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REFRACTORINESS OF RED BLOOD CELLS AND PLATELETS

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The effect of proteolytic enzymes and of arachidonic acid on aggregation of red blood cells and platelets was studied. These substances were found to stimulate aggregation of the blood cells. Preliminary incubation of fibrinolysin, trypsin, and arachidonic acid with suspensions of blood cells, however, is followed by a marked decrease in their ability to aggregate, i.e., by the development of a refractory state. The possible mechanism of this phenomenon is discussed.
KEY WORDS: blood cells; aggregation; refractoriness; proteolytic enzymes; arachidonic acid.

The refractoriness of platelets began to be studied only in the last decade [11, 13]. The essence of this phenomenon is that incubation of platelets with ADP causes them to lose their ability to react to fresh doses of ADP. The state of refractoriness lasts at least 4 h and, according to the authors cited, it is not connected with adenosine formation.

Although in recent years new investigations confirming the possible development of refractoriness of platelets during their incubation with ADP have been published [2, 3, 9], the mechanism of this phenomenon remains unexplained. As yet, moreover, the possibility of development of refractoriness of platelets has been described only in relation to ADP. The only exception has been the work of Eika [7], who showed that the second wave of adrenalin aggregation in heparinized plasma is depressed and collagen aggregation of the platelets is reduced; this is attributed to the development of a refractory state of the platelets. It is also not clear whether refractoriness is a specific property of platelets or whether other blood cells, in particular red blood cells (RBC), can also develop it.

The investigation described below was devoted to a study of these problems.

Arachidonic acid and the proteolytic enzymes fibrinolysin and trypsin were used as the aggregating agents. These substances were chosen for the following reasons. Increased proteolytic activity in the blood is a fairly frequent phenomenon and can take place in many different pathological states (shock, hypoxia, etc.) as well as during the treatment of thromboembolism by thrombolytic substances (plasmin, streptase, urokinase). Data showing the possible aggregating action of these enzymes on platelets have been published [10], whereas virtually no attempt has been made to study their effect on RBC. Arachidonic acid, which aggregates platelets, can be regarded in the opinion of several workers as the key factor of hemostasis and thrombosis [15]. The possibility of aggregation of RBC by arachidonic acid likewise has not been established.

EXPERIMENTAL METHOD

Aggregation of RBC and platelets was investigated photometrically in an aggregometer. The degree of aggregation was estimated by measuring the maximal amplitude of the aggregatogram (Ma). Donors' RBC (1 :400) and platelets (300,000-400,000/mm^3), washed and resuspended in physiological saline, were studied.

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TABLE 1. Effect of Aggregating Substances on Development of Refractoriness of RBC (M ± m)

Aggregating agents	n	Ma of aggregatogram, mm		P
		without incubation	after incubation for 30 min	
Fibrinolysin	10	21,1±1,48	11,7±1,05	<0,001
Trypsin	11	38,7±1,93	21,0±1,97	<0,001
Arachidonic acid	11	88,0±4,03	46,4±3,96	<0,001

TABLE 2. Effect of Aggregating Substances on Development of Refractoriness of Platelets (M ± m)

Aggregating agents	n	Ma of aggregatigram, mm		P
		without incubation	after incubation for 30 min	
Fibrinolysin	11	20,1±1,92	13,4±1,25	<0,01
Trypsin	10	37,0±3,09	22,0±2,09	<0,001
Arachidonic acid	10	58,8±5,09	33,4±2,75	<0,001

Aggregation of the blood cells was induced by trypsin (0.5 mg/ml), fibrinolysin (500 units/ml), and arachidonic acid (45 mM). To study refractoriness, the aggregating agents were added to suspensions of blood cells twice (their final concentration corresponded to the control): before incubation, and after incubation but before introduction of a suspension into the aggregometer. Incubation (without mixing) was carried out at room temperature for 25-40 min.

EXPERIMENTAL RESULTS

Fibrinolysin, trypsin, and arachidonic acid caused marked aggregation of both platelets and erythrocytes if studied in the aggregometer immediately after addition of the above-mentioned substances (Tables 1 and 2). Meanwhile, preincubation of the aggregating agents with suspensions of blood cells was followed by a marked decrease in their ability to aggregate, i.e., by the development of a refractory state. Under these circumstances the blood cells did not completely lose their ability to aggregate, but the degree of aggregation fell sharply. Incubation for short periods (10-15 min) did not cause the development of refractoriness. With an increase in the incubation period to 40-50 min, the intensity of refractoriness also increased. This is also shown by the following fact. Aggregation of RBC by arachidonic acid in some cases was accompanied by marked hemolysis. Preincubation of the RBC with arachidonic acid in these cases either prevents hemolysis from developing or delayed its appearance.

As was stated above, the mechanism of development of refractoriness has not yet been explained. However, on the basis of previous investigations [2, 3, 9] and the results now obtained some definite ideas on this question can be put forward.

The most important factor governing aggregation of platelets is a change in their shape (the "protrusion" of pseudopodia, etc.) [4, 14]. According to observations by Odesskaya [4] and Salzman et al. [14], under the influence of aggregating agents ATPase activity of thrombosthenin (the contractile protein of platelets) is inhibited, it relaxes, and as a result, the surface of the platelets is changed.

The shape of the RBC also depends on the contractile protein of their membrane, which possesses ATPase activity [8]. It has recently been shown that hydrolysis of the phospholipids of the RBC membrane is accompanied by a decrease in its ATPase activity [6].

The proteolytic enzymes used activate phospholipase [12], which hydrolyzes phospholipids, and in that way they probably inhibit the ATPase activity of the contractile protein of the blood cell membranes. Arachidonic acid formed by hydrolysis of phospholipids may be one of the agents that directly inhibit ATPase activity, as a result of which the shape of the blood cells is altered [15]. It seems likely that if the blood cells are mixed, deformation of cell membranes will be followed by aggregation. If, however, the blood cells are incubated with

aggregating agents without mixing, aggregation does not arise. Meanwhile ATPase activity falls to a certain critical level, and subsequent addition of aggregating agents can no longer change it significantly. Under these circumstances a state of refractoriness arises. Regions of the membrane responsible for adhesion of blood cells are perhaps "closed" or "blocked" at this time [9]. Restoration of the ATPase activity of the contractile protein, as shown in [9], leads to restoration of the shape of the cells and to disappearance of refractoriness.

The phenomenon of refractoriness as a general response of blood cells to aggregating agents, as established by these experiments, may help to explain the contradictory data for the action of these substances on aggregation. For example, plasmin itself is known to induce aggregation of platelets [10], whereas there is information that following administration of plasmin to patients aggregation of the platelets is reduced [1]. In the second case the platelets were evidently in a state of refractoriness. Data on the aggregating action of plasmin of platelets [10] also become understandable: Incubation of platelets with fibrinolysin for a short time increases the degree of ADP aggregation, whereas prolonged incubation reduces it. The same applies also to heparin, for differences in the conditions of investigation with heparin (experiments in vivo and in vitro, different times of incubation, and so on) have caused some workers to regard it as an antiaggregating substance [5], whereas others declare that heparin stimulates platelet aggregation [7]. The present results explain the reason for these contradictions.

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