Journal of Supramolecular Structure
and Cellular Biochemistry 16:243–258 (1981)
Cellular Recognition 237–252

Monoclonal Antibody Covalently Coupled to Liposomes: Specific Targeting to Cells

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We have evaluated optimal conditions for coupling monoclonal antibody to small unilamellar lipisomes. Coupling of an IgG_{2a} monoclonal anti-β_2-microglobulin antibody, which reacts with human cells, was examined in detail. Liposomes were composed of dipalmitoyl lecithin and cholesterol, and variable quantities of phosphatidylethanolamine substituted with the heterobifunctional cross-linking reagent N-hydroxysuccinimidyl 3-(2-pyridyldithio) propionate (SPDP). They were reacted with antibody derivatized with the same reagent at a 5- to 20-fold molar excess, and activated by mild reduction. This degree of SPDP modification had no effect on the capacity of the antibody to bind to its target antigen. More than 40% of antibody could be reproducibly bound to liposomes, resulting in the coupling of from 1 to 10 antibody molecules per liposome (mean diameter:580 Å). The coupling reaction did not lead to loss of carboxyfluorescein encapsulated within liposomes. At least 80% of liposomes carried nondenatured antibody, as confirmed by precipitation of liposomes and encapsulated carboxyfluorescein by Staphylococcus aureus, strain Cowan I. The liposome-coupled antibody retained its immunological specificity: only cells expressing human β_2-microglobulin bound liposomes in vitro, and the binding was inhibited by the free antibody in solution. Results with antibodies of different antigenic specificity confirm that the technique can be generally applied.

Key words: liposomes, liposome–protein coupling, fluorescence, monoclonal antibody, cell surface antigens

Because of actual or potential applications in cell biology and cancer chemotherapy, the development of methods of targeting liposomes and their contents to specific cell types is an active area of investigation [1, 2]. The most likely candidate molecule to confer specificity on liposomes is antibody, especially with the

Abbreviations used: SPDP, N-hydroxysuccinimidyl 3-(2-pyridyldithio) propionate; CF, carboxyfluorescein; DPPC, dipalmitoyl L-α-phosphatidyl choline; DPPE, dipalmitoyl L-α-phosphatidylethanolamine; DPPE-DTP, dipalmitoyl-L-α-phosphatidylethanolamine 3-(2-pyridyldithio) propionate; TLC, thin layer chromatography; DTT, dithiothreitol; DTP, 3-(2-pyridyldithio) propionate; DTP-protein, protein substituted with SPDP; protein-SH, protein-DTP activated by reduction with DTT; L-buffer, 10 mM Hepes, 145 mM NaCl, pH 7.4; FCS, fetal calf serum; BSA, bovine serum albumin.

Received April 9, 1981; revised and accepted June 15, 1981.

0275-3723/81/1603-0243$04.50

increasing availability of monoclonal antibodies [3]. The problem of linkage of the antibody molecule to liposomes has been the object of a number of studies. Noncovalent coupling [4, 5] tends to denature antibody and is unlikely to be sufficiently stable to be useful. Several recently proposed covalent coupling schemes using conventional cross-linking reagents [6, 7] are highly inefficient: the amount of protein covalently bound is not much greater than that expected by noncovalent association [1, 8, 9]. Coupling of antibodies to phospholipids [10] or to fatty acids [11] requires exposure of reactants to detergents: it is not yet established that liposomes formed under these conditions are sufficiently detergent free to be suitable for the carriage of low molecular weight solutes. Further, these liposomes tend to be nonhomogeneous in terms of the number of antibody molecules bound per liposome [11].

To avoid homopolymerization and subsequent aggregation, other methods have been described [8]. We have recently proposed the use of an heterobifunctional cross-linking reagent N-hydroxysuccinimidyl 3-(2-pyridyldithio) propionate (SPDP) [12].

In this paper, an extensive study was made in order to define the optimal conditions for protein coupling to liposomes using SPDP. Antihuman β_2-microglobulin and antimurine MHC antibodies were used as model systems and the binding of antibody-bearing liposomes to the appropriate target cells is shown. The specificity of the interaction demonstrated that the immunologic specificity of the antibodies is transferred to the liposomes.

EXPERIMENTAL PROCEDURES

SPDP was purchased from Pharmacia Fine Chemicals. A stock solution (40 mM) was made in methanol and stored at $-20°C$. In agreement with Carlsson et al [13], this solution was stable for at least 2 weeks with respect to N-hydroxysuccinimide content, and no change in the content of 2-pyridyldisulphide was observed.

Carboxyfluorescein (CF; Lot C4x) was obtained from Eastman Organic Chemicals and purified as follows: CF was first crystallized from hot absolute ethanol in the presence of activated charcoal, then dissolved in water by addition of three molar equivalents of KOH (per mole of CF) and filtered through a Sephadex LH 20 (Pharmacia) column equilibrated with water. The product was then lyophilized and a stock solution (200 mM in water) was prepared and kept in the dark at 4°C.

Dipalmitoyl L-α-phosphatidylcholine (DPPC: Synthetic, Lot 59C 5388) was purchased from Sigma Chemical Company. Dipalmitoyl L-α-phosphatidylethanolamine (DPPE; Synthetic; Lot 902769) was obtained from Calbiochem-Behring A.G. Cholesterol (Lot 2038) was from Applied Science Laboratories. [^{14}C]DPPC and [^{14}C]DPPE were from New England Nuclear Gmbh. All lipids were periodically monitored for purity by thin layer chromatography on LKDF 5K TLC plates from Whatman in chloroform:methanol:water (65:25:4).

Dipalmitoyl L-α-phosphatidylethanolamine 3-(2-pyridyldithio) propionate (DPPE-DTP) was prepared as follows: DPPE (10 μmol) was mixed in chloroform: methanol (9:1; 700 μl) with [^{14}C] DPPE to a final specific activity of 0.20 μCi/μmol. SPDP (12 μmol in 300 μl of methanol) was then added followed by 20

μmol triethylamine. After 2 hr at room temperature with stirring, the organic phase was washed with 2 ml phosphate buffer (0.1 M; NaCl 0.1 M, pH 7.4), then twice with water. The organic phase was dried under nitrogen, then lyophilized for 2 hr. The product was redissolved in chloroform:methanol (9:1) to a final concentration of 5 mM (based on ^{14}C). Thin layer chromatography on silica gel revealed a single spot under UV illumination with $r_f = 0.58$ (50-μg sample in chloroform:methanol: water, 65:25:4). The radioactivity was recovered in the same spot and no other product was revealed with iodine. The yield (DPPE-DTP) was 90% based on ^{14}C, and the presence of 2-pyridyldisulfide was demonstrated spectrophotometrically after cleavage of the disulfide bond by dithiothreitol, 500 mM in water (DTT, Sigma).

Liposomes. DPPC (11.3 μmol), cholesterol (6.6 μmol), and DPPE-DTP (from 0.05 to 0.7 μmol) were mixed in conical tubes and the organic solvent was evaporated under nitrogen, then by lyophilization for 2 hr. To the lipid film was added 3 ml buffer (L-buffer: 0.01 M Hepes, 0.145 M sodium chloride, pH 7.45) containing 25 to 100 mM CF. Lipids were suspended by vortexing and liposomes were formed by probe sonication under nitrogen for 40 min at 45°C. Tungsten fragments from the sonicator probe and large liposomes were removed by centrifugation (100,000 g; 1 hr). Unencapsulated CF was removed by gel filtration on a Sephadex G50 column (150 × 9 mm). The mean size of these liposomes was 580 Å as assessed by electron microscopy after negative staining with 0.2% phosphotungstic acid in L-buffer. Total lipid concentrations were determined by the measurement of ^{14}C-labeled lipid.

CF leakage was estimated by measurements of the fluorescence with a Farrand Mark I spectrofluorometer (excitation 488 nm; emission 520 nm) before and after releasing entrapped CF with Triton X-100 detergent (final concentration: 1.0%). The quenching ratio, expressed as

$$1 - \frac{\text{(fluorescence before addition of Triton)}}{\text{(fluorescence after addition of Triton)}}$$

is related to the concentration of CF [14] and thus can be used for monitoring the CF concentration in liposomes.

Antibodies. B1.1G6 (monoclonal Balb/c anti-human β_2-microglobulin), a gift from B. Malissen, Centre d'Immunologie de Marseille-Luminy, was made by immunization of Balb/c mice with alloreactive human T cell blasts. Cells from recipient mice were fused with the NS1.Ag4.1 myeloma [3]. After cloning, cells were injected intraperitoneally into Balb/c mice. The antimurine MHC antibody producing cell line 11.4.1 [15] was grown in tissue culture. Culture supernatants and ascites were purified by passage on a protein A Sepharose column (16). Both antibodies are IgG_{2a},κ and are contaminated by NS1.Ag4.1 κ-chain; B1.1G6 also is contaminated by approximately 10% protein A binding normal immunoglobulin from recipient mice (data not shown). The binding specificity of B1.1G6 was confirmed by experiments to be reported in detail elsewhere (B. Malissen et al, manuscript in preparation).

B1.1G6 or 11.4.1 antibodies (180 μg) were iodinated according to Hunter and Greenwood [17] with ^{125}I sodium iodide (0.5 mCi) (carrier free, NEN), then diluted with unlabeled antibody to final specific activities of between 3.3 and 7.1 mCi/μmol.

Prior to SPDP modification, B1.1G6 and 11.4.1 were transferred to phosphate buffer (0.1 M, pH 7.5, NaCl 0.1 M) by gel filtration through Sephadex

TABLE I. Effect of Dithiopyridine (DTP) Substitution of B1.1G6 Protein on Efficiency of Coupling to Liposomes*

Moles of SPDP/mole of protein	Moles of DTP groups/ mole of protein	DPPE-DTP in liposomes (%)	Protein which became liposome associated (%)	Protein/lipid (μg/μmol)	^{125}I protein precipitation[a] (%)	CF precipitation[b] (%)
5	1.8	1	17	7.4	91	83
		0.25	5.5	2.4	80	65
10	2.8	1	27	11.8	90	84
		0.25	9	3.9	86	74
20	4.4[c]	1	45	19.6	92	88
		0.25	18	7.8	91	83

*The protein concentration was 560 μg/ml for the interaction with SPDP, and 109 μg/ml at the time of incubation wtih liposomes, 2.5 mM in total lipid. The concentration of CF in liposomes was 25 mM. Incubation with SPDP and precipitation with S aureus were performed as described under Experimental Procedures.

[a]When incubated without liposomes: 80% of ^{125}I-labeled B1.1G6 or DTP-modified ^{125}I-labeled B1.1G6 precipitated.

[b]When incubated without B1.1G6 antibody 1.5% of liposome-associated CF was precipitated.

[c]Four separate modifications of B1.1G6 protein by SPDP at 20:1 molar excess resulted in 5.0 ± 0.7 mol of DTP/mol of protein.

G25 (PD 10 column, Pharmacia). B1.1G6 was incubated with variable amounts of SPDP (see Table I) for 30 min at room temperature and transferred to acetate buffer (0.1 M, pH 4.5, NaCl 0.145 M) by gel filtration through PD 10 columns. The DTP to protein ratio was determined according to Carlsson et al [13] by measuring for an aliquot the increment of O.D. at 343 nm (25 μl diluted to 500 μl) after addition of DTT (10 μl of 0.5 M DTT in water).

The DTP–protein was activated by conversion of DTP groups to free thiol groups. The DTP–protein was incubated with DTT (50 mM final) for 20 min at room temperature then filtered through a PD10 column in L-buffer. The free thiol-bearing protein will be referred to as protein-SH. It was used immediately for coupling to liposomes.

Cellular radioimmunoassay. B1.1G6 antibody was labeled with ^{125}I to a specific activity of 7.1 mCi/μmol. Unmodified or SPDP-modified antibody was added, in serial twofold dilution, starting at 50 μg/ml, 10^6cpm, in quadruplicate wells of Cooke conical 96-well microtiter trays. To half of the wells, 5×10^5 Ficoll-isolated human peripheral blood lymphocytes were added; to the other half, were added the same number of CBA mouse spleen cells. The incubation medium was L-buffer, supplemented with 0.2% BSA and 0.02% NaN_3. After 1-hr incubation at 4°C, cells were washed four times by centrifugation and counted for ^{125}I in a multichannel gamma-counter. Values represent the mean of the duplicate wells.

Coupling reaction: Protein-SH was added to liposomes containing DPPE-DTP and incubated at room temperature for 24 hr, which was shown to be sufficient for the reaction to come to completion (see Results). For preparative purposes, protein-bearing liposomes were separated from uncoupled protein by gel filtration on a small Sepharose 4B column (5 × 1.5 cm) [12]. The yield of this reaction was estimated as the ratio of ^{125}I counts eluted in the void volume to the

total counts. The same column was used for several experiments and its performance was found to be reproducible. The elution profile of CF was also determined after each experiment before and after addition of Triton X-100. This showed that more than 95% of CF was liposome encapsulated and that the quenching ratio after the coupling reaction was not significantly changed. The protein–liposome association was confirmed by discontinuous sucrose-density gradient ultracentrifugation, by a modification of the technique described by Litman et al [18]. An aliquot (100 μl) from the protein–liposome reaction or protein alone was mixed in 1-ml L-buffer:sucrose to a final concentration of 45% sucrose, and overlaid with 1.5 ml each of 35, 25, and 15% sucrose in the same buffer. After overnight centrifugation at 130,000g, fractions of 0.6 ml were collected from the bottom and analyzed for ^{125}I-labeled protein, and for CF before and after Triton lysis.

Uncoupled liposomes or protein-bearing liposomes isolated from small Sepharose 4B columns were subjected to analytic gel chromatography on a 16 × 1.2 cm Bio Gel A 150-m column as described [19]. The column was preequilibrated with nonfluorescent liposomes (500 μl, 5 μmol total lipid) and unlabeled antibody (500 μl, 1 mg), in order to reduce lipid and protein loss on the column.

Staphylococcus aureus precipitation of antibody bearing liposomes. Paraformaldehyde fixed S Aureus bacteria (Strain Cowan I) were prepared as described [16]. The bacteria were washed three times before use with L-buffer, then suspended to 10% (v/v) in the same buffer. From 50 to 100 μl of protein-bearing liposomes were diluted to 1 ml with 0.2% bovine serum albumin in L-buffer. To some tubes was added 100 μl of the Staphlococcus aureus suspension, to others was added 100 μl of medium. After 30-min incubation at 4°C, tubes were spun 15 min at 2300g. The bacterial pellets were counted for ^{125}I and measured for fluorescence after addition of 50 μl of 20% Triton X-100 and 900 μl of L-buffer, and centrifugation for 15 min. Supernatants were counted and measured for fluorescence before and after addition of Triton. The percentage of liposomes precipitated was calculated as ^{125}I counts or fluorescence found in the pellet divided by total (pellet + supernatant) ^{125}I counts or fluorescence [12].

Cell binding. Raji and Daudi are human lymphoblastoid B-cell lines; Raji expresses large amounts of β_2-microglobulin, whereas Daudi is well known for its defect in expression of both membrane-associated and secreted β_2-m [20, 21]. RDM4 is a murine tumor rich in the K^k molecule [20], which is the target specificity of antibody 11.4.1. Cells were maintained in tissue culture in RMPI 1640 medium (Gibco) supplemented with 5% fetal calf serum (FCS). For incubation with liposomes, tumor cells, human peripheral blood lymphocytes, or CBA ($H\text{-}2^k$) mouse spleen cells were incubated in the same media, in a total volume of 0.1 ml, in duplicate wells of V-bottomed microtiter trays. To the wells was added 25 μl of antibody-bearing or control liposomes without antibody. B1.1G6 or 11.4.1 antibody (final concentration:25 μg/ml) was added to some wells 15 min prior to addition of liposomes. Cells were incubated 1 hr at 4°C, washed four times in L-buffer containing 5% FCS and 0.02% NaN_3, and resuspended in the same buffer. ^{125}I counts and CF measurements of the applied liposomes and of the cell pellets were performed in duplicate. Cells were placed on glass slides and sealed under coverslips. They were examined by fluorescence and phase optics with a Zeiss universal microscope.

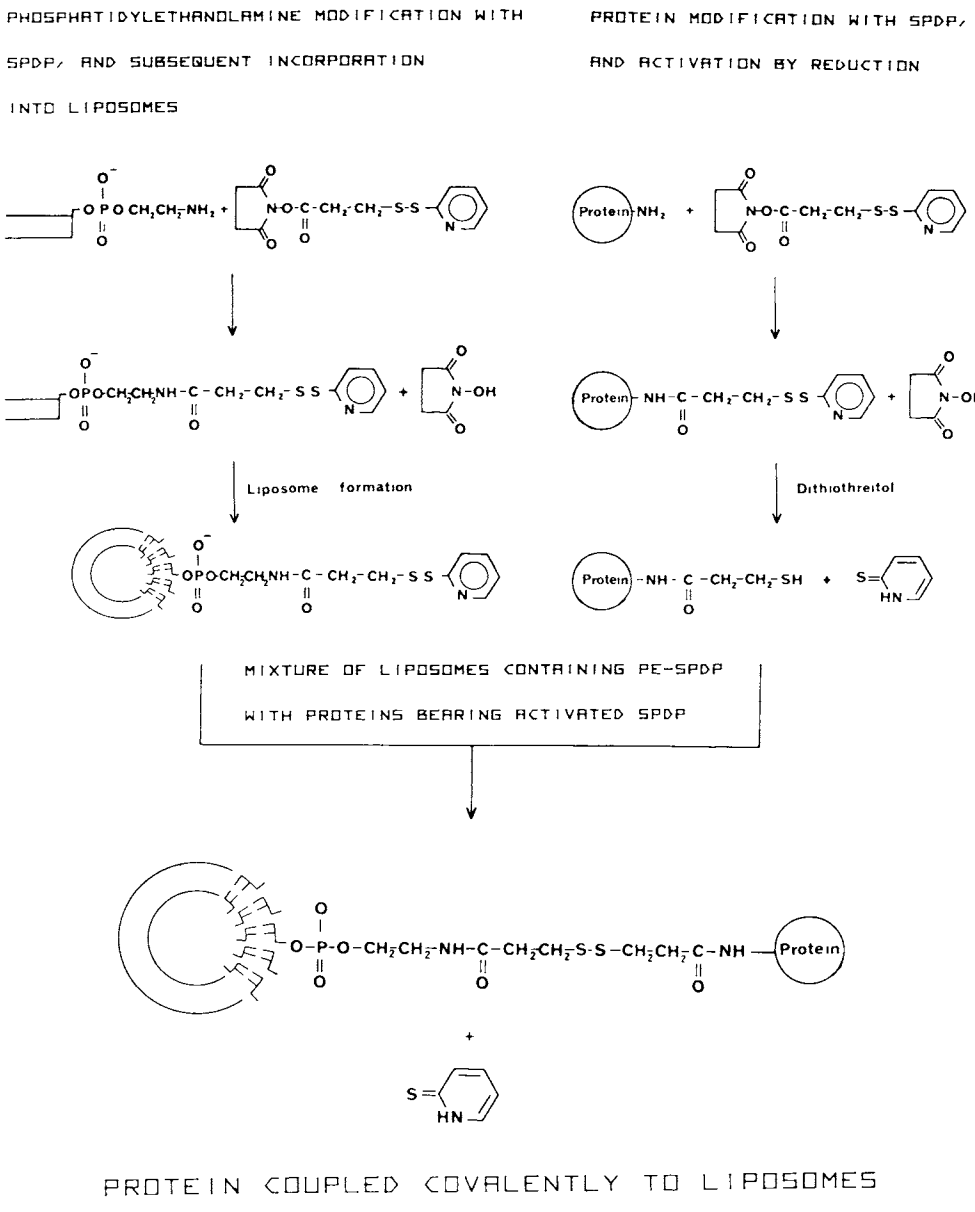

Fig. 1. Schematic of coupling reaction.

RESULTS

Rate and Extent of the Protein–Liposome Coupling Reaction

Liposomes were made and protein coupled to them according to the scheme presented in Figure 1. The protein–liposome association was confirmed by discontinuous sucrose gradient ultracentrifugation (Fig. 2). Liposomes floated at the top

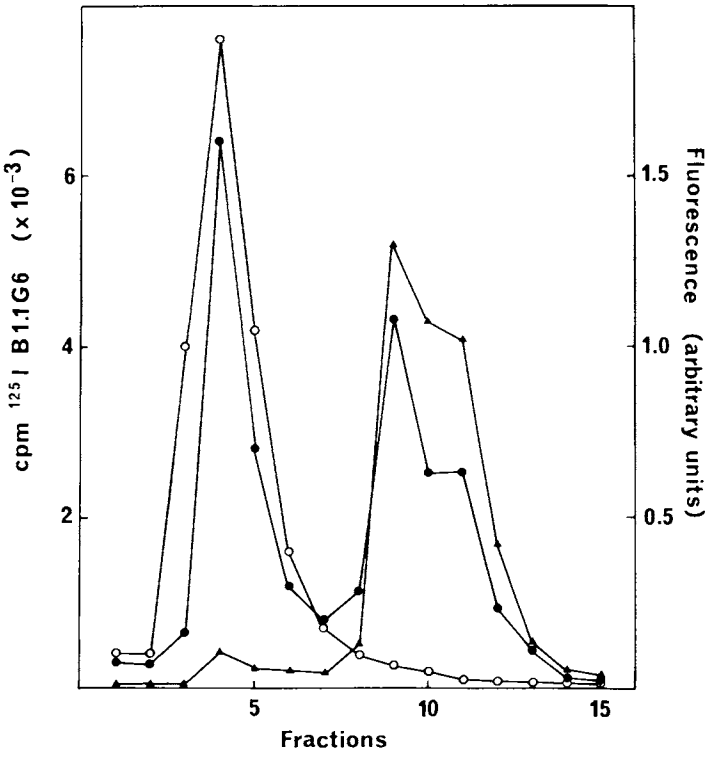

Fig. 2. Discontinuous sucrose-density gradient ultracentrifugation analysis of the reaction between B1.1G6 and liposomes. Details of the technique are described under Experimental Procedures. The quantity of protein which was liposome associated is commensurate with that shown by Sepharose 4B chromatography (Table I), for a protein preparation coupled under similar conditions (20 mol SPDP/mol protein). The bottom of the gradient is to the left. (○) ^{125}I-B1.1G6 alone; (●) ^{125}I-labeled protein from liposome–protein reaction; (▲) CF (after Triton lysis).

of the gradient; protein alone stayed at the bottom but partially coeluted with liposomes after the covalent coupling. To confirm that the coupling technique did not result in aggregation of liposomes, we analyzed on a Bio-Gel A 150-m column an uncoupled liposome preparation and an aliquot of the same preparation coupled to antibody and separated from noncoupled antibody as described below. As shown in Figure 3, the uncoupled and the protein-bearing liposomes eluted from the column in the same position; this type of column has previously been shown to resolve liposome preparations differing only slightly in mean diameters [19]. The extent of the coupling reaction was calculated from the ratio of the ^{125}I-labeled protein counts eluting in the void volume of preparative small Sepharose 4B columns over total counts [12].

We examined the rate of the reaction between DTP–liposomes and protein–SH. At intervals after the mixture of the reactants samples were passed through a small Sepharose 4B column and the extent of the reaction was evaluated. As seen

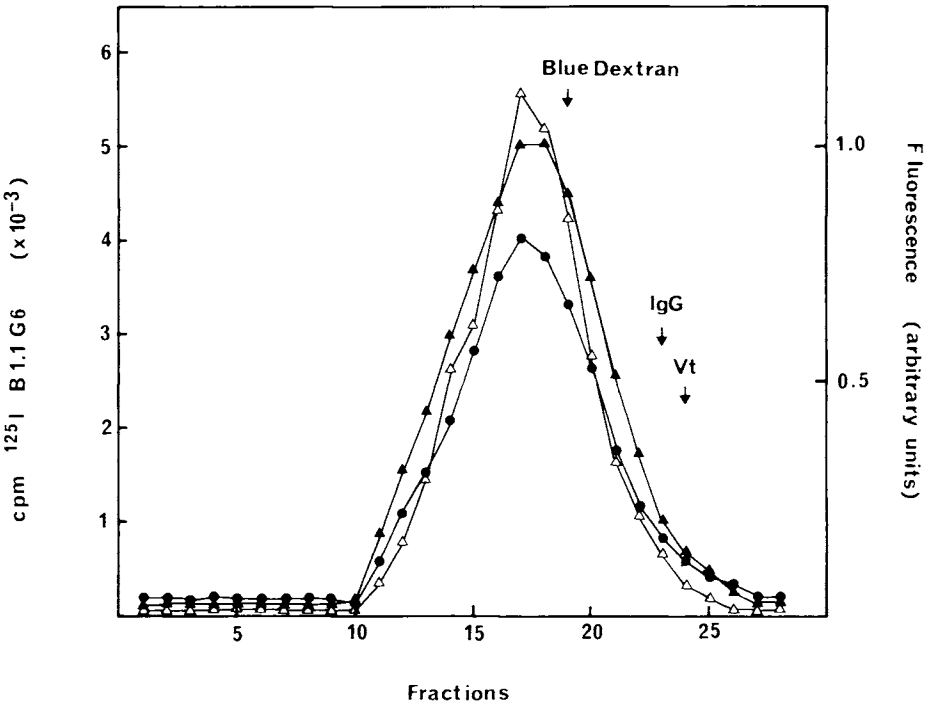

Fig. 3. Bio Gel A 150-m chromatography of protein-bearing and uncoupled liposomes. Liposomes coupled to B1.1G6 modified at 20 mol SPDP/mol of protein were collected from the excluded fraction of a small Sepharose 4B column. At least 80% of these liposomes were bound to protein, as revealed by *S. aureus* precipitation; 200 μl of these liposomes, or an aliquot of the same liposome preparation not coupled to protein were analyzed on a Bio Gel A 150 m column, as described under Experimental Procedures. The column was calibrated using CF, ^{125}I-B1.1G6 and Blue Dextran. (●) ^{125}I counts for liposome-bound protein; (▲) CF after Triton lysis for protein-bearing liposomes; (△) CF after Triton lysis for uncoupled liposomes.

in Figure 4, the reaction reached a plateau level after about 20 hr of incubation. The control incubation of DTP-liposomes with nonreduced DTP protein showed no significant coupling after the same time. In all subsequent studies, protein-liposomes complexes were evaluated having reacted for not less than 24 hr.

Effect of DTP Substitution on Liposome Coupling and Protein Activity

A series of experiments was performed to determine the optimal DTP substitution of the antibody for maximum binding to liposomes with minimal denaturation. B1.1G6 antibody was incubated with SPDP at a 5-, 10- or 20-fold molar excess, resulting in a substitution of 1.8, 2.8, and 4.4 moles per mole of protein, respectively. Identical quantities of protein as confirmed by ^{125}I determination were incubated after DTT reduction with aliquots of two liposome preparations. As seen in Table I, liposome-protein association increased with increasing protein

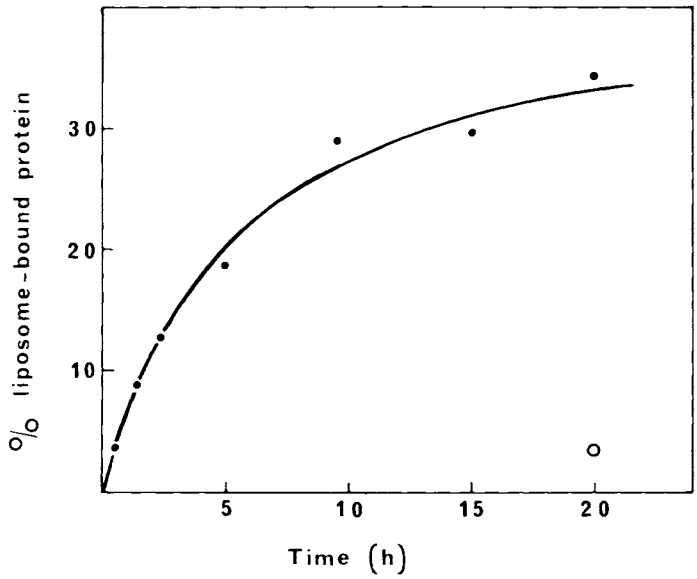

Fig. 4. Kinetics of the reaction between B1.1G6 and liposomes. A lipid mixture of 11.3 μmol of DPPC, 6.6 μmol of cholesterol, and 0.18 μmol of DPPE-DTP was sonicated in 3 ml L-buffer. The 150-μl aliquots were incubated with an equal volume of ^{125}I-labeled B1.1G6 protein substituted with 4.8 mol DTP/mol, and reduced with DTT. The final protein concentration was 72 μg/ml. At the intervals indicated previously, the samples were passed on a Sepharose 4B column and the amount of ^{125}I protein which was liposome associated was determined, as indicated by closed circles. The open circle represents the liposomal association of a similar aliquot made with DTP-substituted B1.1G6 which was not reduced.

substitution. In all cases precipitation of CF in liposomes was commensurate with precipitation of ^{125}I-labeled protein, indicating the reaction had not induced leakage of liposome contents. At an SPDP:protein molar excess of 40 to 1, the protein solution became visibly turbid.

The activity of the modified proteins was evaluated in a radioimmunoassay (Fig. 5). Substitution of as much as 4.4 mol DTP per mole of antibody had no effect on its binding capacity or specificity; the curves of specific binding are superimposable, and the binding to the irrelevant target was not increased as a consequence of the modification.

Effect of Protein Concentration on Lipsome Coupling

Three different concentrations of a single preparation of B1.1G6 substituted with a 20-fold molar excess of SPDP and activated by DTT reduction were incubated with aliquots of a liposome preparation made with 1 mol% DPPE-DTP. Within the range studied, the percentage of liposome-associated protein was essentially constant (Table II), thus the mean number of protein molecules bound per liposome, calculated on the basis of a mean liposome diameter of 580 Å, increased with the concentration of protein from 0.4 to 7.2; at the highest antibody concentration, nearly all liposomes had antibody coupled to their surfaces as demonstrated by CF precipitation.

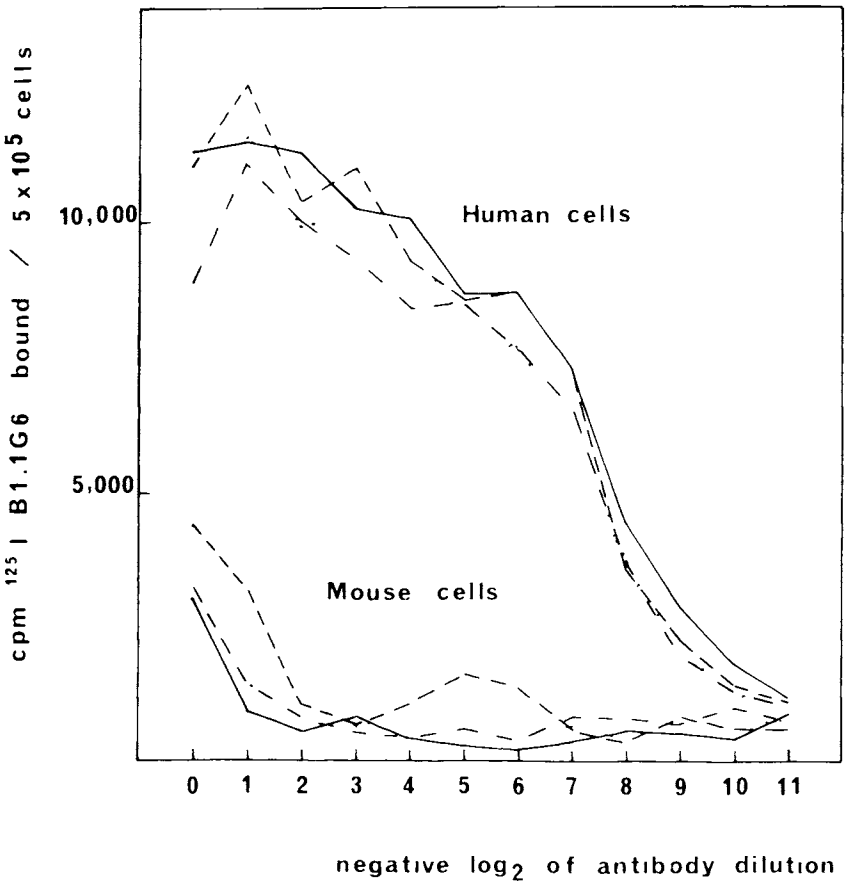

Fig. 5. Cellular radioimmunoassay of DTP-modified B1.1G6. Details are given under Experimental Procedures. ———— unmodified B1.1G6; -------- B1.1G6 modified with SPDP at 5 moles/mole; -•-•-•- SPDP/B1.1G6 at 10 moles/mole; ••••••• SPDP/B1.1G6 at 20 moles/mole.

TABLE II. Effect of the Concentration of DTP-Modified Protein on Liposome Association*

Concentration of protein (μg/ml)	Protein which became liposome associated (%)	Protein/lipid (μg/μmol)	^{125}I protein precipitation (%)	CF precipitation (%)
13	38	2.0	55	42
52	47	9.8	89	86
208	43	35.8	93	94

*The concentration of liposomes was 2.5 mM (expressed as total lipid). B1.1G6 contained 4.75 mol of DTP/mol of protein. Liposomes contained 0.01 mol of DPPE-DTP/mol of total lipid. The concentration of CF in liposomes was 25 mM. Incubation and precipitation were performed as described under Experimental Procedures.

TABLE III. Effect on the Concentration of Liposomes on the Liposome Association of DTP-Modified B1.1G6 Protein*

Concentration of liposomes expressed as concentration of total lipid (mM)	Protein which became liposome associated (%)	Protein/lipid (μg/μmol)	^{125}I protein precipitation (%)	CF precipitation (%)
0.155	21	84.7	77	45
0.625	45	45.0	90	88
2.5	53	13.2	89	84

*The concentration of protein was 62.5 μg/ml containing 5.76 mol of DTP/mol of protein. The concentration of CF in liposomes was 25 mM. Incubation and precipitation were performed as described under Experimental Procedures.

TABLE IV. Effect of the Concentration of DPPE-DTP in Liposomes on Liposome Association of DTP-Modified B1.1G6 Protein*

Moles of DPPE-DTP/ mole of lipid	Protein which became liposome associated (%)	Protein/lipid (μg/μmol)	^{125}I-protein precipitation (%)	CF precipitation (%)
0.0025	26	6.5	79	67
0.010	46	11.5	88	83
0.040	53	13.2	89	84

*The concentration of protein was 62.5 μg/ml containing 5.76 mol of DTP/mol of protein. Total lipid concentration was 2.5 mM. The concentration of CF in liposomes was 25 mM. Incubation and precipitation were performed as described under Experimental Procedures.

Effect of Liposome Concentration and Percentage of Incorporated PE-DTP on Protein Coupling

Experiments were performed at a single protein concentration in order to determine the optimal ratio of protein to liposomes for maximum protein coupling. These experiments were performed in two ways: at various concentrations of liposomes containing a fixed percentage of DPPE-DTP (Table III); or at a constant concentration of liposomes containing variable percentages of DPPE-DTP (Table IV). At very low concentrations of liposomes, the amount of protein which became bound per mole of lipid was quite high (Table III, line 1). However, the efficiency of precipitation was reduced. Similarly, when the substitution was low (Table IV, line 1), the efficiency of precipitation was also low. For the entire series of experiments performed, the optimal range of protein coupling was from 7.4 to 45 μg protein/μmol of lipid which corresponds to 1.5 to 9 antibody molecules per liposome, for liposomes of mean diameter 580 Å.

Cellular Binding

The cellular radioimmunoassay demonstrated that SPDP-modified B1.1G6 not coupled to liposomes would bind to cells expressing the relevant antigen:human

TABLE Va. Binding of B1.1G6 Antibody-Bearing Liposomes to Cells*

Moles of DTP/ mole of protein	^{125}I and CF content of applied liposomes		^{125}I and CF content of cell-bound liposomes			
			Human peripheral blood leukocytes		Mouse spleen cells	
	^{125}I counts in liposome preparation	CF in liposomes (pmol)	(protein counts bound)	(pmol of CF bound)	(protein counts bound)	(pmol of CF bound)
–	–	886	–	0.6	–	0.7
1.8	2903	897	169	31	15	2.5
2.8	4529	820	330	45.6	40	4
4.4	9905	842	598	41.6	58	4.4
4.4[a]	9905	842	11	1	29	0.6

*The total lipid concentration was 0.1 mM. Liposomes contained 2.5 mmol of DPPE-DTP/mol of lipid. The concentration of CF in liposomes was 100 mM. The specific activity of the protein was 7.1 mCi/μmol. Incubation and measurements were performed as described under Experimental Procedures. Each well contained 5×10^6 cells.
[a]Cells were preincubated with B1.1G6 antibody at 25 μg/ml.

TABLE Vb. Binding of B1.1G6 or 11.4.1 Antibody-Bearing Liposomes to Cells*

	Inhibitor added (25 μg/ml)	Binding to RAJI cells		Binding to RDM4 cells	
		^{125}I protein counts bound	CF bound (pmol)	^{125}I protein counts bound	CF bound (pmol)
B1.1G6-bearing liposomes[a]	–	1,030	52.8	40	0.8
B1.1G6-bearing liposomes	B1.1G6	20	1.2	ND[b]	ND
B1.1G6-bearing liposomes	11.4.1	1,135	58.4	ND	ND
11.4.1-bearing liposomes	–	49	0.8	262	11.2
11.4.1-bearing liposomes	B1.1G6	ND	ND	220	9.6
11.4.1-bearing liposomes	11.4.1	ND	ND	60	1.2

*The lipid concentration was 0.6 mM expressed as total lipid. Liposomes contained 10 mmol of DPPE-DTP/mol of lipid. The concentration of CF in liposomes was 40 mM. The specific activity of the protein was 3.3 mCi/μmol for B1.1G6 and 4.8 mCi/μmol for 11.4.1. Incubation and measurements were performed as described under Experimental Procedures. The total amount of ^{125}I-labeled protein and CF was 7,000 cpm and 510 pmol/well for both liposome preparations. Each well contained 2.5×10^5 cells.
[a]The fluorescence of cells incubated with liposomes not coupled to antibody (total CF: 510 pmol) was below the limit of resolution of the measurement (<0.4 pmol).
[b]ND, not done.

β_2-microglobulin. The precipitation of liposomes coupled to B1.1G6 by S. aureus demonstrated that coupled antibody retained an intact protein A binding site. Experiments involving binding of B1.1G6-coupled liposomes to cells bearing human β_2-microglobulin demonstrated that the liposome-bound antibodies also expressed functional antibody-combining sites (Tables Va and b). As shown by both ^{125}I and CF determinations, B1.1G6-bearing liposomes became cell bound only when human

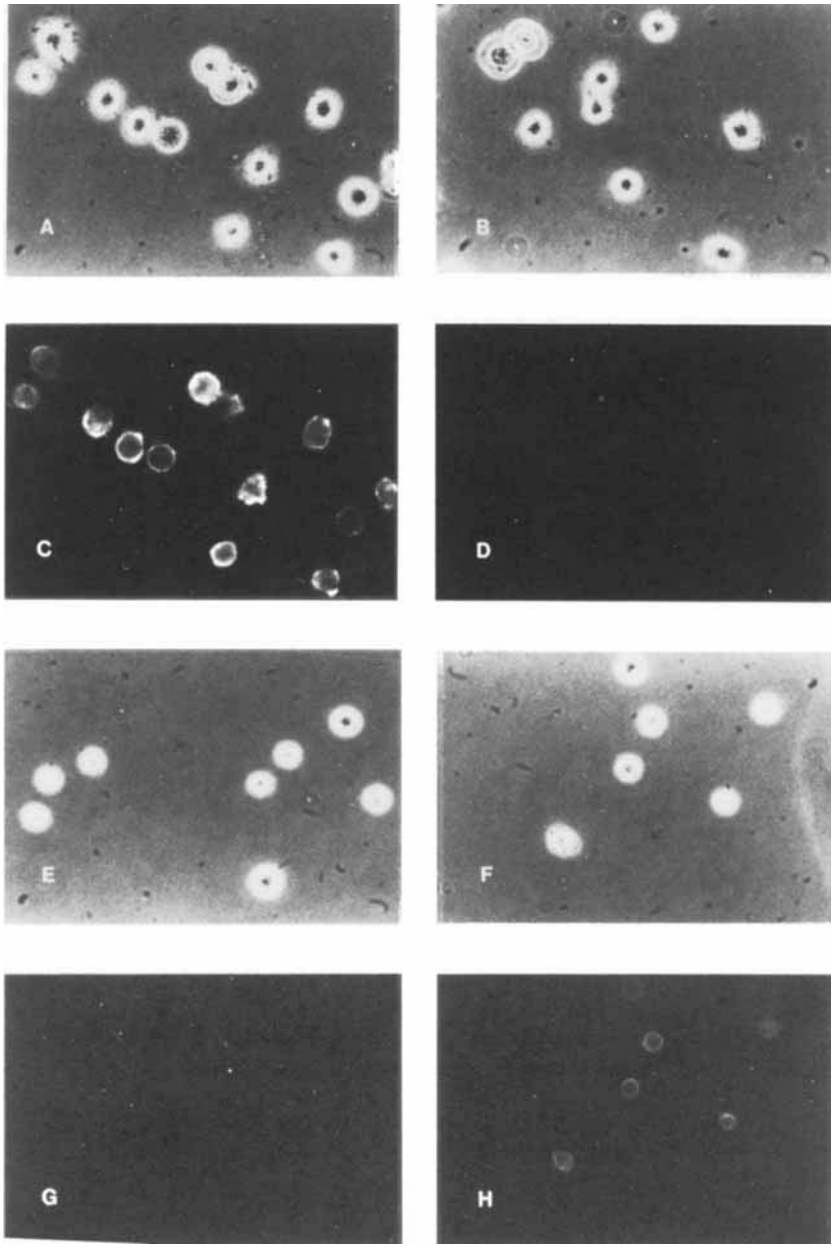

Fig. 6. Binding of fluorescent liposomes bearing B1.1G6 or 11.4.1 antibodies to Raji (A–D) or RDM4 (E–H) tumor cells: (A) and (C) phase contrast and fluorescence micrographs of Raji cells incubated with B1.1G6-bearing liposomes; (B) and (D) phase contrast and fluorescence micrographs of Raji cells incubated with 11.4.1 liposomes; (E) and (G) phase contrast and fluorescence micrographs of RDM4 cells incubated with B1.1G6-bearing liposomes; (F) and (H) phase contrast and fluorescence micrograph of RDM4 cells incubated with 11.4.1-bearing liposomes. All incubations were performed as described under Experimental Procedures. Fluorescence micrographs were taken with a constant 1-min exposure time: All prints were handled identically.

β_2-microglobulin was expressed on the target cells; 11.4.1-bearing liposomes became cell bound only when mouse cells expressing the K^k molecule (RDM4) were the targets. The cell-associated fluorescence is seen on fluorescence micrographs in Figure 6. The ratio of ^{125}I to CF in the applied and bound liposomes (Tables Va, b) was nearly the same, indicating homogeneity of protein distribution in the liposome preparation, and also that there was no major loss of encapsulated CF despite extensive washing and manipulation of cells. The low level of binding of B1.G6-bearing liposomes to mouse cells, despite the use of intact IgG_{2a}, κ immunoglobulin, also demonstrated that the antibody is not markedly aggregated: if this were the case, we would expect a higher level of binding to Fc receptor-bearing mouse spleen cells (Table Va). Figure 6 and Table Vb also demonstrate the reverse incubation, namely that 11.4.1-bearing liposomes bind to mouse, but not human cells. Daudi cells, expressing neither β_2m nor the K^k molecule, bound only background levels of liposomes (data not shown). The complete inhibition of the relevant binding by an excess of free B1.1G6 or 11.4.1 antibodies confirmed the immunologic specificity.

DISCUSSION

General application of targeted liposomes requires methods of coupling soluble proteins to them which are rapid, efficient, and nondestructive of the reactants. The technique we have developed compares favorably with published methods, as determined by the following criteria:

(1) Availability and Stability of the Coupling-Reagent and Reaction

N-hydroxysuccinimidyl 3-(2-pyridyldithio) propionate (SPDP) is commercially available (Pharmacia). The use of SPDP for covalent coupling of proteins to liposomes offers many of the same advantages as the use of that compound [13], and similar compounds [23] for protein–protein coupling. Because SPDP is heterobifunctional, it eliminates homopolymerization of protein or of target phosphatidylethanoamine in liposomes. Especially important in the present technique is the ability to generate, in high yield, the reagent DPPE-DTP in organic solvent. There was no detectable cleavage of thiopyridine from DPPE-DTP after 2 months at $-20°C$ (our unpublished results). DPEE-DTP can thus be introduced in known amount in the constituent lipids of a liposome preparation; use of radiolabeled DPPE confirmed that all DPPE-DTP became liposome associated. We have found that liposomes made with DPPE-DTP and stored at 4°C for several weeks were fully reactive with DTP-bearing proteins. The DTP derivatives of proteins are stable [13]. Thus, if constituents are made in advance, coupling requires only mild reduction of protein-DTP groups, followed by overnight incubation with liposomes. Proteins containing natural thiol groups can also be coupled to DTP-modified proteins [10, 13]. Some proteins in which modification of amino groups led to loss of function (i.e., nerve growth factor) have been thiolated by cystamine derivatization of carboxyl groups [24]. In both of these cases we would expect efficient coupling of these proteins to DTP-bearing liposomes.

(2) Nondestruction of Protein or Liposomes

B1.1G6 antibody modified with SPDP and substituted with as many as 4.4 mol DTP/mol protein showed no reduction in binding efficiency to its target antigen in a sensitive radioimmunoassay (Fig. 5). Similarly, liposomes made with DPPE-DTP were as resistant to leakage of encapsulated CF as liposomes made with DPPC and cholesterol alone. Coupling of these liposomes did not induce additional loss of contents. Liposomes bearing B1.1G6 antibody still contained at least 75% of CF encapsulated after 1 month at 4°C (our unpublished results). It is not certain whether detergent dialysis techniques would permit equivalent encapsulation, because of problems of residual detergent: liposomes are permeable to encapsulated solute at detergent concentrations which are too low to disrupt the bilayer [25].

(3) Homogeneity of the Label

The ratio of antibody to CF in liposomes bound to target cells was nearly the same as in the applied preparation (Table V). This is in contrast to reports of protein–liposome association by detergent dialysis techniques, where micelles of derivatized antibody can form [10, 11], and apparently insert as a bolus into liposomes, resulting in inhomogeneity of label.

(4) Presence of a Spacer Arm

The use of SPDP as a coupling reagent spaces six carbons and a disulfide bridge between the coupled antibody and the amino groups of DPPE and protein lysine residues (see schema in Fig. 1).

In studies of hapten-bearing liposomes it was found that the presence of a spacer markedly enhanced the accessiblity of hapten bound to phosphatidylethanolamine to antibody and also its ability to stimulate antihapten immune responses [26, 27]. Measurements of liposome size and calculations based on moles of antibody bound per mole of lipid indicate that one molecule of coupled antibody was sufficient to bind liposomes to S aureus, or to target cells. This is in contrast to the report of Huang et al, in which 16 molecules of antibody coupled directly to palmitic acid in liposomes were insufficient to bind the liposomes to target cells [11]. This latter result is consistent with steric hindrance based on proximity of the coupled antibody molecules to the liposome membrane.

Another technique for coupling proteins to performed liposomes has recently been published [8]. As presented, it is also a highly efficient method. However, the use of this technique for the binding of liposomes to cells other than erythrocytes has not yet been reported.

We have incubated liposomes with cells in the cold and in the presence of metabolic inhibitors in order to study membrane binding without endocytosis. Liposomes with covalently coupled haptens became bound to cells expressing surface immunoglobulin with affinity for the hapten, but the liposomes contents did not enter the target cells [28]. Hapten-bearing liposomes were endocytosed by IgG Fc receptor-bearing cells, in the presence of antibody to the hapten [29]. The availability of monoclonal antibodies directed at many cell-surface determinants,

together with the technique of coupling proteins to liposomes presented here, will permit a thorough evaluation of the fate of cell-surface molecules, with respect to endocytosis. Liposomes containing encapsulated fluorescent markers and drugs, and bearing covalently coupled hormones or soluble or solubilized cell surface proteins can also be prepared. Studies using these reagents are in progress.

ACKNOWLEDGMENTS

We thank Pierre Cau for electron microscopy, Bernard Malissen for antibody B1.1G6, François Lemonnier for S aureus, François Kourilsky for enthusiastic support, and our colleagues at the Centre d'Immunologie for helpful discussions and criticisms of the manuscript. Supported in part by a grant from Association pour le Développement de la Recherche sur le Cancer, and by a contract under the program INSERM-NCI Recherches Cliniques en Cancérologie.

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