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COVALENT ATTACHMENT OF IMMUNOGLOBULINS TO LIPOSOMES VIA GLYCOSPHINGOLIPIDS

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Summary

We describe a method for covalent binding of proteins to large unilamellar liposomes which involves the periodate oxidation of glycosphingolipids in the vesicle membane. Proteins such as IgG and $F(ab')_2$ may then be attached to the aldehyde groups on the glycolipid by Schiff-base formation at pH 9.5 and reduction with $NaBH_4$, or by reductive amination with $NaBH_3CN$ at pH 8.4. Exposure of the vesicles to periodate, protein coupling and separation from unbound protein by a novel method of flotation in discontinuous dextran gradients does not release the vesicle contents when performed at pH 8.4. Studies on the oxidation of neutral glycolipid-containing vesicles, and on the oxidation of encapsulated glycerol 1-phosphate show that periodate influx into neutral vesicles during a 4 h exposure is appreciable at pH 5.5 but not at pH 8.4. Under optimal conditions, approx. 20% of the protein may be coupled to vesicles, and a ratio of 100—200 μg of protein/μmol of lipid is readily achieved. This method will be of great importance for the antibody-mediated targeting of vesicles to cells.

Introduction

The attachment of specific antibodies to liposomes has often been suggested as a means for their targeting to specific cells [1]. Several investigators have attempted to link proteins to liposomes using glutaraldehyde [2], carbodiimide [3] and diethyl suberimidate [4]. These methods have the inherent disadvantage of producing homopolymers of vesicles, protein or both. In general, the reported extent of protein binding is small and not appreciably greater than the amount of nonspecific binding.

We have previously described the covalent attachment of horseradish peroxidase to vesicles by reaction of aldehyde groups on the periodate-oxidized enzyme with primary amino groups on the vesicle surface [5,6]. This method can attach a large amount of peroxidase to the vesicle surface although it is applicable only to proteins which can be oxidized by periodate. We suggested that antibodies might be coupled to vesicles by a similar method in which appropriate lipids in the vesicle membrane were oxidized by periodate to produce aldehydes. Such a method would require no prior protein modification, but would utilize the proven efficiency of the aldehyde-amino reaction, which cannot produce homopolymers of protein and liposomes and gives rise to well defined products.

The aldehyde-lipid precursors (glycolipids, phosphatidylglycerol or phosphatidylinositol) could be oxidized prior to vesicle formation or in the vesicle membrane by oxidation of intact vesicles. The first approach would expose only the aldehyde-lipid precursor to periodate, but aldehyde lipids would probably be symmetrically arranged in the membrane upon vesicle formation. Encapsulated markers bearing amino groups might subsequently become coupled to the inner surface of the vesicles by imine formation and reduction. The second approach exposes the vesicles and their encapsulated contents to periodate, but could result in the oxidation of only the outer surface of the vesicles if conditions were established in which periodate could not enter the vesicles.

In this paper we describe a two-step method which involves prior oxidation of glycolipids in liposomes to produce aldehyde groups on the vesicle surface, followed by protein coupling by reductive amination with $NaBH_4$ or $NaBH_3CN$. Under optimal conditions, 20% of the protein can be attached to vesicles with a protein : lipid ratio of 100—200 $\mu g/\mu mol$. Periodate treatment at pH 8.4 oxidizes a large proportion of the external glycolipid without oxidizing the internal glycolipid. Encapsulated glycerol 1-phosphate is not oxidized within the vesicles at pH 8.4, and encapsulated carboxyfluorescein is substantially retained.

Materials and Methods

Phosphatidylcholine and phosphatidylglycerol were prepared from egg yolks as previously described [7]. Cholesterol (Fluka) was recrystallized three times from hot CH_3OH. All the above compounds gave a single spot on TLC and were stored in glass ampoules under N_2 or Ar at —50°C. The following glycosphingolipids were purified as described [8]: human neutrophil lactosylceramide, pig intestine globotriaosylceramide, and bovine brain or bovine spinal cord galactosylceramide. Other reagents were purchased from the following suppliers. Bovine γ-globulin (Sigma and Miles); rabbit IgG and $F(ab')_2$ (Cappell Laboratories); rabbit γ-globulin, and mouse IgG (Miles); mixed brain gangliosides, $NaBH_3CN$, $NaBH_4$ and dextran (average mol. wt. 40 000) (Sigma); Sepharose 6B (Pharmacia). $NaBH_3CN$ was purified by precipitation from CH_3CN with CH_2Cl_2 [9]. All other chemicals were reagent grade or better.

Preparation of liposomes. Liposomes were prepared by the reverse-phase

evaporation method of Szoka and Papahadjopoulos [10], and in most experiments they were extruded successively through 0.4 and 0.2 μm pore-size BioRad unipore polycarbonate membranes which have been shown to produce homogeneous particles with a diameter in the range of 0.2 μm [11]. Vesicles were prepared in either 10 mM borate, 60 mM NaCl (pH 8.4) or 10 mM sodium acetate, 60 mM NaCl (pH 5.5).

The reverse-phase evaporation method [10] has not previously been applied to glycolipid-containing vesicles and we will briefly describe some of our observations. Most purified glycolipids are insoluble in ether and some of the lipid mixtures we have used also do not dissolve. This does not interfere with vesicle preparation and we have used isopropyl ether and emulsified the ether/water/lipid mixtures at 45°C by sonication for 5—10 min. Neutral glycolipid-containing vesicles tend to flocculate and will precipitate on standing for even a few minutes. Flocculation does not affect capture efficiency and also does not promote leakage of carboxyfluorescein from the vesicles. Such vesicles may readily be extruded through polycarbonate membranes [11], but immediately reaggregate after extrusion. We have routinely added 3—4 mol% of negatively charged lipid which prevents flocculation.

Protein-vesicle coupling. Vesicles were oxidized by periodate, which was added in an equal volume of isotonic buffered solution. For periodate concentrations below 60 mM, NaCl was added to this concentration. Oxidation was usually allowed to proceed for 30 min in the dark. Subsequently, the vesicles were separated from the periodate on a 1.5 × 10 cm Sephadex G-75 column equilibrated in 20 mM borate, 120 mM NaCl (pH 8.4). The column was discarded after use since periodate oxidizes the Sephadex.

For conjugation with $NaBH_4$, oxidized vesicles in 1—2 ml containing 5—10 μmol total lipid were mixed with 10—30 mg bovine IgG in 1—2 ml of 20 mM borate, 120 mM NaCl (pH 8.4). The pH of the mixture was raised to 9.5 by addition of 0.2 M NaOH; after 2 h at 25°C, $NaBH_4$ was added to a final concentration of 0.3 mg/ml and the mixture incubated further for 1 h at 25°C. The mixture was then dialyzed overnight against borate/NaCl (pH 8.4), concentrated and separated on Sepharose 6B.

For conjugation with $NaBH_3CN$, oxidized vesicles (5—10 μmol lipid in 1 ml) were mixed with 10—30 mg IgG in 0.5 ml borate/NaCl as above. 10 μl of 2 M $NaBH_3CN$ were added per ml of reaction mixture which was left 2–14 h at room temperature prior to separation. For some experiments, coupling was allowed to proceed at 4°C for 24 h. To achieve high protein concentrations in the reaction mixture, some samples were concentrated prior to addition of $NaBH_3CN$; alternatively, solid IgG was dissolved in the oxidized vesicle solution prior to reduction.

Column chromatography. The crude bovine γ-globulins contained appreciable quantities of high molecular weight material which co-chromatographs with liposomes on Sepharose 6B. These preparations were routinely purified on Sepharose 6B prior to use. The main IgG peak was collected, concentrated on an Amicon PM10 membrane and stored at 4°C for up to 3 days before use. When re-chromatographed no high molecular weight material eluted in the void volume. Vesicles were separated from unbound IgG on a 1.6 × 60 cm Sepharose 6B column. The sample was applied in 1 ml and eluted at a static

head of 40 cm water. Vesicles eluted in the void volume; 3.8 ml fractions were collected.

Dextran gradients. The vesicle/protein mixture (0.5 ml) was thoroughly mixed with 1 ml dextran (20%, w/v) in 20 mM borate, 120 mM NaCl (pH 8.4), placed in a 5 ml cellulose nitrate centrifuge tube, and 3 ml of 10% (w/v) dextran were layered over, followed by 0.5 ml buffer. Upon centrifugation at 45 000 rev./min for 15 min in a Beckman SW 50.1 rotor, the vesicles floated to the buffer/10% dextran interface leaving the protein in the lower dextran layer. Fractions were taken starting at the top, by careful aspiration with a pasteur or micropipette. After removal of the buffer, the vesicles were removed in the minimum volume possible (usually 0.5 ml). The rest of the gradient was removed in 1 ml fractions to check that the separation was complete. In many instances, the vesicles were separated on two successive gradients; after the first gradient, the vesicles were removed in 0.5 ml and remixed with 20% dextran.

Estimation of protein and lipid. Liposome-bound IgG was measured by using the method of Lowry et al. [13] in the presence of sodium deoxycholate. A 200 μl portion of the sample was mixed with 60 μl of 20% (w/v) sodium deoxycholate prior to the addition of 1 ml folin reagent and 100 μl of diluted phenol reagent. Column fractions were measured undiluted, but samples from dextran gradients were diluted 4—20-fold prior to measurement. Gradient samples containing less than 2.5% (w/v) dextran produced a colourless turbidity, while 2.5% dextran produced 15% of the colour production of a 200 μg/ml IgG solution. The latter was compensated for with appropriate blanks, and samples requiring more than 4-fold dilution were diluted with 2.5% dextran. Carboxyfluorescein did not interfere with the folin assay, although these samples had a greenish caste. Lipid concentrations in vesicle preparations were measured by phosphorus determination according to the method of Bartlett [14] with minor modifications. Glycolipids were standardized by gas chromatography [15] of the hexoses or by anthrone determination, prior to vesicle preparation.

Determination of glycolipid oxidation. Vesicles were oxidized by periodate, added in an equal volume of buffered isotonic solution. Portions containing 200 μg glycolipid were removed at intervals and mixed with an equal volume of 0.5 M $Na_2S_2O_5$. Unoxidized samples were prepared by mixing periodate and $Na_2S_2O_5$ prior to vesicle addition. CH_3OH was added to the vesicle preparations and the mixture was taken to dryness with N_2. The lipid mixture was hydrolyzed for 18 h at 80°C with 0.75 M anhydrous methanolic HCl and trimethylsilyl derivatives of the methylglycosides were prepared as previously described by Macher et al. [15]. A Bendex 2500 gas-liquid chromatograph (Ronceverte, VW) equipped with a glass column containing 3% SE-30 (Supelco Co., Bellefonte, PA) was used for analysis (temperature programmed from 140 to 200°C at 3°C/min). Vesicles without added glycolipid were analyzed to ensure that other lipid components did not cochromatograph with the trimethylsilyl glycosides.

Encapsulation and efflux of carboxyfluorescein. Vesicles were prepared in 10 mM borate, 30 mM carboxyfluorescein (pH 8.4) as described above. They were desalted on Sephadex G75 equilibrated with 10 mM borate, 60 mM

NaCl (pH 8.4). The carboxyfluorescein content of the vesicles was determined spectrophotometrically after dilution with buffer containing 0.2% (w/v) Triton X-100, assuming a molar extinction coefficient of $E^{1\,cm}_{493\,nm} = 70\,000$ $M^{-1} \cdot cm^{-1}$. Efflux rates were measured by placing 0.5 ml vesicle solution containing approx. 0.1 μmol carboxyfluorescein in dialysis sacs, and dialysing them against successive 5-ml portions of buffer.

Oxidation of glycerol 1-phosphate. Glycerol 1-phosphate was chosen to monitor periodate oxidation of encapsulated material for the following reasons: It is water-soluble, it bears a phosphate group which provides a means for its measurement, and upon oxidation it releases 1 mol/mol HCHO, which can conveniently be measured by the chromotropic acid method [16]. 100 mM glycerol 1-phosphate was prepared in either 10 mM sodium acetate or 10 mM sodium borate and adjusted to pH 5.5 in acetate or 8.4 in borate. Vesicles encapsulating glycerol 1-phosphate were prepared from phosphatidylcholine : cholesterol (50 : 50) * or lactosylceramide : phosphatidylcholine : cholesterol (10 : 45 : 45). Phosphatidylcholine : cholesterol (50 : 50) vesicles were desalted on Sephadex G75 equilibrated with isotonic buffered saline, while the flocculant lactosylceramide : phosphatidylcholine : cholesterol (10 : 45 : 45) vesicles were dialyzed against four changes of isotonic buffer. The vesicles were oxidized with isotonic periodate in the dark, as indicated above. 50-μl aliquots were removed at intervals and mixed with 50 μl of 0.5 M $Na_2S_2O_5$ to stop the reaction. The HCHO released by periodate oxidation was measured by addition of 2 ml chromotropic acid reagent prepared according to the method of McFadyen [16] and the tubes were capped and placed in a boiling water bath for 30 min. Samples were cooled, mixed with 0.5 ml of 12% (w/v) thiourea, and centrifuged at 600 × *g* for 10 min to separate out an oily material. The clear purple solution was removed from below and its absorbance was determined at 570 nm. The measurement was standardized with periodate-oxidized samples of ethylene glycol [17]. Efflux of glycerol 1-phosphate was also measured by dialysing 0.5 ml samples of the vesicles against 5 ml isotonic buffer. The dialysate was changed every hour and glycerol 1-phosphate was measured by its phosphate content. The glycerol 1-phosphate and phospholipid content of the vesicle preparations were determined by phosphate analysis of the appropriate phases [18] of extracted samples. The phosphate in the polar CH_3OH/H_2O phase was taken to represent glycerol 1-phosphate, and that in the $CHCl_3$ phase the phospholipid. All samples were evaporated to dryness in an oven at 60°C prior to digestion for phosphate analysis.

Results

Protein coupling and separation

Fig. 1A shows the separation on Sepharose 6B of vesicles composed of phosphatidylcholine : lactosylceramide (91 : 9) conjugated with IgG. The vesicle fractions contain appreciable amounts of protein in a constant ratio to

* All lipid compositions are expressed as mol ratios, e.g., phosphatidylcholine : phosphatidylglycerol (50 : 50).

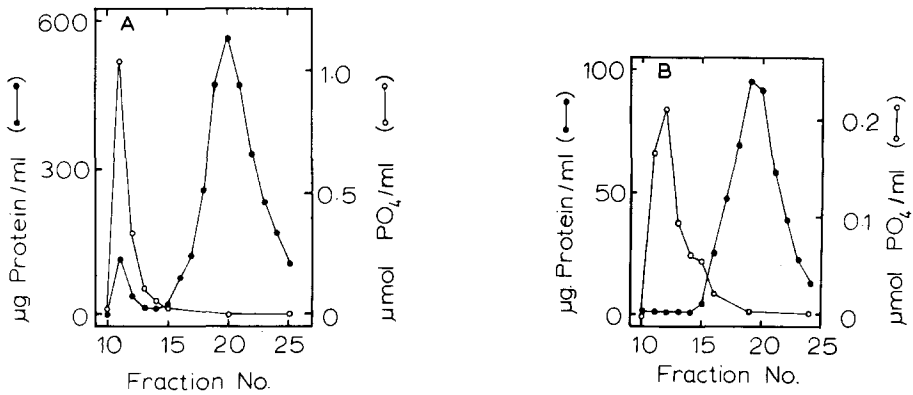


Fig. 1. (A) The separation of IgG-conjugated liposomes from unbound IgG. Coupling was performed by the $NaBH_4$ method using phosphatidylcholine : lactosylceramide (91 : 9) vesicles. The reaction mixture contained 3.6 μmol lipid/ml and 7.1 mg protein/ml. The protein : lipid ratio of the product is 112 μg/μmol lipid. (B) The separation of non-conjugated liposomes from unbound IgG. 2.8 μmol lipid/ml was incubated with 7.1 mg protein/ml with $NaBH_4$. The vesicles were not oxidized and the protein : lipid ratio of the product was 5 μg/μmol.

lipid (112 g/mol). The unbound IgG elutes closely after the vesicles, but the presence of a separate vesicle-associated protein peak demonstrates that protein is bound to the vesicles. Fig. 1B shows a 'control' separation in which the reactants were exposed to all coupling conditions except periodate oxidation. There is very little IgG in the vesicle peak (fraction 12 contains 5 g/mol) and the protein : lipid ratio is not constant. These two elution profiles demonstrate that IgG can be coupled to liposomes by this method.

TABLE I

THE SEPARATON OF VESICLES AND PROTEIN ON DEXTRAN GRADIENTS

Fraction numbers were as follows: 1 = buffer; 2 = buffer — 10% (w/v) dextran interface (vesicles); 3 = 10% (w/v) dextran I; 4 = 10% (w/v) dextran II; 5 = 14% (w/v) dextran I (residual protein); 6 = 14% (w/v) dextran II (residual protein). Complete reaction: vesicles were composed of phosphatidylcholine : cholesterol : lactosylceramide : phosphatidylglycerol (44 : 44 : 9 : 3) and were oxidized at pH 5.5. Coupling was performed with $NaBH_3CN$. The initial protein concentration was 3.98 mg/ml and the efficiency of coupling =25.9. Control was identical to the complete reaction except for the omission of $NaBH_3CN$. The protein concentration was 5.8 mg/ml in the reaction mixture, and the efficiency =1.62.

Fraction number	Protein (μg/ml)	Lipid (μmol/ml)	Protein : lipid ratio (g/mol)
Complete reaction			
1	0	0	—
2	1070	10.37	103
3	0	0	—
4	0	0	—
5	84	0	—
6	92	0	—
Control			
1	0	0	—
2	90	9.54	9.43
3	0	0	—
4	0	0	—
5	124	0	—
6	180	0	—

We have investigated another method of separation of unbound protein based on flotation in discontinuous dextran gradients as a more convenient alternative to gel chromatography. Upon centrifugation, the vesicles float out of the bottom layer through the middle band and form a tight layer at the boundary between 10% (w/v) dextran and buffer. IgG has proved more difficult to separate on dextran than other proteins because it precipitates in dextran. On the first gradient some protein migrates into the middle dextran layer, but a second gradient completes the separation. Table I shows the distribution of protein and lipid in the gradient for a 'coupled' and a control sample. The coupled sample contains appreciable amounts of vesicle-associated protein which floats with the lipid. No protein is present in the 10% dextran layer, and the protein : lipid ratio in the vesicle fraction is 103 g/mol. The control shows a similar pattern, but the protein : lipid ratio in the vesicle fraction is significantly lower. The vesicle fraction contains 9.4 g/mol which is comparable to samples separated on Sepharose 6B. Typically, the recovery of lipids in the vesicle fraction is quantitative; no lipid is detectable in other fractions. We developed the gradient method for separation of dense macromolecules from liposomes, but small amounts of low molecular weight markers such as carboxyfluorescein can also be separated. This separation method is fast and readily applicable to multiple samples. Substances which are difficult or impossible to separate from vesicles by gel chromatography (DNA, ferritin, etc.) can be resolved easily on dextran gradients. Ficoll 400 may also be used, but it produces sufficient colour in the folin assay to make protein determination impossible. We have recently examined metrizamide as a gradient material, and it appears more useful than dextran, since it does not cause protein precipitation or vesicle flocculation.

Conditions for protein coupling

Table II summarizes our experiments on the $NaBH_4$ coupling of IgG to vesicles composed of phosphatidylcholine : lactosylceramide (91 : 9). The nonspecific binding of IgG to vesicles is typically 5–10 g/mol lipid. For coupled samples, ratios between 35 and 134 g/mol are observed. The final ratio is dependent upon the protein concentration, suggesting that we have not achieved the saturation binding previously observed for peroxidase coupling to small unilamellar vesicles [6]. The coupling efficiency (the ratio of coupled protein/mol lipid : protein concentration in reaction mixture) is fairly constant for all protein concentrations used, suggesting that the final protein : lipid ratio is linearly related to the initial protein concentration and can be maximized by using high protein concentrations. The lipid concentration in the reaction mixture affects the percent of protein binding to vesicles, although it does not affect the protein : lipid ratio of the final product.

Table II shows some typical values for protein coupling with the $NaBH_3CN$ protocol. This protocol successfully links protein to liposomes and the results show similar characteristics to those obtained with the $NaBH_4$ protocol. Controls show a nonspecific association of 9 g/mol. The amount of protein coupled to vesicles is dependent on the protein concentration in the reaction mixture over the range we have used, and 2—17% of the protein has been coupled. $NaBH_3CN$ coupling is less efficient than $NaBH_4$ coupling but has certain advan-

TABLE II

IgG-VESICLE COUPLING TO LACTOSYLCERAMIDE-CONTAINING VESICLES

All $NaBH_4$-coupled vesicles were phosphatidylcholine : lactosylceramide (91 : 9) and were oxidized at pH 5.5 Coupling was performed as described in Methods and all separations performed on Sepharose 6B. All $NaBH_3CN$-coupled vesicles were phosphatidylcholine : phosphatidylglycerol : lactosylceramide (87 : 4 : 9) and were oxidized at pH 5.5. Coupling was performed as described in Materials and Methods and vesicles were separated either on Sepharose 6B or on dextran gradients. Efficiency = protein : lipid (g/mol)/initial protein concentration (mg/ml). % bound protein calculated as (protein : lipid ratio) X (initial lipid concentration)/(initial protein concentration) X 10.

Coupling conditions		Extent of coupling		
Lipid (μmol/ml)	Protein (mg/ml)	Protein:lipid (g/mol)	Efficiency	% bound protein
$NaBH_4$ Coupling				
1.26	2.86	31.8	11.13	1
6.64	3.17	37.8	11.89	8
1.26	5.71	74.5	13.06	1.8
2.95	5.71	86.36	15.12	4.5
3.62	7.14	101.81	14.26	5.2
1.26	8.57	104.55	12.20	15
2.95	8.57	121.81	14.21	4.2
2.79 [a]	7.14	10.45	1.46	0.4
$NaBH_3CN$ Coupling				
4.83	4.61	24.54	5.32	2.6
8.29	7.78	54.95	7.06	5.9
29.87	14	79.27	5.66	16.9
12.99 [b]	5.8	9.4	1.62	0.2
6.9 [c]	2.5	34	13.6	9.4

[a] Control experiment. Vesicles were not oxidized.

[b] Control experiment. Vesicles oxidized but $NaBH_3CN$ omitted. Vesicle composition phosphatidylcholine : cholesterol : lactosylceramide : phosphatidylglycerol (44 : 44 : 9 : 3).

[c] Vesicles were oxidized at pH 8.4.

tages. Preincubation of the protein and the vesicles at high pH is not required because $NaBH_3CN$ is stable at neutral pH, and can only reduce the Schiff base linkages and not the aldehydes [19]. $NaBH_3CN$ treatment is also less denaturing to proteins than $NaBH_4$ treatment because disulphide bonds are not reduced [9].

Conditions of oxidation

Periodate oxidation of vicinal hydroxyls proceeds most rapidly under acidic conditions [20], although the oxidation of the glycolipid with $NaIO_4$ at pH 8.4 also permits protein coupling (Table II). Oxidation of the vesicles at 0°C reduces the subsequent coupling efficiency by 50%, but coupling at 0°C for 15 h does not reduce the efficiency of protein binding (not shown).

Variations in lipid composition

Cholesterol may be included in vesicles without appreciably affecting the efficiency of coupling. This is advantageous since cholesterol addition to vesicle membranes reduces membrane permeability and increases vesicle stability in vivo. Glycolipids other than lactosylceramide may also be used for protein coupling (Table III). Vesicles containing 9 mol% globotriaosylceramide

TABLE III

IgG-VESICLE COUPLING USING VARIOUS LIPIDS

All vesicles were oxidized for 30 min at pH 5.5 and were coupled with $NaBH_3CN$. Efficiency was calculated as defined in Table II.

Glycolipid	Protein : lipid ratio (g/mol)	Efficiency
Galactocerebroside [a]	37.7	2.3
Galactocerebroside [b]	67	4.5
Lactosylceramide [c]	55	7.1
Globotriaosylceramide [d]	51.8	16.3
Gangliosides (mixed) [e]	355	35.5

[a] Vesicles prepared from phosphatidylcholine : phosphatidylglycerol : cholesterol : galatocerebroside (44 : 4 : 36 : 16).
[b] Vesicles prepared from phosphatidylcholine : phosphatidylglycerol : cholesterol : galactocerebroside (26 : 7 : 33 : 33).
[c] Vesicles prepared from phosphatidylcholine : lactosylceramide (91 : 9).
[d] Vesicles prepared from phosphatidylcholine : globotriaosylceramide (91 : 9).
[e] Vesicles prepared from phosphatidylcholine : gangliosides (91 : 9).

give protein coupling with comparable efficiency to lactosylceramide, and vesicles containing 9 mol% ganglioside couple protein with an efficiency superior to that of comparable lactosylceramide preparations. Vesicles containing 10 mol% galactocerebroside do not couple proteins, although at 20–33 mol% galactocerebroside appreciable coupling occurs. Galactocerebroside has only one hexose per molecule, which may explain the lower coupling efficiency. The above data suggest that all glycolipids with unsubstituted vicinal hydroxyl groups may form a reactive intermediate for protein coupling.

Phosphatidylglycerol should also be an appropriate lipid for protein coupling, although our results suggest that it is only minimally effective. The $NaBH_4$ procedure produces negligible protein-vesicle association in both control and coupled samples. The $NaBH_3CN$ protocol appears to induce more coupling (47 g/mol), but nonspecific binding appears higher with this protocol, and it is difficult to assess the contribution of covalent and noncovalent binding in these samples. It is likely that the high nonspecific binding is produced by the electrostatic interaction of the protein with the acidic phospholipid.

Retention of encapsulated carboxyfluorescein

It is clearly desirable that the binding of IgG to the liposome membrane should not cause the leakage of encapsulated compounds, which does occur when heat-aggregated IgG binds to liposome membranes [21]. Table IV summarizes data on the encapsulation, retention, and leakage rates of carboxyfluorescein from vesicles of various compositions. The capture of aqueous volume, expressed in l/mol total lipid, is similar for the three compositions used, and compares well with previously pubished data for similarly prepared vesicles composed of phosphatidylglycerol : phosphatidylcholine : cholesterol (10 : 40 : 50) [12]. This high capture suggests that these vesicles are predominantly unilamellar, and negatively stained electron-microscopic preparations reveal vesicles with diameters in the range of 0.2 μm (not shown).

TABLE IV

THE RETENTION AND EFFLUX RATES FOR CARBOXYFLUORESCEIN FROM VESICLES DURING OXIDATION AND PROTEIN COUPLING

The captured volumes for these vesicles were: (a) 2.99 l/mol, (b) 2.67 l/mol, (c) 2.50 l/mol. The carboxyfluorescein capture is calculated from the ratio of carboxyfluorescein to lipid phosphorus. After 4 h of dialysis, 0.5 ml portions were dialyzed for 16 h against 5 ml of buffer. The efflux rate per h is calculated from the leakage during the 16 h dialysis.

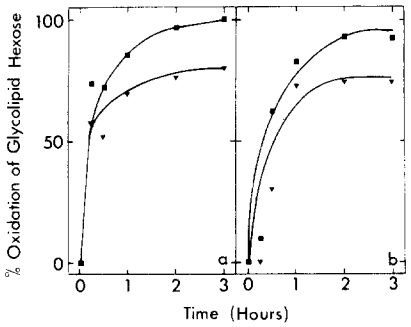
Lipid composition	Treatment	Carboxyfluorescein captured [d] (% of original)	Efflux rate (% total per h)
(a) Phosphatidylglycerol : phosphatidylcholine: cholesterol (10 : 40 : 50)	Untreated	100	0.03
	Oxidized	99	0.04
(b) Lactosylceramide : phosphatidylglycerol: phosphatidylcholine : cholesterol (10 : 4 : 43 : 43)	Untreated	100	0.41
	Oxidized	87.4	0.10
	Protein-coupled	85	0.11
(c) Gangliosides : phosphatidylcholine: cholesterol (10 : 45 : 45)	Untreated	100	0.13
	Oxidized	100	0.09
	Protein-coupled	100	0.08

The efflux rate of carboxyfluorescein from phosphatidylglycerol-containing vesicles is approx. 0.03% per h. Oxidation and protein coupling do not alter the rate of efflux and the carboxyfluorescein : lipid ratio also remains unaltered throughout the process, suggesting a quantitative retention of encapsulated carboxyfluorescein. Vesicles composed of ganglioside : phosphatidylcholine : cholesterol (10 : 45 : 45) similarly exhibit a low efflux rate (0.1%/h) which is not altered by oxidation and protein coupling. The encapsulated carboxyfluorescein is also quantitatively retained within the vesicles during oxidation and coupling. Although 1—2% of the encapsulated carboxyfluorescein may be lost, such losses would not be detectable. Vesicles composed of lactosylceramide : phosphatidylglycerol : phosphatidylcholine : cholesterol (10 : 4 : 43 : 43) appear at first more leaky than the other two compositions (approx. 0.4% per h) though after oxidation and coupling, the efflux rate is comparable to that for the ganglioside mixture (approx. 0.1% per h). In consequence, approx. 10% of the contents is lost during the oxidation procedure, although the subsequent leakage is small.

Oxidation of vesicle glycolipid

We have previously reported the oxidation of phosphatidylglycerol at pH 5.5 in reverse-phase evaporation vesicles [12]. Approx. 50% of the phosphatidylglycerol is oxidized within 30 min and the remainder is resistant to oxidation for several hours. We interpreted this as the oxidation of the external surface of the vesicles, the remaining lipid being resistant to oxidation because periodate could not enter the vesicles.

Periodate treatment of phosphatidylcholine : lactosylceramide (91 : 9) vesicles at pH 5.5 (Fig. 2a) oxidizes 74% of the glycolipid galactose in 15 min and 100% in 3 h. Glycolipid glucose is 60% oxidized in 15 min and 80% oxi-



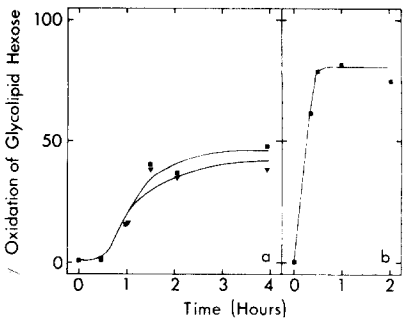


Fig. 2. Periodate oxidation of lactosylceramide-containing vesicles. Vesicles were oxidized with 30 mM $NaIO_4$ in 10 mM acetate, pH 5.5. The extent of glucose (▼——▼) or galactose (■——■) oxidation was measured by determining the residual hexoses as described under Materials and Methods. (a) Vesicles prepared from phosphatidylcholine : lactosylceramide (90 : 10). (b) Vesicles prepared from phosphatidylcholine : cholesterol : lactosylceramide (45 : 45 : 10).

Fig. 3. Periodate oxidation of glycolipid-containing vesicles. Glucose (▼——▼) or galactose (■——■) oxidation was determined from the residual hexoses measured as described in Materials and Methods. (a) Phosphatidylcholine : cholesterol : lactosylceramide (45 : 45 : 10) vesicles oxidized with 7.5 mM $NaIO_4$ in borate, pH 8.4. (b) Galactocerebroside : cholesterol : phosphatidylglycerol (45 : 50 : 5) vesicles were oxidized with 30 mM $NaIO_4$ in acetate, pH 5.5.

dized in 3 h. Whilst 20% of the glucose appears refractory to oxidation, the vesicles seem freely permeable to periodate since all of the glycolipid galactose is available for oxidation. The difference between glucose and galactose oxidation may involve their relative positions in the glycolipid molecule, the glucose being attached to the ceramide. Periodate oxidation of phosphatidylcholine : cholesterol : lactosylceramide (45 : 45 : 10) vesicles at pH 5.5 follows a similar course (Fig. 2b). Oxidation is slower at early times (15–30 min) but by 1 h the extent of oxidation is the same as that for non-cholesterol vesicles. Galactose oxidation is near complete, whilst 25% of the glucose is not oxidized after 3 h. The oxidation of galactocerebroside : phosphatidylglycerol : cholesterol (45 : 5 : 50) vesicles at pH 5.5 (Fig. 3b) shows that 80% of the glycolipid galactose is rapidly oxidized whilst 20% remains unoxidized for up to 4 h. The resistance of 20% of the galactose might be due to a restricted entry of periodate, but is more likely to be comparable to the resistance of lactosylceramide glucose to oxidation at pH 5.5.

Periodate oxidation is known to be slower under alkaline conditions [20], but we have investigated alkaline oxidation since in some circumstances (e.g., the encapsulation of carboxyfluorescein), vesicles cannot be exposed to acidic pH [22]. Oxidation of phosphatidylglycerol : cholesterol (50 : 50) vesicles at pH 8.4 (not shown) is less rapid than at pH 5.5, but the phosphatidylglycerol is 45% oxidized in 2 h and 50% oxidized by 4 h. The remaining lipid requires 170 h for near complete oxidation. Oxidation of phosphatidylcholine : cholesterol : lactosylceramide (45 : 45 : 10) vesicles at pH 8.4 follows a similar course (Fig. 3a), with oxidation 40% complete in 90 min and 50% complete in 4 h. There is little difference between galactose and glucose oxidation, and the close resemblance to phosphatidylglycerol oxidation suggests that periodate cannot permeate these vesicles at pH 8.4. The extent of glycolipid oxidation during the first 4 h (50%) suggests that these vesicles are predominantly uni-

TABLE V

THE RATES OF OXIDATION AND EFFLUX FOR ENCAPSULATED GLYCEROL 1-PHOSPHATE

Oxidation and efflux rates are calculated from the difference in the extent of oxidation between 1 and 4 h after the commencement of treatment. Efflux rates are calculated from a 65 h dialysis of duplicate samples. n.d., not determined.

Lipid composition	pH	Oxidation rate (% per h)	Efflux rate (% per h)
Phosphatidylcholine : cholesterol (50 : 50)	5.5	3.81	n.d.
	8.4	0	n.d.
Lactosylceramide : phosphatidylcholine : cholesterol (10 : 45 : 45)	5.5	2.5	0.12
	8.4	0	0.08

lamellar, which agrees with other evidence mentioned above. This also suggests that the lipid distribution within the membrane is symmetric. Further characterization of the glycolipid distribution and vesicle morphology is in progress.

Oxidation of vesicle contents

Entry of periodate into vesicles can also be examined through the oxidation of an encapsulated marker such as glycerol 1-phosphate. Glycerol 1-phosphate encapsulated in phosphatidylcholine : cholesterol (50 : 50) vesicles is not oxidized at pH 8.4 during the first 4 h of exposure to periodate but at pH 5.5, 3.8% is oxidized per h (Table V). Glycerol 1-phosphate encapsulated in vesicles composed of lactosylceramide : phosphatidylcholine : cholesterol (10 : 45 : 45) at pH 5.5 is oxidized at 2.5% per h, indicating that periodate influx into these vesicles occurs (Table V). At pH 8.4, glycerol 1-phosphate oxidation is not detectable during 4 h of exposure to periodate, suggesting again that periodate does not enter vesicles at pH 8.4. The efflux of glycerol 1-phosphate from lactosylceramide : phosphatidylcholine : cholesterol (10 : 45 : 45) vesicles is extremely low at pH 5.5 and 8.4, demonstrating that its oxidation is due to periodate influx and not to glycerol 1-phosphate efflux. These observations appear to confirm the more rapid influx of periodate into vesicles under acidic conditions, which was suggested by our experiments on glycolipid oxidation.

Discussion

The mechanism of conjugation via aldehyde reaction with amino groups (Fig. 4) initially involves the reversible formation of a Schiff base (II) via a hydrated intermediate (I). Schiff-base formation is only appreciable at pH 9.5 or higher, and the reactants become irreversibly linked if II is reduced to an amine which occurs upon additon of $NaBH_4$ or $NaBH_3CN$. $NaBH_4$ is stable only at alkaline pH and can reduce aldehydes to alcohols although reduction of the imine is more rapid. $NaBH_3CN$ is stable and can reduce imines at neutral pH, while it reduces aldehydes only at pH 2 or lower [19]. These factors determine the precise sequence of our two coupling methods. $NaBH_4$ coupling is achieved by an incubation at a high pH to promote imine forma-

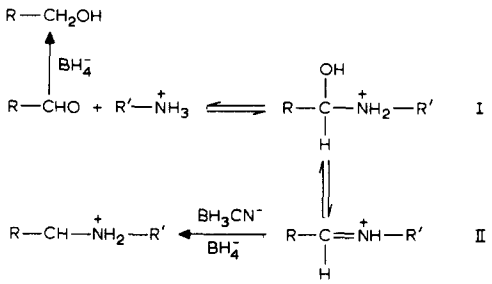


Fig. 4. The reaction of aldehydes with amino groups.

tion, with subsequent reduction at alkaline pH. $NaBH_3CN$ coupling is achieved by a single incubation at neutral pH. The imine concentration is negligible, but those present are rapidly reduced, thereby forcing the reaction to completion. Reduction at neutral pH is made possible by the stability of $NaBH_3CN$. Coupling can occur via a primary or secondary amine, since either can form an imine. Upon reaction, primary amines become secondary amines, and secondary amines become tertiary amines, which do not react further because imine formation is impossible. Lysine residues, the principal amine involved in this protein coupling, may therefore form a double link with the glycolipid molecules. While several methods have previously been described for antibody coupling to vesicles [2,3], the procedure described here is superior in several respects. Protein coupling is efficient and produces antibody-coated vesicles with the highest protein : lipid ratio so far reported. Unlike those methods which employ carbodiimide, glutaraldehyde or diethylsuberimidate, this procedure is not prone to artefacts arising from the polymerization of either proteins or liposomes. Importantly, the quantitative retention of encapsulated materials, together with the low permeability of the vesicles after protein conjugation which we have observed with this procedure, are key factors in the development of antibody-mediated intracellular delivery of vesicle contents.

The choice of either the $NaBH_3CN$ or $NaBH_4$ coupling procedure depends upon the requirements of the particular experiment. We first used $NaBH_4$, since we had previously used it for coupling horseradish peroxidase to liposomes [6], and coupling with $NaBH_4$ appears to be more efficient than with $NaBH_3CN$. With the $NaBH_3CN$ procedure, the reactants are not exposed to alkaline conditions, which may denature proteins and hydrolyse phospholipids, and it is not necessary to change the pH which may cause leakage of vesicle content by osmotic swelling. $NaBH_3CN$ does not reduce disulphide bonds at concentrations at which $NaBH_4$ would [9]. Rabbit, bovine and mouse IgG, and rabbit $F(ab')_2$ can be coupled efficiently to vesicles by these methods, which should apply to any protein bearing sufficient free amino residues. We have used glycolipid vesicles bearing a negative charge, though if a positive charge were required to prevent electrostatic vesicle-protein interactions, this could be achieved with quaternary ammonium compounds such as didodecyldimethylammonium bromide, or tertiary amines.

It is important to consider the efficiency of such coupling methods in

terms of the maximum amount of protein which can be linked to vesicles. We have previously performed such calculations for peroxidase coupled to homogeneous populations of small unilamellar vesicle [6], though for more heterogeneous vesicles such calculations are more difficult. If we assume that reverse-phase evaporation vesicles are unilamellar [10], then 50% of the lipid surface area should be available to couple protein. The amount of protein that can be coupled can be approximated from the surface area per mol lipid and the Stokes' radius of the protein. If we assume an area per lipid molecule of 70 $Å^2$ and a Stokes' radius of 4.47 nm for IgG (horse) [23], then 836 g/mol could be linked to large unilamellar vesicles. Experimentally, we have observed as much as 355 g/mol which is approx. 42% of our theoretical figure and corresponds to 700 molecules per 0.2 μm vesicle. We commonly couple 100 μg antibody per μmol of lipid which for 0.2 μm unilamellar vesicles corresponds to 200 molecules per vesicle.

The coupled protein : lipid ratio appears substantially affected only by the protein concentration of the reaction mixture. For peroxidase coupling to small unilamellar vesicles [6], a cross-linking of vesicles at high protein : lipid ratios was overcome by reducing the lipid : protein ratio and increasing the volume of the reaction mixture. Such cross-linking was presumably due to the collision of vesicle-peroxidase conjugates with other vesicles, permitting the attachment of peroxidase molecules to more than one vesicle. Since the vesicles used in this study are so much larger, the likelihood of such cross-linking is much smaller. High lipid concentrations could be used to increase the percentage of protein coupled, without promoting undesirable cross-linking. This may be important for valuable proteins, and our maximum observed coupling of 20% of the antibody to the vesicles might be increased.

All the glycolipids we have used appear to be useful for this coupling method, and others should also be appropriate. The mole fraction of glycolipid required to produce coupling seems to relate inversely to the number of aldehydes produced per molecule by oxidation. Galactocerebroside produces coupling only if at least 20 mol% is present in the membrane. Lactosylceramide, trihexosylceramide and gangliosides all produce efficient coupling when 9 mol% is in the membrane. Phosphatidylglycerol produces relatively low coupling even with 50 mol% in the membrane. The accessibility of the aldehydes for effective coupling may be a factor, which on molecules such as lactosylceramide will extend away from the bilayer and be more accessible for interaction with proteins in solution. Nonspecific binding of IgG to phosphatidylglycerol vesicles has made it difficult to assess how much protein is covalently linked, although phosphatidylglycerol may yet prove useful for coupling proteins which do not bind nonspecifically to negative vesicles at neutral pH. Phosphatidylglycerol vesicles are oxidized asymmetrically under all conditions used which makes them an attractive prospect for protein coupling.

Neutral vesicles appear more permeable to periodate at pH 5.5 than at 8.4, while negatively charged vesicles are highly impermeant to periodate under all conditions examined. The periodate anion bears a single negative charge at pH 5.5 and one to two negative charges at pH 8.4 [20], which may explain these observations. One would expect negatively charged membranes

to be impermeant to anions, and the acquisition of an extra charge would also be expected to decrease the ability of periodate to permeate neutral membranes. If conditions are required which permit glycolipids in the outer surface of vesicles to be oxidized by periodate while encapsulated markers are protected, the vesicles should contain 50 mol% cholesterol and be oxidized for approx. 2 h at pH 8.4. If the influx of periodate into the vesicles is not detrimental, they may be oxidized for shorter periods at pH 5.5, and the resultant coupling will be somewhat more efficient.

Our experiments with encapsulated carboxyfluorescein show that encapsulated materials of low molecular weight may substantially be retained in vesicles during the manipulations required for protein coupling. The covalent attachment of a protein to the membrane does not affect the efflux rate of carboxyfluorescein, nor do any of the procedures cause a transient high leakage of encapsulated material. It is clear that the lipid mixture which we have investigated most extensively, lactosylceramide : phosphatidylglycerol : phosphatidylcholine : cholesterol (10 : 4 : 43 : 43), is more permeable to carboxyfluorescein and periodate than the phosphatidylglycerol-containing vesicles we have examined. Nonetheless, this composition is sufficiently impermeant to periodate and carboxyfluorescein at pH 8.4 to prevent oxidation of the internally located glycolipid and of encapsulated material which remains within the vesicles. The ganglioside-containing vesicles appear less permeant to carboxyfluorescein than the lactosylceramide-containing vesicles of similar composition.

Glycolipid-containing vesicles have received relatively little attention, particularly with reference to their permeability to small molecules. Cestaro et al. [24] have examined the neuraminidase susceptibility of gangliosides in small unilamellar vesicles. They showed that with 20 mol% ganglioside or less, the reaction of phosphatidylethanolamine with picrylsulphonic acid is not complete, showing that these vesicles are impermeant to the membrane probe. They also found that gangliosides are distributed symmetrically in the vesicle membrane. Glycolipid-containing vesicles prepared by the reverse-phase evaporation procedure [10] will require further characterization, though the observations described here suggest that they are very similar to pure phospholipid vesicles prepared by the same procedure.

The availability of a method for oxidizing specifically the lipids located in the outer monolayer of impermeable vesicles provides the opportunity of detailed characterization of the morphology of such vesicles. For example, this method can be used to estimate the percentage of lipid molecules localized in the outer surface of liposomes. This can be useful for determining whether a population of liposomes consists of unilamellar vesicles [12], for estimating the total surface area of a liposome suspension, or for determining asymmetry of an oxidizable lipid molecule across the membrane of a unilamellar vesicle.

Protein coupling to vesicles should not inactivate the proteins concerned. We previously demonstrated that peroxidase was not completely inactivated by coupling to vesicles [6]. More recently, we have found that the anti-fluorescein IgG retains the ability to quench fluorescein after covalent binding to liposomes [25]. We have also examined the interaction of anti-human erythrocyte F(ab') coated vesicles with human erythrocytes [26]. In this case, we have

observed that a high proportion of vesicles (80–100%) binds to the target cells, while control vesicles, coated with non-specific immunoglobulin F(ab') fragments or vesicles without conjugated protein bind much less efficiently (approx. 1%). Moreover, the vesicles appear to bind intact, since vesicle contents also become cell-associated [26]. We believe that this method will prove of considerable value for studies of protein-mediated vesicle targeting to specific cells.

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