Immunospecific Targeting of Liposomes to Cells: A Novel and Efficient Method for Covalent Attachment of Fab′ Fragments via Disulfide Bonds†

Francis J. Martin,* Wayne L. Hubbell, and Demetrios Papahadjopoulos

ABSTRACT: An efficient method for covalently cross-linking 50K Fab′ antibody fragments to the surface of lipid vesicles is reported. Coupling up to 600 μg of Fab′/μmol of phospholipid (about 6000 Fab′ molecules per 0.2-μm vesicle) is achieved via a disulfide interchange reaction between the thiol group exposed on each Fab′ fragment and a pyridyldithio derivative of phosphatidylethanolamine present in low concentration in the membranes of preformed large unilamellar vesicles. The coupling reaction is efficient, proceeds rapidly under mild conditions, and yields well-defined products. Each vesicle-linked Fab′ fragment retains its original antigenic specificity and full capacity to bind antigen. We have used Fab′ fragments, coupled to vesicles by this method, to achieve immunospecific targeting of liposomes to cells in vitro. Vesicles bearing antihuman erythrocyte Fab′ fragments bind quantitatively to human erythrocytes (at multiplicities up to 5000 0.2-μm vesicles per cell) while essentially no binding is observed to sheep or ox red blood cells. Vesicle–cell binding is stable over a pH range from 6 to 8 and is virtually unaffected by the presence of human serum (50%). Cell-bound vesicles retain their aqueous contents and can be eluted intact from cells by treatment with reducing agents (dithiothreitol or mercaptoethanol) at alkaline pH.

Lipid vesicles have been used as carriers for delivering normally impermeable molecules to the interior of cells (Papahadjopoulos et al., 1974; Weinstein et al., 1977; Weissmann et al., 1977) and for inserting new components into the surface membranes of cells (Magee et al., 1974; Huang & Pagano, 1975; Martin & MacDonald, 1976a,b; Struck & Pagano, 1980). However, formidable obstacles remain to be overcome before liposome-delivery systems achieve their projected usefulness in biology and medicine. A means of directing vesicles to specific target cells will certainly be required for many clinical applications such as drug delivery. A number of recent studies have focused on the use of macromolecular ligands as a means of promoting specific liposome–cell binding in vitro. Liposomes bearing antigenic lipids (Lesserman et al., 1979), heat-aggregated IgM molecules (Weissmann et al., 1975), noncovalently associated immunoglobulins (Gregoriadis & Neerunjun, 1975; Weinstein et al., 1978; Lesserman et al., 1980), and lectins (Juliano & Stamp, 1976) have been shown to interact selectively with target cells. However, the enhancement of cell binding observed in these studies has been limited; only a small proportion of available ligand-bearing vesicles actually attach to cell surface receptors.

Huang et al. (1980) have demonstrated specific attachment of vesicles bearing anti-H-2 monoclonal antibodies to mouse L-929 cells. In their system, intact antibody is covalently coupled to palmitic acid and the resulting complex incorporated into vesicle membranes during detergent dialysis. Such vesicles are heterogeneous with respect to the number of antibody molecules per vesicle and as a result exhibit a differential capacity to bind to target cells. Moreover, it is difficult to entrap material in the aqueous compartment of vesicles prepared by this method. Heath et al. (1980) have recently devised a method for liposome–protein conjugation which they have used to bind up to 200 μg of rabbit IgG per μmol of vesicle lipid. Coupling antihuman erythrocyte $F(ab')_2$ fragments to preformed vesicles by this method enhances vesicle binding to human erythrocytes 200-fold and results in the nearly quantitative association of vesicles (and their contents) with cells.

In the present study, we describe a novel method for the covalent attachment of Fab′ antibody fragments to the surfaces of lipid vesicles. Fab′ fragments are generated by reduction of the inter-heavy-chain disulfide bond of $F(ab')_2$ fragments (obtained by pepsin digestion of the IgG fraction of whole rabbit serum). As a result of such treatment, each Fab′ fragment contains a single reactive thiol group at a predictable position on the molecule. Coupling is achieved via a disulfide interchange reaction between this thiol group and a sulfhydryl-reactive derivative of phosphatidylethanolamine present at low concentrations in the membranes of large preformed vesicles. The coupling reaction is efficient, and we show that each bound Fab′ molecule is oriented on the vesicle surface in such a way that its antigen binding site is available for interaction with water-soluble haptens. Moreover, we find that vesicles bearing Fab′ fragments raised against specific cell surface antigens bind selectively to their target cell under physiological conditions.

Experimental Procedures

Materials

Transesterified egg phosphatidylethanolamine (PE) was obtained from Avanti Polar Lipids. Phosphatidylcholine (PC) was extracted from egg yolks and purified by using standard techniques (Mayhew et al., 1979). Cholesterol (Fluka) was recrystallized 3 times from methanol. ^{125}I-Labeled (*p*-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester (2000 Ci/mmol), [^{14}C]sucrose, and [^{3}H]dipalmitoylphosphatidylcholine (DPPC) were purchased from New England Nuclear. Thioctic acid, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), 2-thiopyridinone, 2,2′-dithiodipyridine, and carbonyldiimidazole (CDI) were obtained from Aldrich. Rabbit antihuman erythrocyte $F(ab')_2$ fragments and goat antirabbit IgG serum were supplied by Cappell Laboratories and nonspecific rabbit IgG was from Pentex. Rabbit antifluorescein IgG was a gift from Dr. Edward Voss. Human erythrocytes and serum were obtained from fresh whole blood and used within 2 days. Sheep and ox erythryocytes preserved in Alsever's solution were provided by Mi-

† From the Cancer Research Institute (F.J.M. and D.P.) and the Department of Pharmacology (D.P.), University of California, San Francisco, California 94143, and the Department of Chemistry, University of California, Berkeley, California 94720 (W.L.H.). *Received November 14, 1980.* This investigation was supported by Research Grant CA-25526 from the National Cancer Institute.

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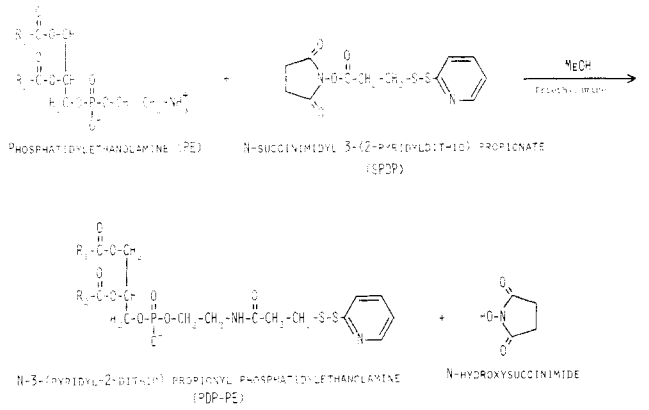
Scheme I: Synthesis of *N*-[3-(Pyridyl-2-dithio)propionyl]phosphatidylethanolamine



crobiological Media Inc. Pepsin, dithiothreitol (DTT), fluorescein, protein A–Sepharose, and dextran T-20 were from Sigma. *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and Sephadex gels were purchased from Pharmacia. Silica gel (Bio-Sil, HA) was purchased from Bio-Rad. All other chemicals were reagent grade. Water was twice distilled, the second time from glass.

Methods

Protein was estimated by the method of Lowry et al. (1951) as modified by Dulley & Grieve (1975). Lipid phosphate was estimated by the calorimetric method of Fiske & SubbaRow (1925). Sulfhydryl groups were determined by the method of Grassetti & Murry (1967) using 2,2′-dithiodipyridine. Thin-layer chromatography of lipid was carried out as described earlier (Mayhew et al., 1979). For the visualization of thiols on chromatographs, a spray containing DTMB was used (Glaser et al., 1970). Fluorescein fluorescence was measured at 515 nm by using a SLM 4000 fluorometer (SLM Instruments, Urbana, IL) at an excitation wavelength of 492 nm.

Synthesis of N-[3-(2-Pyridyldithio)propionyl]phosphatidylethanolamine (PDP-PE). PE (50 μmol) was dissolved in 3 mL of anhydrous methanol containing 50 μmol of triethylamine and 25 mg of SPDP (Scheme I). The reaction was carried out at 25 °C under an argon atmosphere. Following 5 h, TLC of the reaction mixture revealed quantitative conversion of the PE to a faster running product. Methanol was removed under reduced pressure, and the products were redissolved in chloroform and applied to a 10-mL silica gel column which had been activated (150 °C overnight) and prewashed with 100 mL of chloroform. The column was washed with an additional 20 mL of chloroform followed by 20 mL of each of the following chloroform–methanol mixtures 40:1, 30:1, 25:1, 20:1, and 15:1 and, finally, with 60 mL of 10:1 chloroform–methanol. The phosphate-containing fractions eluting in 15:1 and 10:1 chloroform–methanol were pooled and concentrated under reduced pressure.

Analysis by TLC (silica gel H; solvent chloroform–methanol–acetic acid, 60:20:3) indicated a single phosphate-positive, ninhydrin- and sulfhydryl-negative spot. Identification of the product as the (pyridyldithio)propionyl derivative of PE was confirmed by our observation that a stoichiometric amount of 2-thiopyridinone (2-TP) is released upon the addition of excess DTT. No detectable decomposition of PDP-PE was observed for periods up to 6 months when stored in glass ampules under argon at −50 °C.

Preparation of Vesicles. Vesicles were prepared by the reverse-phase evaporation method of Szoka & Papahadjopoulos (1978). Briefly, 10 μmol of cholesterol, 9 μmol of PC, 1 μmol of PDP-PE and a trace amount of [^{3}H]DPPC were dissolved in 1 mL of freshly distilled diethyl ether. Buffer I[1] (pH 6.0) (0.3 mL) was added, and the two phases were emulsified by sonication for 2 min at 25 °C in a bath-type sonicator. Ether was removed under reduced pressure at 30 °C. The resulting vesicle dispersion was extruded through 0.4- and 0.2-μm pore Bio-Rad unipore polycarbonate membranes (Olson et al., 1979). For determination of internal volumes, vesicles were prepared in the presence of 0.3 M sucrose and a trace amount of [^{14}C]sucrose. The internal volume was calculated from the amount of sucrose (specific activity of [^{14}C]sucrose) remaining after removal of unentrapped solute by gel filtration on Sephadex G-25. Sucrose efflux, expressed as the proportion of sucrose remaining entrapped for periods up to 24 h, was determined by dialysis.

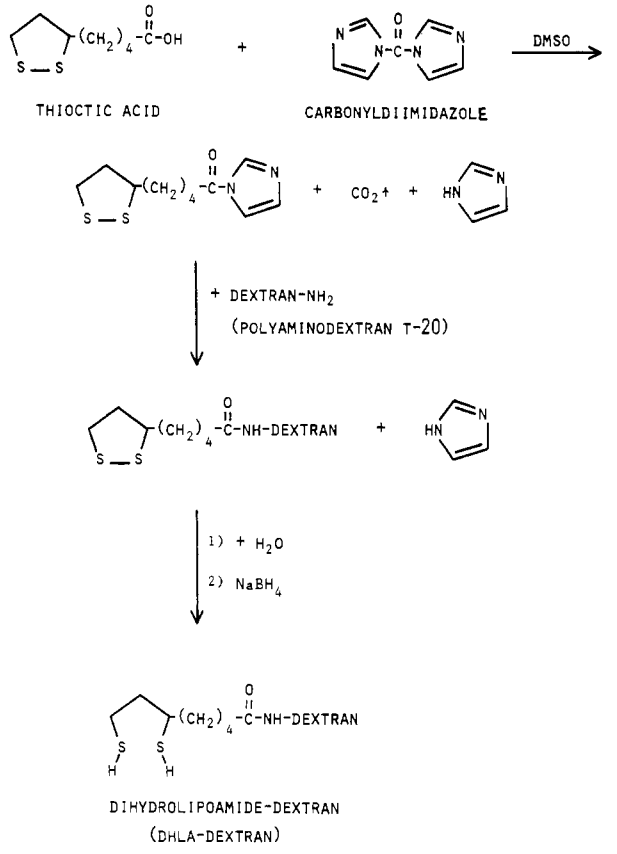
Preparation of Antibody Fragments. Rabbit F(ab′)$_2$ and Fab′ fragments were prepared by using a modification of the method of Nisonoff & Rivers (1961). Pepsin (final concentration 250 μg/mL) was added to a solution of rabbit IgG (10 mg/mL 0.1 M acetate buffer, pH 4.5) and the mixture incubated at 37 °C overnight. The pepsin and other insoluble material were removed by centrifugation (10000 rpm for 20 min). The supernatant was dialyzed against TBS for 18 h and undigested IgG removed by passage through a 5-mL Staph A–Sepharose column at pH 8.0. The resulting F(ab′)$_2$ dimers were further purified by chromatography on a column of Sephadex G-150 preequilibrated with buffer I. The material present in the void volume of such a column was discarded. Fractions containing F(ab′)$_2$ fragments (which elute as a broad peak with the included volume) were pooled and concentrated to 10 mg/mL by using an Amicon ultrafiltration device (YM-10 membrane). Lyophilized antihuman RBC F(ab′)$_2$ fragments were reconstituted with distilled water, dialyzed against buffer I for 12 h, and purified as described above. Antifluorescein F(ab′)$_2$ fragments were prepared by the same methods from immunopurified rabbit antifluorescein IgG.

Nonspecific rabbit F(ab′)$_2$, antihuman erythrocyte F(ab′)$_2$, and antifluorescein F(ab′)$_2$ fragments were radiolabeled with ^{125}I to a specific activity of about 8×10^7 cpm/mg by using the method of Bolton & Hunter (1973). Fab′ fragments were generated from the above F(ab′)$_2$ preparations (10 mg/mL buffer I, pH 5.5) by reduction with DTT (final concentration 20 mM) for 90 min under an argon atmosphere at 25 °C. DTT was removed during chromatography of the reduced fragments on Sephadex G-50 preequilibrated with deoxygenated buffer I (pH 5.5). Fab′ appearing in the void volume of such a column (approximately 2.5 mg/mL) was maintained under argon and used immediately in coupling experiments described below.

Coupling Fab′ Fragments to Vesicles. Vesicles prepared as described above (2–6 μmol of phospholipid in 0.5 mL of deoxygenated buffer I) were mixed with an equal volume of Fab′ fragments (final concentration 1–12.5 mg/mL) under argon. The pH was adjusted to 8.0 with NaOH and the mixture stirred under an argon atmosphere at 25 °C. After 2 h, the mixture was applied to a Sephadex G-150 column (1.5 × 60 cm) preequilibrated with buffer I (pH 6.0). Vesicles, which appear in the void volume, were free of unreacted Fab′ and F(ab′)$_2$ fragments. Protein and phospholipid concentrations were determined directly or calculated from the specific

[1] Abbreviations used: buffer I, 100 mM NaCl, 100 mM borate, 50 mM citrate, and 2 mM EDTA; buffer II, 35 mM NaCl, 100 mM borate, 50 mM citrate, and 2 mM EDTA; TBS, Tris-buffered saline (145 mM NaCl and 10 mM Tris, pH 7.5); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

Scheme II: Synthesis of Dihydrolipoamide-Dextran T-20



activities of ^{125}I-labeled protein and [^{3}H]DPPC, respectively.

Hemagglutination (HA) Titer. The HA titer of liposome dispersions was determined by a modified Salk pattern method (Salk, 1944). Serial dilutions of liposome dispersions (25 μL, buffer II) were made in V-shaped microtiter wells (Falcon Plastics). An equal volume of a 2% suspension of washed human, sheep, or ox erythrocytes in buffer II was added to each well. The lowest concentration of liposomes which produced agglutination, i.e., a positive pattern on the well bottom after 2 h at 25 °C, was scored as 1 HA unit/mL. Duplicate assays gave identical titers.

Cell Binding. A 2% suspension of washed human, sheep, or ox erythrocytes (0.25 mL in buffer II, pH 8.0) was mixed with an equal volume of [^{14}C]sucrose-containing vesicles dispersed in the same buffer. The mixture was allowed to incubate at 25 °C for 30 min with agitation every few minutes. The cells were washed 3 times by pelleting (2000 rpm for 5 min) and resuspended in buffer II (pH 8.0). The final pellet was extracted with chloroform–methanol (Bligh & Dyer, 1959), and the chloroform and aqueous phases were analyzed for radioactivity (^{3}H-labeled phospholipid and [^{14}C]sucrose, respectively).

Synthesis of Dihydrolipoamide–Dextran T-20 (DHLA–Dextran). Thioctic acid (1 g) was mixed with a 20 molar excess of CDI in 1 mL of dimethyl sulfoxide (Me_2SO) and incubated for 3 h at room temperature. Lyophilized polyamine-dextran T-20 (1 g, 6–7 amino groups per molecule) was added and the mixture incubated at 37 °C for 3 h (Scheme II). An equal volume of distilled water was added followed by 5 mL of 2:1 chloroform–methanol. The unreacted CDI remained in the chloroform phase while the derivatized dextran partitioned into the aqueous phase. Me_2SO and low molecular weight contaminants were removed by extensive dialysis against buffer I (5 days, the buffer being changed twice daily). The product was reduced by treatment with sodium borohydride (5 mg/mL) at pH 8.0. Excess $NaBH_4$ was eliminated by shifting the pH to 5.5 for 15 min (sodium borohydride is unstable at acidic pH). The disulfide-reducing capacity of the DHLA–dextran, determined by titration with DTNB (see above), was 40 nmol/μL.

Measurement of 2-Thiopyridinone (2-TP) Release. The concentration of 2-TP was measured spectrophotometrically at 343 nm by using a molar extinction coefficient of 7.06×10^3 (Grassetti & Murray, 1967). 2-TP is difficult to measure in solutions containing high concentrations of vesicles and/or protein. Where such interference was a problem, the protein and/or vesicles were separated from 2-TP by using the following technique: the sample to be tested (0.5 mL) was applied to a calibrated G-25 Sephadex column (10 mL) and the effluent monitored at 343 nm by using a spectrophotometer equipped with a flow cell. The 2-TP concentration was calculated by relating the maximum intensity of the 2-TP absorbance peak obtained for unknown to values obtained when known amounts of 2-TP were chromatographed on the same column.

Electron Microscopy. Vesicles, dispersed in buffer I, were applied to carbon-strengthened formvar grids, stained with 2% sodium phosphotungstate (pH 7.0), and examined in a Phillips 300 electron microscope as described earlier (Martin & MacDonald, 1974).

Results

Characterization of Vesicles. Electron microscopic observations reveal that vesicles composed of PC, cholesterol, and PDP-PE (45:50:5), prepared by the reverse-phase evaporation method and extruded through 0.2-μm pore membranes, are spherical in shape and range in diameter from about 500 Å to 0.5 μm. The vast majority of vesicles, however, fall in the size range of 1000–3000 Å, the mean diameter being about 1900 Å. Occasional multilamellar vesicles are visible in such EM preparations.

The encapsulated volume of such vesicles, calculated from the specific activity of [^{14}C[sucrose remaining associated with vesicles following removal of the unentrapped solute by gel filtration (see Methods), is 4.5 ± 0.3 μL/μmol of vesicle phospholipid, slightly less than the predicted value of 6.4 μL/μmol, assuming that all vesicles are single layered and 0.2 μm in diameter (Enoch & Strittmatter, 1979). The permeability of these vesicles to sucrose was found to be quite low. The rate of sucrose efflux is less than 1%/h at 25 °C.

The low value for sucrose encapsulation together with the EM results suggests that a small proportion of the PC–cholesterol–PDP-PE vesicles used in this study are multilamellar. In order to determine more precisely the average number of lamellae per vesicle, we have synthesized a reducing agent, DHLA–dextran T-20, which cannot permeate vesicle bilayers but is capable of reducing the pyridyl disulfide moiety of PDP-PE molecules that are exposed in the outer monolayer of vesicles. We have measured the appearance of 2-TP, which is released as a product of PDP-PE reduction, to determine the proportion of PDP-PE molecules present in preformed vesicles that are accessible to this impermeable reducing agent. As shown in Figure 1, 54.5 nmol of 2-TP is released within 5 min following the addition of excess DTT (which freely permeates vesicle bilayers) to a suspension of PDP-PE-containing vesicles (0.5 μmol of total phospholipid). This corresponds closely to the expected value of 50 nmol (0.1 mol fraction of the total phospholipid in these vesicles is PDP-PE). However, when the same amount of vesicles is exposed to excess DHLA–dextran T-20, only 17.5 nmol of 2-TP is released. When the DHLA–dextran T-20 is followed by DTT

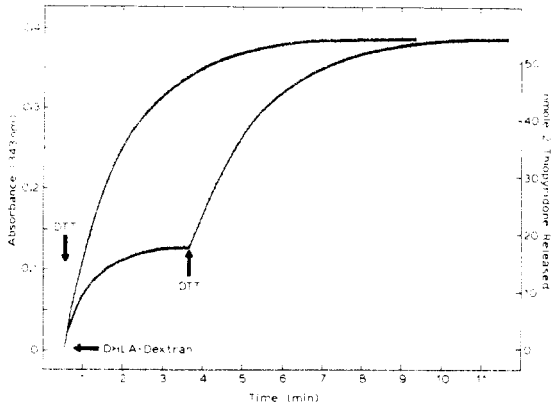


FIGURE 1: Release of 2-thiopyridinone from PDP-PE-containing vesicles following the addition of DTT (upper curve) or DHLA–dextran (lower curve). An excess of either DTT or DHLA–dextran (dissolved in 25 μL of buffer I) was added to a dispersion of PC–cholesterol–PDP-PE vesicles (45:50:5; 1.0 μmol of total lipid in 0.5 mL of buffer I) at time 0. Absorbance was measured at 343 nm. In the case of the lower curve, excess DTT was added after DHLA–dextran-induced 2-TP release had plateaued (approximately 4 min).

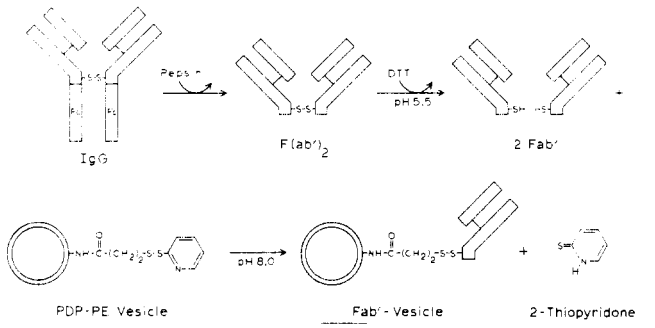


FIGURE 2: Covalent coupling of Fab′ fragments to PDP-PE vesicles. $F(ab')_2$ dimers are prepared by pepsin digestion of the IgG fraction of whole rabbit serum. Fab′ monomers are generated from these by reduction with DTT at low pH. Immediately following the removal of DTT, Fab′ fragments are mixed with PDP-PE-containing vesicles, and the pH is adjusted to 8.0 with NaOH. The mixture is stirred under an argon atmosphere for several hours. Coupling is the result of a disulfide exchange reaction between the thiol group on each Fab′ fragment and the pyridyldithio moiety of PDP-PE molecules present in vesicle membranes. The chromophore, 2-TP, is released as a product of the reaction.

(Figure 1) or sodium cholate (final concentration 2%, not shown in figure), the balance of the 2-TP (37.0 nmol) is released.

Characterization of Fab′ Antibody Fragments. In the following section, we present a method for the covalent attachment of antibody fragments to the surfaces of lipid vesicles. The method depends on the availability of thiol groups on the antibody fragments capable of participating in a disulfide interchange reaction with the (pyridyldithio)propionyl moiety of PDP-PE molecules present in the outer monolayer of preformed vesicles. For minimization of vesicle aggregation due to cross-bridging, a single thiol group per antibody fragment would be desirable. In this section, we described conditions for the preparation of 50000-dalton Fab′ antibody fragments, each of which contains a single reactive thiol group at a defined position on the molecule.

Treatment of rabbit $F(ab')_2$ antibody fragments with DTT (20 mM) at low pH (5.5) for 90 min at 25 °C results in the selective reduction of the inter-heavy-chain disulfide bond of this molecule and thereby produces two monomeric Fab′ fragments (see Figure 2). Titration of Fab′ fragments with Ellman's reagent (Habeeb, 1971) reveals that each monomer produced by this method contains, on the average, 0.95 sulfhydryl group. Gel filtration on Sephadex G-75 indicates that greater than 95% of the $F(ab')_2$ fragments is converted to the 50K Fab′ during such a reduction (not shown). Moreover, when antihuman erythrocyte $F(ab')_2$ fragments are subjected to similar DTT treatment, the capacity of the fragments to agglutinate human erythrocytes is reduced 64-fold (the HA titer of a 10 mg/mL solution falls from 8192 to 128).

Upon the removal of DTT, Fab′ monomers are unstable and tend to re-form $F(ab')_2$ dimers as the result of an oxidative reaction between the sulfhydryl groups exposed on each Fab′ fragment. The rate of $F(ab')_2$ formation (measured as the reduction in the number of titratable thiol groups) is dependent on the pH and the availability of molecular oxygen. Two hours after the removal of DTT, in the absence of O_2, the number of thiol groups per Fab′ monomer is reduced to 0.75 at pH 6.0 and to 0.50 at pH 8.0. In the presence of molecular oxygen, the rate of $F(ab')_2$ formation is accelerated, and essentially complete reannealing is observed within 2 h at pH 8.0.

The hemagglutinating activity of antihuman erythrocyte antibody fragments also reflects the tendency of monomeric Fab′ fragments to spontaneously reanneal following DTT removal. As mentioned above, the HA titer of antihuman erythrocyte $F(ab')_2$ fragments (10 mg/mL) is reduced 64-fold (from 8192 to 128) following reduction with DTT. However, 2 h after the removal of this reducing agent, the HA titer of the same antihuman RBC fragments (maintained under an atmosphere of argon) increases to 1536 at pH 6.0 and 4096 at pH 8.0. In the presence of molecular oxygen, greater than 80% of the original HA activity is regenerated within 2 h following removal of the DTT.

Despite the tendency of Fab′ molecules to recombine into the dimer form at alkaline pH, this competing reaction does not appear to be rate limiting with respect to vesicle coupling. The addition of freshly reduced Fab′ fragments at 30-min intervals during the course of a coupling reaction (see below) does not significantly improve coupling ratios.

Coupling of Fab′ Fragments to PDP-PE-Containing Vesicles. Figure 2 illustrates the protocol we have followed in order to obtain covalent coupling of Fab′ antibody fragments to PDP-PE-containing vesicles. PDP-PE vesicles are mixed with Fab′ fragments (about 3 μmol of phospholipid and 1–12.5 mg of Fab′) immediately following the removal of DTT (see preceding section). The pH is adjusted to 8.0 and the coupling reaction allowed to proceed for 2 h under argon. Unreacted antibody fragments are then removed by gel filtration.

Figure 3A shows the elution profile obtained when a mixture of control vesicles (PC-cholesterol, 50:50) and nonspecific rabbit Fab′ fragments is chromatographed on Sephadex G-150. The vesicles appear in the void volume of such a column while the antibody fragments elute with the included volume. No binding of Fab′ fragments to control vesicles is evident. However, when 5 mol % of PDP-PE is included in the vesicle membrane, a significant proportion (approximately 30%) of the added Fab′ coelutes with the vesicles (Figure 3B). When fractions 15–30 from Figure 3B are pooled, concentrated, and rechromatographed on Sephadex G-150, all of the Fab′ coelutes with the vesicle peak (Figure 4A), indicating a stable association between Fab′ molecules and vesicles. This Fab′–vesicle binding in completely reversible, however, in the presence of 50 mM DTT at pH 8.0 (Figure 4B). These results suggest that Fab′ binding results from the formation of reversible disulfide cross-linkages between Fab′ fragments and vesicles.

Negative-stain electron microscopy revealed little difference in the morphology of Fab′–vesicles compared to the protein-

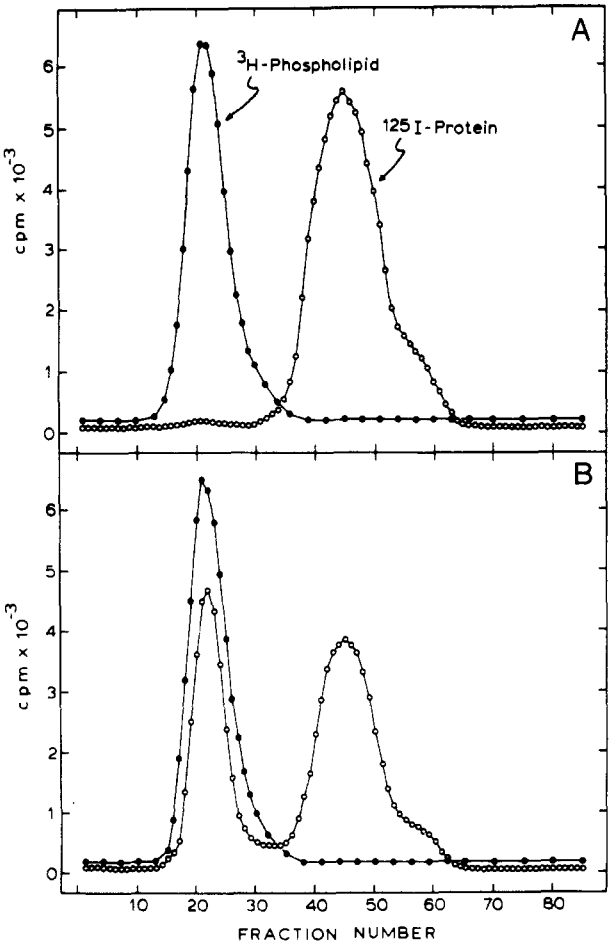


FIGURE 3: Separation of vesicles from unreacted antibody fragments. Control vesicles (PC–cholesterol, 50:50) were mixed with freshly reduced Fab′ fragments and allowed to incubate for 2 h at 25 °C under argon, and the mixture was chromatographed on Sephadex G-150 (A). The vesicles (^{3}H-labeled phospholipid) appear in the void volume while unconjugated antibody fragments (^{125}I-labeled protein) elute with the included volume. No binding of the antibody fragments to such control vesicles is detectable. The elution profile in panel B is for vesicles containing 5 mol % PDP-PE treated as in (A). In this case, about 30% of the initial Fab′ coelutes with vesicles.

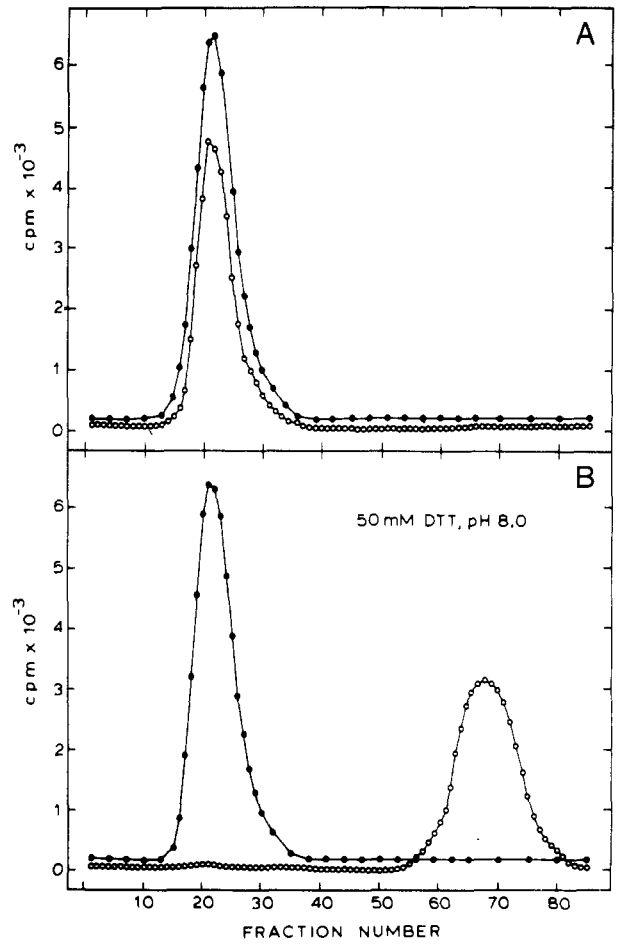


FIGURE 4: Reversibility of Fab′–vesicle coupling. Vesicles eluting in fractions 15–30 of Figure 3B were pooled, concentrated, and rechromatographed on Sephadex G-150 (A). All of the ^{125}I-labeled protein (open circles) coelutes with vesicles (^{3}H-labeled phospholipid, filled circles). In (B), identical vesicles were treated with 50 mM DTT at pH 8.0 for 15 min prior to chromatography on a Sephadex G-150 column preequilibrated with buffer I containing DTT (50 mM). Under such reducing conditions, none of the Fab′ remains associated with vesicles.

free vesicles described above. Moreover, Fab′-bearing vesicles appear to be homogeneous with respect to protein–lipid ratio. As shown in Figure 5, the ^{125}I-labeled antibody and ^{3}H-labeled phospholipid cosediment when vesicles collected from the peak fractions of the Sephadex G-150 column shown in Figure 4A are subjected to velocity centrifugation in a linear sucrose gradient. Moreover, when these same vesicles are exposed to goat anti-rabbit IgG serum (1:16 dilution), greater than 95% of both the ^{125}I-labeled protein and ^{3}H-labeled phospholipid coprecipitates.

If, as we propose, Fab′–vesicle coupling is the result of a disulfide interchange reaction, one would expect the chromophore, 2-TP, to be released during the coupling reaction in numbers equal to the numbers of Fab′ fragments bound to vesicles (see Figure 2). The data plotted in Figure 6 indicate that this is indeed the case. The closed circles in Figure 6 show the time course for coupling of Fab′ fragments to PDP-PE-containing vesicles (expressed as micromoles of Fab′ bound to vesicles with time, assuming a molecular weight of 50 000 for each Fab′ monomer). The open squares show the corresponding release of 2-TP measured spectrophotometrically as described under Methods. Notice that a small percentage of 2-TP is released from PDP-PE vesicles in the absence of any Fab′ coupling (Figure 6, open circles). It is therefore helpful to correct 2-TP release to reflect the actual amount of the chromophore released as a result of Fab′–vesicle coupling. This can be done approximately by subtracting control values (open circles) from apparent 2-TP release (open squares). The dashed line thus obtained corresponds rather closely to the number of Fab′ molecules bound to vesicles (closed circles). The slight deviation of corrected 2-TP release from Fab′ binding may be the result of too high an approximation for the molecular weight of each Fab′ fragment. If 45K rather than 50K is used to calculate Fab′ binding, the Fab′ binding and 2-TP release curves in Figure 6 become coincident. These results further support the disulfide interchange mechanism for coupling we have proposed above (see Figure 2).

We find a linear relationship between the amount of Fab′ bound to vesicles (in 2 h) and the concentration of Fab′ present in the reaction mixture. At an initial Fab′ concentration of 1 mg/mL, approximately 100 μg of the antibody fragments become coupled to vesicles. Up to 600 μg of Fab′ is bound per μmol of vesicle phospholipid at a Fab′ concentration of 12.5 mg/mL. With the assumption of an average vesicle diameter of 0.2 μm, this latter value corresponds to approximately 6000 Fab′ molecules bound to each vesicle. These data also indicate that the coupling efficiency (i.e., the percentage of the total Fab′ present in the reaction mixture that becomes coupled to vesicles) falls off slightly at high Fab′ concentra-

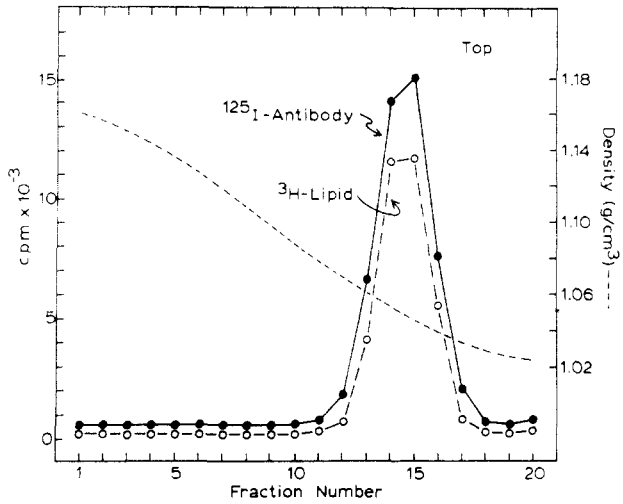


FIGURE 5: Sucrose density gradient centrifugation of Fab'–vesicles. Fab'–vesicles (0.5 mL) collected from the excluded peak of the Sephadex G-150 column in Figure 4A were layered on top of a linear 5–40% sucrose gradient and centrifuged (200000g) for 12 h. Fractions (0.25 mL) were collected from the bottom of the centrifuge tube and analyzed for radioactivity. The ^{125}I-labeled antibody and ^{3}H-labeled phospholipid cosediment at a density of about 1.05 g/cm^3. In parallel gradients, free antibody fragments and protein-free vesicles sedimented at densities of 1.12 and 1.03 g/cm^3, respectively.

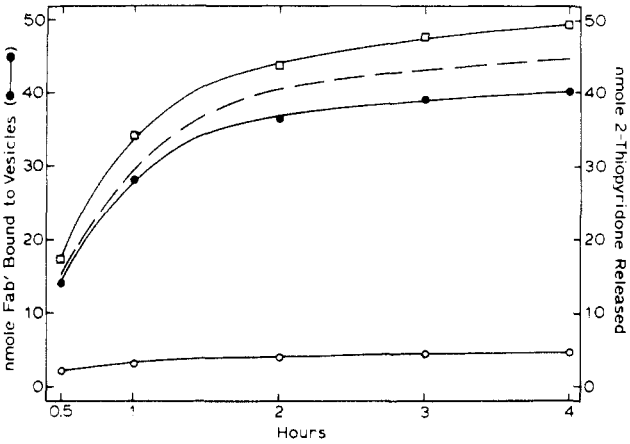


FIGURE 6: Time course of Fab'–vesicle coupling (filled circles) and 2-thiopyridinone release. Vesicles (5 μmol) (PC–cholesterol–PDP-PE, 45:50:5) were mixed with freshly reduced Fab' fragments (7.5 mg) in 1 mL of buffer I at pH 5.5. The pH was shifted to 8.0 at time 0, and coupling was allowed to proceed for 4 h at 25 °C under argon. Aliquots were removed at various time intervals, and the concentration of 2-TP was determined as described under Methods (squares). The corresponding amount of 2-TP released from vesicles alone (i.e., in the absence of Fab') was also determined (open circles). The dashed line represents corrected values for 2-TP release (apparent release, squares; minus control values, circles). The amount of Fab' conjugated to vesicles at each time point was calculated following separation of vesicles from unconjugated antibody fragments on discontinuous dextran gradients of the type described by Heath et al. (1980).

tions. About 26% of the total Fab' is coupled to vesicles at a Fab' concentration of 1 mg/mL, compared to 14% at a Fab' concentration of 12.5 mg/mL. Some aggregation of vesicles is observed within 30 min at the higher Fab' concentrations (above 10 mg/mL). Moreover, as shown in Figure 7, the rate of Fab'–vesicle coupling (expressed as the amount of Fab' bound to vesicles in 2 h) is dependent of the pH of the reaction mixture.

As shown in Table I once formed, the Fab'–vesicle disulfide linkage is quite stable. For example, greater than 92% of the original Fab' remains associated with vesicles during an 8-h incubation at pH values ranging from 6.0 to 8.0. In the presence of fresh human serum, some dissociation of Fab' from

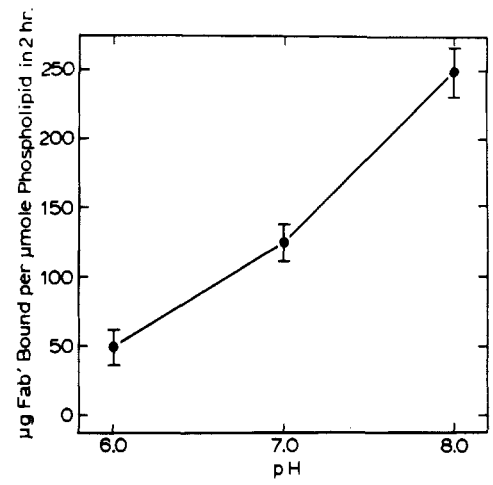


FIGURE 7: pH dependence of Fab'–vesicle coupling. The rate of Fab'–vesicle coupling is expressed as the amount of Fab' bound to vesicles in 2 h. Coupling ratios at 2 h were determined as described in the legend to Figure 6 for reactions carried out at pH 6.0, 7.0, and 8.0. Bars represent standard errors for three measurements.

Table I: Stability of Fab'–Vesicle Disulfide Linkage[a]

	μg of Fab'/μmol of phospholipid		Fab' remaining coupled after 8 h (%)
	start	8 h, 25 °C	
pH 6.0	286	277	97
pH 7.0	286	272	95
pH 8.0	286	263	92
25% human serum	286	212	74
50% human serum	286	177	62

[a] Fab'–vesicles (286 μg of Fab'/μmol of phospholipid) were incubated for 8 h under indicated conditions. The amount of Fab' remaining coupled to vesicles was calculated following separation of vesicles from eluted Fab' fragment on discontinuous dextran gradients of the type used by Heath et al. (1980).

vesicles is observed (Table I). This effect is undoubtedly related to the susceptibility of the Fab'–vesicle linkage to reduction by serum components such as glutathione.

Binding of Fluorescein by Vesicle-Linked Antifluorescein Fab' Fragments. In the preceding section, we have shown that it is possible to cross-link several thousand Fab' fragments to a single 2000-Å vesicle. Here we describe our effort to determine whether specific vesicle-linked Fab' fragments retain their capacity to bind water-soluble antigens.

The binding of fluorescein by antifluorescein F(ab')$_2$ fragments in aqueous solution is accompanied by a decrease of the fluorescein fluorescence emission at 515 nm. For example, the addition of 20 μg of immunopurified antifluorescein F(ab')$_2$ fragments to a solution of fluorescein (0.05 mM) in 0.5 mL of buffer I results in quenching of the total flourescence signal by 42.0% (Table II). There is no detectable difference in the quenching capacity of antifluorescein F(ab')$_2$ fragments and the Fab' fragments produced from them (not shown).

The quenching capacity of vesicle-linked antifluorescein Fab' fragments is also shown in Table II. Under conditions identical with those used to determine antifluorescein F(ab')$_2$ quenching, 20 μg of vesicle-linked antifluorescein Fab' quenches 39.9% of the total fluorescein fluorescence. These results indicate that vesicle-linked antifluorescein Fab' fragments retain up to 95% of the quenching capacity exhibited by the same amount of soluble antifluorescein F(ab')$_2$ fragments. Similar

Table II: Fluorescein Fluorescence Quenching by Antifluorescein Antibody Fragments before and following Coupling to Vesicles

	total fluorescein fluorescence[a] quenched by 20 μg		quenching capacity retained following coupling (%)
	soluble $F(ab')_2$	vesicle-linked Fab' [b]	
nonspecific $F(ab')_2$	0.5	0.8	
antifluorescein $F(ab')_2$ [c]	42.0 ± 1.3[d]	39.9 ± 1.8	95
5:1 mixture nonspecific $F(ab')_2$ + antifluorescein $F(ab')_2$	8.0 ± 0.4	7.5 ± 0.7	94

[a] Fluorescein fluorescence emission was measured at 515 nm by using an excitation wavelength of 492 nm; 100% fluorescence is defined as the measured emission of a solution of fluorescein (0.05 mM) in 0.5 mL of buffer I at pH 8.0. [b] Corrected for quenching by vesicles alone. [c] Prepared from immunopurified antifluorescein IgG. [d] Mean value plus standard deviation for four measurements.

Table III: Specificity of Binding of Fab'-Bearing Vesicles to Cells[a]

		total label cell associated (%)			
		following TBS wash[b]		following DTT wash[c]	
cell type	ligand	^{3}H-labeled phospholipid	[^{14}C]sucrose	^{3}H-labeled phospholipid	[^{14}C]sucrose
human RBC	anti-human RBC Fab' [d]	93.2	91.0	12.6	13.0
	nonspecific Fab' [e]	2.1	3.0	2.0	3.1
	vesicles only	3.2	2.8	2.5	2.0
sheep RBC	anti-human RBC Fab'	8.7	11.0	2.6	2.0
	nonspecific Fab'	3.0	2.6	2.6	1.6
	vesicles only	4.1	3.4	3.2	2.8
ox RBC	anti-human RBC Fab'	6.0	7.1	2.5	2.0
	nonspecific Fab'	2.6	2.5	2.0	3.1
	vesicles only	3.0	2.5	3.0	1.8

[a] Sucrose-containing vesicles at a concentration of 75 nmol of phospholipid were mixed for 30 min at 25 °C with 1.6×10^7 erythrocytes. Binding is expressed as the percentage of the total added label that is associated with the cells following washings. [b] Cells were washed 3 times with TBS. The final pellet was extracted according to Bligh & Dyer (1959), and the chloroform phase was sampled for ^{3}H-labeled phospholipid and the aqueous phase for [^{14}C]sucrose. [c] Same as (b) with 50 mM DTT and 2 mM EDTA included in the TBS (pH 8.0). [d] 180 μg of antihuman Fab' fragments per μmol of phospholipid. [e] 162 μg of nonspecific Fab' fragments per μmol of phospholipid.

results are obtained for a mixture of nonspecific and antifluorescein antibody fragments (Table II). These data suggest that essentially all of the antigen binding sites present on vesicle-linked Fab' fragments which exhibit a specificity toward fluorescein are accessible for interaction with this hapten.

Binding of Vesicles Bearing Antihuman Erythrocyte Fab' Fragments to Human Erythrocytes. In the preceding section, we demonstrate that vesicle-linked Fab' fragments retain their capacity to bind water-soluble antigens. Here we present evidence that vesicle-linked Fab' fragments can also serve as effective ligands for the binding of vesicles to specific antigenic determinants present on cell surfaces.

Figure 8 shows the binding of sucrose-containing vesicles bearing antihuman erythrocyte Fab' fragments to human and sheep erythrocytes. At vesicle concentrations less than 75 nmol of total phospholipid, essentially quantitative binding of vesicle lipid and contents to human erythrocytes is observed. Within the same concentration range, no more than 10% of the vesicles bind to sheep erythrocytes.

Table III compares the binding of control vesicles (lipid alone), vesicles bearing nonspecific Fab' fragments, and vesicles containing covalently attached antihuman erythrycyte Fab'-fragments to human, sheep, and ox erythrocytes. In the case of antihuman erythrocyte Fab'–vesicles, greater than 93% of the ^{3}H-labeled phospholipid and greater than 91% of the encapsulated [^{14}C]sucrose remain associated with the human cells after extensive washing with buffer. Under identical conditions, less than 10% of both labels becomes associated with sheep or ox erythrocytes. Background binding of vesicles alone, or vesicles bearing nonspecific Fab' fragments, does not exceed 5% for all three cell types.

Most of the bound antihuman erythrocyte Fab'–vesicle elute from cells during washing with buffer containing 50 mM DTT (Table III). Eluted vesicles retain their aqueous contents

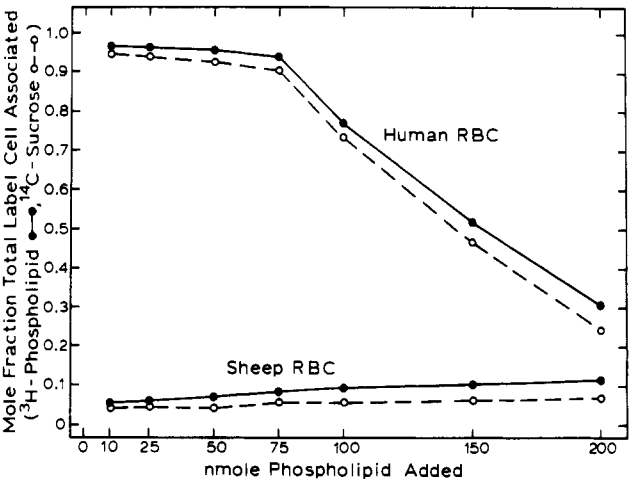


FIGURE 8: Binding of vesicles bearing antihuman erythrocyte Fab' fragments to human and sheep erythrocytes. Sucrose containing antihuman erythrocyte Fab'–vesicles (10–200 nmol of phospholipid in 0.8 mL) was mixed with an equal volume of a 2% suspension of washed human or sheep erythrocytes in buffer II at pH 8.0. Unbound vesicles were removed, and the amount of bound phospholipid and sucrose was determined as described under Methods. Binding is expressed as the fraction of the total added [^{14}C]sucrose or ^{3}H-labeled phospholipid that remains cell associated following washing.

([^{14}C]sucrose), suggesting that neither binding nor subsequent elution with DTT causes damage to the vesicle membrane (data not shown).

The hemagglutination activity of vesicles alone, vesicles bearing nonspecific Fab' fragments, and antihuman erythrocyte Fab'–vesicles is shown in Table IV. Vesicles alone and nonspecific Fab'–vesicles exhibit no HA activity for the three cell types tested. Vesicles bearing antihuman erythrocyte Fab' fragments, on the other hand, strongly agglutinate human

Table IV: Hemagglutination Activity of Fab'-Vesicles[a]

cell type	hemagglutination titer: vesicles alone[b]	nonspecific Fab'[c]	antihuman RBC Fab'[d] −serum	antihuman RBC Fab'[d] +serum
human RBC	<1	<1	384	512
sheep RBC	<1	<1	<1	<1
ox RBC	<1	<1	<1	<2

[a] Hemagglutination titer is expressed as hemagglutinating units/mL, using 8.1×10^7 erythrocytes per well. [b] The initial concentration of vesicles was 1.5 μmol of phospholipid. [c] Same lipid concentration as (b); vesicles contain 190 μg of nonspecific Fab'/μmol of phospholipid. [d] Same lipid concentration as (b); vesicles contain 180 μg of antihuman erythrocyte Fab' fragments per μmol of phospholipid, with and without human serum (50%).

Table V: Stability of Binding of Antihuman RBC-Vesicles to Human Erythrocytes[a]

	total label cell associated[b] (%): ^{3}H-labeled phospholipid start	8 h	DTT[c]	[^{14}C]sucrose start	8 h	DTT
pH 6.0	96	90	14	90	88	6
pH 8.0	95	87	11	91	85	5
25% human serum, pH 7.4	95	83	9	93	86	5
50% human serum, pH 7.4	96	81		88	80	

[a] Sucrose-containing vesicles (0.05 μmol of phospholipid) bearing antihuman erythrocyte Fab' fragments (180 μg/μmol of phospholipid) were mixed with 1.6×10^7 human erythrocytes for 30 min prior to the start of the experiment. [b] Expressed as the percent of total added ^{3}H-labeled phospholipid or [^{14}C]sucrose that remained associated with cells following 3 washings with TBS. [c] Samples at 8 h were washed 3 times with buffer containing 50 mM DTT at pH 8.0.

RBC (both in the presence and absence of human serum), while essentially no agglutination of the sheep or ox erythrocytes is observed under identical conditions. Preincubation of antihuman RBC Fab'–vesicles with DTT (50 mM in TBS) abolishes their capacity to agglutinate human erythrocytes (not shown).

The data presented in Table V indicate that binding of antihuman erythrocyte Fab'–vesicles to human erythrocytes is quite stable. Less than 8% of the bound vesicles dissociate from cells during an 8-h incubation at pH 6–8. Moreover, little entrapped sucrose is released from bound vesicles during the same period. Binding of vesicles in the presence of human serum exhibits similar stability. Less than 12% of the bound ^{3}H-labeled phospholipid and less than 8% of the aqueous marker ([^{14}C]sucrose) are lost from the cells in 8 h of incubation (25% human serum in TBS). Once again, however, the vast majority of vesicles are eluted, intact, by treatment with DTT.

Discussion

We have described an efficient method for covalently attaching Fab' antibody fragments to lipid vesicles. The chemical mechanism involves the formation of a disulfide linkage between the free thiol group on each Fab' fragment and a pyridyl disulfide derivative of PE present in low concentrations in the membranes of preformed vesicles. In addition we have shown that vesicles bearing specific Fab' fragments bind selectively to target cells in vitro.

If we assume that PC, cholesterol, and PDP-PE randomly distribute in vesicle membranes and that each vesicle is bounded by a single bilayer, then 50 mol % of the total PDP-PE molecules would be expected to be present in the outer monolayer and, therefore, susceptible to reduction by the impermeable reducing agent, DHLA–dextran. Our finding that only 32 mol % of the PDP-PE molecules are reduced by such treatment (Figure 1) suggests that about one-third of the total lipid present in such vesicles is in the form of internal bilayers, i.e., that each vesicle, on the average, contains about 1.5 lamellae.[2] This explanation would also account for the low sucrose encapsulated observed here. If one-third of the total lipid is internal, the amount of sucrose encapsulated per micromole of phospholipid would be one-third less than that predicted for single-layered vesicles. Our value of 4.5 μL/mol of phospholipid is about one-third less than the theoretical value of 6.4 μL/mol calculated for single bilayer 2000-Å vesicles (Enoch & Strittmatter, 1979).

The reaction of SPDP with PE leads to the conversion of positively charged amino groups into uncharged amide bonds. This results in a change in the net charge of the phospholipid from essentially neutral (PE) to a molecule containing a net negative charge (the phosphate moiety of PDP-PE retains its negative charge; cf. Figure 2). However, the presence of this charged phospholipid does not adversely affect entrapment efficiencies or increase the rate of leakage of vesicle contents.

Fab' molecules appear to be well suited for cross-linking to PDP-PE-containing vesicles. A single reduction is the only modification required to generate Fab' fragments from rabbit dimeric $F(ab')_2$. Each Fab' fragment so produced contains a single thiol group at a predictable position on the molecule (i.e., at the site of reduction of the inter-heavy-chain disulfide of the $F(ab')_2$ fragments). This property represents an important advantage over other potential ligands such as native IgG molecules or $F(ab')_2$ fragments which contain no free thiol groups. Although sulfhydryl groups can be introduced into these molecules with amino-reactive thiol reagents such as homocysteine thiolactone or SPDP, it is difficult to regulate the site and degree of substitution (White & Sandoval, 1962; Carlsson et al., 1978).

The disulfide exchange reaction used here for Fab'–vesicle conjugation appears to be quite efficient at alkaline pH. Under our conditions, 15–30% of the total Fab' present in the reaction mixture becomes coupled to vesicles. At an initial Fab' concentration of 12.5 mg/mL, we have obtained a coupling ratio in excess of 600 μg of Fab' per μmol of vesicle phospholipid. This value corresponds to about 6000 Fab' fragments for each 0.2-μm vesicle. However, as mentioned above, at initial Fab' concentrations above 10 mg/mL, aggregation of vesicles becomes a problem. The highest coupling ratio achieved without significant aggregation is approximately 400 μg/μmol (at an initial Fab' concentration of 7.5 mg/mL). At this value, greater than 15% of the total Fab' has become coupled to vesicles and about 25% of the PDP-PE molecules present in the outer monolayer of vesicles has reacted (assuming that 32% of the total PDP-PE molecules present in such vesicles are in a position to interact with soluble Fab' fragments; cf. Figure 1). In terms of the number of Fab' molecules coupled per vesicle, a ratio of 400 μg/μmol corresponding to approximately 4000 Fab' monomers per each 2000-Å vesicle.

Once formed, the Fab'–vesicle linkage is quite stable. Only a few percent of coupled Fab' is lost from vesicles in 8 h of

[2] In all likelihood, the large majority of vesicles is unilamellar, with only a small proportion containing multiple bilayers. We find that most of the multilayered forms can be removed by centrifugation at 10000*g* for 30 min.

incubation at pH 6–8. These observations indicate not only that the disulfide linkage is stable but also that the hydrocarbon chains of the derivitized PE are sufficient to anchor the phospholipid–Fab′ complex into the vesicle membrane. In the presence of excess DTT or mercaptoethanol, the Fab′–vesicle coupling is completely reversed. This presumably is due to the susceptibility of this type of disulfide linkage to reduction by low molecular weight thiols (Carlsson et al., 1978).

The release of the chromophore, 2-TP, during the course of Fab′–vesicle coupling offers a convenient means of monitoring the progress of the reaction. The molar extinction coefficient of this chromophore is quite high (7×10^3 at 343 nm), and it is released in numbers equal to the number of Fab′ molecules coupled (Figure 6).

Although maximal at alkaline pH, it is interesting to note that a resonable rate of Fab′–vesicle coupling is observed at a pH of 6.0 (Figure 7). This is in contrast to thiol–disulfide exchange reactions involving aliphatic thiols and aliphatic disulfides where virtually no reaction occurs at acidic pH. The thiolate ion, rather than the thiol, is the reactive species in such nucleophilic displacement reactions (Lindley, 1960; Brocklehurst & Little, 1972). The pK for the protonation of aliphatic thiols is 7–9 (Brochlehurst & Little, 1972). The thiolate ion concentrations, and thereby the rates of such reactions, are thus very low at acidic pH. In the type of thiol–disulfide exchange reactions of concern here, involving aliphatic thiols and 2-pyridyl disulfides (the Fab′ and PDP-PE, respectively), the situation is different. Pyridyl disulfides increase their electrophilicity at acidic pH values owing to protonation of the ring nitrogen atom (pK = 2–3; Brochlehurst & Little, 1972). A reasonable rate of reaction is thus obtained in spite of the low thiolate ion concentration at acidic pH.

Hemagglutination and cell binding results presented here indicate that Fab′ fragments retain their antigenic specificities following coupling to vesicles. Antihuman erythrocyte Fab′–vesicles, for example, bind selectively to human erythrocyte while only background levels of binding are observed to sheep or ox erythrocytes (Table II). Similarly, antihuman erythrocyte Fab′–vesicles agglutinate human cells and not those of related species (Table IV).

Our finding that vesicle-linked antifluorescein Fab′ fragments retain their full capacity to bind fluorescein in aqueous solution indicates that each Fab′ monomer is oriented on the vesicle surface in such a way that its antigen binding site is accessible to low molecular weight haptens. In light of the mechanism of Fab′–vesicle coupling outlined above, this observation is not unexpected. The sulfhydryl group on Fab′ fragments (generated by reduction of the inter-heavy-chain disulfide of $F(ab')_2$ fragments) is believed to be located at a position on the molecule some distance from the antigen binding site. In fact, in the absence of gross conformational changes during Fab′ production, the thiol group would be at the opposite end of the molecule to the antigen binding site. The coupling of Fab′ to vesicles via this thiol, then, would not be expected to restrict the availability of the antigen binding site to small water-soluble molecules.

The avidity of vesicle binding is reflected in the titration curve presented in Figure 8. When a constant number of human erythrocytes is mixed with increasing amounts of antihuman erythrocyte Fab′–vesicles, binding is essentially quantitative at concentrations below 5×10^{-6} nmol of phospholipid per erythrocyte. Above this level, binding decreases sublinearly with increasing numbers of vesicles. With the assumption once again that each vesicle contains 1.5 lamellae, saturation occurs at a multiplicity of about 5200 vesicles (0.2 μm) per erythrocyte. We have calculated that a typical human erythrocyte (average surface area approximately 150 μm^2) can accommodate approximately 4600 close-packed 0.2 μm diameter spheres on its surface. This is in reasonably close agreement with our value of 5200 vesicles per cell and suggests the possibility that at saturation erythrocytes become effectively coated with the antihuman erythrocyte Fab′–vesicles.

The stability of vesicle–cell binding is apparent from the data presented in Table V. The vast majority of vesicles remains attached to cells following extensive washing with buffer and for periods up to 8 h. Bound vesicles retain their aqueous contents under the same conditions. The presence of human serum does not appear to cause severe vesicle elution nor does serum induce leakage of vesicle contents. It is worthwhile to note here the results of a pilot experiment in which 6-carboxyfluorescein was entrapped in antihuman erythrocyte Fab′–vesicles. When such vesicles are mixed with human resealed erythrocyte ghosts, extensive binding ensues. However, no detectable delivery of the fluorophore to the interior of the ghosts was observed during incubation for periods up to 4 h.

Bound vesicles are eluted from cells by treatment with excess DTT at pH 8.0. The ability to remove attached vesicles represents an advantage in in vitro experiments which are designed to study membrane–membrane interactions such as cellular uptake of vesicles by membrane fusion and phagocytosis. The presence of unreacted vesicles at the cell periphery often interferes with assays designed to determine the extent of vesicle internalization.

Fab′ fragments offer several additional advantages over other ligands for use in vesicle–cell targeting. The Fab′ monomer is relatively small (50K) by comparison to intact IgG molecules (150K), $F(ab')_2$ fragments (100K), and many lectins. The size of the ligand would be expected to be important in experiments aimed at vesicle–cell membrane interactions leading to fusion. Bulky molecules or macromolecular complexes may inhibit such interaction by sterically preventing close juxtaposition of vesicle and cell membranes (Martin & MacDonald, 1976b). Finally, unlike intact IgG molecules, Fab′ fragments lack an Fc region and are therefore incapable of complement activation in vivo. Complement activation by vesicle-born IgG is not an unlikely possibility, and should it occur, lysis of vesicle and/or target cell membranes could result.

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