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Effects of Phospholipase C on Human Erythrocytes*

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Summary. Previous studies have shown that the bacterial exoenzyme phosph lipase C permanently alters the chemical structure of erythrocyte ghosts. The prese investigation has shown some of the functional, chemical and structural changes th sequentially occur when intact human red blood cells are lysed by this enzyme. Followir exposure to the enzyme, membrane phospholipids were hydrolyzed with the removal lipid phosphorus. This resulted in a shrinkage of cell size, sphering, and increased su ceptibility to osmotic stress. Progressive hemolysis ensued, leaving ghosts which we characterized by focal electron-dense areas intimately associated with each membran These findings illustrate that the degradation of exposed phospholipids results in chemic and morphological damage to the cell membrane, which in turn alters its function capabilities and results in lysis of the cell. Finally, these data support a newly propose structural model of the cell membrane.

The selective degradative action of the enzyme phospholipase C has bee used recently by several investigators in studying the molecular structure c biological membranes [6, 7, 11]. In each of the aforementioned studie erythrocyte ghosts were subjected to the hydrolytic action of the phosphc lipase enzyme. A permanent alteration in the chemical structure of th membrane consistently resulted.

Since it is known that phospholipase C can cause hemolysis [5], it seeme imperative to investigate the effect of this enzyme on physiologically intac erythrocytes by methods similar to those employed in the study of ghos membranes. The results reported herein describe the sequence of changes i

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selected functional, chemical and morphological parameters as lysis by phospholipase C occurs. The data is consistent with the hypothesis that a rapid focal loss of membrane integrity leads to osmotic instability and ultimate lysis.

Materials and Methods

Preparation of Cells

Normal human blood, type 0, Rh +, was drawn, heparinized, centrifuged at 2,000 rpm for 10 min, and the plasma and buffy coat discarded. The packed red cells were washed three times by resuspension in physiologic saline and centrifugation as before. After the last washing, 3 ml of packed red cells was suspended in 97 ml of physiologic saline to produce a final 3 % suspension.

Enzyme

Phospholipase C from *Bacillus cereus* was prepared, purified and assayed as described by Ottolenghi [10]. Immunological analysis indicated a preparation giving a single precipitation band by immunodiffusion [11].

Incubation

A 1-ml sample (5,000 enzyme units) [10] of the phospholipase C was incubated, with agitation, at 37 °C with 50 ml of the 3 % red cell suspension. For control, 1 ml of physiologic saline was incubated similarly with another 50 ml of the red cell suspension. Aliquots (10-ml) were taken from each reaction mixture after incubation for 0, 20, 40 and 50 min and added to 5 ml of 1 % o-phenanthroline to inhibit the enzyme [10].

Red Cell Studies

Each sample was examined by selected standard hematologic techniques and biochemical analysis. Hematocrits were obtained on each sample by means of a micromethod. Red cell counts were performed with a Model B Coulter counter. Methemoglobin levels were determined spectrophotometrically [12].

Osmotic fragility of the red blood cells was determined with the Osmotic Fragilograph as described by Danon [1]. Cumulative and derivative curves were inscribed in each study and values expressed as the salt concentrations at which 50 % hemolysis occurred.

Red cell lipids were extracted according to the method of Dodge, Cohen, Kayden and Phillips [2], and phosphorus values determined from the lipid extracts by the procedure of Lowry, Roberts, Leiner, Wu and Fair [9]. The final value for cellular lipid phosphorus was expressed as micrograms phosphorus per red cell.

Fresh preparations from the control and experimental samples were periodically examined by phase microscopy to determine the extent of ghost formation. For further morphological study, a 1-ml sample was taken from the control and the experimental portions at the end of 50 min of incubation. These samples were prepared for ultrastructural examination by fixation with cold buffered 2.5 % glutaraldehyde, washing in buffer, and post-fixation in cold buffered 1 % osmium tetroxide. After dehydration, the specimens were embedded in Dow Epoxy Resin [8]. Sections were cut with a Porter-Blum MT-2 ultramicrotome, picked up on uncoated 300-mesh copper grids, stained with uranyl acetate and lead citrate, and observed in an RCA EMU-3 electron microscope.

Results

The hemolytic effect of phospholipase C is illustrated in Fig. 1. A noted, phospholipase-treated red cells began to lyse at some time afte 20 min of incubation. This hemolysis continued, progressively, throug 50 min of incubation, by which time 90% of the original cells had lysed In contrast, the control sample showed no evidence of hemolysis at an time throughout the course of incubation.

The quantities of red cell membrane lipid phosphorus in control an phospholipase-treated samples during the course of incubation are show in Fig. 2. By 20 min of incubation, the phospholipase-treated sampl showed a 26% decrease while the control sample remained essentiall unchanged. By 50 min of incubation, the enzyme-treated sample showe a 65% decrease in cellular lipid phosphorus, again contrasting with th unchanged control sample.

The sequential changes in other erythrocyte parameters during in cubation with phospholipase C are summarized in the Table. At the initia tion of incubation, mean corpuscular volume (MCV) and osmotic fragilit were normal. After 20 min, the MCV had decreased slightly. An increase i

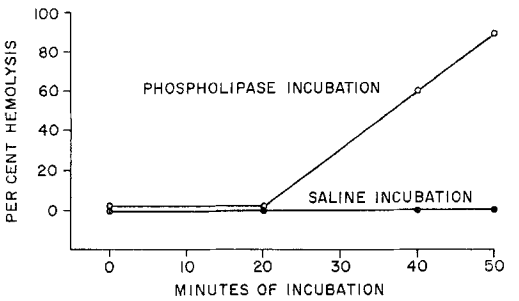

Fig. 1. Hemolytic effect of phospholipase C on human red blood cells. Each poin represents the result of a single determination

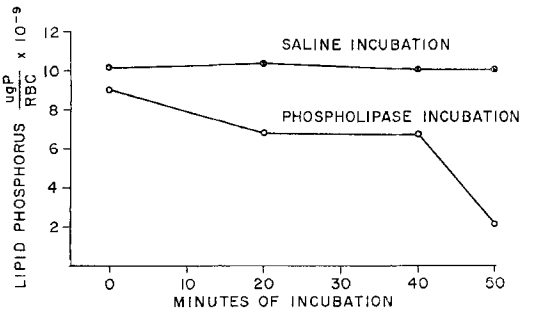

Fig. 2. Effect of phospholipase C on human red blood cell lipid phosphorus. Each poin represents the result of a single determination done in duplicate

Table. *Changes in erythrocytes during incubation with phospholipase C. In the osmotic fragility column, the number indicates the salt concentration (%) at which 50% lysis occurred, and the curves depict the derivative of the cumulative curve, indicating one or more distinct cell populations with respect to osmotic fragility. Sample incubated with saline showed no change in MCV or osmotic fragility during the 50 min*

INCUBATION TIME	MCV	OSMOTIC FRAGILITY
0 min.	97	0.43
20 min.	90	0.48
40 min.	81	0.56
50 min.	48	0.56

cumulative osmotic fragility was noted at this time and was apparently caused by a distinct population of osmotically fragile cells as identified by the derivative curve inscribed by the Fragilograph. These changes progressed with incubation as seen by the data obtained at 40 and 50 min.

The morphology of the erythrocytes in the control sample appeared normal throughout the incubation period. In contrast, the red cells of the sample treated with phospholipase C underwent progressive visible hemolysis. Observation of the phospholipase-treated sample after 20 min of incubation showed erythrocytes which appeared slightly shrunken. There was no evidence of lysed cells at this time. Beginning shortly thereafter

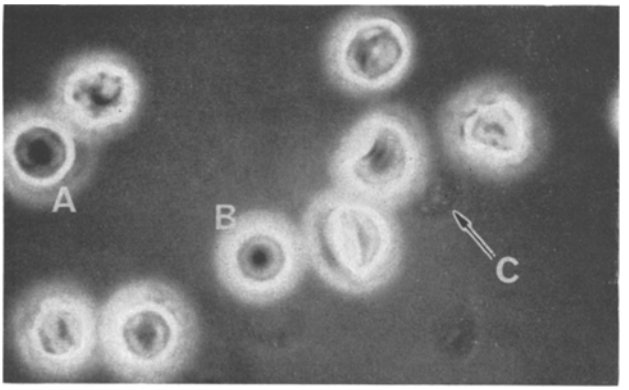

Fig. 3. A phase photomicrograph showing various stages in the lysis of normal human erythrocytes by *B. cereus* phospholipase C. *A* Cell at the beginning of hemolytic sequence: normal size but profile appears rough. *B* Later stage of red lysis is exhibited by shrunken cell with smooth profile. This was observed to occur just prior to loss of hemoglobin. *C* Terminal stage of erythrocyte hemolysis: red blood cell ghost with discrete areas of digestion (white spots). These areas appear either white or black depending on the focal plane. (×2,500)

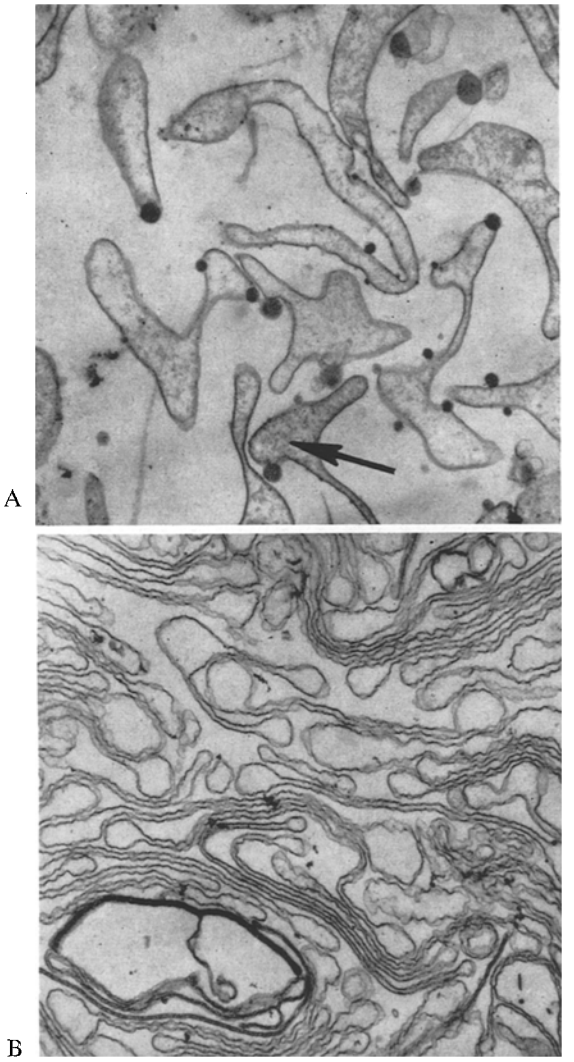

Fig. 4. (A) An electron photomicrograph demonstrating ghosts produced by incubation of normal erythrocytes with phospholipase C for 50 min. Spherical electron-dense blebs can be noted in intimate association with the membranes. A small portion of the red cell's hemoglobin remains within the ghosts (arrow). (×9,216). (B) An electron photomicrograph illustrating ghosts produced by a routine procedure (Dodge, Mitchell & Hanahan, 1963). (×9,216)

(20 to 25 min of incubation) and continuing throughout 50 min, individual red cells were observed to become progressively spherocytic and to decrease in optical density (as the hemoglobin was lost). As this lysis continued, small dark membrane-bound blebs became discernible on each ghost (Fig. 3).

Such events continued throughout the experimental sample until, by 50 min of incubation, most of the original erythrocytes had been lysed. Furthermore, the ghosts produced by the action of phospholipase C showed a decrease in profile diameters when compared with red cell ghosts prepared by a standard procedure.

Electron microscopy of the ghosts resulting from 50 min of phospholipase treatment showed the presence of numerous, discrete electron-dense areas intimately associated with the membranes (Fig. 4A). Electron microscopy of the routinely prepared ghosts showed no such areas (Fig. 4B).

Discussion

The results of this study have described the sequence of events when phospholipase C produced by *Bacillus cereus* is incubated with intact human red blood cells. These data show that phospholipase C catalyzes the hydrolysis of red cell membrane phospholipids with the release of phosphorus into the supernatant. The percentage of phosphorus liberated from intact red cells is in essential agreement with those values reported by Lenard and Singer [7] and by us [11] for red cell ghosts similarly treated. This implies that (1) the quantity of phospholipids available for digestion is consistent for red cells, and (2) this quantity is not altered by osmotic lysis of erythrocytes. This study furthermore reveals that the loss of membrane phosphorus occurred prior to any detectable hemolysis. The concomitant finding of an early decrease in MCV suggests that the action of the enzyme in causing a loss of membrane lipid phosphorus secondarily results in a shrinkage of cell size. This would be expected to cause sphering, which was observed under the phase microscope, and is further supported by the simultaneous appearance of a population of cells with increased sensitivity to osmotic change. It has also been demonstrated that this shrinkage occurred well before lysis began and therefore cannot be attributed to a lytic phenomenon.

In interpreting the changes observed in the different parameters which have been studied, paramount consideration must be given to the influence which might be exerted on the observations by the "enzyme concentration/available site" ratio or by the "number of sites affected/release of hemoglobin" relationship.

A crucial question to be answered is how many sites must be affected and to what extent before release of hemoglobin is observed. Even under the best circumstances, this can only be a statistical average of the cells present in the suspension.

Another and as important a question is that which relates to the decrease in cell size which has been observed. The decrease in cell size occurs before release of hemoglobin as does the release of a small amount of phosphorus (again on a whole population basis). Thus, one might ask: "How much phosphorus has to be released from a given cell before a change in size occurs and how much before release of hemoglobin might be observed?"

The data presented here do not finally answer these questions. The most that can be said is that on a *whole population basis* only a partial release of the phosphorus available to the enzyme is required for both reduction in MCV and hemoglobin release.

In addition, the ghosts resulting from enzyme-induced lysis were noticeably smaller than ghosts prepared by osmotic shock. Such shrinkage has also been noted in similarly treated muscle microsomes [4] and red cell ghosts [11].

Although phospholipase C caused the release of a large percentage of membrane phosphorus, the apparent shrinkage of membrane profiles, and the eventual lysis of the erythrocyte, nonfragmented red cell ghosts were consistently observed after the complete reaction had occurred. This concurs with other investigations [7, 11] in which erythrocyte ghost membranes remained intact after phospholipase C treatment. This illustrates that the enzyme does not degrade the entire membrane.

In the present study, red cell ghosts were consistently observed under phase microscopy to have discrete dark spots associated with the membranes. Electron microscopy of these ghost preparations showed isolated, non-coalescing electron-dense areas in intimate association with all of the membranes. We interpret these areas, seen at the ultrastructural level, to be equivalent to the membrane spots observed under phase microscopy. A recent examination of red cell ghosts treated with phospholipase C [11] revealed dense membrane-associated areas, at both the light- and electron-microscopic levels, that are indistinguishable from the dense areas reported here, and thus imply that the dense areas observed in the present study are not hemoglobin accumulations. In the same study [11], we suggested that diglyceride is the major component of such areas produced by phospholipase C treatment of red cells ghosts. The apparent identity of these areas in both studies lead us to believe that the dense membrane-associated areas produced in the current study represent the same diglyceride accumulations reported for red cell ghosts. This completely agrees with other investigators [4] who found electron-dense accumulations of diglyceride on the membranes of muscle microsomes similarly treated. Finally, the isolated nature of these

areas further substantiates the concept of a focal digestion of phospholipids by the enzyme.

The data presented here furthermore suggest that the lesion produced in the membrane of the red cell is not instantly destructive. Instead, it appears only to alter the functional capabilities of the cell membrane, which in turn irreversibly destines the cell to release hemoglobin. Loss of lipid phosphorus, decreased cell volume, and the appearance of a discrete population of red cells which showed an increased susceptibility to osmotic stress, all occurring before lysis, add credibility to this hypothesis.

Finally, we believe that this study lends support to the newly proposed molecular model of the cell membrane [6, 13]. The susceptibility of the membrane to the degradative activity of the phospholipid-specific enzyme substantiates the hypothesis that the polar group of phospholipid molecules is exposed to the external environment.

It is not possible, however, to decide on the exact localization of the phospholipid molecules since the diglyceride droplets which are seen both by light and electron microscopy might have developed from either the *in situ* digestion of phospholipid or the random migration of lipid produced at other sites to a final accumulation limited by unknown factors. Our present inclination is toward the *in situ* digestion because it appears hard to justify the apparent uniformity observed with red cell ghosts and mitochondria [11] on the basis of random migration.

The localization of the lipid droplets as observed cannot, however, be used with assuredness to determine the final mosaicism of the membrane. First, because our technique involves statistical consideration in relation to the number of enzyme molecules vs. the number of available sites, and second, because the planes of section and observation will affect the apparent size of the bleb or droplet as well as the frequency on any given membrane, no assurance can be given that all the sites are digested that can be digested or seen that are present on any given membrane.

It is true that in this paper, as well as in a previous publication [11], the figure of 65 to 70% loss of lipid phosphorus after digestion with phospholipase is in good agreement with the figures given by Lenard and Singer [7], but it is not possible to determine whether the remaining 30% of lipid phosphorus stays in the lipid fraction unavailable to the enzyme because of its localization or its chemical configuration.

Because of these objections, we must for the present hold the position that membranes probably have areas of phospholipid exposed to the external milieu, but we cannot with present techniques describe the exact arrangement of these areas.

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