BINDING OF ACTIVE AND INACTIVE HEMOLYTIC FACTOR OF SEA ANEMONE NEMATOCYST VENOM TO RED BLOOD CELLS

David A. Hessinger* and Howard M. Lenhoff

Departments of Molecular Biology and Biochemistry,
and Developmental and Cell Biology
University of California, Irvine, California 92664

Received May 31,1973

SUMMARY

Sea anemone nematocyst venom, in the presence of Ca^{2+}, induced the lysis of red blood cells after an induction period. In the absence of Ca^{2+}, however, no lysis occurred, but the hemolytic factor was shown to bind to the cells. This binding was shown to be requisite for the Ca^{2+} dependent lysis to ensue. After freeze thawing, the venom proteins responsible for lysis lost their hemolytic activity, yet still bound to the cells. The freeze-thawed inactivated venom competitively blocked hemolysis by active venom.

INTRODUCTION

Several diverse types of lytic toxins, including snake venom direct lytic factor (1), Pyrmnesium lysin (2), the complement system (3), and lymphotoxin produced by activated human lymphocytes (4) have been shown to bind to their respective "target" cells. Each of the aforementioned lytic substances is chemically unique and interacts with cell membranes to effect cytolysis by apparently different mechanisms.

Hemolysis induced by sea anemone (Aiptasia pallida) nematocyst venom is the result of a series of time ordered reactions involving venom, Ca^{2+} and red blood cells (5). The primary interactions of these components occur during the prolytic phase of lysis, that is, before the ensuing lytic phase. The prolytic phase consists of three distinguishable events, the first two of which can occur in the absence of added Ca^{2+} and seem to involve the independent and sequential interaction of two venom components with the red cell membrane (6). This report describes the binding of active and inactive venom hemolytic material to red cell membranes during the early part of the prolytic phase. This binding is obligatory for lysis and does not require Ca^{2+}.

* To whom inquiries should be addressed. Present address: Department of Biology, University of South Florida, Tampa, Florida 33620

MATERIALS AND METHODS

Preparation and isolation of nematocysts: Sea anemones (Aiptasia pallida) were obtained off the dock pilings at Flipper's set on Key Biscayne in Miami, Florida, and maintained en masse in the laboratory in sea water aquaria. Nematocysts were obtained by Blanquet's (7) modification of Yanagita's method (8).

Preparation of nematocyst venom: The isolated nematocysts were washed and caused to discharge osmotically (9), thereby extruding the nematocysts' soluble contents or venom. This mixture of soluble toxic proteins was dialysed against 0.144 M saline containing 0.01 M Tris, pH 7.4 and then frozen and stored in aliquots until used.

Preparation of red blood cell suspensions: Fresh blood was drawn by cardiac puncture into heparinized syringes from lightly ether-anesthetized male Sprague-Dawley rats. The blood was centrifuged, and the plasma and buffy coat aspirated off. The red cells were suspended in cold isotonic saline buffered with Tris and washed three times. The concentration of red cells used was 0.625% (v/v). At this concentration osmotic lysis of all the cells with water yielded a supernatant with an absorbance of 1.20 at 570 nm on the Zeiss Q II spectrophotometer.

Hemolysis reactions: Hemolysis reactions were carried out in flasks containing 20 ml red cell suspensions (0.625%) in isotonic saline-Tris at 30 °C. The extent of lysis was determined by taking 2 ml aliquots of the reaction mixture at timed intervals, centrifuging at 10 000 x g for about one min and saving the supernatant for spectrophotometric determination of released hemoglobin. The results were expressed as percent hemolysis using an osmotic hemolysate as a 100% lysis reference.

RESULTS

Binding to red blood cells of hemolytic activity from active nematocyst venom: To demonstrate the binding of active venom to intact red blood cells, the Ca^{2+} must be eliminated from the incubation medium; without added Ca^{2+} lysis does not occur (5). The experiment was carried out as follows: (a) Fresh

venom solution was prepared and found to have significant hemolytic activity (Table 1,A). (b) Aliquots of the venom solution were incubated with 1 ml and 2 ml red cells for 30 min, following which the red cells were centrifuged and removed, and the resultant supernatant tested for hemolytic activity with fresh red cells (0.625%) and Ca^{2+}. The results (Table 1,B and C) show that during the incubation 73% and 85% of the lytic activity was removed by the red cells. (c) Finally, the pelleted red cells from the original incubation mixtures were washed three times in Tris-buffered saline lacking Ca^{2+}, and then placed into fresh saline solution containing Ca^{2+} so that venom-induced lysis would occur. The results show (Table 1,D and E) that most of the hemolytic activity lost from the original venom solution was adsorbed to the red cells and caused lysis of those cells when Ca^{2+} was added.

The binding of the venom components to the red cells appears to be rapid and with great avidity because comparable experiments showed that maximal binding occurred in less than 15 min, and repeated washings did not remove significant amounts of the bound hemolytic activity.

Hence, these experiments show that the venom contains a material that both binds to the red cell in the absence of Ca^{2+} and then lyses the red cell when Ca^{2+} is added. The next two experiments show that the binding capacity of this material can be retained even after the hemolytic activity is destroyed by freeze-thawing.

Inactivation of venom hemolytic activity by freeze-thawing: Repeated freeze-thawing of venom caused a progressive change in the pattern of hemolysis (Fig. 1); with each successive freeze-thawing, the rate of lysis diminished and the final extent of lysis leveled off at limits progressively lower than 100%. Although the decrease in rate of lysis can be accounted for by a diminution in the amount of hemolytic activity in the venom, the leveling off of the extent of hemolysis below 100% cannot be simply explained; with less hemolytic activity 100% lysis would still be reached, but only more slowly. Thus it appears that something more than loss of hemolytic activity is involved. We interpret

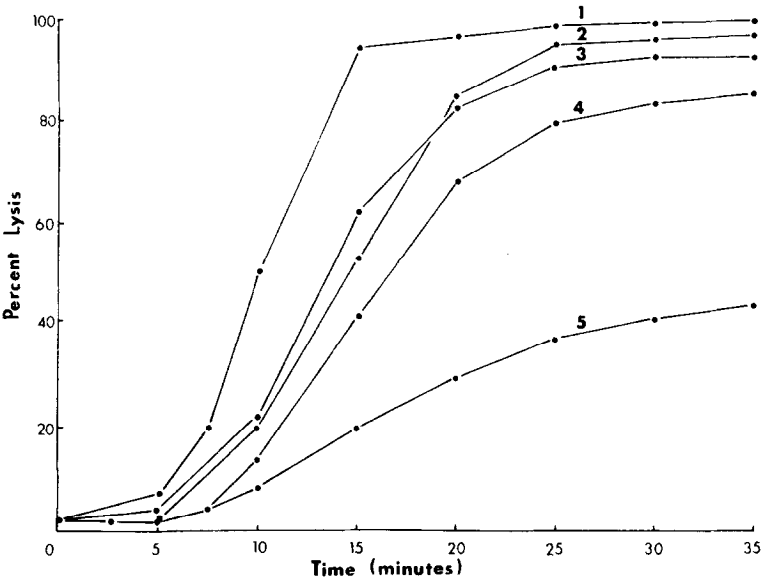

Fig. 1. The effect of repeated freeze-thawings on the ability of nematocyst venom to lyse red cells. Hemolysis was performed in isotonic saline-Tris solution using the same sample of venom (0.230 µg/ml) freeze-thawed a different number of times. The number of freeze-thawings that the venom was subjected to is indicated by the number above each curve.

Table I

Hemolytic Activity Remaining in Solution and Bound to Red Blood Cells

Exp.	Conditions	Hemolytic Activity (% Lysis/min)
A	Venom solution before incubation with red cells	4.09
B	Venom solution after incubation with 1 ml red cells	1.13
C	Venom solution after incubation with 2 ml red cells	0.63
D	Resuspended red cell pellet from exp. (B) plus Ca^{2+}	1.92
E	Resuspended red cell pellet from exp. (C) plus Ca^{2+}	3.64

Incubation times were 30 mins. All red cell suspensions added to the incubation mixtures (20 ml final vol.) were from a 12.5% (v/v) stock suspension. The amount of venom used was 0.230 µg protein/ml (10) and the concentration of Ca^{2+} was 3.3 mM. Red cells treated as in exp. (b), but with no venom present, underwent no significant lysis (0.05%/min).

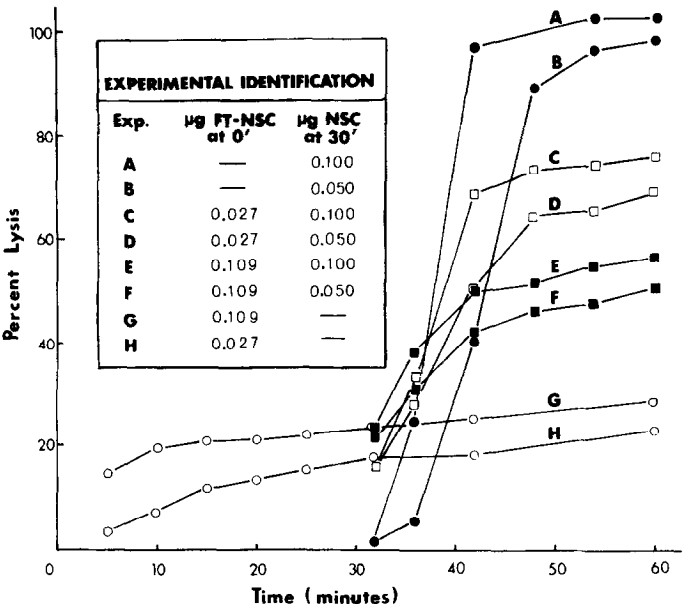

Fig. 2. The effect on hemolysis of pre-treating red blood cells with freeze-thawed venom. Hemolysis reactions were carried out under standardized conditions with 5.5 mM Ca^{2+}. Each of the eight experiments is identified on the table in the upper left corner. The freeze-thawed venom (FT-NSC) was added to the indicated experimental sample at zero minutes and fresh venom (NSC) was added 30 min later as indicated. Note: NSC here refers to nematocyst soluble contents, i.e., the venom.

these data to indicate that the inactivated hemolytic material is binding to the red cells and thereby occupying sites that otherwise would be taken by active hemolytic material.

Binding of freeze-thawed venom to red blood cells: To determine if our interpretation was correct, the following experiments were carried out: Two aliquots of red cells were incubated with different concentrations of freeze-thawed venom and the rate and extent of hemolysis was followed (Fig. 2, Curves G and H). In contrast to the small amount of lysis occurring in the freeze-thawed venom treated cells, the lysis observed in cells exposed to fresh venom preparations was typically rapid and complete (Curves A and B).

To determine if freeze-thawed venom actually binds to red cells and thus prevents additional active venom from binding, the following experiments were carried out. The red cells were first preincubated with the freeze-thawed venom for 30 min, and then fresh venom was added (note that two concentrations

of freeze-thawed venom and of fresh venom were used). The results showed that: (a) in no case did the addition of fresh venom cause 100% lysis to occur (Curves C,D,E and F); (b) the greater the concentration of the freeze-thawed venom in the preincubation mixture, the lower the final extent of hemolysis (compare Curves E and F to C and D), and the greater the requirement for fresh venom to offset this lower final extent of lysis (Curves C and E).

By a series of washing experiments, it was shown that the freeze-thawed venom tightly binds to red cells. Red cells treated with freeze-thawed venom for 30 min, as in Fig. 2, (Curves G and H) were washed three times before being exposed to fresh venom; essentially the same extents of lysis occurred as in Fig. 2, Curves C. D. E and F. Hence, it appears that the inactivated venom is tightly bound and prevents subsequently added fresh venom from binding.

DISCUSSION

A plot of extent of hemolysis induced by nematocyst venom as a function of time is sigmoidal (Fig. 1) consisting of three phases (5): an induction or prolytic phase, during which no lysis occurs; a lytic phase, during which lysis follows first-order kinetics (6); and a terminal phase, during which the remaining cells lyse. It is in the prolytic phase that the initial interactions between venom, Ca^{2+} and red cell membrane occur to produce a configuration of the cell membrane which leads to lysis.

Previous results (6) showed that during the prolytic phase, the following order of events occurred: (a) hemolytic factors of the venom interact directly with the red cell membrane; and (b) the involvement of Ca^{2+} allows the hemolytic factors to alter the membrane so that lysis ensues. The nature of the interaction of the hemolytic factors with the red cell membrane is described in the present paper.

Table 1 showed that in the absence of Ca^{2+}, the hemolytic activity of nematocyst venom binds to the red cell; on the addition of Ca^{2+} lysis takes place. Figs. 1 and 2 showed that the hemolytic activity of the venom is destroyed by freezing and thawing, yet this material retained its capacity to bind to the

red cells. Furthermore, the bound inactive material prevented active hemolytic factors from binding and acting. The more freeze-thawed venom used the more inhibitory it was to added fresh venom. Hence, active hemolytic factors must bind to red cells before they can cause lysis. Binding can occur in the absence of Ca^{2+}, whereas lysis requires Ca^{2+}.

At the present one of the hemolytic factors has been identified as the enzyme phospholipase A_2 (5). Future investigations are being directed toward characterizing and isolating the sites on the red cell membranes to which the hemolytic factors bind in an attempt to probe the molecular nature of the nematocyst venom-cell membrane interaction which results in lysis.

REFERENCES

1. E. Condrea, J. Kendzersky and A. deVries, Experientia, 21 (1965) 461.
2. D. F. Martin and G. M. Padilla, Biochim. Biophys. Acta., 241 (1971) 213.
3. H. J. Müller-Eberhard, in P. A. Meischer and H. J. Müller-Eberhard, Textbook of Immunopathology, Grune and Stratten, New York, 1968, p. 33.
4. D. A. Hessinger, R. A. Daynes and G. A. Granger, Proc. Nat. Acad. Sci. (USA), 1973, in press.
5. D. A. Hessinger, H. M. Lenhoff and L. Kahan, Nature, N.B., 241 (1973) 125.
6. D. A. Hessinger and H. M. Lenhoff, submitted.
7. R. S. Blanquet and H. M. Lenhoff, Science, 154 (1966) 152.
8. T. M. Yanigita and T. Wada, Cytologia, Int. J. Cytol., 24 (1959) 81.
9. R. S. Blanquet, Comp. Bioch. Physiol., 25 (1968) 893.
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. Biol. Chem., 193 (1951) 256.