# LIPID VESICLE-CELL INTERACTIONS

## III. Introduction of a New Antigenic Determinant into Erythrocyte Membranes

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ABSTRACT

The introduction of a new antigenic determinant, 2,4-dinitrophenyl-aminocaproyl-phosphatidylethanolamine (DNP-Cap-PE), into the surface membranes of intact human erythrocytes is described. Fresh cells were incubated in the presence of liposomes composed of 10% DNP-Cap-PE, 5% stearylamine, 20% lysolecithin, and 65% lecithin. Such liposome-treated erythrocytes are shown to be susceptible to immune lysis by anti-DNP serum in the presence of complement. Uptake of DNP-Cap-PE by erythrocyte membranes is also demonstrated by immunofluorescence using indirect staining with rabbit anti-DNP serum followed by fluorescein-conjugated goat anti-rabbit IgG and by electron microscopy using ferritin-conjugated antibody. Antigen uptake did not occur at low temperatures or from vesicles lacking lysolecithin and stearylamine. Fluorescence microscopy shows that the antigen-antibody complexes are free to diffuse over the cell surface, eventually coalescing into a single area on the cell membrane. Electron microscopy suggests that a substantial proportion of the lipid antigen is incorporated by fusion of vesicles with the cell membrane. There are indications that vesicle treatment causes a small proportion of cells to invaginate.

The transfer of lipid molecules from bilayer vesicles to cell membranes has been shown to occur in a variety of situations. The surface membranes of mycoplasma (5), amoebae (2), erythrocytes (10), and cultured mammalian cells (7, 9, 14, 15) acquire labeled phospholipids from externally applied vesicles. Cholesterol is also known to exchange between bilayer vesicles and cell membranes (8). The mechanism underlying the uptake of exogenous lipid by cell membranes, however, has not always been clearly defined. It has been suggested that acquisition by cell membranes of lipid molecules originating in vesicles involves fusion, but such observations do not exclude the possibility that these molecules simply diffuse out of the liposomal membrane and become inserted into the surface membranes of cells. Attempts to visualize liposome-cell fusion have met with only limited success. Electron micrographs have been published from a number of laboratories, including our own, which show blebs on the surfaces of liposome-treated cells, and it has been suggested that these represent fusing liposomes. The possibility remains, however, that such blebs are actually artifactual evaginations of the cell membrane induced by liposome treatment or subsequent preparation for electron microscopy.

In the present study, we have sought to clarify

the question of whether membrane fusion is involved in the uptake of lipid from vesicles by cells. We have utilized liposomes containing an antigenic lipid which is not normally found in cell membranes. Uptake of the antigen by erythrocyte membranes was first established by immunofluorescence and immune hemolysis. Electron microscopy using a ferritin-conjugated antibody indicates an affirmative answer to the question of whether fusion is involved in the transfer process; but it remains to be determined what proportion of the uptake of antigen may be due to other mechanisms.

This work constitutes a continuation of that presented in the preceding papers in this series (12, 13), aimed at using lipid vesicles to mimic the membrane-associated properties of a paramyxovirus such as Sendai virus. The procedures of the previous work were largely those used to investigate virus-cell interactions, and here we continue to rely on such technology, utilizing, in this case, immunological methods that have been effective in demonstrating transfer of viral antigens to cell surfaces.

## MATERIALS AND METHODS

### *Cells*

Freshly drawn human group O erythrocytes were washed twice in cold Dulbecco's phosphate-buffered saline (PBS) and resuspended at a concentration of 10% (vol/vol) in 0.3 M sucrose containing 10 mM Tricine-NaOH (pH 7.8).

### *Immunological Reagents and Miscellaneous Chemicals*

Sources of reagents were as follows: rabbit anti-DNP serum (1.2 mg antibody per milliliter, Miles Laboratories, Elkhart, Ind.); rabbit anti-(human erythrocyte) serum, ferritin-conjugated (1 mg/ml) and fluorescein-conjugated (10 mg/ml) goat anti-rabbit IgG (Microbiological Associates, Bethesda, Md.); stearylamine (SA, 99.5%, Lachat Chemicals, Inc., Mequon, Wis.); and lyophilized guinea pig serum (Grand Island Biological Co., Grand Island, N.Y.). Egg lecithin (L) and lysolecithin (LL) were prepared as previously described (12). Phosphatidylethanolamine (PE) was extracted from egg yolk and purified by silica gel chromatography. Normal rabbit serum (NRS) was obtained from healthy adult rabbits not immunized with DNP. Dinitrophenylated aminocaproyl-phosphatidylethanolamine (DNP-Cap-PE) was prepared as described by Six et al. (16) by acylation of PE with DNP-aminocaproic acid chloride. It was purified by chromatography on silica gel. The final product showed a single yellow spot on thin-layer analysis which stained positive for phosphate and negative with ninhydrin. Its spectrum corresponded to that reported (16). Antibodies are, of course, easily raised against liposomes containing DNP-Cap-PE (19), but for convenience we have used commercially available antiserum produced with DNP as a hapten conjugated to hemocyanin.

### *Preparation of Liposomes and Treatment of Cells*

Liposomes were prepared essentially as described by Bangham et al. (1). Lipids were combined in 4:1 chloroform-methanol in a 10-ml culture tube. Removal of the solvents by rotary evaporation yielded a 5-mg film of lipid composed of, by weight, 10% DNP-Cap-PE, 5% SA, 20% lysolecithin, and 65% lecithin. The dry lipid was dispersed in 1 ml of sucrose (0.3 M) containing 10 mM tricine-NaOH (pH 7.8) by ultrasonic irradiation with a probe type apparatus for either 5 or 15 min, depending upon whether a small or large proportion, respectively, of unilamellar vesicles was desired. The sonicated vesicles were then centrifuged in a clinical centrifuge for 15 min at 3,000 rpm to remove most of the titanium from the probe tip, and, more importantly, to eliminate very large vesicles that might otherwise cosediment with erythrocytes during the cell washing steps described below.

### *Electron Microscopy*

0.1 ml of the liposome dispersion was added to 5 ml of the erythrocyte suspension, and the cells were allowed to agglutinate in the cold for a few minutes. Cells and vesicles were incubated at 37°C for 1–5 min unless otherwise noted. Vesicle-treated erythrocytes were fixed for 8 h at 4°C in a solution of 0.3 M sucrose containing 4% glutaraldehyde. The erythrocytes were subsequently washed three times by pelleting at 2,000 rpm for 5 min in a clinical centrifuge and resuspending in 0.3 M sucrose buffered to a pH of 7.8 with 10 mM tricine-NaOH. After the final wash, the cells were resuspended in 0.5 ml of PBS which contained 0.1 ml of the rabbit anti-DNP serum. After incubation for 1 h at 37°C, the cells were washed (2,000 rpm) three times in PBS to remove excess antibody and resuspended in 0.5 ml of PBS containing 0.1 ml of ferritin-conjugated goat anti-rabbit IgG. After a 2-h incubation at 37°C, the glutaraldehyde-fixed, ferritin antibody-treated cells were washed three times by pelleting and resuspending the cells in PBS. The cells were then postfixed in a solution of 1% osmium tetroxide. Osmium fixation was carried out at 4°C for 48 h to insure maximal fixation of liposomal membranes. The cells were subsequently stained *en bloc* in an aqueous solution of 2% uranyl acetate for 12 h. The cells were then dehydrated in alcohol and embedded in resin (17). Thin sections were cut on a LKB Ultratome (LKB Instruments, Inc., Rockville, Md.) and stained with alco-

holic uranyl acetate. Sections were examined in a HU 11-E electron microscope. Procedures used for negative staining have been given elsewhere (10).

### *Immunofluorescence*

Erythrocytes treated with liposomes (sonicated for 15 min) for 30 min at 37°C were washed five times by pelleting and resuspending the cells in PBS. After the final wash, the cells were resuspended in 0.5 ml of PBS containing 0.1 ml of either NRS or rabbit anti-DNP serum and allowed to incubate for 1 h at 4°C. Excess rabbit serum was removed by three successive washings in PBS. The cells were then resuspended in 0.5 ml of PBS containing 0.1 ml of fluorescein-conjugated goat anti-rabbit IgG and incubated for various times at either 4°C or 37°C. Excess goat IgG was removed by washing the cells twice in cold PBS. The cells were placed on glass slides and examined under incident fluorescence illumination.

Erythrocyte ghosts, prepared according to Dodge et al. (3), were treated with lipid vesicles as described above for whole cells. Ghosts were then treated with anti-DNP serum at 1:10 dilution for 30 min and with anti-rabbit IgG at 1:10 dilution for 5 min, both at room temperature. The washing procedures were as described for whole cells except that the final washing was omitted.

### *Immune Lysis of Liposome-Treated Erythrocytes*

1 ml of the erythrocyte suspension was incubated in the presence of 20 $\mu$l of the liposome dispersion at 37°C for 30 min. The cells were then pelleted (2,000 rpm) and resuspended in PBS. This procedure was repeated three times to insure removal of excess liposomes. The cells were resuspended in 1 ml of PBS containing 10 mM $Ca^{++}$, and 5 mM $Mg^{++}$ and a 1:10 dilution of the rabbit anti-DNP serum (except in control experiments where NRS or anti-erythrocyte serum was substituted for anti-DNP serum). The cells were incubated in the presence of the serum for 10 min at room temperature, after which time 0.5 ml of freshly reconstituted guinea pig serum was added. Hemolysis of the cells was measured from the time that complement was added, as previously described (12).

## RESULTS

### *Antibody- and Complement-Mediated Hemolysis*

Erythrocytes treated with lipid vesicles consisting of L, LL, SA, and DNP-Cap-PE become sensitive to lysis by anti-DNP serum in the presence of complement. The time-course of hemolysis is shown in Fig. 1. It can be seen from the figure that some hemolysis is observed in the presence of

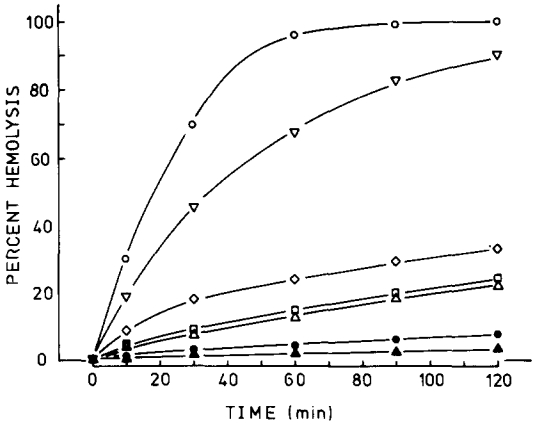



FIGURE 1 Immune lysis of lipid vesicle-treated erythrocytes. Erythrocytes incubated with lipid vesicles were sequentially treated with serum and complement. Addition of complement represents zero time. Except as noted, lipid vesicles consisted of, by weight, DNP-Cap-PE:SA:LL:L, 10:5:20:65. (filled triangles), not treated with lipid vesicles, but treated with an anti-DNP serum and complement. (filled circles), cells were treated with DNP-Cap-PE:L (10:90) and subsequently with anti-DNP serum and complement. (open triangles), cells treated with vesicles of PE:SA:LL:L (10:5:20:65), then with anti-DNP serum and complement. (open squares), non-immune rabbit serum substituted for anti-DNP serum. (open diamonds), complement heated at 50°C for 1 h. (reverse open triangles), complete. (open circles), cells treated only with anti-erythrocyte serum and complement.

nonimmune serum (open squares), but this can be reasonably ascribed to the after effects of the liposome treatment. As shown in the first paper of this series (12), at a dose about 50 times higher than that used in these experiments, erythrocytes are completely lysed by liposomes alone. For the same reason, a similar degree of hemolysis is found when the cells were treated with lipid vesicles containing PE instead of DNP-Cap-PE and subsequently treated with immune serum and complement (open triangles). When complement is inactivated by heating, hemolysis is again drastically reduced (open diamonds), but is still somewhat greater than that attributable to vesicle treatment alone. This difference is probably due to the small amount of complement present in the immune serum. When lipid vesicles contained no LL or SA, the DNP-Cap-PE did not become associated with the erythrocytes, and hemolysis (filled circles) was only slightly above that found with untreated erythrocytes (filled triangles). Hemolysis

induced by antiserum to human erythrocyte stroma (open circles) was somewhat more rapid than that of vesicle-treated erythrocytes induced by anti-DNP serum. This difference does not appear significant, given that the immune serum in the latter case was anti-(DNP-hemocyanin) rather than antiserum to the DNP-Cap-PE vesicle *per se*, as well as the probability that membrane area increases upon liposome treatment because of the additional lipid incorporated (which would tend to delay lysis).

### *Immunofluorescence and Agglutination of Vesicle-Treated Erythrocyte Membranes*

Liposomes containing 10% DNP-Cap-PE clump when incubated with serum from rabbits immunized with DNP-hemocyanin but not with normal rabbit serum. The vesicles disaggregate upon the addition of DNP-lysine. (Lysine is the amino acid to which the DNP is attached in the antigen, DNP-hemocyanin.) Liposomes that do not contain DNP-Cap-PE are not agglutinated by either anti-DNP serum or normal rabbit serum. When exposed to rabbit anti-DNP serum followed by fluorescein-conjugated goat anti-rabbit IgG, liposomes sensitized with DNP-Cap-PE exhibit intense fluorescence. Vesicles containing no DNP-Cap-PE, on the other hand, do not stain when treated with anti-DNP serum followed by fluorescent anti-rabbit IgG. DNP-Cap-PE-sensitized liposomes incubated with normal rabbit serum rather than with anti-DNP serum likewise do not stain upon addition of fluorescein-conjugated anti-rabbit IgG.

Since immune lysis of erythrocytes treated with DNP-Cap-PE-containing lipid vesicles suggested that the lipid antigen is incorporated into the cell membrane, it was of interest to determine whether, like liposomes themselves, such treated erythrocytes could also be agglutinated and labeled with fluorescent antiserum. As the photographs of Fig. 2 A–C reveal, this is indeed the case. Fig. 2 A is a photograph of aggregated erythrocyte ghosts (ghosts rather than whole cells were used only because they photograph well) which had been treated with DNP-containing vesicles and subsequently with anti-DNP serum and anti-rabbit IgG. Similarly treated erythrocytes become highly fluorescent in the presence of a fluorescein-conjugated antibody to the anti-DNP (Fig. 2 B). It is of some interest with regard to membrane fluidity (4) that the fluorescent label does not remain evenly distributed over the erythrocyte membrane. During incubation, the label becomes aggregated into patches and, in about 1 h at 37°C, the patches on 20–30% of the cells coalesce into a single region on the cell surface (Fig. 2 C).

### *Electron Microscopy of DNP-Sensitized Erythrocytes Labeled with Ferritin-Conjugated Antibody*

The immune hemolysis and fluorescent labeling experiments described above revealed that lipid from lipid vesicles of the appropriate composition becomes transferred to cell membranes. In order to gain insight into the mechanism by which the lipid is transferred to the cell, we carried out electron microscopy of vesicle-treated cells.

Our initial investigation utilized negative staining. Erythrocyte ghosts treated with highly sonicated liposomes consisting of L, LL, and SA revealed frequent protuberances on the cell membrane such as that shown in Fig. 3 A (see also reference 9). Since such blebs were about the same size as the vesicles used to treat the cells, and because large numbers of vesicles were found adhering to the cell membrane, it was not unreasonable to identify such structures as liposomes fusing with the cell membrane. Similar features were also observed in thin sections. Fig. 3 B shows one such structure partially filled with hemoglobin, which may, in this case, be leaking out of the cell through the putative vesicle.

Since it is well known that a variety of treatments may induce protuberances of the erythrocyte membrane, the possibility remained that the features seen in electron micrographs of vesicle-treated cells could represent evaginations of the cell membrane caused by, but not directly representing, interaction of vesicles with cell membranes. We therefore turned to ferritin-conjugated antibody to identify DNP-Cap-PE in membranes of cells treated with vesicles containing the DNP lipid. Preliminary experiments verified that lipid vesicles containing DNP-Cap-PE could themselves be readily identified with ferritin-conjugated antibody; DNP-liposomes were found to be heavily labeled with ferritin after treatment with rabbit anti-DNP and ferritin-conjugated goat anti-rabbit IgG. That such labeling is specific was indicated by the absence of ferritin from vesicles not containing DNP-Cap-PE. In addition, vesicles containing DNP-Cap-PE that were treated with

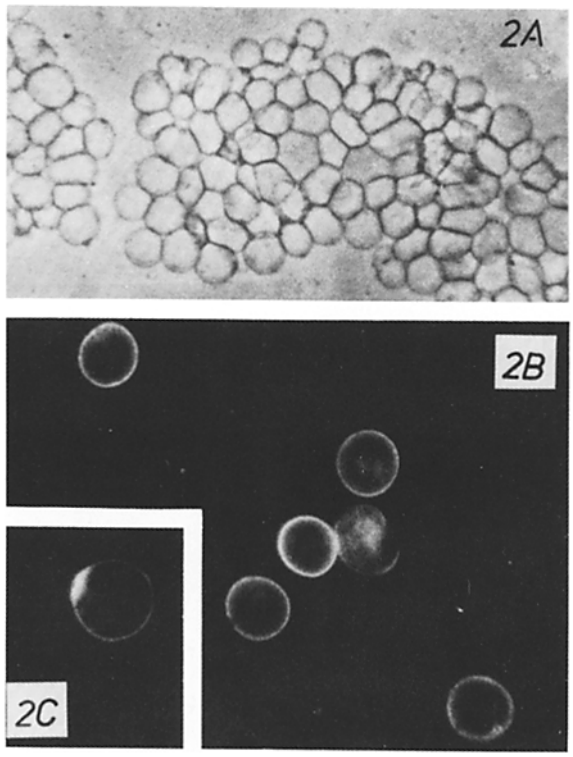
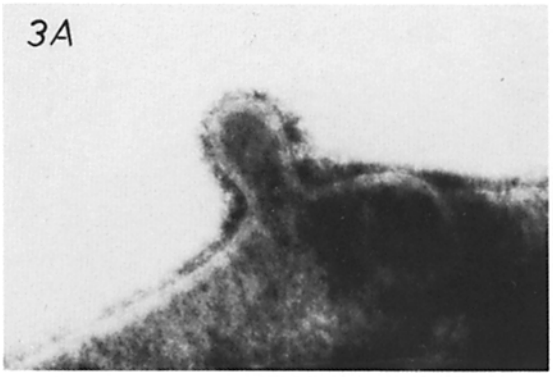



FIGURE 2 (A) Phase-contrast photomicrograph of DNP vesicle-treated erythrocyte ghosts incubated for 30 min at room temperature with anti-DNP, then treated with anti-(anti-DNP), and photographed. × 590. (B) Fluorescence micrograph of DNP vesicle-treated erythrocytes incubated with anti-DNP and with fluorescent anti-(anti-DNP) each for 1 h at 4°C. × 1300. (C) Same as B, except that second incubation was 1 h at 37°C. Vesicle composition in all cases was as given in the legend to Fig. 1.

nonimmune rabbit serum followed by ferritin-conjugated goat anti-rabbit IgG were likewise devoid of ferritin. As expected, the morphology of the vesicles depended upon the length of time the liposomes were sonicated. After sonication of about 1 min or less, the vast majority of the population consists of large (on the order of a micrometer) liposomes having large numbers of concentric bilayer lamellae. Sonication for 1–5 min reduces the average size of the vesicles, and, in addition to vesicles with concentric layers, those with one or more outer layers and several nonconcentric inner vesicles become common. Further sonication increases the proportion of small (about 50 nm) vesicles without, however, completely eliminating larger multilayered and multivesicular structures.

Given the pleomorphic nature of lipid vesicles, it was anticipated that the results would not be so uniform as those of the study that inspired the present application of a ferritin-conjugated antibody, namely, the investigation of fusion of Sendai virus with host cell membranes (6). Nevertheless, features were frequently seen that corresponded to expectations based on the appearance of the vesicle-treated cells described above. Fig. 3 C shows a lipid vesicle, clearly identified as such by a halo of ferritin molecules, attached to a cell membrane such that the contents of the vesicle (sucrose solution) and that of the cell (hemoglobin) have begun to mix. Features which may be interpreted as a later stage in the integration of such a vesicle into the cell membrane are also seen. One of these is the subject of Fig. 3 D. A cluster of ferritin molecules is seen at the right of the bump on the membrane and, although the cell membrane has been obliquely sectioned and is indistinct, ferritin molecules are also spread out along the cell surface to the left of the bump. Such features could still be interpreted as evaginations of the cell membrane occurring specifically at sites of transfer of lipid from vesicle to cell, by, for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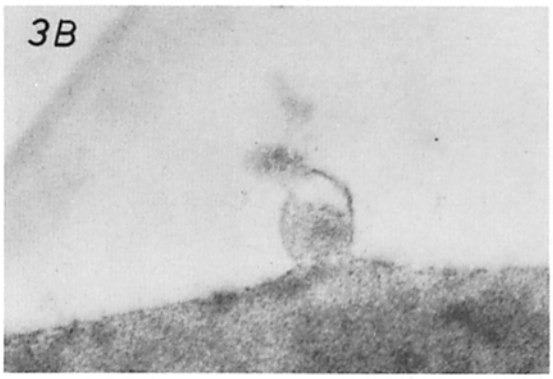
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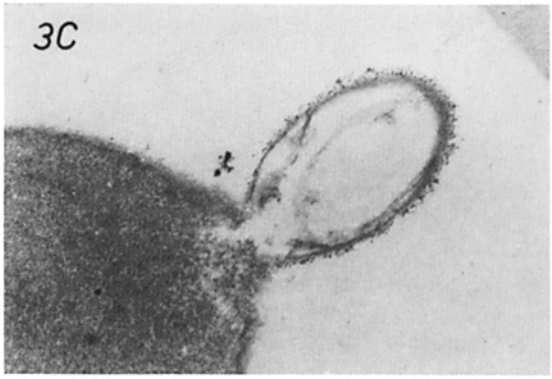
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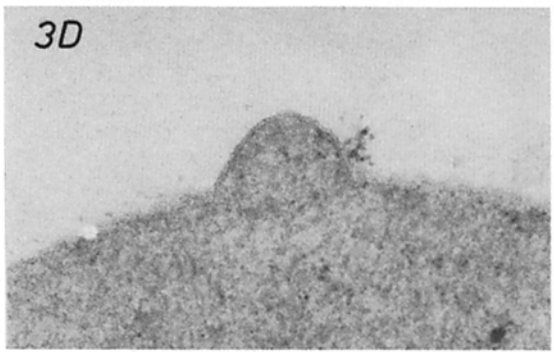
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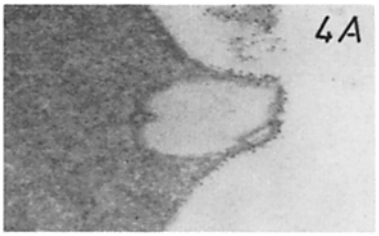
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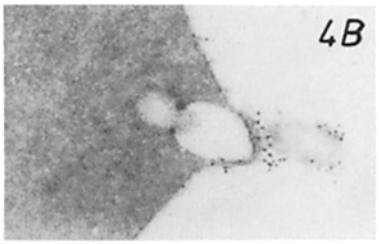
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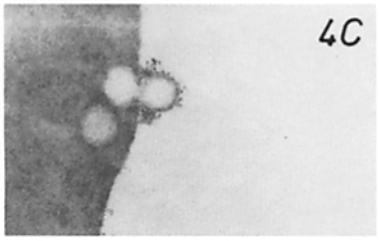
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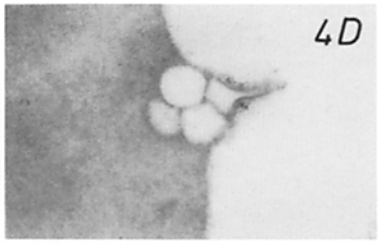
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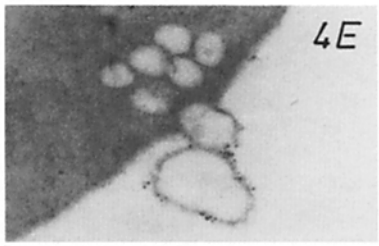
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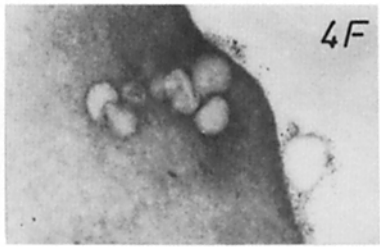
4F

example, simple collision. It was therefore important that more complicated images were obtained when less extensively sonicated vesicles were used to treat cells. Figs. 4 A–F are a selection of micrographs of features abundant in such preparations. Figs. 4 A and B are features most readily interpreted as the result of fusion of a two-layered vesicle with the cell membrane. The features of Figs. 4 C–F would be expected from fusion of vesicles consisting of a single bilayer surrounding a number of smaller vesicles. If the transfer of lipid from the outer bilayer of a multivesicle has occurred by simple diffusion into the cell membrane, the smaller inner vesicles would not be expected to have entered the cytoplasm. It is possible that transfer by diffusion of lipid from the outer bilayer would stimulate the cell membrane to engulf the smaller inner vesicles, but, were that the case, the clusters of vesicles of Figs. 4 C–F would be surrounded by a membrane, which they are not. After brief treatment with lipid vesicles, isolated clusters of ferritin molecules are very frequently seen on surfaces of cells where there are no other distinguishing characteristics such as bumps on the surface or vacuoles underneath the surface. Although those areas could represent a region where the membrane has flattened out subsequent to fusion of a single-layered vesicle but before diffusion of the DNP-Cap-PE away from the site, this interpretation is by no means unambiguous, so such areas are of no help in determining the mode of lipid transfer.

Fig. 5 A shows a vesicle such as those of Fig. 4 A and B at higher magnification. The ferritin-covered portion of the membrane is clearly continuous with the cell membrane although the membranes of the presumptive vesicle are not themselves clearly defined. How the smaller vesicle at the bottom of the larger one in this figure arises is not clear, although it is possible that fusion between cell membrane and liposome does not always occur at a single point of contact. If, for example, the cell membrane indented slightly and fusion occurred at the ring defined by the intersection with the liposome of the plane of the cell membrane outside of the indentation, one would expect such a small vesicle, the upper hemisphere of which would consist of the outer membrane of the liposome and the lower hemisphere of the cell membrane. The fusion mechanism proposed by Pagano and Huang (14), discussed below, could also be modified slightly to account for such a feature. Purely lipid vesicles, it should be emphasized, do not fix and stain well.

Cells treated with lipid vesicles often contain single membrane-bounded vacuoles near the cell membrane. If Fig. 5 A represents a double-layered liposome fusing with the cell membrane, then a number of arrangements frequently seen in electron micrographs of such preparations could represent transitional stages between the fusion of the outer bilayer with the cell membrane and the release of the inner vesicle. Such a possible sequence is shown in Figs. 5 B and C. Features such as that shown in Fig. 5 C are common, even after short exposures of cells to vesicles. Multilayer vesicles are also found at the surface of cells, the outer bilayer being continuous with that of the cell membrane (Fig. 6 A) as well as within the cell (Fig. 6 B). Close examination of Fig. 6 A reveals that almost all of the lamellae are composed of two bilayers. It is, in fact, rather common for bilayers of these lipids to associate pairwise, and this tendency may explain why features such as those of Figs. 6 A–C are seen frequently.

Fig. 7 A shows the result of longer incubations of cells with DNP vesicles; the surface of such cells is heavily labeled with ferritin. If the cells are not

FIGURE 3 (A) Negatively stained preparation of erythrocyte lysed at the air-water interface in the presence of lipid vesicles consisting of L:LL:SA (75:20:5). × 84,000. (B) Thin section of erythrocyte treated with lipid vesicles (composition as in A). (C) Thin section of erythrocyte treated with lipid vesicles consisting of DNP-Cap-PE:L:LL:SA (10:65:20:5), fixed with glutaraldehyde, treated with anti-DNP serum, and then with ferritin-conjugated anti-(anti-DNP) IgG. × 35,000. (D) As C, except × 88,000. Vesicles were sonicated for 45 min for A and B and 15 min for C and D.

FIGURE 4 Electron micrographs of ferritin-labeled, lipid vesicle-treated erythrocytes prepared as described for Fig. 3 C, except that liposomes were sonicated for a shorter time (5 min). (A) × 28,000. (B) × 49,000. (C) × 27,000. (D) × 29,000. (E) × 45,000. (F) × 29,000.

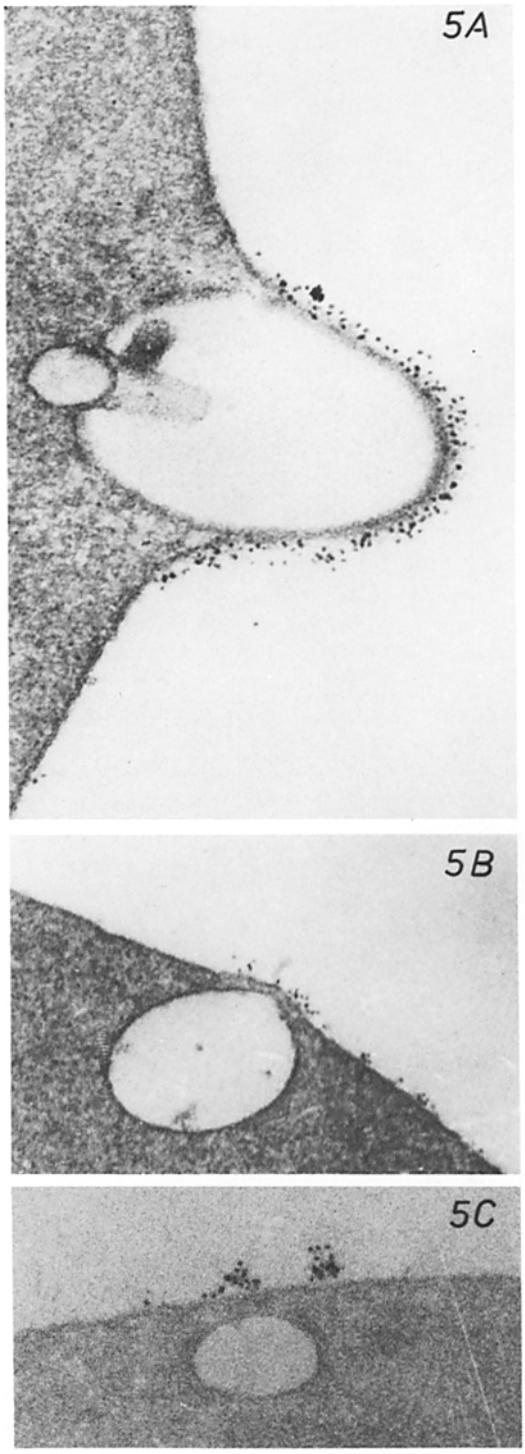


FIGURE 5 Electron micrographs of ferritin-labeled, lipid vesicle-treated erythrocytes. Conditions were as given for Fig. 3 C. (A) × 150,000. (B) × 61,000. (C) × 113,000.

incubated with vesicles at temperatures above 4°C, little ferritin becomes associated with the cell surface (Fig. 7 B). Localization of antibody-antigen complexes upon long-term incubation were not seen in ferritin-labeled cells as they were in the case of fluorescein-labeled cells. The rate of fluorescence aggregation is highly dependent upon the concentration of anti-rabbit IgG; aggregation was not observed within 1 h at the lower concentrations of IgG used for electron microscopy. Moreover, the cells for electron microscopy were fixed with glutaraldehyde before IgG treatment.

Cells which contain invaginations are found, albeit rather uncommonly. One such cell is shown in Fig. 8. This cell also contains a number of structures, devoid of hemoglobin, that are either invaginations from above or below the plane of the section or are vacuoles that have pinched off from the invaginations. Given the fact that almost all of those structures contain ferritin, and that the cells were fixed with glutaraldehyde before antibody treatment, the former appears most likely. Whether they are vacuoles or transverse sections of invaginations, the presence of ferritin molecules at a higher density along the surface of these structures than along the external (unevaginated) cell surface is a characteristic of cells in which any invagination is visible. Another characteristic of such cells, particularly those fixed very soon after vesicle treatment, is the location of the circular structures in the interior of the cell rather than near the cell membrane. Vacuoles such as those of Figs. 6 B and C that are found near the surface of cells (such cells are seldom invaginated) almost never contain ferritin. It appears that a small proportion of the cells in the population respond to lipid vesicle treatment by invaginating at areas that have acquired lipid from vesicles. It is not clear in such cases how the transfer of lipid from vesicle to cell membrane occurs, although it need not necessarily be different from the way other cells acquire the antigen. One of the invaginations of the cell of Fig. 8 contains what apparently is a lipid vesicle, although there is hardly any ferritin associated with it. Vesicles, labeled or not, have been seen in invaginations only in a very few instances.

## DISCUSSION

The present results demonstrate that phospholipid vesicles may be used to introduce a new antigenic determinant into the plasma membranes of intact human erythrocytes. By several criteria, the lipid antigen, introduced into cells by the methods de-

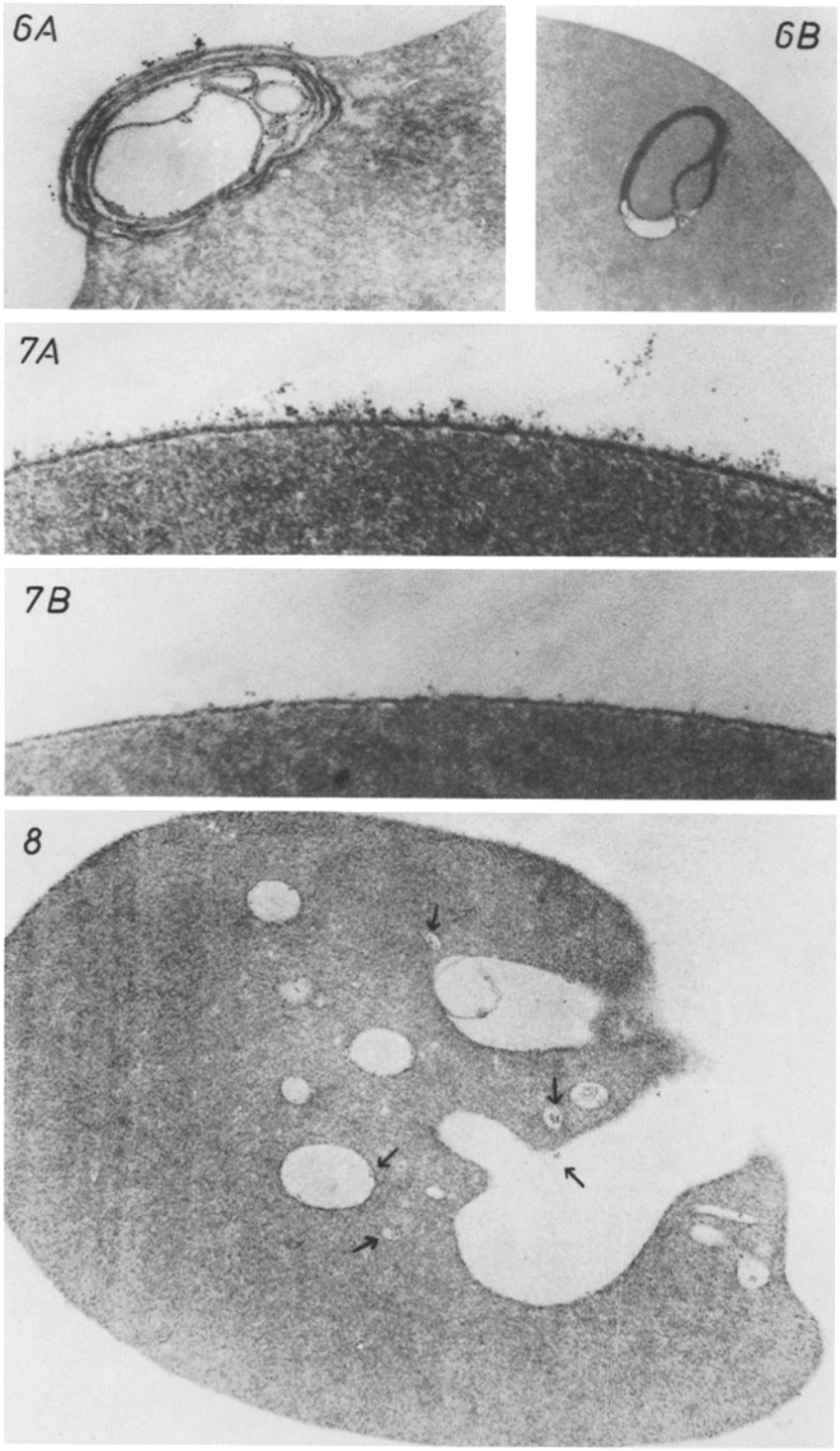


FIGURE 6 Multilayered liposomes in erythrocytes. Conditions were as described for Fig. 3 C. (A) × 63,000. (B) × 20,000.

FIGURE 7 Plasma membrane of erythrocytes incubated with lipid vesicles for 30 min at 37°C (A) or 0 min at 37°C (B). Ferritin labeled. (A) × 86,000. (B) × 81,000.

FIGURE 8 Erythrocytes invaginated after treatment with lipid vesicles and antibodies. Conditions were as described for Fig. 3 C. Some clusters of ferritin are marked with arrows. × 21,000.

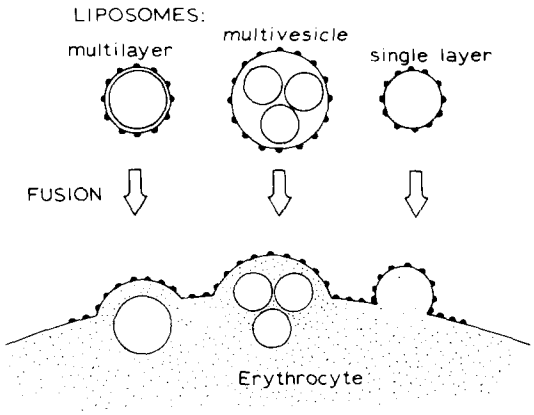


FIGURE 9 Diagram illustrating the expected results of fusion of three types of lipid vesicles with an erythrocyte membrane. In all three cases, as a result of membrane fusion, the outermost layer of the vesicle becomes part of the erythrocyte membrane. When ferritin-conjugated antibody is added, those sections of the membrane derived from the vesicle are labeled with ferritin (represented by black semicircles).

scribed here, is associated with the cell membrane in much the same way as other hydrophobically bound components of the cell membrane: it is not removed by extensive washing of the cells; it interacts with antibody such that, in the presence of complement, lysis ensues; and antibody-antigen complexes diffuse over the cell surfaces.

The conditions under which antigen from lipid vesicles becomes incorporated into the cell membrane parallels those described in the preceding papers in this series for vesicle-induced hemolysis (12) and vesicle-induced cell fusion (13). (The major difference between the present experiments and those intended to demonstrate hemolysis by lipid vesicle treatment *per se* is that very much lower concentrations of liposomes were used in the former than in the latter experiments.) For all of these activities, attachment of the vesicle to the cell membrane requires the vesicle to have an electrostatic charge opposite to that of the cell surface and to possess sufficient lysolecithin to perturb the vesicle bilayer.

The choice of DNP-Cap-PE as the lipid antigen was prompted by the work of Six et al. (16, 18) who have used this compound in studies of complement-induced lysis of liposomes. They demonstrated that immune lysis of liposomes depends on the incorporation of DNP-Cap-PE into the lipid bilayer. The mere presence of the DNP-Cap-PE in solutions containing preformed liposomes was insufficient to permit lysis upon subsequent treatment with antibody plus complement. This means that DNP-Cap-PE does not exchange between membranes to a detectable extent within 1 h or so at incubation temperatures of 37°C. The sign of the membrane potential has little effect on immune lysis of liposomes. At present, there is no reason to expect that the erythrocyte membrane should differ from liposome membranes either with respect to its ability to receive DNP-Cap-PE by diffusional transfer or with respect to sensitivity to antibody-complement action external to the membrane. We therefore suggest that DNP-Cap-PE incorporated into erythrocyte by vesicle treatment is associated with the lipid bilayer portion of the erythrocyte membrane. It must be recognized, however, that we have no *direct* proof as to the disposition of DNP-Cap-PE.

Fig. 9 depicts several permutations of a mechanism of interaction of lipid vesicles with cells that is consistent with most of our electron microscope observations. On the right, a single-layered vesicle fuses with the cell membrane. Initially, a bump on the cell membrane, which would gradually fill with hemoglobin, would be seen. Fig. 3 C may represent such events. The middle sequence of Fig. 9 illustrates the image expected if a multivesicular liposome (several small vesicles contained within a single bilayer membrane) were to fuse with the cell membrane. The electron micrographs of Figs. 4 C–F suggest this sequence of events. The scheme on the left of Fig. 9 depicts what we would expect to see in the electron microscope if the outer layer of a two-layered vesicle were to fuse with the cell membrane. Figs. 4 A, 4 B, and 6 A–B may represent such a process. If the outer layer of a multilayered liposome fused with the cell membrane, the result would be similar except that an $n - 1$ multilayered vesicle would appear in the cytoplasm, as has apparently occurred in the subjects of Figs. 5 A and B. All three types of internal leaflet arrangements, in addition to vesicles with many concentric internal leaflets, are common in the lipid preparations used for the experiments of Figs. 4–6.

Other mechanisms of interaction of vesicles with cell membranes in addition to the simple fusion sequence depicted in Fig. 9 are possible. Pagano and Huang, for example, have suggested that the outermost monolayer of a vesicle could fuse with both monolayers of the cell membrane, giving rise to a vesicle attached to the cell membrane at the equator of the vesicle by a branched

bilayer (14). Although the angle at which we find the cell membrane meeting the vesicle is not a right angle, as would be predicted by the mechanism of Pagano and Huang, it is possible that in reality pressure inside the cell would cause the cell membrane to meet the vesicle membrane at an acute angle (see, e.g., Fig. 6 A). Some of the vacuoles we find inside cells could result from a vesicle budding from the inside of the membrane in a reversal of the process by which it fuses with the cell membrane from the outside. It is also possible that a vesicle in an intermediate position in the scheme of Pagano and Huang, or in the situation illustrated on the left of Fig. 9, could, before being released into the cytoplasm, bud into smaller vesicles. Given that liposomes in simple salt solutions are frequently seen under the light microscope to exhibit a variety of bizarre behavior, including budding and fusion, it would not be unexpected that sudden contact of a lipid bilayer with a concentrated hemoglobin solution would provoke some morphological changes because of surface tension imbalance across the bilayer.

The suggestion that lipid vesicles may fuse with cells is by no means new, having been made previously (2, 5, 9, 14, 15). The evidence that cells acquire lipid from vesicles is indisputable. Evidence as to what proportion of this uptake is due to a particular mechanism is less convincing. There are many possible ways for molecules from vesicles to enter cells (fusion, phagocytosis, simple engulfment, collisional transfer, partial fusion, and exchange), and demonstration of the operation of a single mechanism requires some unusually discriminating techniques. In many previous instances, the methods that have been used have not been sufficiently definitive, although all possibilities for transfer have not been recognized until recently. Grant and McConnell (5), for example, found vesicle-mediated lipid transfer, but no incorporation of vesicle contents by cells. Such results would now probably be interpreted as indicating contact transfer, as has been rather convincingly demonstrated to occur between vesicles and cultured cells (7, 14), or as simple equilibration, which can occur between lipid vesicles without contact (11). The possibility, indeed the likelihood, of operation of these mechanisms of lipid transfer vastly complicates the task of defining which mechanism may predominate in a particular situation. Most cells can be expected to phagocytize vesicles so, for example, a combination of phagocytosis and molecular transfer of lipid could be incorrectly interpreted as fusion with a degree of leakage during fusion, according to how much the (aqueous phase)/(lipid phase) uptake ratio falls below unity. Even when phagocytosis is ruled out (2), invagination and subsequent engulfment driven by surface tension effects (as opposed to metabolic energy-driven phagocytosis) remain possible. Some evidence for such a process has been obtained in the present work, and, although it appears to be a relatively insignificant occurrence in the case of erythrocytes, it would seem judicious to consider engulfment, independent of a chemical energy source, to be more and possibly very much more significant in the case of other cells.

Our immune lysis and fluorescent labeling experiments do not settle the question of the mechanism of antigen transfer. They merely demonstrate that transfer can occur and indicate simple procedures for maximizing transfer of lipid to the cell membrane (as opposed to the cytoplasm). The experiments presented in the first two papers of this series (12, 13) suggest, but do not prove, that vesicles of the composition most effective in the phenomena studied there fuse with cell membranes. We therefore turned to microscopy, which is probably the least ambiguous method of demonstrating fusion, to determine whether this was indeed the case. The evidence, particularly when taken together with that of the preceding papers, strongly supports the inference that fusion of L-LL-SA vesicles with cell membranes occurs. None of our data, however, rule out the possibility of some transfer by other mechanisms. Vesicles of the composition we used do, as opposed to those of lecithin alone, rather closely resemble those of natural membranes that exhibit a propensity toward fusion.

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