Upsala J Med Sci 86: 1–8, 1981

Jet Expulsion of Cellular Contents from Red Cells during Photodynamic Hemolysis

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Dedicated to Torsten Teorell

ABSTRACT

When red cells are incubated in the dark in the presence of the dye Rose Bengal and subsequently irradiated with visible light, they hemolyse. Under certain conditions some of the hemoglobin is expelled in the form of a convective jet and appears as a transient cloud beside the cell. Elastic contraction of the membrane is not a sufficient driving force for the jet. A plausible mechanism (an osmotic "pump") is presented.

INTRODUCTION

A landmark in our acquisition of knowledge concerning the red blood cell was the finding of Teorell (21) in 1952 that posthemolytic ghosts obtained by mild osmotic hemolysis retained some permselectivity and were not merely the inert leaky remnants of red cells. Implicit in the discovery is the ability of the red cell membrane to reseal after a transient period of high porosity. Resealing, as Teorell showed, can restore the cell, for a time at least, almost to its original state even though during hemolysis the porosity is such as to allow the escape of molecules heavier than 100.000 daltons (10).

The liberation of hemoglobin (Hb) during hemolysis seems to depend, at least in the initial stage, in a convective outflow of water, whereas in the latter stages it is probably only diffusive (6, 9, 17). Heedman (personal communication) reported that occasionally hemolysis was accompanied by the emission of a cloud of Hb. We too, observed this phenomen and attempted, in vain, to find a hemolytic system which would reproducibly yield cloud formation. It was quite by accident when studying photodynamic hemolysis induced by Rose Bengal (RB) that we observed cloud emission occurring in nearly all the cells in the microscopic field. The phenomen was moreover reproducible. This paper describes these clouds and discusses the mechanism which creates

them.

Photodynamic hemolysis is a mode well suited to microscopy since it can be triggered off at will by merely illuminating the cells with the required wavelength and intensity.

Photodynamic hemolysis

If red cells are incubated in the dark with a low concentration ($\sim 10^{-4}$ M) of a xanthene dye such as RB (5) (or fluorescein, eosin or erythrosin) hemolysis can be induced by irradiation with visible light (3). This photodynamic effect is oxidative (2, 4, 15) and all effective photosensitizing dyes are thought to act because of the formation of long-time excited states (16). The structure oxidised is probably a hydrophobic side chain of a membrane protein. The amino-acids susceptible to attack are histidine, tyrosine, tryptophan, methionine and cysteine (2).

METHODS

Blood was taken by a finger prick from apparently healthy individuals and either blood or washed red cells were suspended in 0.16 M NaCl containing about $3.5 \cdot 10^{-4}$ M RB (Mol. Wt. = 1017, obtained from G.T. Gurr, London, U.K.). The cells were allowed to stand for at least 20 min in subdued light. The RB concentration was critical for cloud formation and had to be carefully adjusted whenever a new solution was made up. Hemolysis was induced by illuminating a red cell suspension sealed between a slide and a coverslip. The microscope-camera system was first adjusted in readiness in subdued light, using a neutral filter. For photography the filter was removed and after about 20 s hemolysis usually began. The cells were photographed in either phase or interference contrast (Zeiss) with a 16 mm Arriflex ciné camera on a special stand fixed to a bracket on the wall. The camera was thus not in mechanical contact with the microscope.

RESULTS

In the great majority of cells hemolysis was not manifest as a gradual change but started with explosive suddenness in the form of the ejection of a cloud of refractile material (see Fig. 1). Those clouds extended when maximal for up to about one cell diameter ($\sim 6 \mu m$). Occasionally, as shown in Fig. 2, two clouds were emitted from one cell. The time during which the jet is observed is the real time of its existence because it is so small that it dissipates rapidly by diffusion. Thus for a cloud having the diameter of a red

cell (6.6 μm) the half time of disappearance is about 5 ms (13). The average time during which a cloud was visible was about 200 ms. This is about 5% of the half time for complete hemolysis.

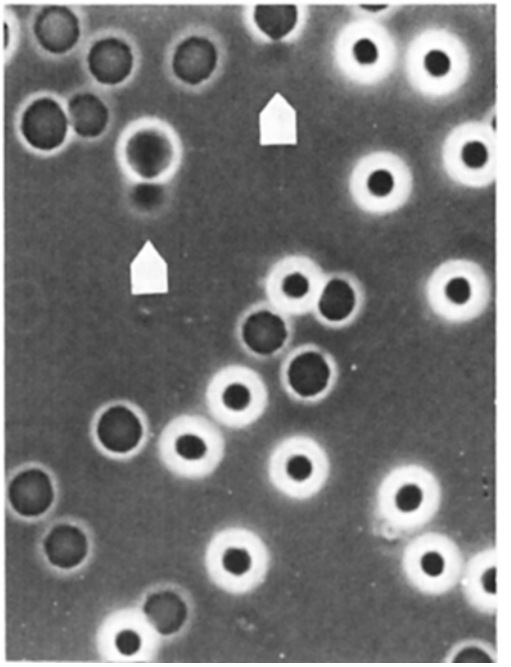

Fig. 1: View of a field in phase contrast showing red cells undergoing hemolysis. The bright cells with dark centres are unhemolysed cells. The dark cells with faint haloes are ghosts. The two cells indicated with arrows are in different phases of jet ejection; in the upper one the cloud has nearly dissipated.

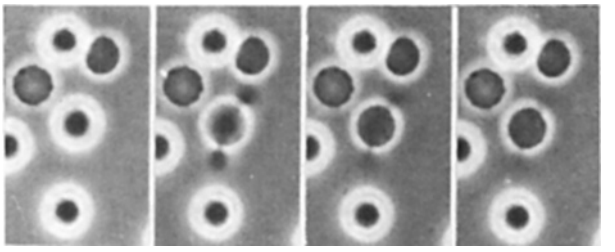

Fig. 2: Four frames from a ciné-film in phase contrast. One cell initially unhemolysed is seen to be emitting simultaneously two clouds about 180^{o} apart.

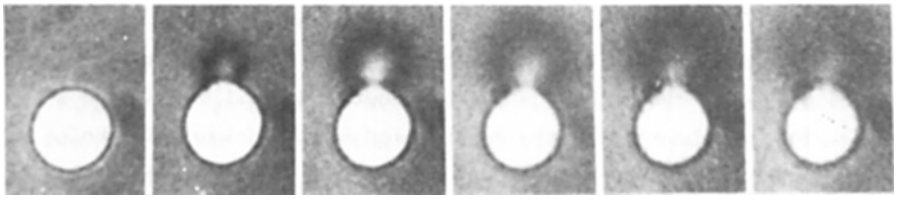

Fig. 3: Six consecutive frames taken in interference contrast at intervals of 23 ms showing cloud formation outside a red cell. The optical path difference (proportional to the amount of material traversed by the beam, i.e. mass/unit area) is maximal in the 3rd frame shown (t=46 ms).

A typical sequence of events is shown in Fig. 3, where the frames are taken at 23 ms intervals. The fact that the cloud in Fig. 3 may have a white centre and a dark periphery is of no importance here; it is merely a consequence of the setting of the interferometer; the white centre having a larger phase retardation than the dark periphery. This film shows only the first 115 ms of hemolysis. The cell will eventually change from white to black as it loses more Hb, but, at the stage of the sixth frame, Hb loss has not yet become sufficiently great for contrast reversal to occur.

The clouds emitted from the cell in Fig. 2 appeared apparently synchronous, but the frame speed (44 s^{-1}) does not give good time resolution. Photographs were therefore taken at a rate of 600 frames$\cdot s^{-1}$ (Stålex camera) with a resolution better than 2 ms. One cell was seen emitting two clouds asynchronously.

DISCUSSION

The clouds are undoubtedly due to bulk ejection from the cell because they have two properties of jets; (a) they can propel the emitting cell in the opposite direction to that in which the cloud is emitted and (b) a cloud impinging on a neighbouring cell can move it. These phenomena are not always observed, presumably because some cells adhere to the microscope slide.

It seem generally accepted that hemolysis occurs when the cell has swollen to reach a critical volume (11, 19) at which the rather inelastic membrane (20) is fully extended. At this stage the continued entry of water causes a rise in internal pressure which produces leakiness of the membrane and Hb leaks out, usually over the whole surface, or exceptionally, as in the present case, from a localised site.

The clouds must be generated by an internal pressure excess and it would seem at first sight that the reason for cessation of cloud formation is a falling internal pressure. Since the cloud is a bulk outflow there must also be a hole in the membrane sufficiently large to allow this.

The effect of RB is to cause the red cell to sphere (19) and then to swell and eventually hemolyse. RB associated with the membrane absorbs a number of light quanta successively transferring their energy to the cell components to oxidise them (15). It is thus feasible that "such a disorganization at a strategic point can bring the whole structure down like a house of cards" (19). This may be an adequate description of the origin of a hole, but apparently it is not necessarily a permanent defect as discussed below.

It has been suggested (19) that RB induced photodynamic hemolysis is colloid osmotic in nature (22). This mechanism divides hemolysis essentially into two phases; there is an initial equilibration of ions due to inability of the cell to maintain the normal dyssymetry and finally the colloid osmotic

pressure of the macromolecular cellular contents, mainly Hb, causes the cell to swell and liberate the latter. Resealing may take place afterwards as demonstrated many years ago by Teorell (21).

When cloud emission is not seen, Hb escape presumably occurs at many points scattered over the surface. This is probably also true for the cell which emits a cloud; the cloud cannot account for more than about 20% of the total cellular Hb. Diffusive Hb loss may occur after the jet phase through the same hole or elsewhere over the surface. Often the cell appears to flicker as hemolysis begins (18). This may be due to movements of the surface, but it could reflect transient inhomogeneities in the refractive index outside the cell due to small convective outflows through numerous small holes. In the special case of cloud formation one or sometimes two larger holes open. In seeking a driving force to maintain the jet, two possibilities may be put forward.

Since hemolysis is presumably initiated because the membrane can no longer remain intact due to the rising internal pressure, the membrane must stretch before the hole appears. If the membrane were very elastic, the jet could be driven by subsequent contraction of the membrane. Now Rand (20) found that Young's modulus of the membrane substance was approximately 10^5-10^7 $N \cdot m^{-2}$, a value which means that the membrane cannot be stretched to any significant extent before hemolysis begins and thus elastic contraction of the membrane cannot account for the jet.

As soon as there is a hole in the membrane the internal excess pressure will vanish almost instantaneously since the shock wave, generated by contact of the internal contents with the lower external pressure travels with the velocity of sound in water; i.e. about 1.500 $m \cdot s^{-1}$ (8). This would thus cross a spherical prehemolytic erythrocyte (diameter $6.6 \cdot 10^{-6}$ m) in 4.4 ns, which is less than 1 millionth of the duration of the cloud.

In the absence of an elastic driving force the only other possiblity seems to be an osmotic driving force due to the intracellular contents. When jet expulsion occurs there is one (or two) large hole(s) permeable to all species of particles with the local reflexion coefficients zero. The permeability of the rest of the membrane is not known; it may be normal or more likely increased by the action of RB. The reflexion coefficients must thus vary, that of Hb may still be unity or possibly already lower. As long as the reflexion coefficient for Hb over the rest of the membrane is greater than zero, there will be an inflow of water to the cell. With a constant volume of the distended cell this inflow must be matched by an equal outflow through the hole. The ratio of the velocities of these two flows will be simply that of the cell surface area to the area of the hole. The factors which will determine the velocity of the jet are the internal effective osmolarity, the permea-

bility of the membrane to water and the size of the hole. The internal solute concentration will of course be continuously reduced and the jet must inevitably decay. Although at present this postulate lacks a sound quantitative basis, there is already strong evidence for a convective outflow in the initial stages of hemolysis also in red cells where jets are not apparent.

This evidence is both direct and indirect. From the microinterferometry of individual red cells undergoing hemolysis induced by different kinds of stimuli, hypotonic, lytic, photodynamic and immune, Heedman (9) concluded that in the initial phase Hb liberation was too fast to be accounted for by diffusion. He observed that hemolysis began abruptly and "... when the fading time (optical) was short the impression was obtained that the cell content was ejaculated". Results by Anderson and Lovrien (1) support the finding of Heedman. They concluded that Hb liberation from the cell occurred in two phases with different rate coefficients.

Indirect evidence was provided by Davis et al. (6) who studied hemolysis in hypotonic systems where the Hb loss was reduced by the presence of dextran. The latter apparently exerts its effects by virtue of its colloid osmotic pressure being able to balance that of the intracellular contents (23). Colloid osmotic balance can occur when Hb loss is only partial, because the low molecular species, i.e. ions etc., which are responsible for most of the osmolarity of the unhemolysed cell, escape while most of the Hb still remains within the cell (6, 23). Such a situation can arise from the differences in diffusion coefficients (23), but a changing porosity may also play a role.

A peculiarity of the dextran-inhibited loss is that although the Hb remaining in the cell is roughly proportional to the external dextran concentration, the residual Hb is never greater than about 80%. Now dextran below a molecular weight of about 150.000 daltons can enter the cell during hemolysis (14, 17). Östling (17) also showed that the uptake of dextran was nearly a linear function of the Hb loss, the more Hb lost, the more dextran entered. However, it appeared that the entry of dextran approached zero with an Hb loss of 20%. This observation is consistent with a non-diffusive escape of the first 20% of the Hb; dextran which can only enter by diffusion is unable to do so until the outward fluid stream has slowed down sufficiently.

It is true that this indirect evidence is from hypotonic and not colloid osmotic hemolysis but, apart from the initially higher osmotic differences in the former mode, in both processes ionic equilibration is probably largely separated in time from Hb liberation (12). If a non-diffusive, convective outflow is a normal event in the first stage of hemolysis, with a number of small holes scattered over the cell surface (7), cloud emission may be just a special case of this, rather than an unique event.

It is perhaps not surprising that cloud emission, which must involve a more than usually large local effect of a lysin on the membrane, is associated with a photosensitive dye capable of oxidising many times its equivalent of substrate.

The jets exist for only about 0.2 s. Whereas the half time for the complete hemolysis of a cloud emitting cell is usually several seconds, Heedman's (9) data indicate that the first 20% of the Hb escapes in about 0.5 s. This presumably sets the time limit for jet ejection, since Hb escape becomes diffusive after this.

There is, however, also evidence that the jet may cease for another reason. One cell was observed to emit two jets asynchronously. If the interior of the cell is homogenous, the driving force will be the same everywhere.

The decay of one jet before the other suggests a membrane controlled process in which the hole is becoming smaller and closing. Possibly the holes are subject to a relaxation process in which opening is pressure-generated but closing is time-dependent.

ACKNOWLEDGEMENTS

We thank Professor Sune Berndt for very helpful discussions.

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Received November 18th 1980

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