BRIEF REPORT

Freeze-Cleaving of Red Cell Membranes in Paroxysmal Nocturnal Hemoglobinuria

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PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) is a rare hemolytic anemia thought to be due to an acquired red cell membrane defect.1,2 Although these patients are apparently abnormally sensitive to serum complement,3,4 the chemical nature and precise location of the membrane defect responsible for this increased sensitivity is unknown.5-7 Numerous efforts have been made to visualize the initial acquired lesion with the electron microscope, but these attempts have produced conflicting results, with some investigators claiming to find lesions8-11 and others finding essentially normal red cell membrane ultrastructure.12,13 The technic used in previous studies involved lysis of cells and subsequent drying of cell ghosts onto thin films prior to examination in the electron microscope, an approach criticised by Robertson for producing drying artifacts and likened by him to studying mud at the bottom of a dried-out pond.14 Freeze-cleave and freeze-etch methods of tissue preparation for electron microscopy15,16 have recently been employed in studies on the fine structure of red cells17 and, according to some authors, these preparative technics allow for the examination of relatively “artifact free” preparations of partially hydrated biological materials.15,17 In this study, the simplified freeze-cleave technic of Bullivant and Ames20 is applied to studies of PNH red cell membranes. Since reports of primary defects in PNH cell membranes have actually referred to lesions in isolated red cell ghosts, the appearance of PNH cell membranes from intact cells (at the time of fracture) is compared with the appearance of freeze-cleave PNH ghosts prepared by gradual osmotic lysis.21

MATERIALS AND METHODS

Blood was drawn by venipuncture from a previously reported thirteen year old girl with PNH2 and stored at 4°C for periods up to one week in Citrate-Phosphate-Dextrose (CPD) solution.2 Typically, previous studies on PNH cells have revealed only some of the cells to be abnormal,21 necessitating examination of large numbers of cells in this investigation to insure adequate sampling. At the time of collection, 70 percent...
Fig. 1.—Carbon-platinum replica of part of a typical PNH cell. The cell membrane is partially covered with a particular component. The cytoplasm appears to be filled with tightly packed small particles. 38,000 X. Insert. Schematic representation of a red cell showing the area of the cell illustrated in Figure 1.

of this patient's blood could be lysed by the sugar water test\(^2\) as determined by measuring total blood hemoglobin and free hemoglobin before and after hemolysis, indicating that a large proportion of red cells were abnormal. Cell membranes of over 200 of the patients red cells were examined. Control samples of blood were collected into CPD from six professional donors and membranes of over 400 donor cells were surveyed.

When ready for use, PNH or control blood samples were centrifuged lightly and the supernatant and buffy coat removed. Red cells were washed three times with saline and then carried stepwise through 10, 20 and 40 percent glycerol solutions containing phosphate buffer (pH 7.2).\(^3\) Following glycerination, cells were packed by centrifugation and
freeze-cleaved as described in detail by Bullivant and Ames.\textsuperscript{17,20} With this technic, fracture planes through frozen packed cells follow paths of least resistance and tend to be deviated by red cell membranes. The frozen fracture face was replicated \textit{in vacuo} with carbon following shadowing with carbon-platinum evaporated at a 45\textdegree angle. Following replication, the packed red cells were dissolved in strong household bleach (Chlorox\textsuperscript{®}). Fragments of carbon-platinum replica were rinsed with distilled water. Replicas were picked up on uncoated copper grids and examined directly in a Siemens Elmiskop I electron microscope.

Hemolyzed cells were prepared by placing 4 ml. of whole blood in a cellophane bag and dialysing against 1.5 liters of 0.025N NaCl for 2 hours\textsuperscript{21} and against 1 liter of 0.01N NaCl for 30 minutes. Ghosts were packed in an International refrigerated centrifuge at 10,000 \textit{g} for 30 minutes and then washed with several changes of physiologic saline solution. The isolated stroma was packed by spinning for 30 minutes at 14,000 \textit{g}. The supernatant was removed and the resulting pellet of packed PNH cell ghosts was suspended in an equal volume of 40 percent glycerine containing phosphate buffer. This suspension was placed in the well of the Bullivant-Ames brass block and freeze-cleaving and carbon-platinum replication were carried out as before.

Photomicrographs used to illustrate this paper are printed as positives so that shadows produced by platinum shadowing appear white.

RESULTS

Since a description of the appearance of replicas of freeze-cleaved red cells has been previously published,\textsuperscript{17} only a brief summary of the morphology of red cell membranes is included in this report. The fracture plane through frozen red blood cells can pass either directly through the cytoplasm of individual cells or be deviated by the cell membrane revealing large areas of cell membrane in three dimensional relief (Figure 1). The external (Figure 2) and internal (cytoplasmic) (Figure 3) aspects of red cell membranes in both PNH and in normal donors appear finely granular and are partially covered with a particulate component.\textsuperscript{17} These particles may appear singly but also tend to form clusters or chains (Figure 2). External aspects of PNH and normal red cell membranes tend to be covered with more particles than internal aspects of red cell membranes. The percent of total surface area covered by particles varies from cell to cell in a single donor. However, cells were present in control bloods which were indistinguishable from any cells in the blood of this patient with PNH.

Planes of fracture through frozen osmotically lysed PNH cells may also pass along membranes or may pass abruptly into the region formerly occupied by the cytoplasm. The cytoplasm of intact cells appears to be filled with small tightly packed particles (Figure 5) which may represent replicas of molecular hemoglobin.\textsuperscript{25} The cytoplasmic region of lysed cells contains only a few small scattered particles (Figure 6). The ultrastructure of lysed cell membranes is similar to that of intact membranes with the same apparent thickness (approximately 100\textdegree A) and with similarly distributed surface associated particles (Figure 4). There was no evidence of previously described pits, clefts or depressions in intact hydrated PNH cell membranes or in isolated PNH ghosts examined with this freeze-cleave technic.

DISCUSSION

Although it is generally agreed that there is a fundamental defect in the
red cell membrane in PNH, attempts at identifying this defect morphologically have resulted in a controversial literature. Mattes, Schubothe and Lindemann\textsuperscript{11} were the first to note an abnormality in osmotically lysed, dried PNH cell ghosts consisting of many clefts giving the membrane a patchy appearance, and this was subsequently confirmed by some groups\textsuperscript{10,11} but not by others.\textsuperscript{12,13} Believing that the variability in results obtained by others could be attributed in part to membrane damage caused by the drastic osmotic
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Hemolysis of cells used by earlier workers, Danon, Nevo, and Marikovsky, devised a method of gradual hemolysis in which cells are dialyzed against hypotonic saline solutions. They conclude that the ghosts thus obtained were relatively undamaged and, using this method, described an unusual pitting of dried PNH membranes. They recognized that the pitting could represent an artifact of preparation.

The freeze-cleave approach used in this study has the advantage that cell membranes are partially hydrated and intact at the time of fracture and replication thereby eliminating artifacts which may be introduced during cell lysis and, probably more important, drying onto supporting membranes. As seen in replicas of freeze-cleaved preparations, both PNH cell membranes and isolated hydrated PNH ghosts are indistinguishable from cell membranes observed in the control series. However, this does not necessarily invalidate the conclusion that real differences exist between PNH and normal cell membranes. It is possible, for example, that drying of ghosts reveals hidden structural differences between PNH and normal red cell membranes which are inapparent when membranes are hydrated and therefore are not seen in replicas of freeze-cleaved cells. The results of this study do suggest that specific structural abnormalities such as pitting previously reported in osmotically lysed, dried PNH cell ghosts represent an artifact of preparation and that a counterpart of these lesions has not been demonstrated in intact hydrated PNH cell membranes.

SUMMARY

Electron microscopic studies on dried isolated red cell ghosts have been reported to show lesions associated with cell membranes in paroxysmal nocturnal hemoglobinuria (PNH). In this study, carbon-platinum replicas of membranes of freeze-cleaved, partially hydrated PNH red cells and isolated PNH cell ghosts failed to confirm the existence of these abnormalities. This suggests that the previously described lesions are the products of drying artifacts, although they may reflect hidden structural differences between PNH and normal red cell membranes.

Fig. 2.—Replica of the external aspect of a PNH cell membrane. The membrane is covered with many particles which tend to form chains and clusters. 60,000 x.

Fig. 3.—The internal or cytoplasmic aspect of a PNH cell membrane appears finely granular and is also partially covered with particles which are fewer in number than those seen on external surfaces (Figure 2). 60,000 x.

Fig. 4.—The fracture plane (larger arrow) through a pellet of PNH ghosts has passed along the external surface of one cell membrane (left), through the membrane of an adjacent ghost (small arrows), and along its internal or cytoplasmic aspect (right). As in intact cells, external surfaces of lysed membranes are covered with more particles than internal aspects of membranes. 54,000 x.

Fig. 5.—Cytoplasm of an intact PNH cell showing packed small particles filling the cytoplasm. 68,000 x.

Fig. 6.—Replica of the area formerly occupied by cytoplasm in a ghost prepared by gradual osmotic lysis. The area appears nearly empty, containing only a few small particles which may represent residual hemoglobin (arrows). 68,000 x.
SUMMARIO IN INTERLINGUA

Es reportate in le litteratura que studios per microscopia electronic in desiccate phantomas isolate de erythrocytos ha revelate lesiones associate con le membranas cellular in nocturne hemoglobinuria paroxysmic. In le presente studio, replicas ex carbon-platinum del membranas de cryofissionate e partialmente hydratate erythrocytos e isolate phantomas de cellulas in nocturne hemoglobinuria paroxysmal non ha confirmate le existentia del mentionate anormalitates. Isto suggesta que le previemente descripte leso es le producto de artefactos del desiccation, sed il es possibile que illos reflete occulte differentias structural inter le membranas de erythrocytos de nocturne hemoglobinuria paroxysmal e illos de erythrocytos normal.

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REFERENCES

15. Steere, R. L.: Electron microscopy of
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