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ULTRASTRUCTURE OF HEMOGLOBIN-DEPLETED HUMAN ERYTHROCYTE RESEALED GHOSTS

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Summary

Both negative-stain and freeze-fracture electron microscopic techniques revealed that the ultrastructure of resealed white ghosts prepared at high dilution during the hemolysis step is very different from that of resealed ghosts prepared at low or moderate dilution (pink ghosts). The negative-stained resealed white ghosts showed light halo substructures on membrane surfaces and protrusions at the edge of the ghosts. Freeze-fracturing of these ghosts showed that membrane blebbing had occurred and that fragments of the membranes resealed to form small right-side-out vesicles ranging from 0.1 to 0.3 μm in diameter.

The use of resealed erythrocyte ghosts as a prototype plasma membrane has gained wide popularity because ghosts are relatively easy to prepare and the cell contents can be varied widely by selectively trapping solutes. The ghost preparations most commonly used involve one hemolysis step to a final dilution of between 5- and 30-fold. The mean cell volumes and membrane morphology of the resealed ghosts with these slight dilutions do not deviate appreciably from those of the intact red cells. However, more recently, many investigators have increased the magnitude of the dilution during the ghosting process. Cavieres and Glynn [1,2] used a 4000-fold dilution during cell lysis in an attempt to decrease endogenous adenylate kinase activity to a level inhibitable by P^1, P^5 diadenosine 5′-pentaphosphate. Kennedy et al. [3] employed a double-hemolysis scheme to achieve the same results. Kaplan (personal communication) has used a 200 000-fold hemolysis dilution in sequential hemo-

lyses to rid his ghost preparation of endogenous nucleotide diphosphokinase activity.

These biochemical and physiological studies were in general not supplemented by morphological studies describing the state of the membrane after resealing. A recent report by Bjerrum [4] has shown some scanning electron micrographs and negative staining of hemoglobin-depleted human erythrocyte resealed ghosts prepared by low-dilution hemolysis (20-fold) followed by extensive washing of the ghosts before resealing. However, the membrane morphology of ghosts prepared with high dilution is not available. Thus, the present study was undertaken to examine the changes in membrane ultrastructure and mean cell volumes of resealed ghosts prepared after high dilution hemolysis. Results from conventional negative stain methods and freeze-fracture techniques using ultrarapid freezing of the ghost suspension in the absence of fixatives or cryoprotectants indicate that the morphology of the native red cell membrane is drastically disrupted after a high dilution hypotonic hemolysis. These membranes bleb, fragment and form many vesicles which remain in the extracellular space after resealing.

Freshly drawn heparinized human blood was washed three times in 165 mM NaCl at 0—4°C. The buffy coat was carefully removed after each centrifugation. All ghost preparations described are modifications of a procedure originally described by Passow and co-workers [5—7]. Pink resealed ghosts (commonly referred to as Passow type ghosts) were prepared by adding 3 ml of 45—47% hematocrit red cell suspension to 30 ml hemolysing solution (2 mM ATP; 4 mM $MgSO_4$; pH 3.6—4.0). The pH of the hemolysate was adjusted to 6.0 with 0.1 M acetic acid or Tris base. After 5 min, 3 ml of restoring solution (1.8 M NaCl; 25 mM Tris base) were added to restore isotonicity and the pH was titrated to approx. 7.2. After 15 min, resealing was performed by incubation at 37°C for 1 h. The resealed ghosts were concentrated and layered on a 43% sucrose cushion and centrifuged for 45 min at 27 000 × *g*. The ghosts remaining on top of the cushion were washed three times and resuspended in 165 mM NaCl, 2 mM Tris-HCl at pH 7.2 and 0°C.

Singly lysed white ghosts were prepared by adding 12 ml of 45—47% hematocrit red cell suspension to 912 ml of hemolysing solution and the suspension was left for 5 min in the ghosting chamber at pH 6.0. The hemolysate was then concentrated by centrifugation and resuspended in 30 ml of supernatant. Subsequent steps are identical to the preparation of pink resealed ghosts.

Initial hemolysis of doubly lysed resealed white ghosts was carried out analogously to that described for singly lysed white ghosts except the initial dilution was greater (13 ml of cell suspension into 1.0 l of hemolysing solution). After restoring to isotonicity, ghosts were concentrated and added directly to a second hemolysis medium at a dilution of 1 : 10 at pH 6.0. The ghosts were subsequently prepared in exactly the same manner as that used for pink ghosts.

Dilute ghost suspension was negatively stained with 1.0% phosphotungstic acid (adjusted to pH 7.0 with KOH). Freeze-fracturing was carried out as follows: small samples (0.1 μl) of packed resealed ghosts were sandwiched between two copper strips (giving a sample of 10 μm thickness and about

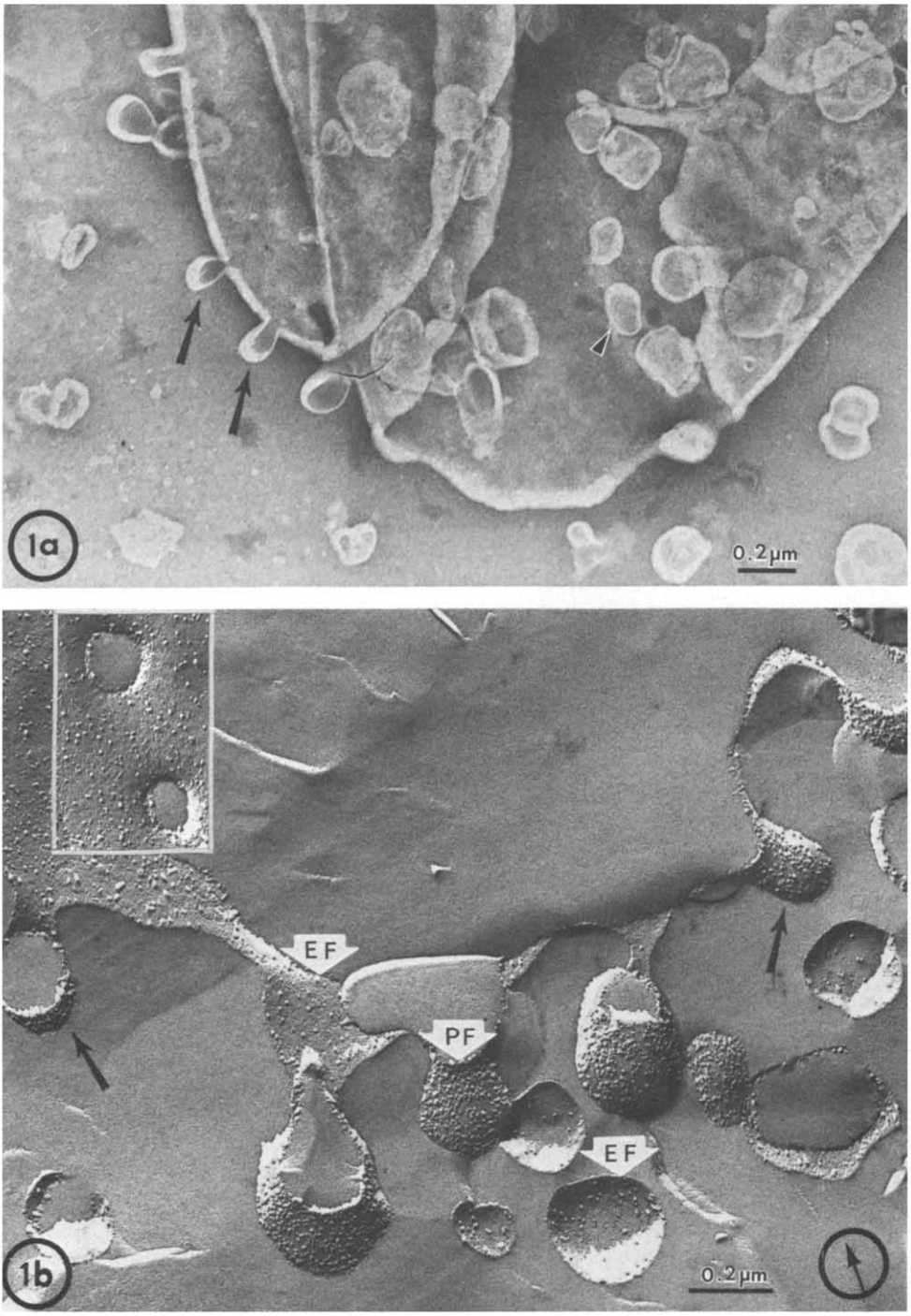

Fig. 1. (a) Phosphotungstic acid negative-stain preparation of resealed singley lysed white ghosts showing the protrusions (arrows) and halo substructures (arrowhead) on the membrane surface. Magnification, ×40 000. (b) Freeze-fracture micrograph of singly lysed resealed white ghosts. The arrows indicate protrusions from the membrane. The protoplasmic fractured faces (PF) are covered by many 80—100 Å particles while the extracellular (EF) surfaces have fewer particles. Similar membrane fragmentation is observed for doubly lysed resealed ghosts. Arrow at the lower right-hand corner indicates the direction of shadowing. The membrane indicates the direction of shadowing. The membrane evaginations viewed from the cytoplasmic space are shown in the insert. Magnification, ×58 500.

2.5 mm in diameter) and frozen in liquid propane at −190°C using the 'guillotine' dropping device developed by Costello and Corless [8]. Specimen cooling rates for this technique exceed 10 000 K/s (cooling from 273 to 173 K; see Refs. 8—10). Frozen samples were loaded in a specially designed hinged device [10] under liquid N_2 then transferred to the precooled stage of a Balzers BA-360 freeze-fracture instrument. The samples were fractured at −150°C and at about 10^{-7} torr. Samples were immediately shadowed with Pt/C at 45°, the carbon at 90°. The replicas were folated off the copped strips onto distilled water, cleaned with Clorox and picked up on naked 400 mesh copper grids. All specimens were examined in a Philips 300 or 301 electron microscope at 80 kV.

The resealed singly lysed white ghosts stained with 1% phosphotungstic acid (Fig. 1a) show distinct light halo substructures on the ghost surface (arrowhead), ranging from 0.15 to 0.3 μm in diameter. Protrusions (arrows) are also seen at the edge of the ghosts. Some of the protrusions pinch off to form vesicles. Similar membrane alterations were observed for doubly lysed white ghosts (micrograph not shown). Neither intact red cells nor pink resealed ghosts show these characteristic substructures when stained under similar conditions (micrographs not shown). The depressions in the middle of the halos are not transverse holes in the membrane, since these ghosts are impervious even to sucrose. However, these depressions may be equated to pits created by protrusion (exocytosis) of the membrane. Mean cell volumes of pink and white ghosts calculated from negatively stained specimens are shown in Table I. The mean cell volumes of white ghosts are about 1/2 of those of intact red cells whereas the mean cell volumes of red ghosts and intact red cells are not appreciably different. White ghosts prepared by Bjerrum [4] using low dilution hemolysis and extensive washing also have the same mean cell volume as our white ghosts. Although he did not mention blebbing, some blebs can be seen upon careful examination of his scanning electron micrographs. This is consistant with our interpretation that a decrease in mean cell volume is due to membrane blebbing and fragmentation.

The distinct morphological changes due to fragmentation and blebbing can also be observed in white ghosts using freeze-fracture techniques (Fig. 1b). In addition to the extended fracture surfaces, as normally observed with intact red cell membrane and pink ghosts, we see a heterogeneous population

TABLE I

CHARACTERISTICS OF RESEALED HUMAN ERYTHROCYTES AND GHOST PREPARATIONS

Preparation	Dilution factor (-fold)	Hemoglobin concentration (g/l)	Mean cell volume (μm^3)	Lysis steps
Red blood cell		319 *	94 ± 14 **	
Pink ghost	27	11.8 ± 0.2	84.9 ± 0.6	one
Singly lysed white ghost	200	1.59 ± 0.05	50—60	one
Doubly lysed white ghost	2000	0.16 ± 0.01	50—60	two

* Data from Funder and Weith [15].

** Data from Evans and Fung [16].

of vesicles in the extracellular space or blebs (arrows) from the membrane; they both range from 0.15 to 0.3 μm in diameter. The vesicles are similar in size to the halos observed in negatively stained specimens and to evaginations of the plasma membrane (insert of Fig. 1b). These vesicles formed are not all intramembranous particle-free vesicles as reported for those exocytosed from ghosts which have been pretreated to remove most of the spectrin [11]. It should be noted, however, that in an earlier report of Steck et al. [12] vesicles prepared from ghosts were greatly depleted of spectrin, yet the freeze-fracture micrographs showed that these vesicles were covered with particles.

By the criteria established for red cell membranes [13], the small vesicles we see on Fig. 1b are mostly right-side-out vesicles which are covered by many 80—100 Å particles on the protoplasmic fracture faces whereas fewer particles appear on the external fracture faces. This is also consistent with the finding that most vesicles formed in the presence of $MgSO_4$ during the ghosting process are right-side-out [12]. All the ghosting procedures used in this investigation utilized 4.1 mM $MgSO_4$ in the hemolysing solution.

The exact mechanism of membrane fragmentation is not known. However, it could have resulted from the mechanical stress brought about by an osmotic pressure especially in large dilution hypotonic hemolysis or ATP depletion [14]. In our case, ATP depletion does not play a role because our hemolysing media contained 2 mM ATP.

To the best of our knowledge, no direct correlation between the membrane transport properties and the morphology of white resealed ghosts has been made during membrane blebbing. However, when passive cation permeabilities were examined, Bjerrum [4] found that only half of his white ghosts were resealed to cations, although all of them had the same permeability to mannitol and water as intact red cells. Further experiments are underway to characterize the functional properties of our resealed white ghosts.

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References

1 Cavieres, J.D. and Glynn, I.M. (1976) J. Physiol. 263, 214—215
2 Cavieres, J.D. and Glynn, I.M. (1979) J. Physiol. 297, 637—645
3 Kennedy, B.G., Lunn, G. and Hoffman, J.F. (1980) Fed. Proc. 39, 1978
4 Bjerrum, P.J. (1979) J. Membrane Biol. 8, 43—67
5 Bodemann, H. and Passow, H. (1972) J. Membrane Biol. 8, 1—26
6 Lepke, S. and Passow, H. (1972) Biochim. Biophys. Acta 255, 696—702
7 Schwoch, G. and Passow, H. (1973) Mol. Cell. Biochem. 2, 197—218
8 Costello, M.J. and Corless, J.M. (1978) J. Microsc. 112, 17—37
9 Corless, J.M. and Costello, M.J. (1979) Biophys. J. 25, 289a
10 Costello, M.J. (1980) Proc. Scanning Electron Microsc. Soc. II, 361—370
11 Elgsaeter, A., Shotton, D.M. and Branton, D. (1976) Biochim. Biophys. Acta 426, 101—122
12 Steck, T.L., Weinstein, R.S., Straus, J.H. and Wallach, D.F.H. (1970) Science 168, 255—257
13 Weinstein, R.S. (1969) in Red Cell Membrane Structure and Function (Jamieson, G.A. and Greenswalt, T.J., eds.), pp. 36—82, J.B. Lippincott Co., Philadelphia
14 Lutz, H.U., Liu, S.-C. and Palak, J. (1977) J. Cell Biol. 73, 548—560
15 Funder, J. and Weith, J.O. (1966) Scand. J. Clin. Lab. Invest. 18, 167—180
16 Evans, E. and Fung, Y.-C. (1972) Microvasc. Res. 4, 335—347