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ATTACHMENT TO MEMBRANES OF EXOGENOUS IMMUNOGLOBULIN CONJUGATED TO A HYDROPHOBIC ANCHOR

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Summary. A novel method has been developed for the attachment of exogenous protein to liposomal and other membranes. A new phospholipid-containing alkylating reagent, N-(N$^{\alpha}$-idoacetyl, N$^{\epsilon}$-dansyl lysyl)-phosphatidylethanolamine, was synthesized for conjugation to sulfhydryl groups of proteins. Model experiments were carried out with a Bence-Jones dimer which was reduced to generate one sulfhydryl group per monomer. After alkylation with the reagent the modified protein spontaneously attached to pre-formed liposomes and red cell ghosts. The attachment and accessibility of the protein were demonstrated by the association of the protein with the liposomal fraction in gel filtration and the agglutination of treated vesicles and red cell ghosts with antisera to human λ chain and to the dansyl group.

Liposomal membranes have provided a useful model for the investigation of a variety of membrane processes. In particular, the attachment of ligands to such membranes has allowed the detailed molecular analysis of interactions occurring on membrane surfaces (1,2). The incorporation of proteins into liposomal membranes has, however, been largely restricted to integral membrane proteins (3,4,5,6). More recently a general method for the covalent attachment of protein to the liposomal membrane has been described using α-chymotrypsin and multilamellar liposomes (7). In this report we present a new method for the attachment of exogenous protein to liposomal and other membranes. It was developed for the purpose of attaching immunoglobulins to pre-formed membranes in order to confer specific reactivity on liposomes and mammalian cells. The method is based on the

Abbreviations: DNS, D and dansyl are 5-dimethylamino-naphthalene-1-sulfonyl; t-BOC, t-butyloxycarbonyl; DCCI, dicyclohexylcarbodiimide; PEA, phosphatidyl-ethanolamine; DLPEA, N-(N$^{\epsilon}$-DNS-lysyl)-PEA; iodo-DLPEA, N-(N$^{\alpha}$-iodoacetyl, N$^{\epsilon}$-DNS-lysyl)-PEA; DOL, dioleoylphosphatidylcholine; DML, dimyristoyl-phosphatidylcholine; λ, monomeric light chain derived from dimeric light chain, lambda-type Bence-Jones protein; DPH, diphenylhexatriene.

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coupling to specific sites on the immunoglobulin of a hydrophobic moiety which serves to anchor the protein to the membrane. This moiety is a sulfhydryl-reactive alkylating reagent whose synthesis utilizes N^{ϵ}-(5-dimethylaminonaphthalene-1-sulfonyl)-L-lysine (N^{ϵ}-DNS-lysine) and phosphatidylethanolamine (PEA).

MATERIALS and METHODS: Dipalmitoylphosphatidylethanolamine (PEA), dicyclohexylcarbodiimide (DCCI), dioleoylphosphatidylcholine (DOL), dimyristoylphosphatidylcholine (DML), diphenylhexatriene (DPH) and N^{ϵ}-dansyl lysine were purchased from Sigma (USA). t-Butyloxycarbonyl (t-BOC) azide was a product from Aldrich Chemical Co. [^{3}H] acetic anhydride (400 mCi/mmole) was obtained from New England Nuclear.

A schematic diagram for the synthesis of the alkylating agent N-(N^{α}-iodoacetyl, N^{ϵ}-DNS-lysyl)-PEA (iodo-DPLEA) is shown in Fig. 1. The preparation of N^{α}-t-BOC, N^{ϵ}-DNS-lysine has been described elsewhere (8). The intermediate compound DLPEA was crystallized from ethyl acetate and further purified by passage through a silicic acid column using $CHCl_3$:CH_3OH:H_2O (80:19:1) (CCH) as the solvent. It gave a single fluorescent spot on thin layer chromatography (TLC) with CCH as the solvent (R_f=0.49). Phosphate (9) and fatty acid (10, 11) analyses of the compound revealed a purity of 98-99%. TLC in CCH of the iodo-DLPEA preparation showed the presence of one major fluorescent component (R_f=0.70) that was ninhydrin negative and iodine positive and one minor fluorescent component which was ninhydrin positive and iodine negative. The latter was identified as unreacted DLPEA. The preparation was used without further purification since unreacted DLPEA did not interfere in the alkylation step.

The preparation of the modified immunoglobulin was carried out with a dimeric, lambda-type Bence-Jones protein (λ_2). The interchain disulfide bond of the protein (10 mg) was first reduced with 5 mM dithiothreitol (DTT) in 2 ml of 0.2 M Tris-HCl, pH 8.0 for 1 hour under N_2 at room temperature to produce one sulfhydryl group per chain. The pH of the reaction mixture was lowered to 4.0 with glacial acetic acid and the protein precipitated by addition of a 1% solution of sodium dodecyl sulfate (SDS) in an amount sufficient to give a ratio of protein to SDS of 5:1 (w/w). Excess DTT and other components were removed by washing the ppt with 0.1 N acetic acid and the washed protein dissolved in 2 ml of 0.2 M Tris-HCl, pH 8.0 containing 0.1% SDS. The alkylating agent (iodo-DLPEA) in 0.1 ml ethyl acetate was then added in a mole ratio of 1.5-2 per sulfhydryl group. The reaction was carried out with stirring in the dark for 2 hours at room temperature under N_2. The protein was precipitated by lowering the pH to 3.0 with 1 N HCl. After one washing with 0.1 N acetic acid it was dissolved in 0.06 M Tris-HCl, pH 6.8, 0.1% in SDS. Monomeric DLPEA-conjugated light chain (DLPEA-λ) was separated from dimeric Bence-Jones protein and free alkylating agent by preparative SDS-polyacrylamide gel electrophoresis. The detergent in the DLPEA-λ preparation was removed by dialysis against Rexyn 1-300 resin (Fisher Scientific Co.) for 48 hours. The concentration of SDS in the final preparation was found to be less than 0.005% as measured by the method of Karush and Sonenberg (12).

For preparation of tritiated DLPEA-λ (DLPEA-[^{3}H]λ) the protein was first labeled using [^{3}H] acetic anhydride and purified by gel filtration and dialysis. The reduction and alkylating procedure then followed that given for the DLPEA-λ preparation. Tritiated Bence-Jones monomer ([^{3}H]λ) was prepared by reduction of the tritiated dimer with 5 mM DTT under N_2 at room temperature for one hour, alkylation with 50 mM iodoacetamide and purification by gel filtration.

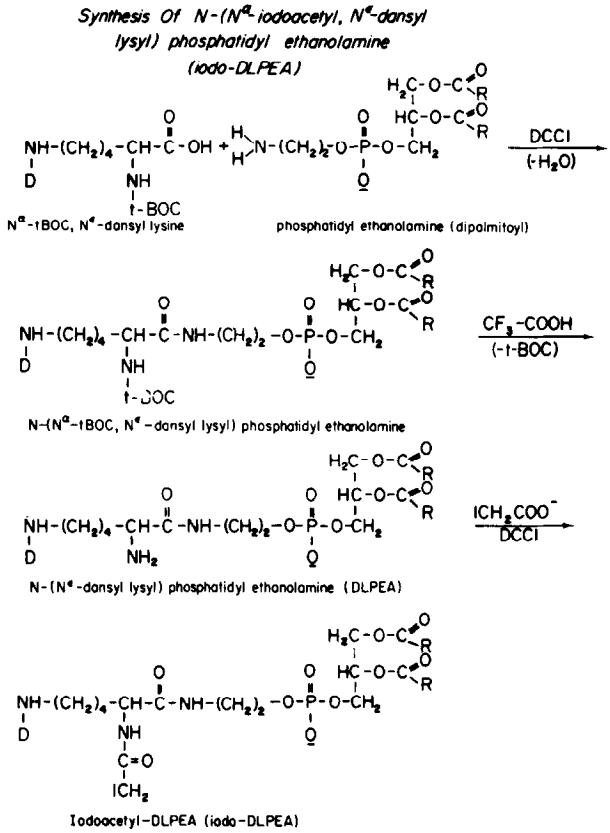

Fig. 1. Series of chemical reactions and intermediates involved in the synthesis of the alkylating reagent iodo-DLPEA.

To prepare liposomes the organic solvent from DOL was evaporated with nitrogen. Lecithin at a concentration of 1 mg/ml was suspended in 0.1 M Tris-acetate, pH 8.1 containing 2 mM EDTA. The suspension was mixed by vortexing for several minutes and then sonicated under nitrogen at room temperature for 3-5 minutes with the microtip of a Branson Sonicator (LS75). The preparation was centrifuged at 14,000 rpm (23,500 g) and the clear supernatant used in the experiment. The attachment of DLPEA-[^{3}H]λ to the membrane of lipid vesicles was studied by incubating 0.5 ml (1 mg lipid/ml) of either dioleoyl phosphatidylcholine (DOL) or dimyristoyl phosphatidylcholine (DML) vesicle preparations with 50 µl of DLPEA-[^{3}H]λ (2×10^{-5} M) or 50 µl of [^{3}H]λ-monomer (2×10^{-5} M) for 24 hours at room temperature. The incubation mixture was then subjected to gel filtration on a Bio-Gel A-5m column (0.8 x 54 cm) equilibrated with 0.1 M Tris-acetate, pH 8.0 containing 0.02% NaN_3. In the case of DOL vesicles gel filtration was done at 4-5°C and N_2 was bubbled through the buffer to avoid oxidation of the lipid. Fractions of 0.4 ml were collected and 0.2 ml of each fraction were mixed with 10 ml Scintisol (ISOLAB) for measurement of the radioactivity in a liquid scintillation counter. Relative concentrations of the lipid in the eluates were measured by adding 0.25 ml of a suspension of diphenyl-hexatriene (DPH) (10^{-4} M) in the same buffer to the remaining 0.20 ml of each eluate. The mixture was incubated for 2 hours at room temperature and then the fluorescence intensity measured (excitation at 357 nm, emission at 420 nm) using a Perkin-Elmer 512 spectro-

fluorimeter. The fluoresence emission of DPH is totally quenched in the aqueous system but reappears as it enters the lipid vesicle membrane (13). In the range of concentrations used in our experiment the fluorescence intensity was found to be linearly related to the concentration of the lipid.

The utility of the DLPEA moiety for attaching immunoglobulins to mammalian membranes was evaluated with human erythrocyte ghosts. These were prepared from freshly drawn blood. Ghosts at a concentration of 5×10^8 cells/ml in PBS were incubated with either DLPEA-λ (0.30×10^{-6} M) or [^{3}H]λ-monomer (0.32×10^6 M) for 48-72 hours at 4-5°C. The ghosts were then centrifuged and thoroughly washed with PBS and finally resuspended in 5 ml of the same buffer. DLPEA-λ on the ghosts was quantitated by measuring the DNS fluorescence at 480 nm (excitation at 340 nm). To quantitate the [^{3}H]λ-monomer bound to the ghosts, 0.25 ml of the washed suspension was mixed with 10 ml of Aquasol-2 (New England Nuclear) and the radioactivity counted in a liquid scintillation counter.

RESULTS and DISCUSSION: The elution profiles of DLPEA-[^{3}H]λ with or without incubation with the liposomal phospholipid are shown in Fig. 2. There was a large shift in the elution pattern of DLPEA-[^{3}H]λ when it was incubated with the DOL vesicles and there was complete overlap of the lipid and protein peaks. The preparation of vesicles, containing 1 mM lecithin, was sufficient to bind almost all of the DLPEA-[^{3}H]λ at a concentration of 10 μM. The virtual irreversibility of the binding was evident from the rechromatography of the liposomal peak since no free DLPEA-[^{3}H]λ was detectable. Similar results were obtained when dimyristoyl lecithin (DML) was used instead of DOL. On the other hand the association with the lipid of the monomer without the DLPEA group ([^{3}H]λ) was practically neligible (Fig. 2). The difference in the elution positions of DLPEA-[^{3}H]λ and [^{3}H]λ is presumably due to the formation of micelles in the former case, estimated to contain about 20 molecules per micelle from its elution position with a calibrated column.

The results for the attachment of DLPEA-λ and [^{3}H]λ-monomer to the ghost membrane are compared in Table 1. It is estimated that as many as 1×10^5 molecules of DLPEA-λ could be attached per ghost compared to 1×10^4 molecules of [^{3}H]λ. The results obtained both with the lipid vesicles and the ghosts demonstrate that the attachment of the protein to the membrane is greatly enhanced by the hydrophobic anchor.

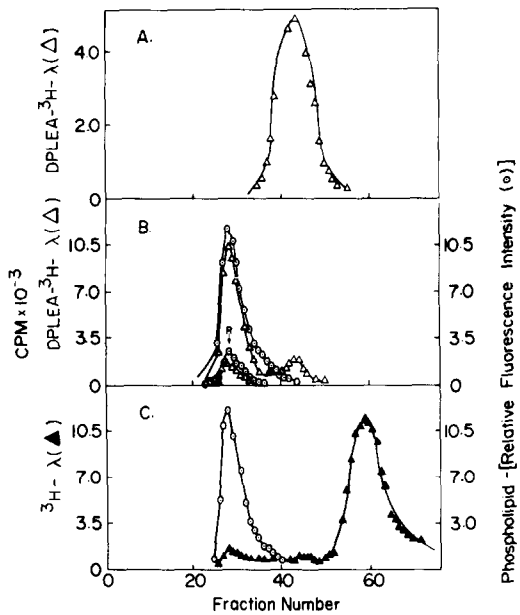

Fig. 2. The attachment of DLPEA-[^{3}H]λ to DOL liposomes. Samples in 0.5 ml of 0.1 M Tris-acetate buffer, pH 8.0 and 2 mM EDTA contained (A) 10 μM DLPEA-[^{3}H]λ, (B) 10 μM DLPEA-[^{3}H]λ and 1 mM phospholipid (liposomal) and (C) 10 μM [^{3}H]λ-monomer and 1 mM phospholipid (liposomal). Samples (B) and (C) were incubated for 24 hours at room temperature before gel filtration. The samples were applied to a column (0.8 x 54 cm) of Bio-Gel A-5m in 0.1 M Tris-acetate buffer, pH 8.0 and 0.02% NaN_3 and fractions of 0.4 ml collected. The elution patterns of the proteins were obtained by assay of radioactivity and those of the phospholipid by fluorescence (see text). The profiles labeled R in panel B are those observed by rechromatography of fraction 28 (peak tube) of the original gel filtration.

Table 1. Attachment of DLPEA-λ and [^{3}H]λ to erythrocyte ghost membranes

Cell Conc. (ghosts/ml)	Incubation period (hrs)	% attached DLPEA-λ	% attached [^{3}H]λ
5×10^8	48	23	4
5×10^8	72	32	4

The total concentrations of DLPEA-λ and [^{3}H]λ in the incubation mixtures were 3.0×10^{-7} M and 3.2×10^{-7} M, respectively. The amount of DLPEA-λ attached was measured by fluorescence (see text) and that of [^{3}H]λ by radioactivity.

The presence of DLPEA-λ on ghosts and lipid vesicles was further confirmed by the agglutination reaction. DLPEA-λ-treated ghosts (2×10^8 cells/ml) were incubated with either rabbit anti-DNS serum (1:10) or rabbit anti-human λ serum (1:10) or normal rabbit serum for 20 minutes at room temperature. Examination under phase-contrast showed the formation of clumps with anti-λ and anti-DNS serum but not with normal rabbit serum. Similarly, DLPEA-λ-treated vesicles (0.5 mM phospholipid) were found to clump when incubated with rabbit anti-human λ serum or rabbit anti-DNS serum but not with normal rabbit serum. Neither untreated nor [^{3}H] λ-treated vesicles exhibited clumping when incubated with either of the antisera.

The method described here for the attachment of immunoglobulins and other proteins to membranes has a variety of potential applications. With antibody molecules or specifically reactive fragments derived from them, for example, liposomes and cells may be directed to interact with target cells exhibiting specific surface antigens, e.g., malignant cells. Thus liposomes containing chemotherapeutic drugs could be targeted for transport of these drugs to a specified cell type (14, 15). The potential feasibility of this technique has been greatly enhanced by the recent developments in hybridoma technology since it is now possible to prepare monoclonal antibody specific for individual antigenic determinants (16). The development of human hybridoma lines would, of course, greatly expand the proposed application of our procedure.

Furthermore, liposomal-bound antibody provides a useful experimental system for the study of the interaction of membrane-bound antibody with monovalent and multivalent ligands and for the evaluation of the dependence of the interaction on fluidity, composition of the membrane and surface density of antibody. Finally, we would suggest that the attachment of exogenous antibody to viable T and B lymphocytes is also feasible. With such surface-modified lymphocytes it may be possible to examine initial events associated with the binding by the natural receptor of its homologous antigen.

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