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Effect of sickling on dimyristoylphosphatidylcholine-induced vesiculation in sickle red blood cells

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To study the effect of sickling on dimyristoylphosphatidylcholine (DMPC)-induced vesiculation, sickle (SS) red blood cells were incubated with sonicated suspensions of DMPC under either room air or nitrogen. Like normal red cells, when sickle cells were incubated with DMPC under oxygenated conditions, incorporation of DMPC into the erythrocyte membrane occurred, followed by echinocytic shape transformation and subsequent release of membrane vesicles. On the other hand, when SS cells were induced to sickle by deoxygenation, DMPC-induced vesiculation of these cells was dramatically reduced. However, upon reoxygenation, release of vesicles from these sickle erythrocytes occurred immediately. When SS cells were incubated under hypertonic (500 mosM) and deoxygenated conditions (where hemoglobin polymerization occurs but red cells do not show the typical sickle morphology), a similar decrease in the extent of vesiculation was observed. Experiments with radiolabelled lipid vesicles indicated that incorporation of DMPC into erythrocyte membranes occurred in all cases and therefore was not the limiting factor in the reduction of vesiculation in deoxygenated SS cells. Taken together, these results indicate that cellular viscosity and membrane rigidity, both of which are influenced by hemoglobin polymerization, are two important factors in process of vesicle release from sickle erythrocytes.

Introduction

Although vesiculation of human red blood cells, particularly pathologic erythrocytes, has been reported to have physiologic implications, very little is known about the underlying mechanism of this cellular phenomenon. Release of vesicles can be induced in normal (AA) red cells in vitro by a variety of methods [1–4]. Many of these procedures are believed to act via alterations of the membrane skeleton that underlies the lipid bilayer, whereas others affect only the lipid moiety of the membrane [5,6]. Dimyristoylphosphatidylcholine (DMPC)-induced vesiculation is an example of the latter case. Upon treatment with DMPC, erythrocytes undergo shape change from discocytes to echinocytes and subsequently release spectrin-free vesicles [5].

Furthermore, spontaneous vesiculation of red cells in vitro and in vivo has been observed in stored blood [7] as well as in many hemolytic disorders such as sickle cell anemia, hereditary spherocytosis, protein 4.1 deficiency and hemoglobin H disease [8]. Vesicles released from sickle (SS) red cells during repeated cycles of deoxygenation and reoxygenation [9] can enhance in vitro clotting activity [10,11] which, in turn, may contribute to the occurrence of microvascular occlu-

Abbreviations: AA, normal erythrocytes; DMPC, dimyristoylphosphatidylcholine; HbS, sickle hemoglobin; SS, sickle erythrocytes.

sion, a prominent pathological feature in sickle cell anemia.

The vesicles spontaneously released from red cells have very similar lipid and protein compositions to those induced by multiple in vitro manipulations [1,2,5,7–9,11,12]. It is possible that the vesiculation process in all human erythrocytes has, in part, the following common underlying mechanism. (1) A shape change from discocytes to spiculated cells; (2) A reorganization of membrane components involving dissociation of the intrinsic domain from the membrane skeleton; (3) A membrane-fusion process.

We used DMPC which has been well characterized as a model system for vesiculation in normal red cells [5,12,13] to study vesicle release in sickle erythrocytes. Sickle cells were chosen since several acquired membrane defects have been reported in those cells (reviewed in Ref. 14) and since sickle erythrocytes are known to undergo vesiculation in vivo [8,9]. We found that deoxygenation of sickle red cells greatly reduced DMPC-induced vesiculation and that internal cellular viscosity and membrane rigidity are important factors in the process of vesicle release from red cells.

Materials and Methods

Materials. After informed consent, fresh human blood samples from patients with sickle cell disease or from healthy normal controls were collected in heparinized tubes. The cells were separated from plasma (2500 rpm for 4 min at 4°C) and the buffy coat was removed by aspiration. The resulting red cell suspension was washed three times with Tris-buffered saline (10 mM Tris-HCl, (pH 7.4), containing 144 mM NaCl and 2 g/l glucose; 298 mosM; referred to as buffer throughout). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was from Sigma, St. Louis, MO, and showed a single spot when subjected to two-dimensional thin-layer chromatography. [^{14}C]DMPC and glycerol tri[9,10(n)-^{3}H]oleate were purchased from Amersham, Arlington Heights, IL. Acqua Mix aqueous liquid scintillation fluid was obtained from WestChem, San Diego, CA. All other chemicals were reagent grade from standard sources.

Release of vesicles. Acetylcholinesterase activity was determined according to Ellman et al. [15]. Oxygenated or deoxygenated red cells (8% final hematocrit in buffer) were incubated with sonicated suspensions of DMPC (0.5 mg/ml final concentration) according to Ott et al. [5]. At appropriate time intervals, an aliquot was removed from the incubation mixture and chilled on ice in order to stop the vesiculation process. Erythrocytes were then spun down at 2500 rpm for 4 min at 4°C and vesicle release was monitored by measuring acetylcholinesterase activity in the supernatant. Oxygenated conditions were maintained by incubating under room air. Deoxygenated conditions were maintained by performing the incubations in sealed tubes constantly flushed with humidified nitrogen. All buffers used for the experiments under nitrogen were deoxygenated with N_2 for at least 1 h before use. High-tonicity media (500 mosM) were obtained by adding appropriate amounts of NaCl to the buffer.

Shape change. Red cells were fixed in glutaraldehyde (3%) and shape change of erythrocytes was monitored by light microscopy using Zeiss Nomarsky-optics. Samples for scanning electron microscopy were prepared as described elsewhere [16] without critical point drying.

[^{14}C]DMPC incorporation into normal and sickle red cells. Incorporation of exogenous lipid into red cells was determined by adding [^{14}C]DMPC and glycerol tri[^{3}H]oleate (0.89 and 0.02% of total lipid, respectively) to the DMPC suspension before sonication. Otherwise, conditions for DMPC-induced vesiculation were the same as described above. At appropriate time points, 250-μl aliquots were removed from the incubation mixture, suspended in 10 ml of ice-cold isotonic saline and centrifuged at 2500 rpm for 4 min at 4°C. The pellet was washed two more times as mentioned above and subsequently lysed in 200 μl H_2O. Membrane lipids were extracted with cold isopropanol and radioactivity was measured in a liquid scintillation spectrometer.

Results and Discussion

Like normal red cells, when sickle red cells were incubated with sonicated DMPC suspensions under fully oxygenated conditions they underwent echinocytic shape change (Fig. 1A and B) and

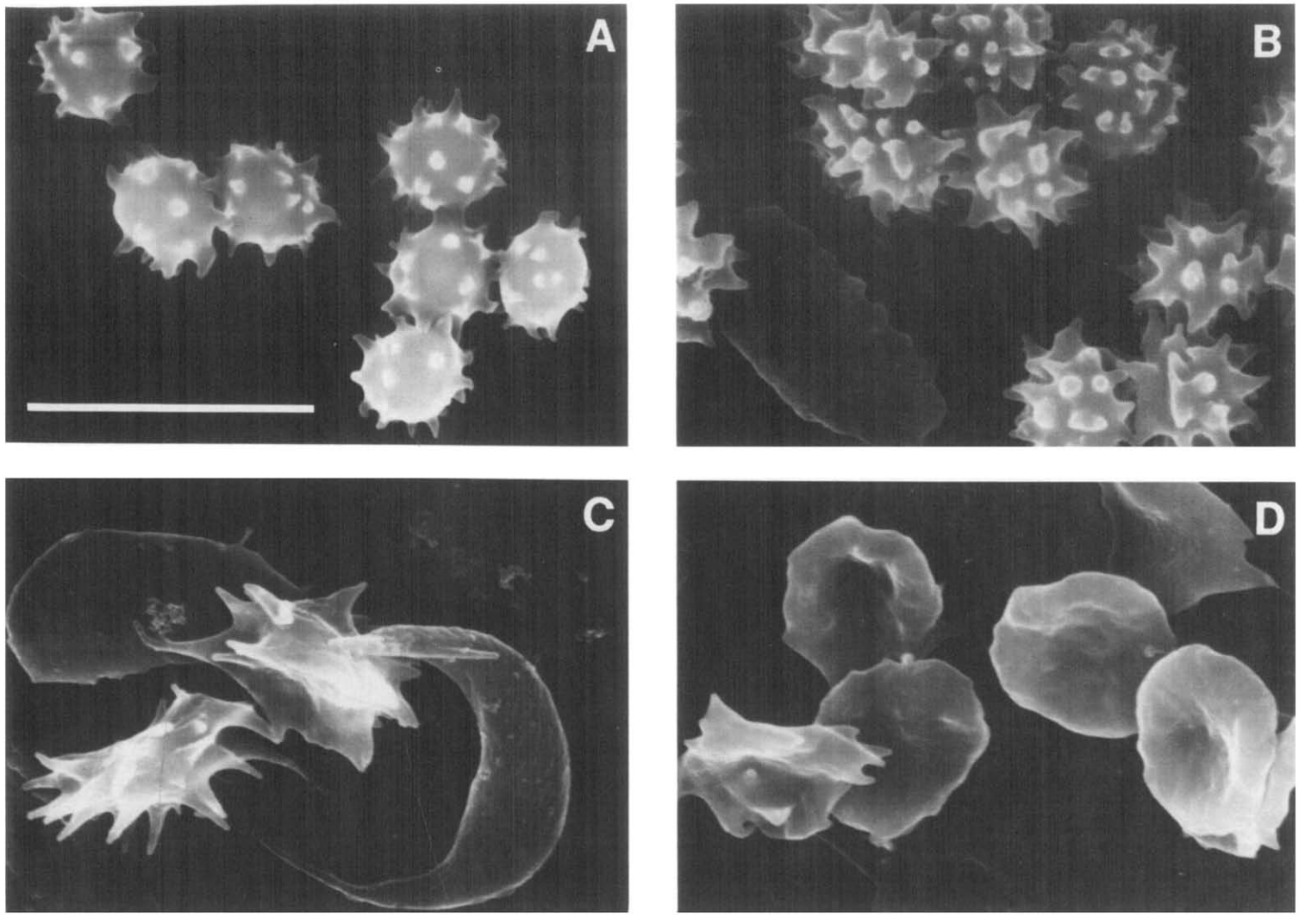

Fig. 1. Scanning electron micrographs of normal and sickle red cells. Erythrocytes were incubated with DMPC under isotonic and hypertonic conditions as described in Materials and Methods. Aliquots were removed from each incubation mixture after 60 min of incubation and fixed in 3% glutaraldehyde. Samples were prepared for electron microscopy basically as described in Ref. 16. (A) Normal red cells; (B) oxygenated and (C) deoxygenated sickle cells under isotonic (298 mosM) conditions; (D) deoxygenated sickle cells under hypertonic (500 mosM) conditions. The bar represents 10 μM.

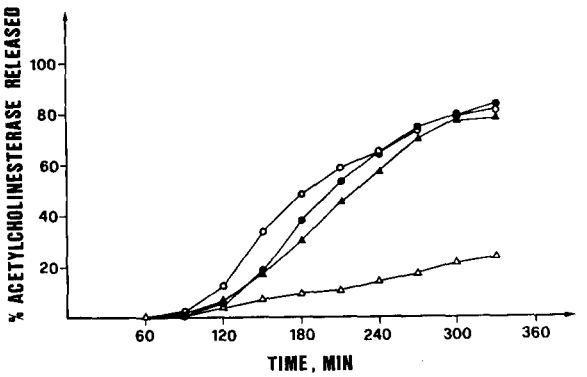

Fig. 2. DMPC-induced vesiculation of normal (●, ○) and sickle (▲, △) erythrocytes under oxygenated (filled symbols) and deoxygenated (open symbols) conditions. Oxygenated or deoxygenated red cells (8% final hematocrit) were incubated with sonicated suspensions of DMPC (0.5 mg/ml final concentration) as described in Materials and Methods. Vesicle release was monitored by measuring acetylcholinesterase activity in the supernatant after low-speed centrifugation. Acetylcholinesterase activity in the whole incubation mixture was taken as 100% and stayed constant throughout the experiment (±4.5%, S.D.).

subsequently released membrane vesicles after a lag time of about 90 min (Fig. 2). Approx. 70–80% of total acetylcholinesterase activity could be recovered in the vesicle fraction (supernatant) within 3 h after initiation of vesicle release. No major difference in DMPC-induced vesiculation was observed between samples from various sickle cell patients. The protein composition of the released vesicles was similar to that of vesicles released from normal cells (see Ref. 12).

In contrast to DMPC-induced vesiculation under fully oxygenated conditions, when SS cells were induced to sickle under nitrogen, vesiculation was dramatically reduced. Less than 25% of total acetylcholinesterase activity from sickle cells were found in the supernatant. Release of acetylcholinesterase activity from normal cells was not affected by deoxygenation (Fig. 2). The characteristic echinocytic shape change of erythrocytes which follows addition of DMPC to normal red cells occurred in oxygenated SS cells but not in deoxygenated SS cells (Fig. 1C). This observation is analogous to an earlier finding that treatment with bee venom phospholipase A_2 causes echinocytic transformation in normal and oxygenated SS red cells, but not in deoxygenated sickled cells [16].

To determine whether reduction of DMPC-induced vesiculation of deoxygenated SS cells is reversible, fully deoxygenated SS cells were incubated with DMPC as mentioned above. After 3.5 h, when less than 10% of acetylcholinesterase activity was released in the supernatant, red cells were reoxygenated by exposure to a stream of oxygen for 5 min. Upon reoxygenation, the cells transformed immediately to echinocytes, and vesiculation started instantaneously (Fig. 3). In less than 10 min after reoxygenation, the extent of vesiculation reached a similar level as in the control, where the SS cells were kept oxygenated throughout the experiment.

Extensive studies by others [9,10,17] have shown that SS cells release membrane vesicles upon several deoxy-/reoxygenation cycles in vitro. In our experiments, red cells underwent at most one such sickling-unsickling cycle and the extent of spontaneous vesiculation was low since less than 1% of total acetylcholinesterase activity in the suspension was released in the vesicle fraction (result not shown). To rule out the possibility that the vesiculation process due to repeated cycles of sickling had an effect on DMPC-induced vesiculation, DMPC was added to deoxygenated SS cells only shortly (2 min) before they were reoxygenated. The vesiculation pattern as measured

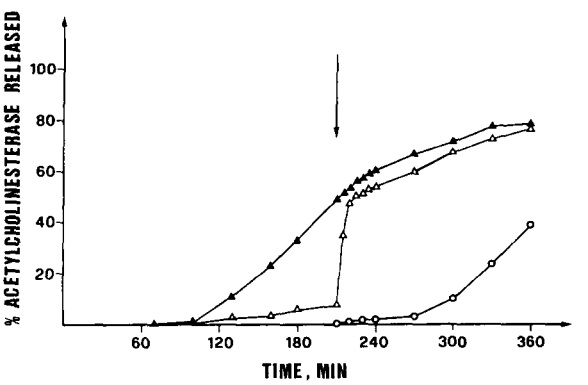

Fig. 3. Reversibility of the reduction of DMPC-induced vesiculation of deoxygenated sickle cells. Oxygenated (▲) and deoxygenated (△) sickle cells were incubated with sonicated DMPC vesicles as described in Fig. 2. After 3.5 h of incubation, deoxygenated SS cells were reoxygenated by exposure to a stream of oxygen for 5 min (indicated by the arrow). In order to determine the effect of a sickling-unsickling cycle in vitro on DMPC-induced vesiculation, sickle cells were kept deoxygenated for 3.5 h in the absence of DMPC. Shortly (2 min) before the sample was reoxygenated, DMPC was added and vesicle release was monitored as described in Fig. 2 (○).

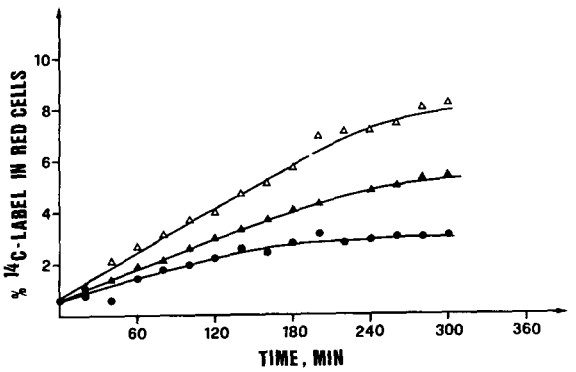

Fig. 4. DMPC incorporation into normal and sickle erythrocytes. Incorporation of exogenous lipid into red cell membranes was measured by adding [^{14}C]DMPC and glyceroltri[^{3}H]oleate to the DMPC suspension before sonication. For more details see Materials and Methods. Otherwise, conditions for DMPC-induced vesiculation were the same as described in Fig. 2. Radioactivity in the whole incubation mixture was taken as 100%. The percentage of ^{14}C label recovered in the red cell pellet after low-speed centrifugation was monitored for AA (●), oxygenated (▲) and deoxygenated (△) SS cells. ^{3}H label found in the pellet was taken into calculation as the amount of sonicated DMPC vesicles sticking to erythrocytes. After 5 h of incubation under the above-mentioned conditions, less than 1% of sonicated DMPC vesicles were found to adhere to AA cells, whereas less than 2 and 4%, respectively, were found with oxygenated and deoxygenated SS cells.

by acetylcholinesterase release was unchanged compared to the control where SS cells had not previously been subjected to a deoxy-/reoxygenation cycle (Fig. 3). In all experiments, hemolysis never exceeded 3.5% after 5 h of incubation.

To determine if the failure of deoxygenated SS cells to undergo echinocytic transformation following incubation with DMPC was due to the inability of these cells to incorporate DMPC or to the inability to transform to echinocytes despite incorporation of DMPC, we incubated deoxygenated SS cells with radiolabelled DMPC vesicles. We were able to demonstrate that DMPC incorporation takes place in AA (see also Ref. 18) as well as in SS cells under both oxy- and deoxygenated conditions (Fig. 4) and therefore is not the limiting factor in vesicle release from deoxygenated SS cells. In fact, we found that SS cells incorporated more [^{14}C]DMPC than AA cells and that deoxygenation of SS cells stimulated additional incorporation of exogenous lipid. A similar observation was made earlier by Schwartz et al. [19]; mixed phosphatidylcholine/phosphatidylserine vesicles interacted to greater extent with sickled cells than with oxygenated SS and AA cells. These results, in turn, further support the finding that membrane phospholipid organization in deoxygenated SS cells is altered [11,16,20] and this could result in a facilitated interaction between phospholipid vesicles and sickled red cells. Moreover, the observation that vesiculation started immediately upon reoxygenation (Fig. 3) provides additional support to the conclusion that the sickling process rather than a limited incorporation of foreign lipid inhibits vesicle release in sickle erythrocytes.

Since the sickling process involves polymerization of sickle hemoglobin (HbS) followed by the interaction of hemoglobin polymers with the red cell membrane leading to morphologic sickling, we determined whether morphologic sickling is necessary for the reduction of DMPC-induced vesiculation in sickled cells. To do this, we carried out similar experiments as described above at high tonicity (500 mosM). Under this hypertonic condition, deoxygenated SS cells do not show the typical sickle morphology (Fig. 1D) although HbS polymerization occurs which, in turn, leads to elevated internal viscosity [21]. The vesiculation pattern of AA cells under these conditions was unchanged and again, a similar reduction in DMPC-induced vesiculation of deoxygenated SS

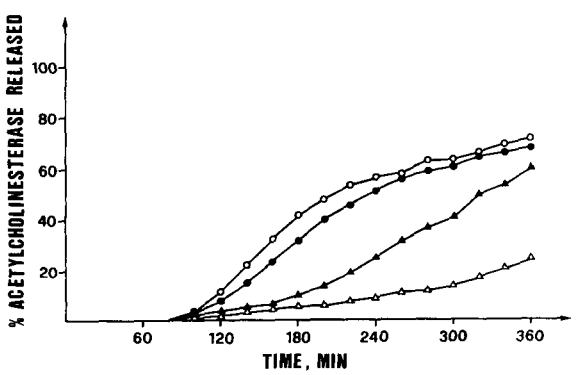

Fig. 5. DMPC-induced vesiculation of normal and sickle erythrocytes under hypertonic conditions. Normal red cells (●) and oxygenated (▲) and deoxygenated (△) sickle cells were incubated at high tonicity (500 mosM) with sonicated DMPC vesicles as mentioned in Fig. 2. Normal erythrocytes under isotonic conditions (298 mosM) served as a control (○).

cells was observed as under isotonic conditions (Fig. 5). This strongly suggests that it is not the transformation of the cells to the sickled configuration but rather polymerization of HbS that results in the reduction of vesiculation.

Interestingly, although similar rates of DMPC incorporation into red cells were observed under both isotonic and hypertonic conditions (results not shown), the acetylcholinesterase release pattern of oxygenated SS cells at 500 mosM was changed compared to SS cells at 298 mosM. The rate of vesiculation was lower during the first 3 h of incubation but accelerated thereafter, reaching similar amounts of acetylcholinesterase released in the supernatant after 6 h of incubation (Fig. 5). Since formation of echinocytes is a prerequisite for DMPC-induced vesiculation, this initial shape transformation may be affected in sickle cells by hypertonic conditions. According to present concepts [22], shape changes can be suppressed by an increase of either static or dynamic rigidity, or by an increase of cytoplasmic viscosity. Furthermore, Haest et al. [23] have shown earlier than an increase of membrane-shear stiffness suppressed shape change of red blood cells. In normally hydrated sickle cells, both static and dynamic rigidities were reported to be nearly normal, whereas in dehydrated SS cells, both parameters were increased [22]. In contrast, dehydration of normal cells resulted only in increased dynamic rigidity, and the values for static rigidity remained unchanged. In our experiments, normal red cells reacted similarly upon treatment with DMPC under isotonic and hypertonic conditions. Considering the results of Evans et al. [22], we suggest that it is an increased static rigidity rather than dynamic rigidity that is responsible for a more sluggish response of dehydrated oxygenated SS cells to DMPC treatment at 500 mosM. In addition, Clark et al. [21] have reported that cell water content may be more important than intrinsic membrane properties in limiting the morphological response of sickle cells to changes in oxygen tension. Since dehydration accompanies sickling as a result of polymerization of HbS [21], and because an increase of the hemoglobin concentration at the cytoplasma/membrane interface and an increase of cytoplasmic hemoglobin concentration are thought to affect rheological properties of red cells [22], these changes may be responsible for restricted shape change of sickled cells and suppressed vesiculation. In addition, increased interaction between HbS and the membrane in dehydrated sickle cells [22] may also result in the observed changes of vesiculation patterns, although our experiments do not provide any direct evidence to support this concept.

In summary, these studies using sonicated DMPC vesicles to manipulate red cell membrane integrity provide new insight into the mechanism of vesicle release from red cells and the behavior of sickle erythrocytes. It appears that sickle hemoglobin polymers have a major influence on membrane rigidity which in turn affects the ability of sickle cells to release membrane vesicles upon treatment with DMPC. In addition, our studies also support the notion that the state of cell hydration may have a profound influence on the interaction between sickle hemoglobin and the red cell membrane.

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