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IMMUNOSPECIFIC TARGETING OF LIPOSOMES TO ERYTHROCYTES

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Abstract—Immunoliposomes were made by covalently linking Fab′ fragments (from rabbit antimouse erythrocyte IgG) to reverse-phase evaporation vesicles (REV) via maleimido-4-(*p*-phenylbutyrate) phosphatidylethanolamine (MPB-PE) as anchor molecule. These immunoliposomes were characterized in terms of size, charge, stability and antigen binding capacity. The effect of the liposomal Fab′ density on the interaction with the target cell was studied. Two isolation methods were tested to separate free Fab′ from liposomally bound Fab′. The necessity of deactivation of remaining reactive sites with dithiothreitol preincubation to increase the specificity of immunoliposome target cell interactions was demonstrated.

During the last decade several reports on the preparation and application of immunoliposomes were published [1–5]. In this paper immunoliposomes specifically directed against mouse erythrocytes were prepared, analysed and tested for their *in vitro* binding specificity. These liposomes were developed to interact with erythrocytes *in vivo* and deliver drugs in the proximity of these target cells. A proper physicochemical characterization of immunoliposomes is not always included in the literature reports. The physico-chemical properties of the immunoliposomes will strongly influence their disposition *in vivo* [6, 7] and their behaviour *in vitro* [6–8]. Therefore it is important to prepare immunoliposomes well characterized in terms of particle size and surface charge, with acceptable long term stability and preservation of immunoactivity. Only then can optimal experimental conditions to study the *in vitro* and *in vivo* interactions with the appropriate target cell exist and solid conclusions be drawn.

For specific targeting IgG fragments (Fab′) were covalently linked to liposomes according to the method of Martin and Papahadjopoulos [4]. This technique was selected from a number of options [1] for the following reasons:

(a) Mild conditions are maintained during coupling avoiding a decrease in the immunoactivity of the coupled antibody fragment.
(b) Fab′ fragments, generated from F(ab′)2 fragments by dithiothreitol (DTT) incubation, are covalently linked to preformed vesicles containing the (for −SH groups) highly reactive maleimido-4-(*p*-phenylbutyrate) phosphatidylethanolamine (MBP-PE). As can be seen in Fig. 1 this method presumably results in the best orientation of the Fab′ fragments on the liposomes: the antigen binding sites are outward directed and fully accessible to antigen binding.
(c) The Fc part of the IgG molecule is removed. This can be of great importance for *in vivo* use of Fab′-vesicles. In this way the Fc receptor of macrophages is not activated and elimination of the vesicle by the reticuloendothelial system (RES) might be reduced or slowed down [9].

In this study we describe the linkage of Fab′ fragments of rabbit antimouse erythrocyte IgG to preformed MPB-PE vesicles. After thorough characterization and optimization of the preparation method we determined the coupling efficiency of Fab′ fragments to reverse-phase evaporation vesicles (REV). Also the effect of the liposomal Fab′ density on the interaction with the target cell was investigated. Two methods for separating unconjugated Fab′ fragments from liposome bound Fab′ were compared. Finally, the necessity of DTT preincubation for the proper evaluation of the results of the targeting efficiency to mouse erythrocytes is discussed.

MATERIALS AND METHODS

Material. Cholesterol, phosphatidylcholine (PC), phosphatidylserine (PS), pepsin and DTT were purchased from Sigma Chemicals (St Louis, MO). Phosphatidylethanolamine (PE) was obtained from Lipid Products (Nutfield, U.K.). Succinimidyl-maleimido-4-(*p*-phenylbutyrate) (SMPB) was obtained from Pierce Chemical Company (Rockford, U.S.A.). Carboxyfluorescein (CF) was purchased from Eastman Kodak Company (Rochester, NY) and was purified by the method described by Ralston *et al.* [10]. Dextran T 40, Sephadex gels and Protein A-Sepharose CL-4B were purchased from Pharmacia Fine Chemicals (Uppsala,

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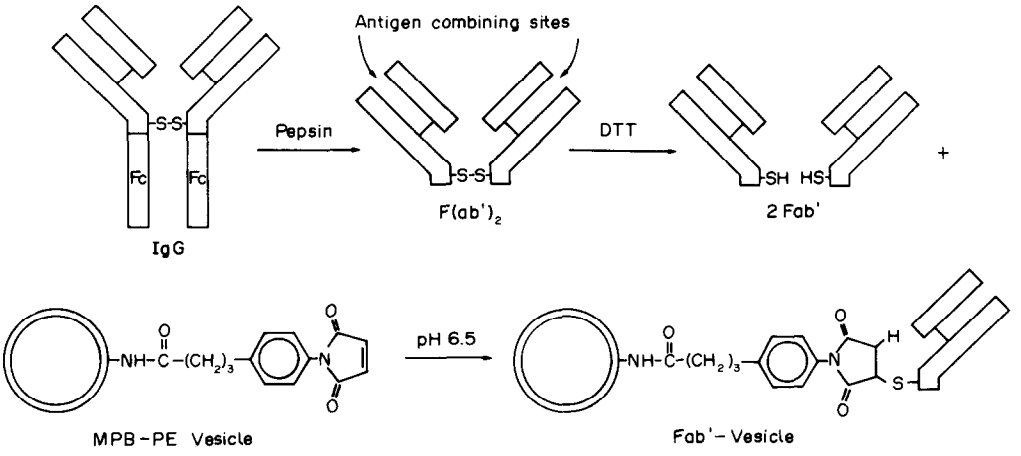

Fig. 1. Covalent coupling of Fab' fragments to *N*-(4-(*p*-maleimidophenyl)butyryl) phosphatidylethanolamine (MPB-PE) vesicles. F(ab')2 dimers are prepared by pepsin digestion of the IgG fraction of whole rabbit serum. Fab' monomers are generated from these by reduction with dithiothreitol (DTT) at low pH. Immediately following the removal of DTT, Fab' fragments are mixed with MPB-PE containing vesicles and the pH adjusted to 6.5. Addition of the Fab'-SH to the double bond of the maleimide moiety of MPB-PE molecules present in vesicle membranes results in a stable thioether cross-linkage (from Martin and Papahadjopoulos [4], with permission).

Sweden). Silicagel (70–325 mesh) was obtained from Merck (Darmstadt, F.R.G.) and SE-23 from Servacel (Heidelberg, F.R.G.). Mouse erythrocytes were obtained from fresh heparinized whole blood and used the same day. Sheep erythrocytes, preserved in Alsevers solution, were supplied by the Faculty of Microbiology, Utrecht. All other reagents were of analytical grade.

Methods. Protein concentration was determined by the method of Peterson [11] and expressed as μg of protein per μmole of phospholipid (PL). Lipid phosphate was estimated by the colorimetric method of Fiske and SubbaRow [12]. The quality of phospholipids and the synthesis of MPB-PE was tested by a TLC method [13]. CF fluorescence was measured at 516 nm in a Kontron Instruments spectrofluorimeter, Model SFM 25 (Watford/Herts, U.K.) at an excitation wavelength of 489 nm. A standard fluorescence curve was produced under the same conditions [14].

Particle size analysis was performed by dynamic light scattering with a Malvern PCS 100 SM (Malvern Ltd, Worcestershire, U.K.) equipped with a particle analyzer processor (Model 7027) and a 100 mW helium/neon laser, at 632.8 nm (NEC Corp., Tokyo, Japan).

The electrophoretic mobility of the vesicles and the Fab'-vesicles was determined with a Mark II microelectrophoresis apparatus (Rank Brothers, Cambridge, U.K.). Zeta potentials were calculated from averaged mobilities of at least 20 particles in both directions (variation coefficient was less than 15%). Care was taken to focus on the stationary levels [15].

The number of bilayers was determined by ^{31}P-NMR measurements as described by Jousma *et al.* [16].

Mouse erythrocytes were purified by applying the heparinized whole blood (1 ml) onto a column (5 ml) containing three volumes of Sephadex G-150 Superfine and one volume of SE-23 to remove white blood cells [17].

Synthesis of MPB-PE. This synthesis was carried out as described earlier by Martin and Papahadjopoulos [4]. Briefly, 100 μmole PE was dissolved in 5 ml of anhydrous methanol containing 100 μmole of freshly distilled triethylamine and 50 mg SMPB. The reaction was carried out in a nitrogen atmosphere at room temperature for 2 hr. After establishing the complete conversion of PE of MPB-PE by TLC [4], methanol was removed under reduced pressure and the products were redissolved in chloroform. After extraction (twice) of the chloroform phase with a 1% sodium chloride solution, to remove unreacted SMPB and other water soluble products, further purification was carried out by silicagel chromatography as described earlier for *N*-3-(pyridyl-2-dithio) propionyl phosphatidylethanolamine (PDP-PE) [3]. Stability of MPB-PE was checked with TLC. MPB-PE stored at −35° in chloroform solution and in sealed glass ampules under nitrogen was stable for at least 6 months.

Preparation of vesicles. REV were prepared by the method of Szoka and Papahadjopoulos [18] with minor modifications. Briefly, 40 μmole cholesterol, 40 μmole PC and 4 μmole PS (in cases where MPB-PE vesicles were prepared 38 μmole PC and 2 μmole MPB-PE instead of 40 μmole PC was used) were dissolved in freshly distilled diethylether (4.0 ml). After addition of glass beads, 1.2 ml buffer (100 mM sodium acetate, 88 mM sodium chloride, pH = 7.4) was added and the two phases were emulsified by sonication for 5 min in a bath type apparatus (50 Hz) and subsequently mixed on a vortex mixer (Janke and Kunkel K.G., Staufen, F.R.G.) for 1 min. The diethylether was removed under reduced pressure in a nitrogen atmosphere with a rotary evaporator (Model Buchi RE 011, Buchi Laboratoriumstech-

niek A.G., Flawil, Switzerland) at room temperature. The resulting vesicle dispersion was extruded through 0.4 μm and 0.2 μm polycarbonate membranes (Nucleopore Corp., Pleasanton, U.S.A.). In cases where CF was entrapped in the vesicles, 1.2 ml CF solution (70 mM CF in buffer) was used instead of buffer alone.

After extrusion, free CF was separated from liposomal CF by gel filtration chromatography on a Sephadex G-50 Fine column (0.8 × 20 cm) pre-equilibrated with buffer.

Preparation of antibody fragments. Antimouse erythrocyte antibodies were raised in New Zealand white albino rabbits. They received i.v. injections of 10^9 mouse erythrocytes repeatedly until high haemagglutination (HA) titers (titer 15 in a serial twofold dilution test; see further below) were obtained. IgG was isolated by Protein A-Sepharose CL-4B affinity chromatography [19]. Rabbit F(ab')2 and Fab' were prepared and purified as described earlier [3], except that nitrogen instead of argon was used and the buffer used for chromatography on a Sephadex G-50 Fine column, to remove DTT from the Fab' fragments had a pH = 6.5.

Coupling Fab' fragments to the vesicles. Freshly prepared vesicles as described above (0.5–4.0 μmole PL/ml in deoxygenated buffer) were mixed with freshly prepared Fab' fragments (final concentration 0.2–4.0 mg/ml). The coupling reaction was carried out in a nitrogen atmosphere under stirring at room temperature for two hours. To separate vesicles linked to Fab' fragments from unconjugated fragments either density gradient centrifugation on a discontinuous 10 and 30% Dextran T 40 gradient [2] or ultracentrifugal sedimentation of the Fab'-vesicles at 30,000 *g* for 30 min (pellet method) was used. Pellets were resuspended and washed twice with buffer (final supernatant was Fab' free).

Haemagglutination (HA) assay. The HA titer of the Fab'-vesicles and control vesicles was determined by a modified Salk pattern method [20]. Briefly, serial twofold dilutions of the sample (50 μl in phosphate-buffered saline (PBS) containing 0.02% Tween 80) were made in round bottom microtiter 96 wells plate (Flow Laboratories, Irvine, Scotland). 40 μl of a 1% suspension of washed mouse or sheep erythrocytes in PBS was added to each well. Readings were taken after 2 hr of incubation at 37°. As control, Fab' free MPB-PE-vesicles and as reference F(ab')2 solutions were used. Duplicate assays gave identical titers.

Cell binding assay. A 1% (2×10^8/ml) suspension of washed mouse or sheep erythrocytes (0.25 ml in PBS) was mixed with an equal volume of CF (70 mM) containing immunoliposomes, which had been preincubated with 6.5 mM DTT for 30 min at room temperature. It was not necessary to remove any free DTT after this preincubation. The mixture was incubated with repeated agitation at 37° for 30 min. The erythrocytes were washed twice with ice-cold PBS and after centrifugation (700 *g*, 5 min), to separate erythrocyte bound vesicles from unbound vesicles (the final supernatant was liposome free). The resulting pellet was resuspended in 1 ml buffer containing 0.5% Triton X-100 (v/v) and subsequently heated for 20 min at 80° to lyse the immunoliposomes bound to the erythrocytes (thereby quantitatively releasing the CF entrapped in the liposomes) and to denature hemoglobin and other proteins. After centrifugation at 1500 *g* for 10 min the supernatants (900 μl) were analysed for CF as described above.

RESULTS

Coupling of Fab' to REV: isolation of Fab'-vesicles by density gradient centrifugation

The REV and REV-MPB-PE without Fab' were used as controls in the HA test. The mean particle diameters are equal but the zeta potential of the REV-MPB-PE more negative because of the neutralisation of the positive charge of the amine group of PE after coupling with SMPB (Table 1).

Flotation on a dextran gradient was one method used to isolate and concentrate REV-MPB-PE that bound Fab' fragements after incubation. Recovery of Fab'-vesicles by this technique was 90–95%. Results of the analysis of the immunoliposome preparations obtained by this technique are summarized in Table 1, which shows that a considerable amount of Fab' is specifically linked to REV-MPB-PE and

Table 1. Analysis of REV and REV-MPB-PE without Fab' and with Fab', isolated by flotation on a dextran gradient

	Particle diam. (μm)*	Zeta potential (mV)†	Ratio‡ μg Fab'/μmol PL	HA titer§
REV	0.26 ± 0.04	−12.3	0	0
REV-MPB-PE	0.27 ± 0.01	−21.3	0	0
REV-Fab'‖	0.40 ± 0.03	−10.6	60 ± 22	0
REV-MPB-PE-Fab'	>2.5	−19.8	168 ± 33	16

* Measured as described in Materials and Methods (N = 6).

† Determined as described in Materials and Methods (all variation coefficients were less than 15%).

‡ Expressed is the amount of Fab' covalently bound to liposomes after 2 hr of incubation of Fab' (1 mg/ml) with the two liposome preparations, REV and REV-MPB-PE (1 μmol PL/ml).

§ Determined as described in Materials and Methods.

‖ REV (no MPB-PE in bilayer) were incubated with Fab' using the same protocol as for preparation of REV-MPB-PE-Fab'.

§ Represents SD (N = 5).

Table 2. Analysis of antimouse erythrocyte Fab'-vesicles (REV with and without MPB-PE): the effect of Fab' concentration during preparation

μg Fab' added*	Ratio† μg Fab'/μmole PL	Particle diam. (μm)‡	HA titer§
0	0 (0)	0.32 (0.30)	0 (0)
345	24 (0)	0.38 (0.45)	128 (0)
552	60 (0)	0.70 (0.40)	256 (0)
759	87 (0)	0.65 (0.40)	512 (0)
966	105 (0)	0.81 (0.39)	1024 (0)

Expressed are the results for the REV-MPB-PE; the results in parentheses refer to REV.

* 2.84 μmole PL for REV-MPB-PE or 2.08 μmol PL for REV was added to the indicated amount of Fab' in a total volume of 1.5 ml. Incubation and isolation (pellet method) of Fab'-vesicles as described in Materials and Methods.

† μg Fab' associated per μmol PL; protein and PL were determined as described in Materials and Methods. The ratio is expressed as the mean of a duplicate of a typical experiment (N = 3).

‡ Determined as described in Materials and Methods and expressed as the mean of a duplicate of a typical experiment (N = 3).

§ HA titer as described in Materials and Methods.

still has antigen binding capacity. Although the non-specific binding of Fab' to REV is high, these immunoliposomes exhibited no antigen binding capacity as demonstrated by their inability to induce haemagglutination.

Coupling of the Fab' fragments to these vesicles did not change the zeta potential significantly (Table 1).

Coupling of Fab' to REV isolated by the pellet method

Fab' concentration dependence. From the results obtained by particle size analysis (Table 1), it can be concluded that the REV-MPB-PE-Fab' aggregate. There were indications that aggregation was induced by Dextran T 40 [21]. The pellet method (see Materials and Methods) was introduced and used throughout this study to isolate Fab'-vesicles. In Table 2 the effect of varying the initial Fab' concentration at constant PL (2.84 μmole PL for REV-MPB-PE and 2.08 μmole PL for REV in a final volume of 1.5 ml) concentration was investigated.

With the pellet method 50–65% of the Fab'-vesicles (based on phosphate and CF analysis) was recovered. No non-specific binding of Fab' to REV was observed as can be seen in Table 2. This might be explained by the fact that the former experiments (Table 1) were done with Fab' which was isolated from antiserum with a much lower starting HA titer; 2^8 instead of 2^{15}. The Fab'-vesicles showed only little aggregation as is demonstrated by analysing the particle size (Table 2). In a separate experiment we compared the density gradient centrifugation method with the pellet method for the "high titer" Fab'. Again the mean particle diameter of the Fab'-vesicles isolated with the density gradient centrifugation method was larger than 2.5 μm, while the mean particle diameter of the Fab'-vesicles isolated with the pellet method was smaller than 0.6 μm for similar ratios of μg Fab'/μmole PL (results not shown).

Phospholipid concentration dependence. In an alternative experiment the effect of varying the initial PL concentration at a constant Fab' concentration (575 μg Fab' in a final volume of 1.8 ml) was investigated for REV-MPB-PE. Results are presented in Table 3. As was expected the ratio μg Fab'/μmole PL decreases as the PL concentration increases.

The immunoliposomes were studied for their stability over an eight week period of storage under refrigerator conditions in isotonic buffer, pH = 7.4 (under nitrogen atmosphere). They retained their antigen binding capacity (HA test) and CF leakage was less than 6% over the experimental period of time (results not shown). No aggregation was observed during this time (results not shown).

Specificity of the immunoliposomes towards the erythrocytes

From Tables 1–3 it can be derived that Fab'-vesicles interact with the mouse erythrocytes as indicated by the HA titer. The interaction of the Fab'-

Table 3. Analysis of antimouse erythrocyte REV-MPB-PE-Fab': the effect of phospholipid concentration during preparation

μmole PL added*	Ratio† μg Fab'/μmole PL	Particle diam. (μm)†	HA titer†
0.42	587	0.34	16
0.84	159	0.38	32
1.68	64	0.45	48
2.51	36	0.51	64
3.35	59	0.55	128
5.01	50	0.56	128

* 575 μg Fab' was added to the indicated amount of REV-MPB-PE in a total volume of 1.8 ml. Incubation and isolation of Fab'-vesicles (pellet method) as described in Materials and Methods.

† Protein, PL, mean particle diameter and HA titer were determined as described in Materials and Methods. Expressed values are the mean of a duplicate of a typical experiment (N = 3).

Table 4. Effect of DTT on immunospecificity of CF containing antimouse erythrocyte REV-MPB-PE-Fab'

	% binding to mouse RBC‡		% binding to sheep RBC‡	
	−DTT	+DTT†	−DTT	+DTT
REV-MPB-PE-Fab'*	90 ± 8	17 ± 3	0	0
REV-MPB-PE	73 ± 4	0	0	0
REV	0	0	0	0
REV-MPB-PE-Fab'§	62 ± 9	0	0	0

* The ratio of the REV-MPB-PE-Fab', isolated with the pellet method, was 95 μg Fab'/μmole PL and the HA titer was 128.

† Fab'-vesicles and controls (0.4 μmole PL in 0.25 ml buffer) were preincubated with 6.5 mM DTT as described in Materials and Methods. −DTT means not preincubated.

‡ This percentage was calculated from the ratio of CF in the supernatant after Triton X-100 lysis and the amount of CF originally present (in REV) as described in Materials and Methods. The values are expressed as a mean (±range) of a duplicate of a typical experiment (N = 3).

§ As control REV-MPB-PE-Fab', we used Fab', isolated from an anti-idiotypic Avian Leukemia virus IgG1 monoclonal with a ratio of 50 μg Fab'/μmole PL.

vesicles with erythrocytes was investigated in more detail with Fab'-vesicles that contain CF (see Materials and Methods). Fab'-vesicles, control liposomes and control Fab'-vesicles were preincubated with 6.5 mM DTT (pH = 7.4). This preincubation was crucial as it deactivated the highly reactive double bond of the MPB-PE molecules which were not occupied with Fab' molecules and which might be able to interact non-specifically with molecules at the erythrocyte surface (Table 4). When the immunoliposomes were not preincubated with DTT up to 90% was bound to mouse erythrocytes but also liposomes without Fab' (REV-MPB-PE) exhibited a binding of 70% to the mouse erythrocytes. This clearly demonstrated that the reactive double bond of the MPB-PE molecule should be blocked to avoid an overestimation of the immunospecific interaction of the antimouse erythrocyte Fab'-vesicles with their target cells. Table 4 also shows that only mouse erythrocytes interacted with REV-MBP-PE (without Fab') while sheep erythrocytes were unable to interact with these vesicles, indicating that they can discriminate between these two types of erythrocytes. This phenomenon is further investigated for a number of erythrocytes of different species. The results are submitted for publication elsewhere [22].

Experiments to study the effect of the Fab' density on the liposomes upon their interaction with mouse erythrocytes showed a higher binding when the Fab' density was increased (Table 5); this effect was cell type specific. In an additional experiment the antigen binding capacity was tested in samples obtained from incubations of a fixed amount of Fab' with a varying amount of PL (Table 6). Again binding to antigen was specific and depended on the amount of liposome coupled Fab'.

All *in vitro* incubations presented so far were done with 0.5% erythrocyte suspensions (final concentration). In order to create more realistic conditions, higher erythrocyte concentrations were also used with liposomes with a fixed ratio of 27.5 μg Fab'/μmole PL (Table 7). At a final erythrocyte concentration of 2.5% already 52% of the added Fab'-vesicles was specifically bound to the mouse erythrocytes. In a similar experiment a specific binding of 64% was obtained when Fab'-vesicles (ratio: 59 μg Fab'/μmole PL) were incubated with a 25% mouse erythrocyte suspension.

DISCUSSION

In the present paper the feasibility of immunospecific targeting of liposomes, with Fab' fragments of antimouse erythrocyte IgG covalently linked to

Table 5. Binding of CF containing antimouse erythrocyte REV-MBP-PE-Fab' to mouse and sheep erythrocytes

μg Fab' added	Ratio* μg Fab'/μmole PL	% Binding† to mouse RBC	to sheep RBC
0	0	0	0
345	24	7	0
552	56	11	0
759	87	15	0
966	105	16	0

* These ratios were obtained in experiments in which at *fixed* PL concentration (1.6 μmol/ml) the Fab' concentration was varied (as indicated) in a total volume of 1.5 ml. REV-MPB-PE-Fab' were isolated by the pellet method.

† Cell binding assay was carried out as described in Materials and Methods. The values were the mean of a duplicate of a typical experiment (N = 3).

Table 6. Binding of CF containing antimouse erythrocyte REV-MBP-PE-Fab' to mouse and sheep erythrocytes

μmole PL added	Ratio* μg Fab'/μmole PL	% Binding† to mouse RBC	% Binding† to sheep RBC
0.42	587	30	0
0.84	159	26	0
1.68	64	30	0
2.51	36	24	0
3.35	59	16	0
5.01	50	12	0

* These ratios were obtained in experiments in which the amount of PL (as indicated) was varied at a *fixed* Fab' concentration (0.32 mg/ml) in a total volume of 1.8 ml. REV-MPB-PE-Fab' were isolated by the pellet method.

† Cell binding assay was carried out as described in Materials and Methods. The values are the mean of a duplicate of a typical experiment (N = 3).

the surface, to mouse erythrocytes was investigated *in vitro*. The study concentrated on three items:

(a) The effect of varying the Fab' density on the interaction between immunoliposome and target cell.
(b) The use of density gradient centrifugation or ultracentrifugal sedimentation for separation of free Fab' and Fab' coupled to liposomes.
(c) The role of DTT preincubation to increase the specificity of immunoliposome–cell interaction.

Aggregation of the immunoliposomes during flotation on a dextran gradient for separation of free from liposomal Fab' is unfavourable because it changes the behaviour of these immunoliposomes *in vivo* and also *in vitro*. For instance, in the present study large aggregates of liposomes are spun down in the liposome–cell interaction experiments (results not shown) and would therefore cause an artefact suggesting liposome–cell interaction. Aggregation was minimized when the immunoliposomes were collected by ultracentrifugal sedimentation at 30,000 *g* for 30 min (Tables 1–3).

The immunoliposomes show clearly antigen binding capacity as is demonstrated by the HA titers presented in Tables 1–3. The HA titer increased with increasing ratio of μg Fab'/μmol PL (Table 2). No agglutination was observed with REV, REV-MPB-PE (without Fab') containing dispersions with different mean particle diameters (ranging from 0.28 to 2.5 μm) (results not shown). With sheep erythrocytes instead of mouse erythrocytes in none of the samples (including the antimouse erythrocyte Fab'-vesicles) agglutination occurred (results not shown). This clearly demonstrated the specificity of the antigen binding capacities of the antimouse erythrocyte Fab'-vesicles to mouse erythrocytes. The Fab'-vesicles were more potent in haemagglutinating than F(ab')2 fragments. The minimal concentration at which F(ab')2 agglutinated mouse erythrocytes was 8.7 μg/ml in the HA test. The Fab'-vesicles (ratio: 100 μg Fab'/μmole PL) agglutinated at a minimal concentration of 0.10 μg/nmole phospholipid/ml in the HA test. Fab' fragments do not agglutinate at all in these tests [2, 3] because of the inability to form bridges between the target erythrocytes. Compared to F(ab')2, the Fab'-vesicles were 90 times as potent in the HA test. The protein concentration was taken as parameter for comparison. This phenomenon of increased potency was mentioned before by others [23, 24] but was not fully quantified. It is ascribed to the multivalency of the Fab'-vesicle combinations [3, 4]. The results in Table 4 clearly demonstrate that in certain cases blocking the reactive groups of MPB-PE, or other unknown molecular structures, by incubation with a DTT solution is essential to achieve the required specificity for Fab'-liposome-cell interactions. This indicates that only a fraction of the total amount of exposed MPB-PE molecules is actually used for specific and covalent binding to Fab' fragments. A rough estimate by calculating the number of Fab'- and MPB-PE molecules per vesicle supports this suggestion of a relative abundance of exposed MPB-PE molecules that did not react with Fab' molecules. As was determined by ^{31}P-NMR all vesicles were unilamellar (results not shown). Assuming that the vesicles have a diameter of 250 nm, that the surface per PL head (equimolar cholesterol) is 0.96 nm^2 [18] and that MPB-PE is equally distributed over both the inner and outer monolayer of the vesicle, it can be calculated that approximately 9200 MPB-PE molecules per vesicle are outward directed. On the other hand only 800 Fab' molecules per

Table 7. The effect of erythrocyte concentration on the REV-MPB-PE-Fab' erythrocyte interaction

% RBC*	% Binding† to mouse RBC	% Binding† to sheep RBC
0.25	5	0
0.50	14	0
1.00	27	0
1.50	33	0
2.00	40	0
2.50	52	0

* This is the final percentage erythrocytes in the incubation volume (0.5 ml).

† The ratio of the REV-MPB-PE-Fab' was 27.5 μg Fab'/μmole PL and 0.12 μmole PL was added to the indicated erythrocyte suspension (final volume 0.5 ml). The cell binding assay was further carried out as described in Materials and Methods. The values are the mean of a duplicate of a typical experiment (N = 3).

vesicle are bound at a ratio of 100 μg Fab′/μmole PL (Mr Fab′ is 50,000). So at this ratio only 8.5% of the MPB-PE molecules was used for specific binding with the Fab′ molecules.

Tables 5 and 6 show that an increase of the Fab′/PL ratio resulted in a higher interaction with the erythrocytes. Above a certain ratio (105 μg Fab′/μmole PL) there is tendency to level off (Table 5). The same trend was observed for the results in the HA tests (Table 2). When increasing the erythrocyte concentration, the specific interaction of the Fab′-vesicles increased and already at a final erythrocyte concentration of 2.5% half the added amount of Fab′-vesicles specifically bound to the mouse erythrocytes (Table 7). As can be derived from Table 7 approximately 350 Fab′-vesicles per erythrocyte are specifically bound at a final erythrocyte concentration of 2.5% (using the same assumptions as made above). This amount increased as the erythrocyte concentration decreased. Martin and Papahadjopoulos [4] used 50 mM DTT to study the stability of the thioether bond formed in REV-MPB-PE-Fab′ systems. Our liposomes were stable when they were incubated with DTT concentrations in the range of 6.5–20 mM DTT, but at a concentration of 50 mM the REV destabilised, as was checked by measuring the CF release (70% loss within an hour). Martin and Papahadjopoulos [4] used PC-cholesterol vesicles (no PS) in their *in vitro* studies on Fab′-vesicle target cell interactions. They reported also an almost quantitative interaction (95%) of their immunoliposomes to mouse erythrocytes while control vesicles (REV-MPB-PE) showed a binding of less than a few percent even without preincubation with DTT.

Much lower interaction percentages were recently reported by Singhal *et al.* [25] on incubating immunoliposomes with rat erythrocytes *in vitro*. The immunoliposomes were prepared by covalently linking PC/cholesterol (equimolar) liposomes with sodium cyanoborohydride to F(ab′)2 fragments. Unfortunately their results of specific erythrocyte binding are not fully comparable to ours because they used F(ab′)2 instead of Fab′ and carried out their experiments in heparinized whole rat blood while we used washed mouse erythrocytes. They obtained much lower values for specific erythrocyte binding of their immunoliposomes: only 1% per 10^8 rat erythrocytes while our binding percentages ranged between 7 and 30% per 5×10^7 mouse erythrocytes (Table 6), depending on the ratio μg Fab′/μmole PL. In an additional experiment comparable percentages were obtained in heparinized whole blood indicating that there is no effect of plasma and no interaction with other blood cells [22].

Control vesicles with which the results of experiments with immunoliposomes will be compared must be carefully chosen. Salord *et al.* [26] used MPB-PE-vesicles (large unilamellar vesicles) for targeting. The results (5-fold increase of specific interaction) were compared with control liposomes (equimolar PC and cholesterol), lacking MPB-PE. Considering our results (Table 4) this might be an overestimation due to the highly reactive double bond of the maleimido group which could also interact non-specifically with the target cells.

In summary, immunoliposomes based on REV were prepared by an established method. The binding of Fab′ fragments of antimouse erythrocyte IgG to liposomes was done covalently using MPB-PE. The product was stable in terms of size, charge, number of bilayers, Fab′/PL ratio and antigen binding capacity. The Fab′ density on the liposomes could be varied by proper selection of the initial Fab′/phospholipid ratio. This density was an important parameter to achieve liposome–target cell interaction. It was demonstrated that preincubation with 6.5 mM DTT was essential to avoid non-specific immunoliposome–cell interactions. Therefore appropriate controls must be included in the experimental scheme.

REFERENCES

1. Toonen PAHM and Crommelin DJA, Immunoglobulins as targeting agents for liposome encapsulated drugs. *Pharm Weekbl* **5**: 269–280, 1983.
2. Derksen JTP and Scherphof GL, An improved method for the covalent coupling of proteins to liposomes. *Biochim Biophys Acta* **814**: 151–155, 1985.
3. Martin FJ, Hubbel WL and Papahadjopoulos D, Immunospecific targeting of liposomes to cells: A novel and efficient method for covalent attachment of Fab′-fragments via disulfide bonds. *Biochemistry* **20**: 4229–4238, 1981.
4. Martin FJ and Papahadjopoulos D, Irreversible coupling of immunoglobulin fragments to preformed vesicles. *J Biol Chem* **257**: 286–288, 1982.
5. Hashimoto Y, Sugawara M, Kamiya T and Suzuki S, Coating of liposomes with subunits of monoclonal IgM antibody and targeting of the liposomes. *Methods Enzymol* **121**: 817–828, 1986.
6. Senior J, Crawley JCW and Gregoriadis G, Tissue distribution of liposomes exhibiting long-half lives in the circulation after intravenous injection. *Biochim Biophys Acta* **839**: 1–8, 1985.
7. Senior J and Gregoriadis G, Stability of small unilamellar liposomes in serum and clearance from the circulation: The effect of the phospholipid and cholesterol components. *Life Sci* **30**: 2123–2136, 1982.
8. Heath TD, Lopez NG and Papahadjopoulos D, The effects of liposome size and surface charge on liposome-mediated delivery of methotrexate-γ-aspartate to cells *in vitro*. *Biochim Biophys Acta* **820**: 74–84, 1985.
9. Aragnol D and Leserman LD, Immune clearance of liposomes inhibited by an anti-Fc receptor antibody *in vivo*. *Proc Natl Acad Sci USA* **83**: 2699–2703, 1986.
10. Ralston E, Hjelmeland LM, Klausner RD, Weinstein JN and Blumenthal R, Carboxyfluorescein as a probe for liposome-cell interactions: Effect of impurities, and purification of the dye. *Biochim Biophys Acta* **649**: 133–137, 1981.
11. Peterson GL, A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal Biochem* **83**: 346–356, 1977.
12. Fiske CH and SubbaRow Y, The colorimetric determination of phosphorous. *J Biol Chem* **66**: 375–400, 1925.
13. Heath TD, Macher BA and Papahadjopoulos D, Covalent attachment of immunoglobulins to liposomes via glycosphingolipids. *Biochim Biophys Acta* **640**: 66–81, 1981.
14. Szoka FC, Jacobson K and Papahadjopoulos D, The use of aqueous space markers to determine the mechanism of interaction between phospholipid vesicles and cells. *Biochim Biophys Acta* **551**: 295–303, 1979.

15. Crommelin DJA, Influence of lipid composition and ionic strength on the physical stability of liposomes. *J Pharm Sci* **73**: 1559–1563, 1984.
16. Jousma H, Talsma H, Spies F, Joosten JGH, Junginger HE and Crommelin DJA, Characterization of liposomes. The influence of extrusion of multilamellar vesicles through polycarbonate membranes on particle size, particle size distribution and number of bilayers. *Int J Pharm* **35**: 263–274, 1987.
17. Eling WMC, Separation of parasitized erythrocytes from Plasmodium berghei infected mouse blood. *Trans R Soc Trop Med Hyg* **71**: 267–268, 1977.
18. Szoka F and Papahadjopoulos D, Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc Natl Acad Sci USA* **75**: 4194–4198, 1978.
19. Goudswaard J, Van Der Donk JA, Noordzy A, Van Dam RH and Vaerman J-P, Protein A reactivity of various mammalian Immunoglobulins. *Scand J Immunol* **8**: 21–28, 1978.
20. Salk JE, Simplified procedure for titrating hemagglutinating capacity of Influenza-virus and corresponding antibody. *J Immunol* **49**: 87–98, 1944.
21. Schachter D, Aggregation of liposomes by dextrans of high molecular weight. *Biochem Biophys Res Commun* **84**: 840–844, 1978.
22. Peeters PAM, Oussoren C, Eling WMC and Crommelin DJA, Nonspecific interactions of maleimidophenylbutyrate phosphatidyl ethanolamine (MPB-PE) containing (immuno) liposomes with their target cells *in vitro*. *FEBS Lett*, submitted for publication.
23. Bragman KS, Heath TD and Papahadjopoulos D, Simultaneous interaction of monoclonal antibody-targeted liposomes with two receptors on K562 cells. *Biochim Biophys Acta* **730**: 187–195, 1983.
24. Heath TD, Fraley RT, Bentz J, Voss EW, Jr., Herron JN and Papahadjopoulos D, Antibody-directed liposomes: Determination of affinity constants for soluble and liposome-bound antiflourescein. *Biochim Biophys Acta* **770**: 148–158, 1984.
25. Singhal A, Bali A and Gupta CM, Antibody mediated targeting of liposomes to erythrocytes in whole blood. *Biochim Biophys Acta* **880**: 72–77, 1986.
26. Salord J, Tarnus C, Muller CD and Schuber F, Targeting of liposomes by covalent coupling with ecto-NAD-glycohydrolase ligands. *Biochim Biophys Acta* **886**: 64–75, 1986.