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HYPERTONIC CRYOHEMOLYSIS AND THE CYTOSKELETAL SYSTEM

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Hypertonic cryohemolysis is defined as the lysis of erythrocytes in a hypertonic environment when the temperature is lowered from above 15–18°C to below that temperature. This has been found to be a general phenomenon (that is, whether the solute is charged or not), to exhibit interesting temperature characteristics and to be preventable by agents such as valinomycin which tend to dissipate the concentration gradient across the cell membrane. As yet, no clear information is available to translate this phenomenon to the molecular level and to relate it to current structure/function concepts in the erythrocyte membrane. In this study, data are presented which would indicate on the basis of two entirely separate methodologies that the spectrin-actin cytoskeletal framework is involved in this phenomenon. The first of these methodologies is based on radiation-induced ablation of cryohemolysis and indicates that an intact macromolecular complex of an order of 16 000 000 daltons is required for cryohemolysis with hypertonic NaCl. The second methodology is based on selective cross-linking of spectrin and actin in the agent diamide, which resulted in concentration-dependent suppression of cryohemolysis. Polyacrylamide gel electrophoresis of the erythrocyte from diamide-treated cells showed intense protein aggregation with loss of spectrin-actin and bands 4.1, 4.2. We conclude that the spectrin-actin cytoskeletal system possibly including its interaction with phospholipids is the key to the phenomenon of hypertonic cryohemolysis.

Introduction

Cryohemolysis is predominantly an in vitro phenomenon, although the solutions used clinically for hyperalimentation result in massive cryohemolysis in vitro when incubated first at 37°C and then the temperature is lowered to 4°C [1,2]. Fortunately, for human use, any temperature shift would be in the opposite direction, in which case, this phenomenon does not occur. The details of cryohemolysis as a phenomenon have been reasonably well worked out, including parameters of temperature, pH, specific ions and sugars, rate of temperature change, influence of sulfhydryl reagents, cytochalasins, and ATP depletion [1–3]. Unfortunately, these observations have not led in a direction which would eventuate in the elucidation of the molecular basis.

In the present state of knowledge, hypertonic cryohemolysis can only be studied at the intact cell level, thus precluding the use of many techniques of structure/function analysis. Irradiation inactivation [4,5], however, is a technique which is applicable at the whole cell level, yet provides information on the size of the structure central to a particular function. The conditions necessary for this technology to be applicable require that the cells be in a frozen state, which can be easily achieved using the modern cryoprotective technology, that the atmosphere be free of oxygen, and that the membrane barrier function be affected relatively little in comparison to any effect on cryohemolysis. This situation was also found to obtain.

For the second methodology, a selective mem-

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Abbreviation: SDS, sodium dodecyl sulfate.

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brane cross-linking reagent applicable to the whole cell was employed. Diamide (azodicarboxylic acid bisdimethylamide) treatment results in cleavable disulfide bonds being formed from membrane sulfhydryl groups and the effect in intact red blood cells is thought to be predominantly on the cytoskeletal system [6,7].

Both of these methodologies pointed to the spectrin-actin system as being the central site of perturbation in the membrane during hypertonic cryohemolysis.

Methods

Cryohemolysis assay. This was carried out as previously described [1] and consists simply, for the purpose of this study, of incubating the erythrocytes with varying concentrations of NaCl in 0.05 M phosphate buffer, pH 7.4 at 37°C for 10 min, then at 4°C for 10 min centrifugation, and measuring the extent of hemolysis in the spectrophotometer compared with the controls of total lysis of the cells.

Radiation inactivation and target size analysis. This was carried out as previously described [8] and in brief as follows. The freezing solution contained 280 g glycerol, 28 g sorbitol and 721 ml 150 mM NaCl, which was equilibrated for 30 min at 4°C. 2-ml aliquots were plated at a depth of 1.2 mm in open aluminum trays, and the trays were frozen by immersion in liquid nitrogen. The cells were thawed by immersion in 38 ml of 37°C freezing solution diluted 50% with 150 mM NaCl. The thawed cells were centrifuged at $1000 \times g$ for 5 min, were washed twice with 600 mM NaCl followed by 300 mM NaCl containing 10 mM Tris-HCl at pH 7.4. The frozen cells were irradiated under flowing liquid nitrogen with a Van de Graaf generator producing 0.5 mA beam of 1.5 meV electrons. The temperature remained below −40°C at all times as judged by the maintenance of the structure mercury needles placed in the irradiation chamber. Dosimetry was performed with bleaching of a blue acetate film calibrated against a Fricke dosimeter.

After each dose of radiation was applied, the cells were thawed as above and assayed for cryohemolysis. The data were plotted as the logarithm of residual cryohemolysis (in a fraction of control) (S) as a function of the radiation dose (D). This log survival curve typically showed a shoulder at lower doses followed by a steep, almost exponential fall at higher doses. A linear regression line was calculated to fit the steep fall of the curve, assuming that it is linear between log values of −1 and −3, from which the slope, R, and its Y intercept, E (extrapolation number), was estimated.

Data were analyzed based upon the target theory according to the expression [5]:

$$S = 1 - \left\{1 - e^{-D/D_0} \sum_{n=0}^{n-1} \frac{(D/D_0)^{n-1}}{(n-1)!}\right\}^m \quad (1)$$

where S is the fraction of the cell population surviving a dose D, as defined above, and D_0 is the dose causing a single event in the target, m is the number of targets, and n is the number of hits each an individual target must receive for the unit to be inactivated. A Cyber 173 of SUNY at Buffalo University Computing Center was used to calculate log S as a function of D/D_0 from Eqn. 1 for a variety of values of n for $m = 1$, the single target, multi-hit model, and for a variety of values of m for $n = 1$, the single hit, multi-target model. Experimental survival curves were then superimposed on these theoretical curves using a range of numerical values for D_0, about $-0.567/R$ [9]. The value of D_0 which gave best fit of data to a theoretical curve was determined. The molecular weight (M_r) was calculated using this value for D_0 in Mrads according to the empirical relationship [4].

$$M_r = \frac{6.4 \cdot 10^{-11}}{D_0} \quad (2)$$

Membrane barrier function assay. Labelled sucrose and NaCl were used to assess possible loss of the membrane barrier function. Details of this methodology are reported elsewhere [8]. At a radiation dosage up to 4 Mrads, there was no significant loss in this function.

Diamide treatment and peptide analysis. Cells were incubated with the indicated concentrations of diamide at pH 8.0 with 0.025 M phosphate buffer in 0.9% NaCl [6] for 1 h. After centrifugation and washing, the cells were studied for hypertonic cryohemolysis. Membranes were prepared [10] under these conditions and solubilized in sodium dodecyl sulfate for polyacrylamide gel electrophoresis according to Fairbanks et al. [11], whose nomenclature was used

in referring to the bands. The membranes were solubilized both with and without dithiothreitol (40 mM) in order to assay the extent to which cross-linking was reversible.

Results

Fig. 1 shows the generality of cryohemolysis in hypertonic solutions of a number of different salts. Cryohemolysis is defined as the amount of hemolysis observed when the cells were incubated at 37°C, then at 4°C, minus the controls consisting of cells at 37°C and held at that temperature. With NaCl and most salts, the control that needed to be subtracted was only a few percent. Quantitative differences seen among the various salts could be on the basis of specific ion effects and may or may not be directly linked to the primary molecular basis.

Irradiation of cells with an increasing electron beam dose abolished hypertonic cryohemolysis with a distinct dose dependency (Fig. 2). When the log of the postirradiation cryohemolysis is plotted against the radiation dose on a linear scale, a lag was seen followed by a steep in residual activity (Fig. 2). Survival curves of this nature have been described with both multi-target, single hit and multi-hit, single target models [9], and analysis of the steep fall of the curve gave $R = -5.35$ Mrads^{-1} and $E = 2 \cdot 10^2$

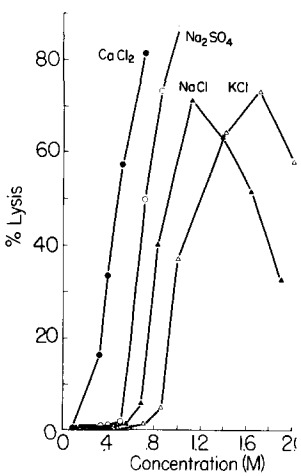

Fig. 1. Cryohemolysis as a function of the concentration of the indicated solutes. The erythrocytes were incubated at 37°C for 10 min and then at 4°C for a further 10 min as described in Methods.

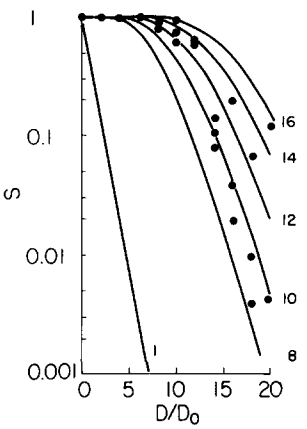

Fig. 2. Theoretical and experimental postirradiation cryohemolysis as a function of radiation dose. The y axis represents cryohemolysis (S) as a fraction of the nonirradiated control in the common logarithmic scale. The x axis represents radiation doses in multiples of D_0. Curves are theoretical predictions based on the single-target, multi-hit model of Eqn. 1, the number of hits (n) being specified for each curve. Solid circles represent experimental data superimposed on the theoretical curve for $D_0 = 0.67$ Mrads.

(see Methods). For the multi-target model, this would imply that each of the 200 individual targets with a molecular weight of $8 \cdot 10^6$ would need to be hit for inactivation. On the other hand, for the multi-hit model, this same set of parameters implies that the inactivation involves a single target with the molecular weight of approx. $15 \cdot 10^6$ which would receive 10–12 hits. It should be noted however that neither of the models precisely simulates the observed survival curve.

Fig. 3 shows the effect of increasing concentrations of diamide on cryohemolysis due to hypertonic NaCl. At 6% NaCl, the control hemolysis was approx. 50%. In erythrocytes from two different donors, there was a reduction in cryohemolysis at 2 mM concentration, and this continued such that the cryohemolysis was reduced by a half at 5 mM concentration. With increasing concentration, there was an increasing suppression effect.

Polyacrylamide gel electrophoresis of the membranes after diamide treatment is shown in Fig. 4. When the solubilizing SDS solution was free of dithiothreitol, there was a marked reduction in spectrin as well as actin and band 4.1. There was also an increase in the large molecular weight aggregates,

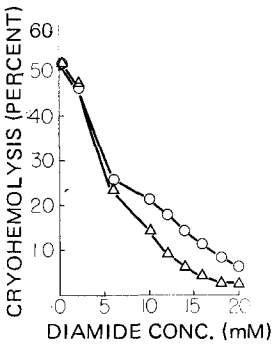

Fig. 3. Suppression of cryohemolysis by diamide treatment. The cells were treated with this agent at the indicated concentrations, washed, and cryohemolysis with hypertonic NaCl was measured.

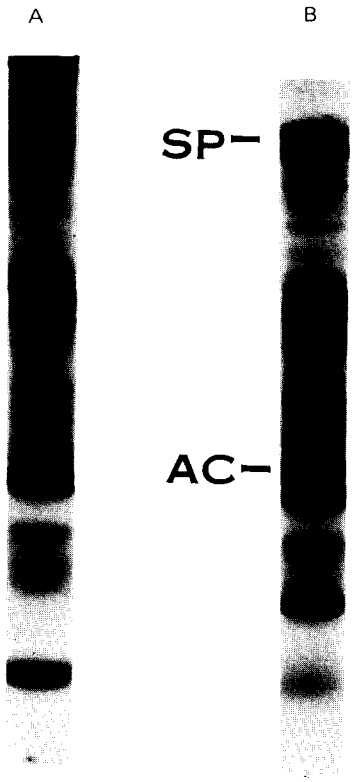

Fig. 4. SDS-polyacrylamide gel electrophoresis of the diamide-treated membranes solubilized both with and without 40 mM dithiothreitol. A, no diamide; B, 10 mM diamide. SP, spectrin; AD, actin.

part of which did not enter the gel. The effect was seen at 2 mM diamide and increased at 10 mM, resulting in the virtual disappearance of spectrin, actin and band 4.1 (Fig. 4). In contrast to these results, when dithiothreitol was included in the solubilizing buffer, the spectrin-actin system appeared normal after diamide treatment (nor shown).

Discussion

Interest in the phenomenon of hypertonic cryohemolysis is not predicated upon its clear relevance to human disease, although pathological erythrocytes have not been studied and such studies could be important. Nevertheless, solutions resulting in cryohemolysis are used in man and conceivably, circumstances might obtain in which this phenomenon could develop. A far more important focus of interest in cryohemolysis is as an indicator of previously unexplored structure/function relationships in the erythrocyte membrane.

A recent review has detailed the evidence of a central structural role of the spectrin-actin skeleton with erythrocyte membrane [12]. Reconstitution experiments of spectrin-deficient mouse erythrocytes have also shown significant functional improvement as compared to untreated spectrin-deficient cells [7]. Momber et al. [13] have reported evidence of phospholipid interaction with the spectrin-actin system and Haest et al. [6] used altered susceptibility to phospholipase under conditions of spectrin aggregation to concluded that spectrin may stabilize phospholipid assymetry in the erythrocyte membrane. We originally speculated that cryohemolysis might be due to interaction of a membrane protein which was intimately bound to phospholipids, and that the reduction in temperature resulted in a phase change of the phospholipids which distorted the lipid-protein interaction. We further speculated on the basis of current knowledge of membrane structure that the hypertonic effect was on the spectrin-actin network [1]. Other workers have also taken up this suggestion [3]. Current concepts of the erythrocyte skeletal structure [14] include spectrin tetramers associated with actin and band 4.1 resulting in a complex of somewhat over $1 \cdot 10^6$, although the exact stoichiometry is not certain. On the basis of the present data and modelling based on published examples of other target

inactivation studies, we concluded that 8–16 such complexes make a higher level unit (see below).

An important aspect of the present observations is that alteration in the spectrin-actin system both by radiation and by cross-linking results in amelioration of the hemolysis rather than a worsening of it. A possible explanation is that damage to the spectrin-actin system by irradiation or cross-linking uncouples the phospholipid-protein interaction and prevents the phase changes in the phospholipids from affecting the protein network. Alternatively such damage could have abolished a temperature effect in the protein itself.

In the case of radiation inactivation of cryohemolysis, the general shape of the survival curve seen in the present study is known, and is predicted by either the multi-target, single hit or the single target, multi-hit model. However, neither of these models precisely simulates the behavior of the observed survival curve and some more sophisticated model may need to be devised. Application of the multi-target model to the present data requires a target number of more than 100, which is too high to be credible. This model also requires the unlikely assumption that the single hit inactivates macromolecules as large as $8 \cdot 10^6$ daltons. The multiple-hit model, however, predicts an inactivation of a $15 \cdot 10^6$ dalton structural unit by 10 or 12 hits, which is entirely plausible. Direct evidence in vitro for the presence of large oligomeric species of spectrin alone (up to 11 dimers) has recently been reported by Morrow and Marchesi [15]. These authors found that the formation of large molecular species was concentration-dependent, and postulated that high concentrations in situ would also favor interaction with phospholipids.

General validation of the target size estimation has been made in a large number of proteins whose molecular weight was independently assessed after purification and isolation [4]. Few studies have been done with cells at the intact level [8]. In order to measure a specific cell function, that particular function alone must be the limiting factor rather than loss of such permissive functions as the permeability barrier. This was fortunately found to be the case, since only a slight change in barrier function to L-glucose was observed, indicating that this function is subserved by a structure of much smaller molecular weight [8].

In the case of the suppression of cryohemolysis by diamide, there cannot be complete certainty that the alteration involves only the spectrin-actin system. However, in the present studies as well as those of other investigators [6,7], it seemed to be the dominating effect. And, indeed the polyacrylamide gel electrophoresis showed that the aggregation was fully reversible with the disulfide-breaking reagent, dithiothreitol.

Although some degree of radiolysis cannot be completely excluded, its extent must be very small, since the water was totally in the frozen state. Additionally, SDS gels failed to show evidence of general cross-linking under these conditions. Finally, the ability to measure specific membrane functions [8] and the preservation of the membrane barrier would tend to exclude diffuse damage. Although it is known that cross-linking can inhibit hemolysis nonspecifically [16], in the present studies we observed, for example, that the procedures employed did not protect against hypotonic hemolysis.

Extension of this work to erythrocytes having known membrane and/or hemoglobin abnormalities may provide further insight into the area of structure/function relationships in the normal as well as the abnormal erythrocyte.

Acknowledgment

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