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Colloidal Iron Stain for Distinguishing Open from Sealed Endocytic Vesicles of Erythrocytes (40889)[1]

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Abstract. Ferritin-labeled antibodies and lectins have been used to visualize endocytic vesicles in erythrocytes. Distinguishing preformed endocytic vesicles from invaginated plasma membrane has proved difficult especially since antibodies and lectins by themselves induce these changes in membrane configuration. We have used a transmission electron microscope technique (TEM) utilizing positive colloidal iron staining, employing as our model erythrocytes treated with cationic amphipathic drugs. Our technique allows fixation of cells at any point during drug treatment before staining. The membrane lining of unsealed but not of sealed vesicles was coated with electron dense ferric oxide grains. The formation of sealed vesicles is evidence for the fluidity of membranes of the erythrocytes of adults. The data also suggest that the mechanism may not be identical with every cationic amphiphile.

Ferritin-labeled antibodies and lectins have been used to visualize the formation of endocytic or pinocytotic vesicles in erythrocytes (1–3). However, distinguishing endocytic vesicles from invaginated plasma membrane in human erythrocytes has proved to be difficult. In addition, antibodies and lectins by themselves may induce shape change and vesicle formation. In this study we employed a transmission electron microscopic (TEM) technique which utilizes the positive colloidal iron staining of plasma membrane (4) to visualize endocytic vesicle formation in erythrocytes treated with amphipathic drugs. This technique allowed fixation of cells at any time point during drug treatment, before staining the membrane and it distinguished easily sealed (endoycytic vesicles) from unsealed (invaginated) membrane surfaces (5).

Materials and methods. Human red blood cells (RBC) from blood samples freshly collected in acid–citrate–dextrose (ACD, NIH Formula B) were washed three times in 0.15 *M* saline, discarding the buffy coat. The donors were volunteers who all signed written consent forms prescribed by the Committee on Human Experimentation.

Drug treatment and fixation of RBC. A ml of 50% RBC prepared as previously described (5) was incubated with each drug for 10 min at 37°C in a volume of 6 ml of 0.1% BSA–0.15 *M* saline. The final concentrations of the drugs in the test suspensions were 1.75 m*M* primaquine diphosphate (Parke–Davis Pharmaceutical Co., Detroit, Mich.), 0.3 m*M* chlorpromazine–HCl (Zenith Laboratories, Inc., Northvale, N.J.), 3.0 m*M* DL-propranolol–HCl, 1.0 m*M* tetracaine–HCl, and 0.5 m*M* dibucaine–HCl (Sigma Chemical Co., St. Louis, Mo.). Duplicate samples were incubated for 120 min at 37°C. The controls were RBC suspensions treated identically with 0.1% BSA–saline without added drugs.

After the incubations the RBC were washed three times in hypertonic 0.1% BSA–1.2% saline to avoid hemolysis of the fragile drug-treated cells. For fixation 8 ml of 2% glutaraldehyde in Hendry's phosphate buffer (6), pH 7.4 (PB), were mixed by rocking for 1 hr at room temperature with 0.2 ml of 50% RBC suspension and stored at 4°C overnight. Prior to staining the RBC were washed five times with PB.

Staining with colloidal iron and TEM. The colloidal ferric oxide sol was prepared

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as described by Gasic *et al.* (4) by adding 50 ml of 0.5 *M* $FeCl_3$ in distilled water in an almost continuous stream to 600 ml of boiling distilled water. The final iron concentration was adjusted to 1.2 g/liter with distilled water. The stock solution, pH approximately 3.5, was prepared by mixing 100 ml of this solution with 100 ml of 0.0012 *N* HCl. The stain contained 10 ml of stock solution mixed with 10 ml of glacial acetic acid and 20 ml of distilled water. Five ml of the stain were added to the centrifuged button of the glutaraldehyde-fixed RBC. The mixture was mixed at room temperature for 15 min with rocking, washed once with 12% acetic acid, followed by distilled water. The cells were prepared for TEM as previously described (5).

Results. Figure 1 illustrates the electron microscopic appearance of normal human erythrocytes fixed with glutaraldehyde prior to staining with colloidal iron. Note that colloidal iron particles localize exclusively at the plasma membrane and do not penetrate it. In drug treated cells, open invaginations were clearly labeled by stain particles which marked areas of cell surfaces where the plasma membrane and the invaginated membrane were continuous (Fig. 2). In all such cases, colloidal iron particles were observed within the surface of the invagination. Figure 3 illustrates the staining pattern of cells sectioned so that morphological discrimination could be made between invaginations with unsealed membrane and true endocytic vesicles with sealed membrane. The presence of colloidal iron particles identified membrane areas exposed to the extracellular medium. In the light microscope, invaginations such as the ones shown in Fig. 3 would be identified probably as vesicles. The micrograph in Fig. 4 illustrates this point further. In the light microscope it would be impossible to differentiate the endocytic vesicles (arrows) from the invaginated plasma membrane (stained with colloidal iron). Figure 5 illustrates one other interesting activity of membrane exposed to the five intercalating drugs used in this study; the formation of membrane vesicles apparently devoid of hemoglobin (arrows). These pieces of membrane were also observed in invaginations and at the periphery of cells, but their number in these locations was reduced probably due to washing of the cells during staining and embedding. The entrapment of these erythrocyte "dust" particles, and not by the invaginated areas stained with colloidal iron particles, is further evidence that the membrane of sealed, endocytic vesicles does not stain with colloidal iron.

Preliminary counts of sealed vesicles and invaginations in random thin sections revealed no differences in numbers in erythrocytes treated with primaquine for 10 or 120 min. The means ± SD of vesicles and invaginations in sections of cells treated with primaquine for 10 min were 7.9 ± 8.1 and 1.7 ± 1.1, respectively, and 5.6 ± 6.7 and 1.6 ± 1.2, respectively, after treatment for 120 min. Similar values were obtained from sections of cells treated with dibucaine or tetracaine for 10 min. However, chlorpromazine-treated cells contained a larger number of sealed vesicles (18.3 ± 15.6), whereas propranolol-treated cells contained fewer vesicles (2.0 ± 3.3); the number of invaginations were 1.2 ± 0.6 and 3.7 ± 2.2, respectively. The data were not tested for statistical significance and were intended only as an indication of endocytosis induced by the different compounds. These results correlate well with our previous studies (5) which indicated that the drugs used could produce different effects in the membrane bilayer of human erythrocytes.

Discussion. The colloidal iron staining technique of Gasic *et al.* (4) is useful for distinguishing between sealed and unsealed endocytic vesicles of erythrocytes using transmission electron microscopy. Other electron opaque markers which label negative charges on cell surfaces such as cationized ferritin (7), alcian blue (8, 9), and ruthenium red (10) might also be useful for the same purpose. Cationized ferritin is not as easy to prepare and offers no advantages. Our preliminary attempts with alcian blue and ruthenium red were complicated by intense staining of vessel surfaces by the former and instability of the solution with the latter and were therefore not pursued.

Using as our model the production of endocytic vesicles in the erythrocytes of

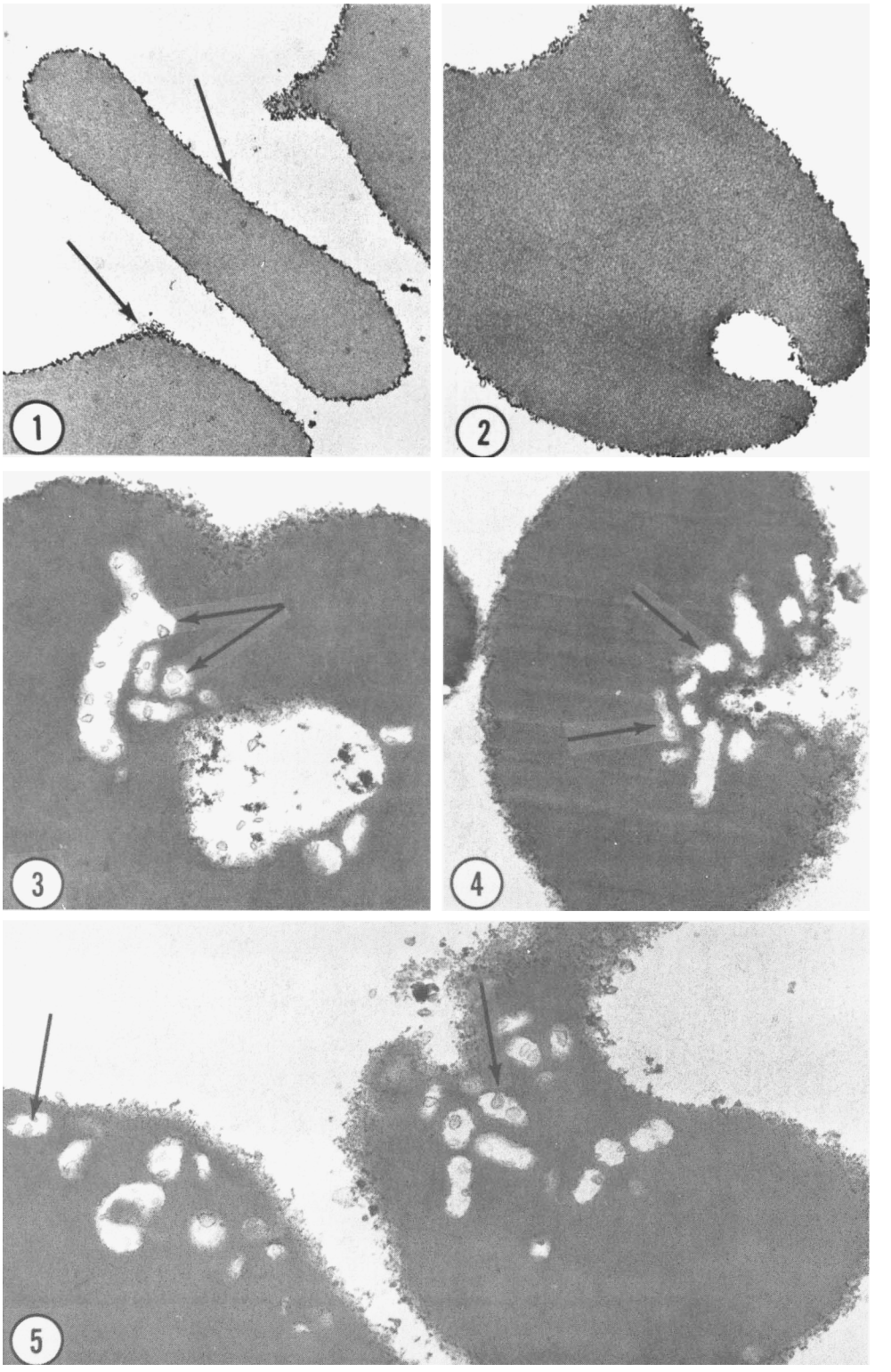
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FIG. 1–5. Transmission electron micrographs of erythrocytes fixed with glutaraldehyde and stained with colloidal iron, before and after treatment with 1.75 m*M* primaquine diphosphate or 0.3 m*M* chlorpromazine hydrochloride.

FIG. 1. Erythrocytes (control) stained with colloidal iron immediately after fixation. Note that colloidal iron particles (arrows) are localized to the outside of plasma membrane. ×11,200.

FIG. 2. Section of an erythrocyte treated with primaquine for 10 min prior to fixation and colloidal iron staining illustrating the marking of invaginations or unsealed, endocytic vesicles by colloidal iron. ×30,400.

FIG. 3. Treatment with primaquine for 120 min resulted in erythrocytes displaying extensive invaginations (colloidal iron stained) and sealed endocytic vesicles (arrows) which were not stained with colloidal iron. ×23,800.

FIG. 4. Erythrocyte displaying extensive endocytic vesicle formation after exposure to chlorpromazine for 10 min. ×24,100.

FIG. 5. All vesicles in drug-treated erythrocytes contained membrane "dust" (arrows). These pieces of membrane were also observed at the cell periphery but their lower incidence is probably due to the washing of cells during preparation. ×28,400.

adults by cationic amphipathic drugs makes it possible to draw some conclusions about the fluidity of the membrane and possibly also the lateral mobility of receptors in adult cells. Blanton *et al.* (1) using ferritin-labeled anti-A antibodies found that the treated erythrocytes of the newborn were frequently vesiculated but those of adults responded only occasionally. Schekman and Singer (3) later made similar observations using ferritin-labeled concanavalin A (Con A) and concluded that the membranes of adult erythrocytes were less fluid than those of newborn erythrocytes. However, Kehry *et al.* (11) were unable to demonstrate any differences between adult and newborn erythrocytes by fluorescence polarization. Voak and Williams (2) observed that pinocytotic activity induced by anti-A antibodies was largely confined to young erythrocytes containing ribosomes. This observation is supported by studies of rabbit reticulocytes by Zweig and Singer (12) with ferritin-labeled Con A which demonstrated that the extent of endocytosis decreased as the reticulocytes matured. Our data suggest that the fluidity of the membranes of the erythrocytes of adults is sufficient to permit fusion to form sealed endocytic vesicles after invagination secondary to drug action. We believe that further studies will show that these vesicles originate in spectin-free domains such as those described by Tokuyasu *et al.* (13) in their studies of endocytosis in neonatal erythrocytes following the action of Con A.

The formation of more sealed vesicles by the action of chlorpromazine than by the action of primaquine, dibucaine, and tetracaine and much more than by the action of propranolol suggests that the mechanism involved may not be identical with every cationic amphiphile. It should be pointed out that although the millimolar concentrations of the drugs used varied, in our experiments, the dosage in each instance was selected as the maximum concentration tolerated without producing visible hemolysis.

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