicksville, NY). The liposome suspension was extruded twice through polycarbonate membrane filters (Nucleopore) of 100 nm pore size yielding vesicles uniformly distributed in size (100 ± 10 nm) as determined by photon correlation spectroscopy using a Coulter model N4SD sub-micron particle analyzer (Coulter Electronics, FL). Liposomal concentrations were determined using the phosphate assay (Rouser et al., 1970) and Ellman's method was used to determine thiol-reactive functions (Riddles et al., 1979). Conjugation of the peptide was performed by mixing freshly prepared liposomes with an equimolar (with respect to the functionalized PE) quantity of peptide, at 4°C for 12 h under argon, under pH conditions similar to those used for the preparation of the antigens (see above). This step was followed by the addition of a ten-fold excess of 2-mercaptoethanol (except for the preparation containing PDP-PE and **2**) to deactivate the remaining maleimide and bromo groups. After 1 h of this treatment, the liposomes were dialyzed extensively against a 50 mM sodium phosphate buffer (pH 7.4) containing 145 mM sodium chloride to eliminate unconjugated peptides and reagents. Estimation of liposome-associated peptide was obtained by hydrolyzing an aliquot of the preparation (50 μl liposome suspension, 50 μl 12 M HCl, 110°C, 12 h) followed by quantification of the number of amino groups with fluorescamine. To that end, 10 μl of the hydrolysis mixture were added to 1.5 ml of 50 mM PBS (pH 8.0) and 500 μl fluorescamine solution (300 μg/ml dioxan) and the fluorescence measured on a Jobin Yvon (Jobin Yvon, France) spectrofluorimeter (excitation wavelength 390 nm, emission 475 nm). Independent hydrolysis of the peptide CGIRGERA provided a fluorescence calibration curve, and similarly hydrolysis of the derivatized PE provided a measure of the contribution of PE to the total fluorescence.

2.6. Immunization

Mice were injected i.p. with the various liposome preparations, containing 1.5 μmol lipid, 3 μg MPL, and about 100 μg peptide/injection per animal. Three injections were given at intervals of 3 weeks and blood was withdrawn from the retroorbital venus plexus 5 days after each injection. Bleedings were repeated at 31, 173 and 223 days after the last injection. As a control, a blood sample was obtained from each mouse before the first injection.

2.7. ELISA procedure

The ELISA procedure used to measure the production of antibodies to each of the heterobifunctional reagents and IRGERA peptide was adapted from previously described procedures (Friede et al., 1993). Briefly, flexible microtiter plates (Falcon, Oxnard, CA) were coated overnight at 37°C with GGIRGERA peptide conjugated to BSA (2 μM, expressed in terms of peptide) or with the heterobifunctional reagent conjugated to BSA (2 μM as expressed in terms of spacer arm) in 50 mM sodium carbonate buffer, pH 9.6. After washing with PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T), the plates were incubated for 1 h with PBS-T containing 1% (w/v) BSA, at 37°C. The plates were washed three times with PBS-T and the coated wells were incubated for 1 h with serial dilutions of mouse antiserum diluted in PBS-T containing 1% (w/v) BSA at 37°C. After washing the reaction was classically revealed by the addition of rabbit anti-mouse IgG conjugated to peroxidase and the peroxidase substrates (Nordic Immunology, Netherlands); the resulting absorbance was measured at 450 nm. The titers are reported as that dilution of serum which was required to give an absorbance of 1.0. All ELISA data are expressed as means ± standard deviations. The in vivo data were also analyzed by a Student's t test for pairwise comparison of means which were estimated to be significantly different at probability levels of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

3. Results and discussion

The aim of this study was: (i) to evaluate the immunogenicity of linkers (Table 1) that permit the conjugation of peptide immunogens to the surface of liposomes (Fig. 1) and (ii) to determine the influence

of these linkers, i.e. structure, length and type of bond, on the immune response against our model peptide. To that end newly synthesized heterobifunctional reagents **1–3** (Table 1) (Frisch et al., 1996), were coupled to DPPE to yield the PE-(**1–3**) conjugates and similarly the DPPE derivatives of bromoacetic acid (i.e. PE-**4**), PDP-PE and MPB-PE were prepared for comparison. These six DPPE derivatives are thiol-reactive reagents which, after incorporation into liposomes, can conjugate cysteine-containing peptides. They differ, however, by several structural and chemical functional features. PE-**3** and MPB-PE contain a maleimide moiety that reacts readily with thiols to give a stable thioether linkage. The same bond is obtained with the bromoacetyl derivatives PE-**1** and PE-**4**. In contrast, PDP-PE and PE-**2**, after reaction with a sulfhydryl containing peptide, yield a thiol-cleavable disulfide bond, i.e. a biodegradable linkage that could release the free peptide during the processing of the liposomal construct. These linkers also differ in their lengths (Table 1), which might be of interest since the recognition of the liposomal surface-bound peptide by competent cells is very probably an important step in the immune response (Frisch et al., 1991; Friede et al., 1993; Friede, 1995). Finally, PE-**3** differs from MPB-PE, which we have used in our previous studies, by the presence of a polyoxyethylene spacer which is assumed to be less immunogenic than an aromatic moiety (Zalipsky, 1995).

3.1. Preparation and characterization of liposome-IRGERA conjugates

SUV (PC/PG/Chol/MPL; 100 nm mean diameter) were prepared containing 15 mol% of total phospholipids of the different thiol-reactive PE derivatives. These preformed vesicles were then reacted with CG-IRGERA, i.e. our model peptide that was extended at its N-terminus by a CG spacer needed for the coupling reaction. As found previously (Frisch et al., 1996) it was important to follow the conjugation step carefully; thus, in sharp contrast with the maleimide and dithiopyridyl moieties which react readily at pH 7.4, the bromoacetyl moiety reacted very sluggishly under these conditions and a more basic medium (pH 9.0) was necessary in order to obtain a good yield under our experimental conditions (12 h at 4°C). The quantity of peptide associated with the liposomes, which was determined by acid hydrolysis and subsequent determination of the number of amino groups by fluorescamine, demonstrated that it represented 50 ($\pm$ 10)% of the total amount of PE derivatives. The coupling of the peptide was, therefore, nearly quantitative with respect to any reactive function present on the surface of the vesicle. After the coupling step, residual thiol reactive functions (i.e. present mostly on the internal surface of the liposome) were deactivated with a ten-fold excess of 2-mercaptoethanol, except for the preparations where CG-IRGERA was coupled via a disulfide linkage (i.e. with PE-**2** and PDP-PE) to avoid the release of the peptide.

3.2. Preparation and characterization of the antigens

Two types of antigen, i.e. BSA-[linker]-unrelated peptide or BSA-IRGERA, were prepared to evaluate the immunogenicity of the linkers or that of IRGERA peptide. In the first case, BSA was reacted with the six heterobifunctional cross-linking reagents (SPDP, SMPB and **1–4**; see Table 1); the determination of the residual amino groups by fluorescamine indicated a coupling efficiency ranging from 12 to 23 mol of cross-linkers introduced per mol of BSA (Table 2). For use in the ELISA, the thiol-reactive moieties were then blocked with a cysteine-containing peptide, unrelated to IRGERA. As described above for the liposomes, the experimental conditions for the coupling took into account the reactivity of the different functions with thiols. In the second case, GG-IRGERA was coupled to BSA using glutaraldehyde (average coupling: 14 $\pm$ 2 mol of peptide per mol BSA).

Table 2
Coupling efficiency of the different heterobifunctional reagents to BSA

BSA derivatives	Number of cross-linkers (per mol of BSA)
BSA-**1**	23
BSA-**2**	14
BSA-**3**	12
BSA-**4**	23
BSA-MPB	22
BSA-PDP	20

3.3. Immunogenicity of the linkers

Mice (four animals/linker) were injected three times at 3 week intervals with the liposome-[linker]-CGIRGERA constructs and the sera tested for anti-linker antibodies. Fig. 2A shows that there was a significant production of IgG against the linkers of MPB-PE and PE-**3**. A high level of response against [MPB] was already apparent after the second injection whereas for [**3**] the highest titers were observed after the third one. 7 months after the last injection (day 270), there was no detectable IgG against [MPB]; in contrast, there was still an important response against [**3**]. Although these results confirm that a phenyl-maleimide type structure induces antibody responses, they are somewhat surprising since it was expected that the replacement of the aromatic ring of [MPB] by a polyoxyethylene chain in [**3**] would result in a decreased immunogenicity of the linker. Indeed it was shown by Peeters et al. (1989) that the immunogenicity of MBS, which like SMPB contains an aromatic ring linked to a maleimide moiety, is much reduced when the ring is replaced by a caproyl moiety, i.e. a straight alkyl chain. In contrast, Jones et al. (1989) have suggested that the conjugation of a peptide to diphtheria toxin, with maleimidocaproyl-*N*-hydroxysuccinimide, which introduces this [maleimidocaproyl] linker, leads also to a sizable anti-linker immune response. Indications that the maleimide moiety itself could trigger an anti-linker immune response was evidenced by cross-reactivity experiments; thus e.g. antisera obtained from mice immunized with liposomes containing MPB-PE coupled to CGIRGERA gave a strong response in ELISA using BSA-[**3**]-unrelated peptide as antigen (not shown).

In contrast, very low and short (in time) responses against the linkers of **1** and **4** were observed (Fig. 2B). As shown in Fig. 2C, an increase of the response against the linkers of PDP-PE and **2** was observed up to the third injection before immediately decreasing. The immunogenicity of the linker of PDP-PE observed in this study is also somewhat at odds with data from the literature (Peeters et al., 1989) which suggest a very weak response. Control experiments have excluded the possibility that the measured immune response could be due to residual dithiopyridyl moieties in the liposomes and on the antigen used in the ELISA: the response against [PDP] and [**2**] was equivalent regardless of the antigen used in the ELISA procedure, namely BSA-[linker]-unrelated peptide and BSA-[linker]-**2**-mercaptoethanol.

Altogether, the lowest immunogenicity was ob-

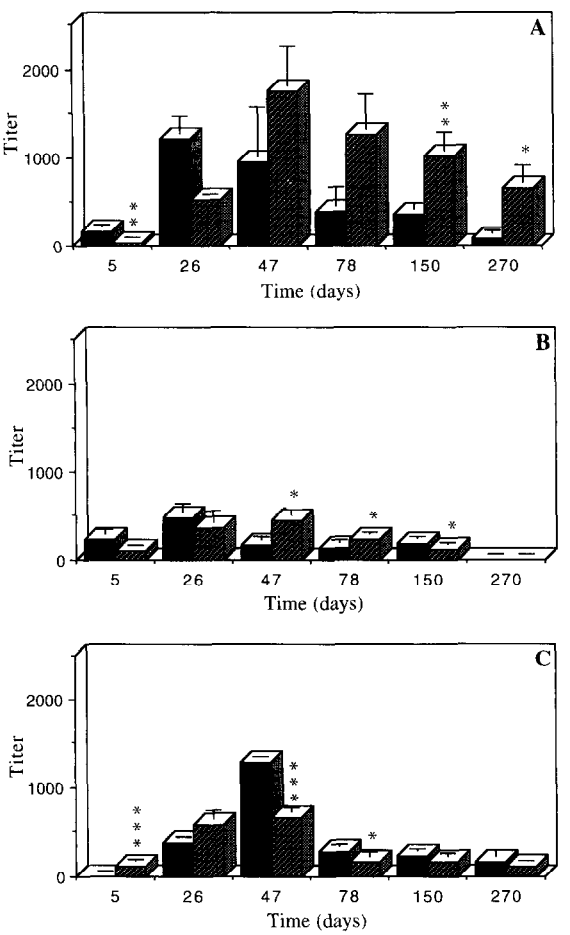


Fig. 2. IgG antibody response to the cross-linkers. BALB/c mice were immunized i.p. with small unilamellar vesicles containing MPL (3 μg per injection) and carrying the peptide CG-IRGERA (100 μg per injection) coupled to their surface via six different cross-linkers: (*A*) [MPB] (solid), [**3**] (hatched); (*B*) [**4**] (solid), [**1**] (hatched); (*C*) [PDP] (solid), [**2**] (hatched). IgG titers were measured by ELISA 5 days after each injection with an interval of three weeks between each injection and also on days 36, 108 and 228 after the last injection, using the corresponding BSA-[cross-linker]-unrelated peptide antigens (see text). The histograms represent the average titers obtained for each group of mice with SD ($n = 4$). Differences were significantly different at probability levels of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

served with the linkers of **1** and **4**. Both originate from bromoacetyl terminated heterobifunctional reagents, the first being characterized by a relatively long polyoxyethylene spacer arm whereas the second is very short. Currently both are of interest when a low immunogenicity for the linker is of importance as, for example, in the development of synthetic vaccines. To explain the immunogenicity observed with the other linkers, one should take into account the fact that the data in the literature were obtained by coupling heterobifunctional cross-linkers to carrier proteins (Peeters et al., 1989; Jones et al., 1989) while in our case the reagents were conjugated to liposomes containing MPL as the immunoadjuvant. Since it is known that such liposomal constructs dramatically increase the immunogenicity of associated haptens (Alving, 1993), one cannot rule out the possibility that the relatively important immunogenicity obtained here with the other linkers was due to such an effect.

3.4. Effect of the linkers on the immunogenicity of IRGERA associated to liposomes

Antisera were also tested by ELISA in order to determine the influence of the different linkers on the immune response against IRGERA peptide. In all six cases (Fig. 3A–C) a strong IgG response was observed. In general, the highest titers were observed after the third injection except when we used linkers [MPB] and [**4**], in which case highest titers were already observed after the second injection. Fig. 3B indicates that a relatively long and flexible linker such as [**1**] between the peptide and the liposome surface, is not a prerequisite for achieving a high antibody response since with the very short linker, namely [**4**], the response was as high if not superior. It seems therefore that for IRGERA the length of the linker is not important and this was borne out (Fig. 3C) by comparing [PDP] and [**2**]. As previously discussed (Frisch et al., 1991; Friede et al., 1993; Friede, 1995), to explain the immunogenicity of peptides linked to the surface of liposomal constructs, we favor a mechanism based on recognition between the peptide and competent B lymphocytes. The fact that a very short linker is so efficient would not, a priori, favor this interpretation. However, the linker between the liposome surface and IRGERA

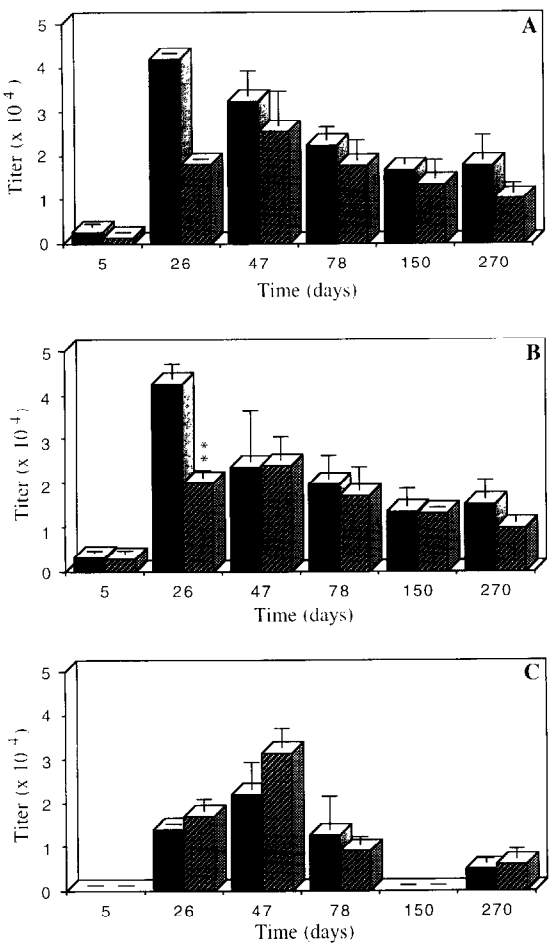


Fig. 3. Effect of the cross-linkers on the IgG antibody response against the peptide IRGERA associated with liposomes. BALB/c mice were immunized three times at 3 week intervals with the peptide CG-IRGERA (100 μg per injection) conjugated to small unilamellar liposomes (containing 3 μg MPL per injection) with the six different cross-linkers: (*A*) [MPB] (solid), [**3**] (hatched); (*B*) [**4**] (solid), [**1**] (hatched); (*C*) [PDP] (solid), [**2**] (hatched). The antibody titers were measured by ELISA using BSA-IRGERA as antigen. The histograms represent the average titers obtained for each group of mice with SD ($n = 4$). Differences were significantly different at probability levels of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

was extended by a CG spacer arm at the N-terminus of the peptide, i.e. by 0.79 nm. Moreover, it was also shown recently that the 3 residue sequence at the C-terminal end is the most immunogenic part of this peptide (Muller, S., unpublished observation).

Comparison of Fig. 3A, Fig. 3B and Fig. 3C does

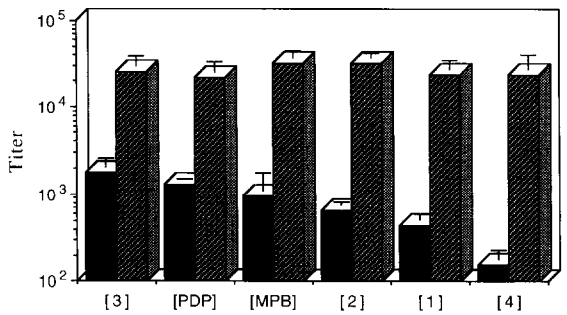


Fig. 4. Comparison of the IgG antibody response obtained against the cross-linkers and IRGERA using the liposome-based constructs for immunization. BALB/c were immunized i.p. with small unilamellar vesicles containing MPL (3 μg per injection) and carrying the peptide CG-IRGERA (100 μg per injection) coupled to their surface with the six cross-linkers: [1–4], [PDP] or [MPB]. Three injections were given at three week intervals. IgG titers were then measured by ELISA against the cross-linkers (solid) and the peptide CG-IRGERA (hatched) using respectively the corresponding BSA-[cross-linker]-unrelated peptide and BSA-IRGERA antigens. The histograms represent the average titers obtained for each group of mice with SD ($n = 4$).

not indicate a marked difference in the immune response observed against the peptide depending on whether it was conjugated to the linker via a stable thioether linkage, such as the one obtained with maleimide (Fig. 3A) or bromoacetyl (Fig. 3B) moieties, or via a biodegradable disulfide bridge, such as the one generated with the dithiopyridyl moiety (Fig. 3C). The only difference was the time span of the immune response, which was slightly less important in the latter case. Since the free peptide is non-immunogenic, these results mean that despite the possibility that a cleavage of the disulfide bond might occur in vivo (see e.g. Russel-Jones et al., 1995), the peptide probably remains linked to the liposomes and/or to its phospholipidic hydrophobic anchor until its recognition and/or processing by the competent immune cells. Related to that point, it was observed that the disulfide linkers are fairly stable in vivo either in targeted chimeric peptides (Bickel et al., 1995) or in immunoliposomes (Park et al., 1995).

Finally, in Fig. 4 are summarized the IgG antibody titers against the linkers and the peptide IRGERA, measured after the third bleeding. Interestingly, the response against the peptide is equivalent regardless of the linker used. Linkers derived from maleimido and dithiopyridyl containing reagents displayed a higher contribution in the antibody response compared to the ones derived from bromoacetyl derivatives; e.g. anti-[3] and anti-[4] IgG antibody titers represented respectively 6.8 and 0.7% of the response against the peptide.

4. Conclusion

Three newly synthesized polyoxyethylene-based cross-linkers ([1], [2] and [3]) and three known cross-linkers ([MPB], [PDP] and [4]) have been used to conjugate a model peptide IRGERA to the surface of SUV containing MPL as adjuvant. An important anti-peptide IgG response was elicited in BALB/c mice with the six liposomal constructs that was independent of the length of the linkers (ranging between 0.3 and 1.6 nm) and of the nature of the linkage between the peptide and the thiol-reactive moieties of the cross-linkers, i.e. stable thioether or bio-reducible disulfide bonds. The least immunogenic linkers ([1] and [4]) bound the peptide via a simple thioether bond. Despite its polyoxyethylene structure, [3] gave the highest anti-linker response and this did not differ statistically from the linker of the classical MPB-PE derivative. Altogether these results suggest that maleimide moieties are immunogenic, at least in our liposomal constructs. In order to limit potential carrier-induced epitopic suppression, bromoacetate-based linkers seem to be more suitable; a similar conclusion was previously reached by Bernatowicz and Matsueda (1986) for the coupling of peptides to protein carriers. Finally, our results illustrate that liposomes containing the non-toxic adjuvant MPL and bromoacetate-based cross-linkers can act as carriers for peptides of low intrinsic immunogenicity in order to induce long-lasting IgG responses. Such an approach may be useful when designing synthetic vaccines.

References

Alving, C.R. (1991) Liposomes as carrier of antigens and adjuvants. J. Immunol. Methods 140, 1.

Alving, C.R. (1993) Lipopolysaccharide, lipid A, and liposome containing lipid A as immunological adjuvants. Immunobiology 187, 430.

Arnon, R. and Horwitz, R.J. (1992) Synthetic peptides as vaccines. Curr. Opin. Immunol. 4, 449.

Benkirane, N., Friede, M., Guichard, G., Briand, J.P., Van Regenmortel, M.H.V. and Muller, S. (1993) Antigenicity and immunogenicity of modified synthetic peptides containing D-amino acid residues. J. Biol. Chem. 268, 26279.

Bernatowicz, M.S. and Matsueda, G. (1986) Preparation of peptide-protein immunogens using *N*-succinimidyl bromoacetate as a heterobifunctional crosslinking reagent. Anal. Biochem. 155, 95.

Bickel, U., Kang, Y.-S. and Pardridge, W.M. (1995) In vivo cleavability of a disulfide-based chimeric opioid peptide in rat brain. Bioconj. Chem. 6, 211.

Böhlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Fluorometric assay of proteins in the nanogram range. Arch. Biochem. Biophys. 155, 213.

Briand, J.P., Muller, S. and Van Regenmortel, M.H.V. (1985) Synthetic peptides as antigens: pitfalls of conjugation methods. J. Immunol. Methods 78, 59.

Buiting, A.M.J., Van Rooijen, N. and Claassen, E. (1992) Liposomes as antigen carriers and adjuvants in vivo. Res. Immunol. 143, 541.

Carlsson, J., Drevin, H. and Axen, R. (1978) Protein thiolation and reversible protein-protein conjugation. *N*-succinimidyl 3-(2-pyridyldithio)propionate, a new heterobifunctional reagent. Biochem. J. 173, 723.

Chai, S.K., Clavijo, P., Tam, J.P. and Zavala, F. (1992) Immunogenic properties of multiple antigen peptide systems containing defined T and B epitopes. J. Immunol. 149, 2385.

Friede, M. (1995) Liposomes as carriers of antigens. In: J.R. Philippot and F. Schuber (Eds.), Liposomes as Tools in Basic Research and Industry. CRC Press, Boca Raton, FL, p. 189.

Friede, M., Muller, S., Briand, J.P., Van Regenmortel, M.H.V. and Schuber, F. (1993) Induction of immune response against a short synthetic peptide antigen coupled to small neutral liposomes containing Monophosphoryl lipid A. Mol. Immunol. 30, 539.

Friede, M., Muller, S., Briand, J.P., Plaué, S., Fernandes, I., Frisch, B., Schuber, F. and Van Regenmortel, M.H.V. (1994) Selective induction of protection against influenza virus infection in mice by a lipid-peptide conjugate delivered in liposomes. Vaccine 12, 791.

Frisch, B., Muller, S., Briand, J.P., Van Regenmortel, M.H.V. and Schuber, F. (1991) Parameters affecting the immunogenicity of liposome associated synthetic hexapeptide antigen. Eur. J. Immunol. 21, 185.

Frisch, F., Boeckler, C. and Schuber, F. (1996) Synthesis of short polyoxyethylene-based heterobifunctional cross-linking reagents. Application to the coupling of peptides to liposomes. Bioconj. Chem., in press.

Gregoriadis, G. (1994) The immunological adjuvant and vaccine carrier properties of liposomes. J. Drug Targeting 2, 351.

Jones, G.L., Edmundson, H.M., Spencer, L., Gale, J. and Saul, A. (1989) The use of maleimidocaproyloxysuccinimide to prepare malarial peptide carrier immunogens. Immunogenicity of the linking region. J. Immunol. Methods 123, 211.

Katz, D.H. (1980) Adaptive differentiation of lymphocytes. Theoretical implications for cell-cell recognition and regulation of immune response. Adv. Immunol. 29,137.

Martin, F.J., Hubbell, W.L. and Papahadjopoulos, D. (1981) Immunospecific targeting of liposomes to cells: a novel and efficient method for covalent attachment of Fab' fragments via disulfide bonds. Biochemistry 20, 4229.

Martin, F.J. and Papahadjopoulos, D. (1982) Irreversible coupling of preformed immunoglobulin fragments to preformed vesicles. J. Biol. Chem. 255, 286.

Park, J.W., Hong, K., Carter, P., Asgari, H., Guo, L.Y., Keller, G.A., Wirth, C., Shalaby, R., Kotts, C., Wood, W.I., Papahadjopoulos, D. and Benz, C.C. (1995) Development of anti-$p185^{HER2}$ immunoliposomes for cancer therapy. Proc. Natl. Acad. Sci. USA 92, 1327.

Peeters, J.M., Hazendouk, T.G., Beuvery, E.C. and Tesser, G.I. (1989) Comparison of four bifunctional reagents for coupling peptides to proteins and the effect of three moieties on the immunogenicity of the conjugates. J. Immunol. Methods 120, 133.

Ricchiuti, V., Briand, J.P., Meyer, O., Isenberg, D.A., Pruijin, G. and Muller, S. (1994) Epitope mapping with synthetic peptides of 52-kDa SSA/Ro protein reveals heterogeneous profiles in human autoimmune sera. Clin. Exp. Immunol. 95, 397.

Riddles, P.W., Blakeley, R.L. and Zerner, B. (1979) Ellman's reagent: 5,5'-dithiobis(2-nitrobenzoic acid) a re-examination. Anal. Biochem. 94, 75.

Rouser, G., Fleischer, J. and Yamamoto, A. (1970) Two dimensional thin layer chromatographic separation of plant lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5, 494.

Russel-Jones, G.J., Westwood, S.W., Farnworth, P.G., Findlay, J.K. and Burger, H.G. (1995) Synthesis of LHRH antagonists suitable for oral administration via the vitamin B_{12} uptake system. Bioconj. Chem. 6, 34.

Verma, J.N., Rao, M., Amselem, S., Krzych, U., Alving, C.R., Green, S.J. and Wassef, N.M. (1992) Adjuvant effects of liposomes containing Lipid A: enhancement of liposomal antigen presentation and recruitment of macrophages. Infect. Immun. 60, 2438.

Wang, R., Charoenvit, Y., Corradin, G., Porrozzi, R., Hunter, R.L., Glenn, G., Alving, C.R., Church, P. and Hoffman, S.L. (1995) Induction of protective polyclonal antibodies by immunization with a *Plasmodium yoelii* circumsporozoite protein multiple antigen peptide vaccine. J. Immunol. 154, 2784.

Zalipsky, S. (1995) Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. Bioconj. Chem. 6, 150.