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Action of Snake Venom Phospholipase A on Isolated Platelet Membranes.* (31632)

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Platelets play a significant part in the coagulation process by contributing a phospholipid (or more likely a phospholipoprotein) to the interaction of coagulation factors, which eventually leads to the formation of a prothrombin activator. This clot-promoting property of platelets is ordinarily present in latent form(1-3), but through an unknown mechanism the latency is lost as the coagulation process gains momentum. The physical form in which procoagulant material from platelets interacts with clotting factors is also unknown. Actual release from platelets has been suggested(4), and alternatively it has been proposed that the activity becomes available to the coagulation process as a catalytic lipoprotein surface on the plasma membrane of the platelet(5). These protein-lipoprotein interactions basically involve problems of cellular lipoprotein availability.

A further example of variations in the availability of cellular lipoproteins is revealed by the studies of Condrea and associates(6) and Kirschmann *et al*(7). These investigators have shown that, whereas the phospholipids of osmotically haemolyzed erythrocytes and intact platelets were hydrolzyed by *N. naja* phospholipase A, intact red cells were unaffected. Phospholipase A from Russell viper venom was also inactive against intact but active against haemolyzed red cells. Palestinian viper venom phospholipase A did not produce hydrolysis of phospholipids in intact platelets, intact erythrocytes, or osmotically haemolyzed red cells, and was only active against red cells disrupted by sonication. The results obtained with *N. naja* and Russell viper venom suggested similarities between lipid availability for the coagulation process and susceptibility to venom action. Specifically, all the cell preparations which resisted the action of venom were also inactive in *in vitro* coagulation systems. On the other hand, venom-sensitive cells were specifically those which were active in coagulation.

It therefore seemed pertinent to investigate the basis for the difference in venom sensitivity of intact platelets and erythrocytes. One of the principal hypotheses to be examined was the possibility that phospholipids of platelet membranes had a specific affinity for the venom enzyme. In line with this approach, the effect of venom phospholipases on isolated platelet membranes was studied and compared to the venom effect on intact platelets, isolated platelet granules, intact red cells, and plasma lipoproteins.

Methods. Washed platelets and isolated platelet membranes and granules were prepared from 500 ml citrated human blood as described by Marcus *et al*(8). Platelet-poor plasma was prepared by centrifuging platelet-rich plasma for 20 minutes at 5,000 × *g*. Red cells were washed 3 times in isotonic buffered saline, pH 7.4(9). The buffy coat was removed after each wash and the cells finally suspended in buffer to a concentration of 50%. Approximately 1 g wet weight of platelets was suspended in 10 ml of buffer. Subcellular platelet particles were dialyzed

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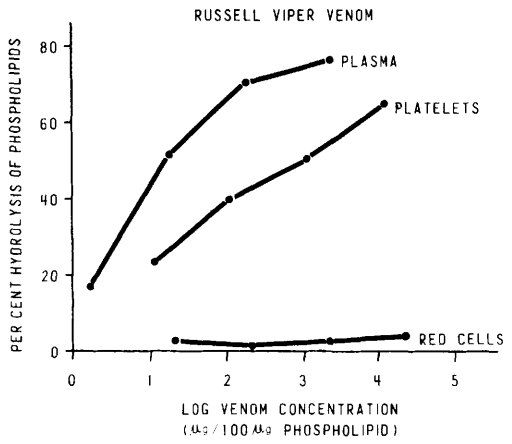

FIG. 1. Action of Russell viper venom on phospholipids of human plasma, intact platelets, and intact red cells.

overnight against 2 changes of buffer and diluted to a concentration of approximately 3 mg protein per ml. Protein concentrations were determined by the method of Miller(10).

Russell viper venom (RVV) was obtained from Sigma Chemical Co. and Palestinian viper venom (PVV) through the courtesy of Dr. E. Kochva of Tel Aviv University. The venoms were dissolved in buffered saline at concentrations of 10 mg to 1 μg per ml (RVV), or 40 mg to 1 μg per ml (PVV). The crude venoms contained approximately 65% protein by weight.

Plasma, cell or subcellular particle suspensions were incubated with crude venom solution for 60 minutes at 37°C (pH 7.4). The mixture consisted of 1 ml plasma or cell suspension in buffered saline, 0.1 ml venom or blank solution and 0.1 ml 0.005 M $CaCl_2$. Lipids were extracted(11) before and after incubation, evaporated to dryness under purified nitrogen and reextracted with chloroform. The phospholipids (usually 1 to 3 mg) were adsorbed onto 300 mg heat activated silicic acid (Mallinckrodt 100 mesh) in a ground glass stoppered tube. The lipids remaining in the chloroform supernatant were evaporated to dryness and suspended in 2.0 ml distilled water. Free fatty acids were then extracted from the aqueous suspension and quantified (12). Finally, the phospholipids were eluted from the silicic acid with successive additions of chloroform:methanol 4:1, and 1:4 followed by methanol. Mean recovery of lipid phosphorus(13) from the silicic acid was 96%. Phospholipids were then separated by thin-layer chromatography(14) and the phosphorus content of each "spot" determined(15).

Percentage hydrolysis of phospholipid substrate was calculated from the amount of fatty acid released. This quantification was confirmed by determining from the thin-layer chromatograms the sum of the decrements in phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl inositol (PI) and the corresponding increment in lysolecithin (LL) concentrations after venom action. The fall in lecithin concentration could not be measured directly since lysophosphatidyl ethanolamine (LPE) overlapped with the lecithin spot.

The activity of different snake venom enzymes was compared by measuring free fatty

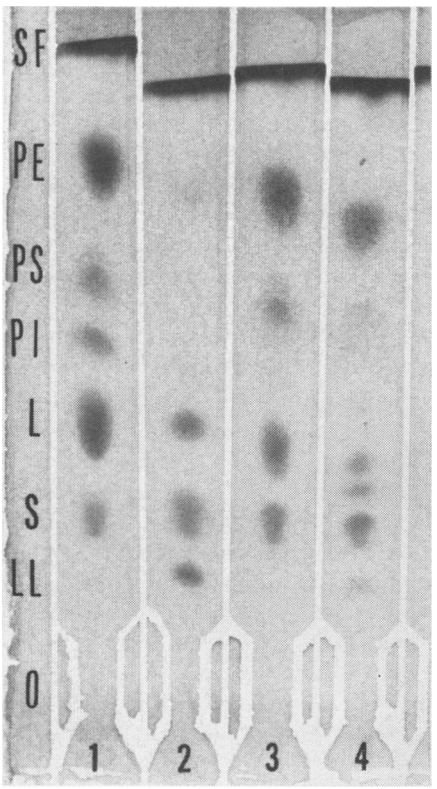

FIG. 2. Thin-layer chromatogram of phospholipids of intact platelets and red cells before and after hydrolysis by Russell viper venom (10 mg/ml). 1) Untreated platelets. 2) Venom-treated platelets. 3) Untreated red cells. 4) Venom-treated red cells.

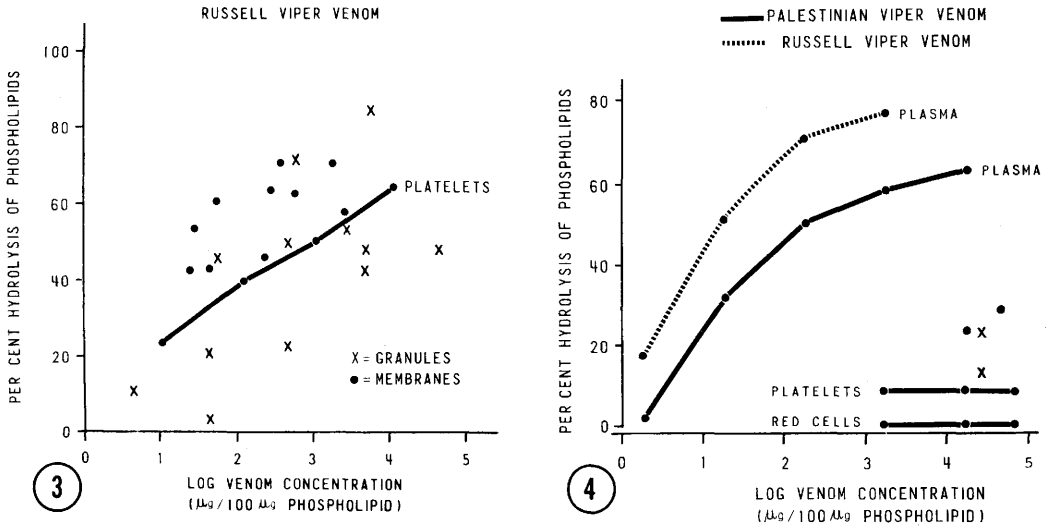

FIG. 3. Effect of Russell viper venom on phospholipids of isolated platelet membranes (●) and granules (x). Solid curve indicates effect of the venom on intact platelets.

FIG. 4. Action of Palestinian viper venom on phospholipids of plasma, intact platelets, red cells, isolated pltelet membranes (●) and granules (x). Effect of Russell viper venom on plasma is also shown.

acid release from a 40% suspension of egg yolk diluted with buffered saline (pH 7.4) after incubation for 60 minutes at 37°C in the presence of 0.5 μMoles calcium per ml egg yolk suspension. These conditions permitted estimation of initial reaction velocity in the presence of excess substrate.

Results. Russell viper venom. The phospholipids of washed, intact platelets were hydrolyzed by the venom at all concentrations used. However, there was virtually no effect on intact red cells (Fig. 1). Hydrolysis of plasma phospholipids was greater than platelet phosphatides. As shown in Fig. 2, there was almost complete hydrolysis of the ethanolamine phosphoglycerides (PE), serine phosphoglycerides (PS), inositol phosphatides (PI) and lecithin (L) in the platelets. Sphingomyelin appeared to be unaffected. The phospholipids of red cells showed only slight hydrolysis, as evidenced by the appearance of a small quantity of lysolecithin.

As illustrated in Fig. 3, the phospholipids of isolated platelet membranes and granules were hydrolyzed by the venom to approximately the same extent as the phosphatides of whole platelets. Although the finding was not consistent in all experiments, there appeared to be a tendency for membrane phospholipids to be hydrolyzed to a greater extent than those of the granules, especially at low concentrations of venom.

Palestinian viper venom. The phospholipase A activity of this venom was slightly less effective than that of Russell viper venom when tested against plasma phospholipids. This is shown in Fig. 4. The finding was confirmed when the effects of equal weights of the 2 venoms were tested against egg yolk in which an excess of substrate was present. The initial reaction velocity of Russell viper venom was 1.2 times greater than Palestinian viper venom. Nevertheless, concentrations of Palestinian viper enzyme with activity comparable to that of Russell viper venom failed to hydrolyze the phospholipids of either red cells or platelets to any significant degree. In Fig. 4 and 5 it is seen that there was some hydrolysis of membrane and granule phospholipids after incubation with Palestinian viper venom, but very much less than in the case of Russell viper venom. The effect on membranes and granules was approximately equivalent.

Discussion. The results indicate that Russell viper venom affects red cells and platelets in a manner similar to *N. naja* phospholipase A(6). The extent of hydrolysis of platelet

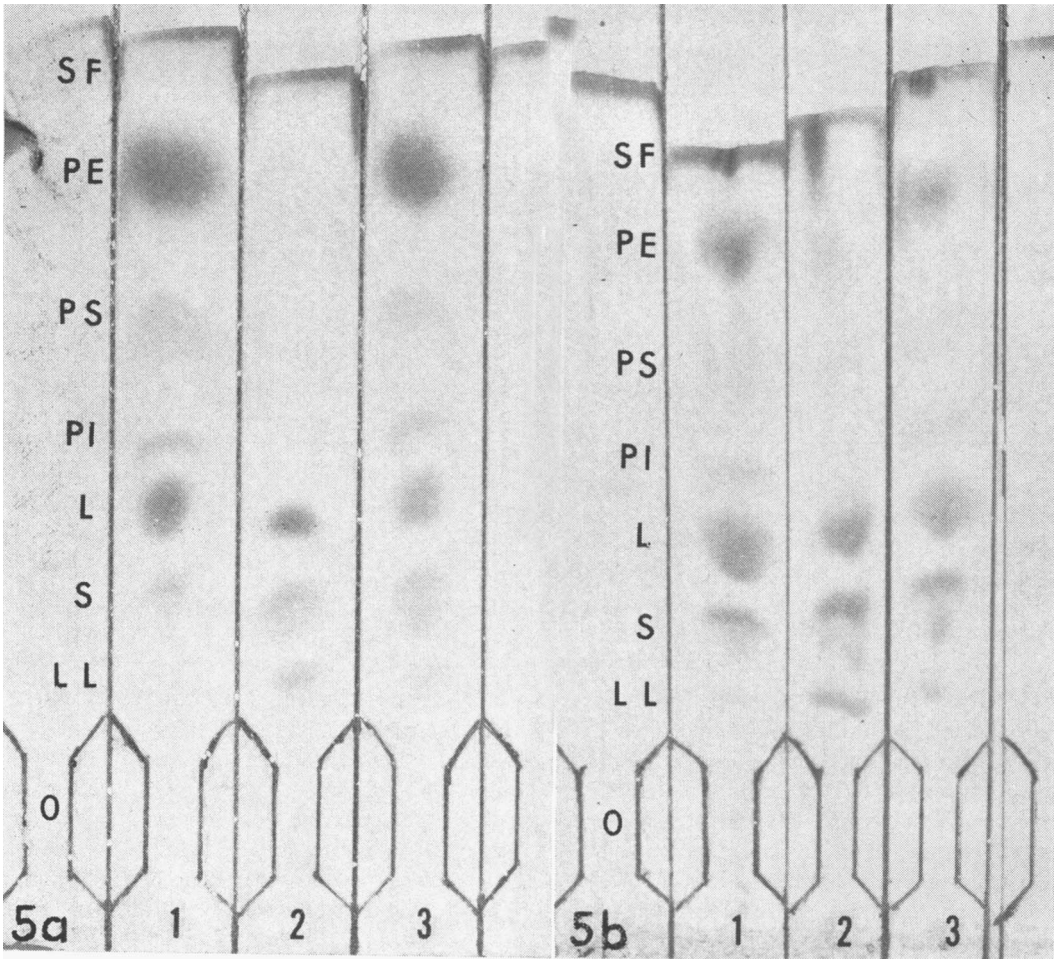

FIG. 5. Thin-layer chromatograms of phospholipids of isolated platelet membranes (A) and granules (B). 1) Untreated particles. 2) RVV-treated particles (10 mg/ml). 3) PVV-treated particles (10 mg/ml).

membrane and granule phospholipids by Russell viper venom was approximately equivalent. The finding that intact platelet phospholipids were not hydrolyzed by Palenstinian viper venom was confirmed(6). It was also demonstrated that isolated platelet membrane and granule phospholipids were both hydrolyzed to a limited but equal extent by this venom.

The data do not support the hypothesis that platelet membrane phospholipids have a specific affinity for venom phospholipase A since the granule phospholipids were hydrolyzed to a comparable extent. This is plausible if one considers that each platelet granule appears to have its own unit membrane.

The striking difference between the action of the venom enzyme on erythrocytes and platelets is of considerable interest. It is unlikely that this difference could be due to variation in lipid composition since available data(5,16) have not demonstrated major differences between the two cells. Presumably, therefore, the platelet lipids are more readily available to the action of the enzyme. This "availability" might be determined by nonlipid components of the membrane (such as proteins and polysaccharides). The phospholipids in the red cell membrane may be covered by proteins or polysaccharide molecules which are absent from or differently orientated in the platelet.

The cells used in the present study were washed in saline. Since washing is known to affect the coagulant properties of platelets(5), this may have affected the venom sensitivity in the present experiments. If so, it may indicate that the washing process exposes phospholipid molecules in the platelet but not in the red cell. It may be that the intact (unwashed) platelet is also resistant to venom action but that washing in saline removes cell components that interfere with the action of the enzyme. The red cell is clearly not affected in the same way, perhaps because the non-lipid membrane components are more firmly bound or qualitatively different. These considerations, admittedly speculative, may also be pertinent with regard to the availability of phospholipids for the coagulation process.

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Phenothiazine Inhibition of Carbon Tetrachloride Cytotoxicity *in vitro** (31633)

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The hepatotoxicity of carbon tetrachloride has been demonstrated by administering the agent to the intact animal(1-4), by perfusing the isolated liver with this agent(5,6), and by exposing isolated liver cell mitochondria to it(7). A number of studies have demonstrated that the hepatotoxic effects of CCl_4 are reflected in the elevation of a number of enzymes in the plasma(8-12). Studies in this laboratory have shown that the cytotoxicity of CCl_4 *in vitro* also can be demonstrated by the leakage of intracellular enzymes from non-hepatic cells (grown in tissue culture) after brief exposure to the agent(13).

The present study was designed to evaluate the suitability of the *in vitro* system employing non-hepatic cells as a model for the study of CCl_4 hepatotoxicity. It demonstrated that agents known(14-18) to protect against CCl_4 hepatotoxicity *in vivo* could prevent manifestations of cytotoxicity *in vitro*.

Methods and materials. The cells studied were the strain of laryngeal carcinoma cells maintained in tissue cultures (H. Ep. #2) originally isolated by Moore, Sabuheurh, and

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