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CELL VOLUME AND OSMOTIC PROPERTIES OF ERYTHROCYTES AFTER COMPLEMENT LYSIS MEASURED BY FLOW CYTOMETRY[1]

JOHANN BAUER[2] AND GÜNTER VALET[3]

From the Max-Planck-Institut für Biochemie, D-8033 Martinsried, Federal Republic of Germany

The changes of volume distribution curves of erythrocytes during and after lysis by complement or nystatin or in hypotonic buffers were measured by flow cytometry. Biconcave and spheroidal ghosts were observed after complement lysis and spheroidal ghosts were seen only after nystatin and hypotonic lysis. The spheroidal ghosts derived from red cells lysed by complement or nystatin were permeable to sucrose; those from hypotonic lysis were sucrose-impermeable. Spheroidal ghosts after complement lysis remained permeable for sucrose whereas spheroidal ghosts after nystatin lysis resealed after removal of the drug by washing. Biconcave ghosts produced by complement lysis were almost impermeable to sucrose initially and therefore responded to osmotic changes, but they became sucrose-permeable upon prolonged incubation at 37°C. The rate of sucrose equilibration increased as the stability of the biconcave shape diminished with increasing numbers of C5b-9 complexes. At 850 C5b-9 complexes/ghost, the biconcave shape and impermeability for sucrose were completely lost. The results support the hypothesis that complement C5b-9 complexes, in addition to the interaction with the lipid bilayer, may interact with the cytoskeleton of the erythrocyte membrane.

Activation at the cell surface of either the classical or the alternative complement pathway effectively lyses erythrocytes or nucleated cells (1). Lysis is caused by C5b-9 complexes[4] (monomeric complex formed from components C5b through C9) assembled after activation of C5 by both complement pathways (2). The C5b-9 complexes are lipophilic dimers with a m.w. of 1.7×10^6 (3, 4) and behave as integral membrane proteins (5–8), requiring detergent extraction for solubilization (9, 10). The embedded C5b-9 dimers are cylindrical protein complexes with a diameter of 10 nm and a length of 15 nm (11). It is not clear whether the complexes have central hydrophilic holes (12, 13). The lipophilic interaction of the protein complex with the membrane occurs via the C9 molecules (14).

There is evidence that the cell membrane-bound C5b-9 dimers increase the passive fluxes of ions and low m.w. compounds through the membrane. The increased solute flux is supposed to occur either via a protein pore (doughnut hypothesis) (15) or via a lipid channel (mixed micelle hypothesis) (16) that is formed around one dimer (5) or between several dimers (17). It is thought that during transmembranous equilibration of low m.w. solutes, colloid osmotic swelling of the cells to their maximum volume occurs with subsequent rupture of the cell membrane and protein release (17–20). Similarly, colloid osmotic cytolysis can be effectively induced by ion channel formers, such as nystatin (21), that do not allow protein exchange. Both complement and nystatin lysis may be delayed or prevented by the presence of high concentrations of bovine serum albumin (BSA) in the suspending medium (22) counterbalancing the colloid osmotic pressure of intracellular hemoglobin.

The fast and continuous measurement of the cell volume of single cells by flow cytometry constitutes a new possibility to investigate the volume changes of erythrocytes during complement lysis (23). Earlier, it was found that both biconcave and spheroidal ghosts were formed after complement lysis and that the transition from the biconcave to spheroidal shape was an abrupt and nonosmotic process (23) if more than 850 C5b-9 complexes were bound to the erythrocyte membrane (24). The finding of two types of ghosts was surprising because one would expect the presumed colloid osmotic lysis mechanism should be similar for all erythrocytes.

The aim of this study was to characterize further the properties of the biconcave and spheroidal ghosts derived from complement lysis of sheep red cells. For comparison, hypotonic lysis and colloid osmotic lysis of erythrocytes by nystatin were investigated. The functional behavior of the erythrocytes during and after complement lysis was of particular interest.

MATERIALS AND METHODS

Buffers. Low ionic strength buffers were obtained by 1 + 1 (v/v) dilution of Veronal- (5 mM) buffered-saline (VBS) (25), pH 7.4, with 5% glucose solution (VBS-G), or by 1 + 1.5 dilution of VBS with 9.7 % sucrose solution (VBS-S). VBS buffer containing (20 mM) EDTA (VBS-EDTA) was obtained by 4 + 1 dilution of VBS with 0.1 M EDTA-buffer pH 7.4. The buffers for the cell assays contained 0.3% gelatin; the sheath fluid in the flow cytometer was without gelatin. All buffers, except VBS-EDTA, contained 0.15 mM Ca^{2+} and 1 mM Mg^{2+}. Buffers with different osmolarities (130, 190, 240, 300, 370 mOsmol) but constant electrical conductivity (60 mM NaCl, 5 mM Veronal, pH 7.4, 6.6 mS/22°C) were prepared by varying the sucrose concentration of the VBS-S buffer (0, 60, 110, 170, 240 mM).

Erythrocytes. Erythrocytes were collected from the jugular vein of sheep or from the cubital vein of humans. They were optimally sensitized with rabbit anti-sheep erythrocyte membrane serum (Behringwerke, Marburg, FRG) or with anti-human erythrocyte membrane serum (Boehringer, Mannheim, FRG). In some experiments, human erythrocytes were covalently fluoresceinated by incubation of sensitized cells at a concentration of 1×10^9 cells/ml with 0.25 mM FITC[4] for 3 hr at pH 9.5 and 25°C.

Lytic assays.

a) Volume analysis during lysis. The erythrocytes (8×10^6 cells/ml) were analyzed during lysis at 25°C. They were suspended in VBS-G and were lysed by the addition of 20 μl guinea pig serum/ml assay or 132 μg/ml nystatin (Sigma, St. Louis, MO). Hypotonic lysis was performed by suspending 5 μl of either human or sheep erythrocytes in 250 μl of a 130-mOsmol VBS buffer. The degree of lysis at various time intervals after the start of the incubation was photometrically monitored by the decrease of light scattering at 660 nm in a cuvette of a Zeiss PMQ 3 photometer (Zeiss,

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[2] Present mailing address of Dr. J. Bauer: Department of Physiology, Box 3709, Duke University, Durham, N.C. 27710.
[3] Correspondence should be addressed to Prof. Dr. G. Valet Max-Planck-Institut für Biochemie, 8033 Martinsried, FRG.
[4] Abbreviations used in this paper: EDTA, ethylene diaminetetraacetate; C5b-9, monomeric complex formed from complement components C5b through C9; CH50, amount of complement that produces 50% lysis in a hemolytic assay; VBS, Veronal-buffered saline; FITC, fluorescein isothiocyanate.

Oberkochem, Germany) at 25°C. The decrease of the scatter paralleled the hemoglobin release that was determined by centrifuging aliquots of the assays for 15 sec at 8000 × G and measuring the released hemoglobin photometrically at 412 nm.

b) Osmotic reactivity of ghosts. The erythrocytes were incubated for 1 hr at 37°C at a concentration of 5×10^6/ml with variable amounts of whole guinea pig complement, with 132 μg/ml nystatin in isotonic VBS-G buffer, or in a 130-mOsmol hypotonic buffer. An aliquot of each assay was centrifuged for 2 min at 8000 × G and the hemoglobin in the supernatant was measured photometrically at 412 nm to determine lysis. Another aliquot was diluted 1/25 in VBS-S buffers of constant electrical conductivity (6.6 mS at 22°C) but osmolarities between 190 and 370 mOsmol to test the osmotic reactivity, i.e., the resealing capacity of the erythrocyte ghosts. These samples were analyzed by flow cytometry.

c) Determination of the number of nystatin and complement complexes in the cell membrane. The number of complement lesions were determined by lysing EA cells in VBS-G at a concentration of 1×10^8 cells/ml with C7-deficient human serum to which ^{125}I C7 was added. The total amount of effective complement was 5 CH50.[4] The number of cellbound ^{125}I C7 molecules was determined from the cellbound radioactivity and the specific radioactivity of the labeled C7 after washing the ghosts twice in VBS (24). The cell membrane-bound nystatin molecules were calculated from the difference between the free NH_2 groups of nystatin in the supernatant after incubation with erythrocytes and of the original nystatin solution. Hence, hypotonically lysed erythrocyte ghosts at a concentration of 5×10^8 cells/ml were washed twice and were incubated for 30 min with 100 μg/ml nystatin in VBS-G at 37°C. The ghosts were sedimented by 7 min at 8000 × G centrifugation. The free amino group of nystatin in the supernatant was stained by 1 mM trinitrobenzene sulfonic acid in VBS. The yellow color of the reaction was determined photometrically at 340 nm (26) after 30 min incubation at 20°C and acidification of the assay to pH 4. The number of the membrane-bound nystatin molecules was determined from the decrease of the yellow color compared with the control assay, when nystatin was incubated without erythrocyte ghosts.

Flow cytometry.

a) Cell volume analysis. The volume distribution curves of erythrocytes or erythrocyte ghosts after complement, nystatin, or hypotonic lysis were measured electrically in a Metricell flow cytometer (27) at a speed of 800 to 1200 cells/sec. The cylindrical orifice had a diameter of 65 μm and a length of 52 μm, and the cell flow was hydrodynamically focused through the center of the orifice. The aperture current was 0.47 mA and the measurements were performed at 25°C.

b) Osmotic reactivity. The volume changes of erythrocytes and ghosts after dilution of the cells in buffers with different osmolarities were measured within 0.5 and 5 min after dilution. The sheath flow in all experiments was 300 mOsmol VBS solution without gelatin that had the same electrical conductivity as the buffers of different osmolarity used for the resealing experiments. The hydrodynamically focused particle beam and the sheath fluid of the Metricell were not mixed during the analysis because the flow conditions in the instruments were laminar and because the time of contact between particle beam and sheath flow was only a fraction of a second (28). This is an important condition for the validity of the size measurements in buffers of different osmolarity because the measurements would not be interpretable if sheath flow and sample flow would mix in the orifice. The maintenance of the osmotic conditions of the erythrocytes in the orifice was experimentally verified by suspending the erythrocytes in an isotonic VBS-S buffer and measuring consecutively the volume distribution curves with a 300 mOsmol and a 130 mOsmol sheath fluid of the same electrical conductivity. The cell volume in both experiments was the same, indicating the sample and sheath fluid did not mix during the erythrocyte transit in the orifice.

c) Cell volume and fluorescence analysis. The FITC-labeled erythrocytes were measured in a Fluvo-Metricell flow cytometer (29), which determined simultaneously the cell volume and the fluorescence intensity of each cell. The cylindrical orifice had a diameter of 110 μm and a length of 100 μm. The particle beam was hydrodynamically focused. The aperture current was 0.9 mA. The fluorescence was excited with an HBO-100 mercury high pressure lamp between 450 and 500 nm. The fluorescence pulses emitted were collected between 500 and 580 nm and were amplified with a photomultiplier tube.

Data storage and handling. The maximal amplitude of the volume and fluorescence pulses of each cell was electrically measured. The pulse data were classified and were displayed as one or two-dimensional histograms in a multichannel analyzer. The data were then transferred to a magnetic tape and were evaluated by Fortran IV computer programs described earlier (30).

RESULTS

The time course of hemolysis was measured in a photometer at 660 nm and 25°C. Figure 1 shows that in the presence of high complement (80 U CH50) or nystatin (132 μg/ml) concentrations, sheep blood cells began to lyse 2 min after the start of the experiment. Fifty percent lysis was obtained after 4 min and 90% after 6 min. In contrast, hypotonic lysis of human red cells occurred within 40 sec after dilution of the cells in a 130-mOsmol buffer. Hypotonic hemolysis was not investigated in sheep red cells because they lysed so fast that the time course of lysis could not be measured.

The volume changes of the red cells during lysis were analyzed with the Metricell. Figure 2*a* shows that between 2.5 and 5.5 min after the addition of complement, i.e., during the period of hemoglobin release (Fig. 1), the sheep erythrocytes swelled from the normal biconcave to the spheroidal volume, which corresponded to an 1.51-fold increase of the apparent volume. The true cell volume, however, is only 1.29-fold higher, if the increase of the apparent volume is corrected for the change of the form factor during cell swelling from the biconcave to the spheroidal state (30). The spheroidal volume was stable for at least 1 day provided the ghosts were stored at 0°C.

In contrast, Figure 2*b* shows that before colloid osmotic lysis in the presence of nystatin, the mean volume of sheep red cells increased from the point of nystatin addition (equivalent to volume class 40) to the beginning of hemoglobin release (equivalent to volume class 48) after 2 min and further to the end of the experiment (equivalent to volume class 80) after 8 min. Hence, the main difference between the volume alteration of sheep red cells during complement and nystatin lysis is that during complement lysis no volume increase was detected before hemoglobin release. A different time course of volume changes was found during hypotonic lysis of human red cells. The second solid curve in Figure 2*c* shows a volume analysis of human red cells that was measured between 15 and 40 sec after dilution in a 130-mOsmol buffer when hemoglobin release occurred (Fig. 1). During this period small *and* large cells were observed. The 3.0 and 4.0 solid curve of Fig. 2*c* show that between 1 and 3 min, only post-hemolytic ghosts with small volume pulses were present in the sample (compare with Fig. 1). Subsequently, the volume pulses increased and after 5 min they corresponded to volume pulses of spheroidal cells.

The observation of small volume pulses suggesting the presence of small ghosts after hypotonic lysis for a short period of about 4 min was surprising because microscopic observations revealed that all hypotonically lysed cells were large spheroids. This apparent discrepancy can be resolved by assuming a transient increase of the electrical conductivity of the cell

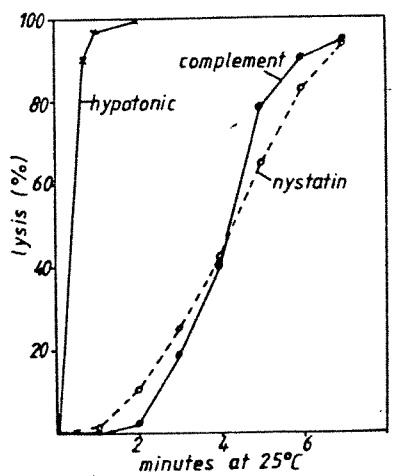

Figure 1. Time course of hemoglobin release from sheep erythrocytes during complement, nystatin, and hypotonic lysis. The progress of hemoglobin release was determined by measuring the decrease of light scattering at 660 nm.

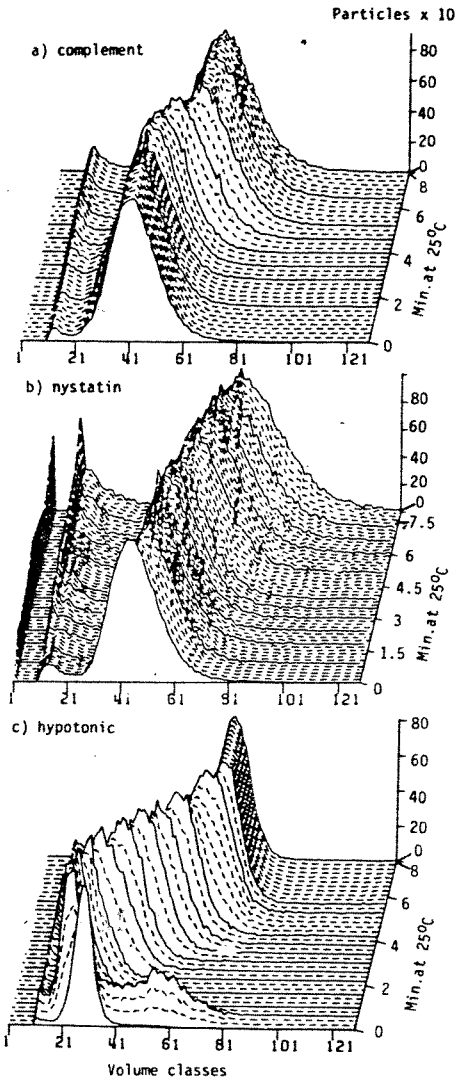

Figure 2. Time course of the changes of volume distribution curves of sheep erythrocytes during complement (80 CH50) (*a*), nystatin (132 μg/ml) (*b*), and hypotonic lysis (130 mOsmol) (*c*). The solid volume distribution curves were measured at different times after the start of the experiment. The dashed curves were linearly interpolated. Cell volume is plotted on the x-axis and time of incubation on the y-axis. Between 20 and 60 × 10^3 cells per curve (z-axis) were measured for this histogram and also in subsequent histogram curves.

membranes due to ion leaks, indicating substantial lesions of the cell membranes. Obviously these ion leaks were resealed several minutes later when the volume pulses of the ghosts corresponded to the actual spheroidal volume. The results in Figure 2 clearly show that during nystatin and hypotonic lysis, but not during complement lysis, cell swelling was detected before hemoglobin release, and that during hypotonic lysis, but not during complement and nystatin lysis, the electrical resistance of the cells was transiently decreased immediately after the onset of lysis. In further experiments, we studied whether also during complement lysis our Metricell technique would detect a fraction of ghosts with reduced electrical resistance. An important prerequisite for such an experiment was to secure that the electrical resistance of the cells was not decreased to such an extent that the volume pulses of these cells disappeared into the background noise. To approach this problem a volume-independent fluorescence marker for the presence of cell ghosts was tested. Covalently FITC-labeled cells were prepared and measured in a Fluvo-Metricell flow cytometer.

We first investigated whether during hypotonic lysis all cells were detected by volume analysis. Figure 3*c* shows that during the first 3 min of hypotonic lysis, the same fluorescence distribution curve with the maximum in channel 22 (y-axis) was detected as in the unlysed control cells (Fig. 3*a*), but a high percentage of ghosts with volume pulses (x-axis) smaller than that of normal red cells (Fig. 3*a*) was seen. Note that no volume pulses were recorded in the background region that is normally below volume class 2. These findings indicate the electrical conductivity of the ghosts was increased, but the ghosts were not completely permeable for the electrical current, suggesting curves obtained by one-parameter analysis represented all cells that passed through the orifice of the Metricell.

We then looked for the fraction of cells that during complement lysis became transiently permeable for the electrical current. Figure 3*b* shows an analysis of human red cells (sheep erythrocytes were too small for this type of analysis). The volume pulses (x-axis) and fluorescence (y-axis) pulses of the FITC-labeled cells were simultaneously recorded between 3.0 and 5.0 min after complement addition when the rate of lysis was maximal (0.5% cells/sec). During this period, volume pulses equal to or greater than those seen with intact erythrocytes were generated, indicating that a fraction of the cells passed the orifice before and another passed after lysis. At the

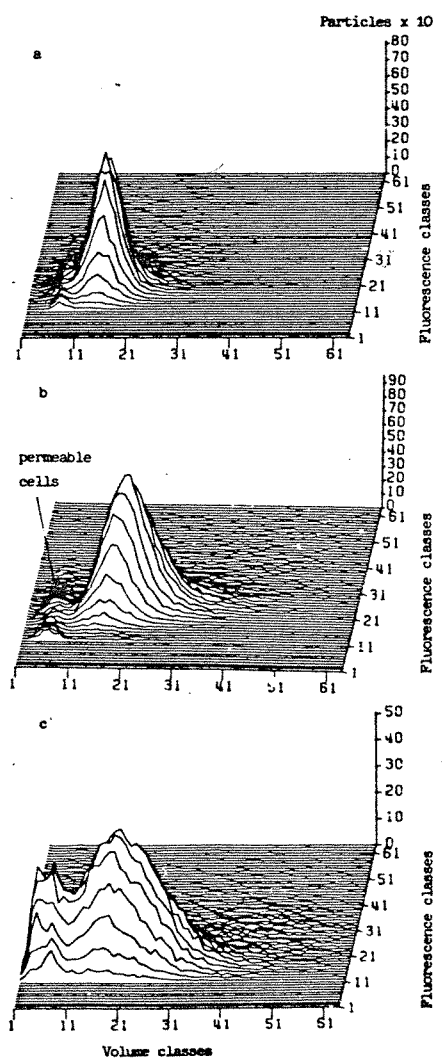

Figure 3. Volume and fluorescence two-parameter analysis of FITC-labeled human erythrocytes without complement (*a*), during complement lysis (*b*), and during hypotonic lysis (*c*). The volume (x-axis) and the fluorescence (y-axis) signals of each single cell were measured simultaneously. The measurements were made between 2 and 6 min after the start of the complement reaction and between 0.5 and 3 min during hypotonic lysis when the lytic rate was maximal in both assays.

moment of lysis, a third fraction may have passed the orifice, because the amount of small cells in Figure 3*b* was increased by 1% when compared with intact erythrocytes of Figure 3*a*. Based on these assumptions, the average time of cell opening (t) was calculated from the ratio of percent open ghosts to percent of cells lysed/sec, and was found to be 2 sec. This result shows the membrane lesion through which hemoglobin escapes remains open for a shorter time period during complement than during hypotonic lysis.

Next, we investigated whether the membrane alterations caused by the various mechanisms of lysis were also different with regard to the osmotic behavior of the ghosts. Figure 4 (curve 4) shows that spheroidal ghosts produced by hypotonic lysis were completely resealed because they shrank upon dilution in hypertonic (370 mOsmol) VBS-S buffer at room temperature. (Similar results were described earlier (31)). Spheroidal ghosts, however, caused by complement or nystatin lysis were apparently permeable for sucrose, because they did not shrink in the hypertonic buffer (Fig. 4, curves 2 and 3).

Although the spheroidal ghosts obtained after complement and nystatin lysis were osmotically inactive with respect to sucrose, important differences between the two kinds of ghosts did exist. Mainly large spheroids were found after nystatin lysis regardless of whether the cells were treated with 70 μg/ml (the lowest nystatin concentration that lysed sheep erythrocytes) or with 235 μg/ml, corresponding to 10^7 to 10^8 bound nystatin molecules/cell. With the removal of nystatin from membranes by 15 min incubation at 37°C in VBS-G followed by washing, the ghosts remained spheroidal but became impermeable for sucrose. Hence, the nystatin-induced sucrose leak was of reversible nature.

After complement lysis with low serum concentration (1 or 2 CH50), the majority of ghosts was biconcave and responded to osmotic manipulations; swelling occurred in hypotonic (240 mOsmol) and shrinking occurred in hypertonic (370 mOsmol) VBS-S buffers (Fig. 5, curves 3 and 4). In contrast to nystatin, the quantity of ^{125}I radioactivity incorporated into the C5b-9 complexes via the ^{125}I C7 was not reduced by incubation at 37°C and by washing the ghosts (data not shown). Hence low complement concentrations caused hemoglobin release but apparently did not abolish other features of the erythrocytes such as the biconcave shape or the impermeability for sucrose. A further analysis, however, using various amounts of complement, i.e., various amounts of C5b-9 complexes bound per ghosts, revealed that even in biconcave ghosts irreversible alterations of the membrane indeed occurred. Figure 5 shows that complement-lysed biconcave ghosts were osmotically active when diluted in buffers with different osmolarities measured

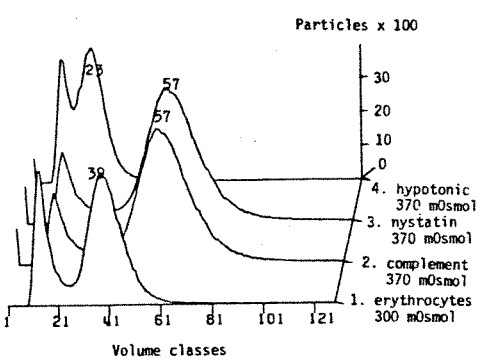

Figure 4. Volume distribution curves of sheep erythrocytes in 300 mOsmol isotonic VBS-S buffer (1) and of erythrocyte ghosts in 370 mOsmol buffers after complement lysis (6CH50) (2), nystatin lysis (132 μg/ml) (3) and hypotonic lysis (130 mOsmol) (4).

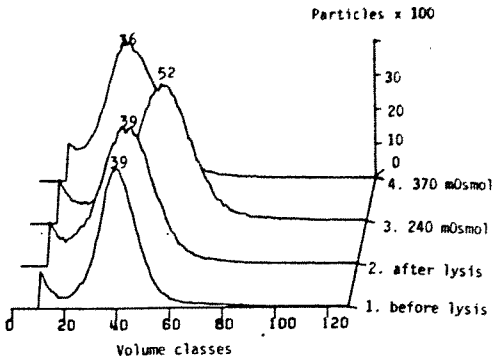

Figure 5. Volume distribution curves of sheep erythrocytes (1) and/or erythrocyte ghosts after complement lysis with 2 CH50 (80% lysis) (2), diluted in an isotonic 300 mOsmol VBS-S buffer, in a 240-mOsmol hypotonic buffer (3), and in 370 mOsmol hypertonic buffer (4).

by the volume distribution curves within 10 min at room temperature. The ghosts, however, slowly increased their volume when incubated during a prolonged time at 37°C in 370 mOsmol hypertonic VBS-S buffer, indicating a significant permeability for sucrose. The effect depended on the concentration of guinea pig serum used during lysis, i.e., on the number of C5b-9 complexes in the cell membrane. When 50% of the erythrocytes were lysed with 1 CH50 (0.25 μl guinea pig serum/ml cell suspension) and the cells were suspended by 1/25 dilution in the 370 mOsmol hypertonic VBS-sucrose buffer, the ghost shrank immediately. One-half of the shrunken ghost (volume class 36), however, gradually swelled to spheroids (volume class 57) during 30 min at 37°C incubation, which means the membranes were permeable to sucrose, a fact that became apparent with prolonged incubation. With 3 CH50, more than 95% of the EA cells were lysed. The biconcave ghost shrank in hypertonic (370 mOsmols) VBS-S buffer, but all swelled within 30 min at 37°C to the spheroidal volume.

We also looked at the spheroidal ghost lysed by 3 CH50, which immediately after dilution in 370-mOsmol hypertonic buffer shrank slightly but returned to the spheroid volume within 3 min at 25°C. Spheroidal ghosts obtained after lysis by 6 CH50 U also shrank but returned to spheroids after 40 sec, indicating quick equilibration of sucrose through the cell membrane. On the other hand, intact erythrocytes when shrunken in hypertonic media from volume class 39 to 36 (Fig. 4, curve 4) maintained their reduced cell volume unchanged for several hours. These results show there is an increase of sucrose permeability in complement-lysed erythrocyte ghosts commensurate to the quantity of complement present during lysis. An additional loss of impermeability to sucrose was detected when the ghosts were spheroid after lysis, suggesting the biconcave shape supports the cells counterbalancing the effects of the C5b-9 complexes.

Because the biconcave shape is lost when more than 850 C5b-9 complexes are bound to the ghost membrane (24), it was of interest to test the effect of less than 850 C5b-9 complexes (i.e., less than 3 CH50). Figure 6 (curves 3 and 4) shows that intact sheep erythrocytes suspended in a 240-mOsmol VBS buffer swelled by 10% until osmotic equilibration. Swelling in hypotonic buffer of 190 mOsmol was required to make them almost spheroid. Similarly, as for intact erythrocytes, biconcave ghosts lysed by 1 CH50 guinea pig serum swelled by 10% in a 240-mOsmol buffer (Fig. 7*a* and *b*, curve 3). Curves 4 and 5 in Figure 7*a* and *b*, however, show biconcave ghosts with 2 CH50 or with 3 CH50 swelled by 33% and 46%, respectively. The results indicate increasing the number

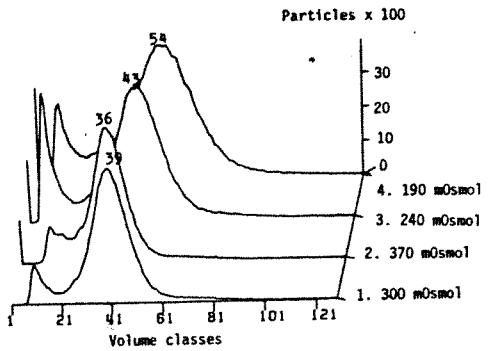

Figure 6. Volume distribution curves of sheep erythrocytes in a 300-mOsmol isotonic (1), 370-mOsmol hypertonic (2), 240-mOsmol hypotonic (3), and 190-mOsmol very hypotonic VBS-S buffer (4).

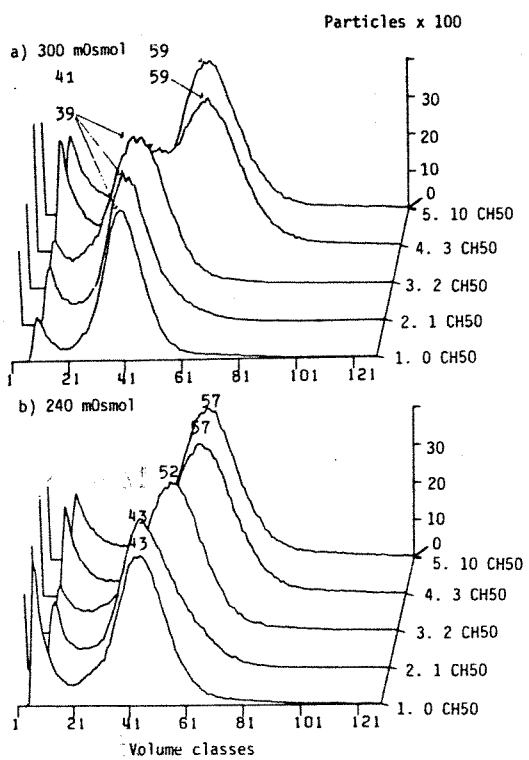

Figure 7. Volume distribution curves of erythrocytes and erythrocyte ghosts diluted in 300-mOsmol isotonic (*a*) and in 240-mOsmol hypotonic (*b*) VBS-S buffers. The cells were measured after 1 hr incubation at 37°C with 0 CH50 (1), 1 CH50 (2), 2 CH50 (3), 3 CH50 (4), and 10 CH50 (5) U guinea pig serum.

of C5b-9 complexes diminishes the stability of the biconcave shape that at 850 complexes/cell cannot be maintained.

DISCUSSION

The flow cytometer measurements show hemolysis of red cells with complement and nystatin, both thought to occur by colloid osmotic forces (17–21), to be similar in several respects but different from hypotonic lysis in others. This is understandable because the cell membrane and the ratio of low to high m.w. substances remains constant during hypotonic swelling until the moment of membrane rupture at maximum swelling. In contrast, complement and nystatin perturbed the cell membrane (5, 21), causing leaks for substances of low m.w. (16, 17).

Hypotonic lysis causes large transient lesions in the cell membrane (32, 33) that in our experiments were manifested by a decrease of the apparent electrical cell volume between 1 and 4 min after hypotonic lysis (Fig. 2*c*), although microscopically the ghosts were spheroid. Later, the hypotonic ghosts resealed as maximally swollen spheroids, because they quantitatively shrank in hypertonic sucrose buffer (Fig. 5, curve 4) and remained at this volume for several hours of incubation, indicating the ghosts were well resealed.

No transient decrease of the apparent cell volume was observed during complement and nystatin lysis. This means that either the erythrocyte membranes resealed within less than 2 sec or the lesions were very small at the moment of hemoglobin release. The latter possibility is supported by the fact the ghosts were correctly measured as spheroids by electrical sizing, although no resealing occurred in complement-lysed spheroids. In this case, hemoglobin release may have occurred at or around the areas of membrane weakening through the insertion of complement or nystatin complexes. Membrane weakening could especially occur in membrane areas in which the complement complexes are not randomly dispersed on the surface of the erythrocytes but are accumulated in some areas as patches (17).

Although complement and nystatin lysis show similarities, they are not identical. The number of hexameric nystatin complexes (21) required to lyse a cell is 10^3 to 10^4-fold higher than the number of C5b-9 complexes found on biconcave or spheroidal ghosts after lysis (24). Biconcave ghosts were generated during complement lysis (23) (Fig. 5, curve 2) but were not observed during nystatin lysis. Even at the lowest hemolytic nystatin concentrations, only osmotically inactive spheroidal ghosts were generated. The membrane-bound nystatin complexes could be removed by incubation at 37°C, which caused resealing of the membrane. C5b-9 complexes, in contrast, cannot be removed from sheep and human erythrocyte membranes by the same incubation and washing procedures; they are firmly anchored in cell membranes—detergents are required for solubilization (9, 10).

The shape of erythrocyte membranes is stabilized by the cytoskeleton (33–35). The removal of the membrane lipids by detergents leaves the biconcave cytoskeleton of the erythrocyte intact (34). C5b-9 complexes mediate the dose-dependent transition of biconcave to spheroidal shape. It is conceivable that C5b-9 complexes do not only interact with the lipid bilayer but that the local interaction with the cytoskeleton is of importance for the observed shape transition from biconcave to spheroidal ghosts and also for hemoglobin release. An alteration of the cytoskeleton by complement lysis is also supported by the osmotic properties of complement-lysed ghosts. Intact erythrocytes swell in a 240-mOsmol hypotonic buffer by 10% and are then in osmotic equilibrium. Complement-lysed biconcave ghosts swell in the same buffer between 33 and 46%, depending on the complement dose. This means complement tends to spheroidize the erythrocyte membrane, destroying its ability to maintain the energetically unstable biconcave shape.

The membrane damage observed may be due to physicochemical (5, 36) or enzymatic effects produced by C6, whose natural substrate is hitherto unknown (37). At the critical number of 850 C5b-9 complexes, when the shape transition occurs and the ghosts lose their osmotic reactivity, only about 1% of the membrane fatty acids are bound or reoriented by the lytic complexes (4, 5, 24). This low value suggests these complexes specifically attack structures that stabilize the membrane but do not randomly disorder large parts of the lipid bilayer; this occurrence is more likely during nystatin lysis.

This nonosmotic transition of the erythrocyte ghosts from biconcave to spheroid (23), together with the sudden increase in the permeability of the cell membrane, may be of general

importance, especially for the survival of nucleated cells under complement attack.

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