Biochemical Pharmacology, 1962, Vol. 11, pp. 9–15. Pergamon Press Ltd., Printed in Great Britain.

EFFECTS OF SNAKE VENOM ON RABBIT BASOPHIL LEUKOCYTES*

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(*Received* 17 *May* 1961; *accepted* 29 *July* 1961)

Abstract—Rabbits respond to treatment with *Crotalus atrox* venom with a biphasic basopenic response. The lecithinase A content of snake venom may satisfactorily account for the rapid depletion of basophils in rabbit blood, while other mechanisms, possibly endocrine, may account for the delayed response. Constituents other than lecithinase A are responsible for the major portion of the lethal action of *Crotalus atrox* venom in rabbits.

FOLLOWING the demonstration of the phenomenon of histamine release from mast cells by 48/80, considerable study of this process of granule release has results.[1–4] In parallel with these studies on the mast cell, we have been able to demonstrate that rabbit basophil leukocytes undergo degranulation when the animals are treated with histamine-liberator 48/80.[5] This occurs synchronously with the development of the shock-like syndrome that follows intravenous administration of this drug.

In considering the mechanism of degranulation by 48/80, studies *in vitro* have revealed only a de-staining phenomenon to occur.[6, 7] This phenomenon is probably not important in producing the destructive effects seen *in vivo*. More importance may attach to the concept advanced by Hogberg and Uvnas[3, 4] for an indirect enzymic mechanism of mast cell disruption involving the action of a lecithinase. The present report is an attempt to determine whether this theory of the mechanism of mast cell degranulation can be extended to include the degranulation of basophil leukocytes. By determining the effect of snake venom and venom fractions upon rabbit blood, we conclude that lecithinase may have a role in this process.

MATERIALS AND METHODS

Male albino rabbits weighing approximately 2 kg were used. Injections were made intravenously into one ear and blood samples were obtained from the opposite ear. Serial vein punctures were performed upon segments of vein separated widely enough so that a puncture was not made through an area of inflammation resulting from previous sampling. Blood was diluted in conventional leukocyte counting pipettes with a modified Moore and James[8] solution and counted in Fuchs–Rosenthal double-thickness chambers. The simplified dilution fluid consisted of 20 ml of 0·67 M phosphate

* Supported by Grant RG-2606, National Institutes of Health, U.S. Public Health Service. Reported in part at the meeting of the Ohio Valley Section, Society for Experimental Biology and Medicine, Lexington, Ky., October, 1960.

buffer (pH 7·4), 7 ml of 95 per cent ethanol, and 0·3 ml of 1 per cent aqueous toluidine blue. With this solution one obtains complete red cell hemolysis, good differential staining, and freedom from sediment in the counting chamber for a long enough period of time to enable accurate basophil and total leukocyte counts to be made. Fresh diluting solution must be made weekly.

In vitro studies were performed upon blood withdrawn with siliconized syringes, placed in siliconized sterile bottles and treated with 50 mg of sodium citrate per ml of blood. The bottles were kept in a Dubnoff shaker at 37 °C for the duration of each experiment.

Venom of *Crotalus atrox* (Western Diamondback Rattlesnake Venom, Ross Allen Reptile Farm) was dissolved in saline and used directly in these studies. The venom was also used as a source of lecithinase. A lecithinase preparation was made by the method of Slotta and Fraenkel-Conrat[9] and its activity was tested by a hemolytic method.[10] Proteolytic activity was tested by the method of Anson.[11]

Each animal served as its own control in the experiments *in vivo*, several control blood counts being taken before any drug administration. Each blood sample in the *in vitro* experiments was divided into two equal portions, one of them serving as the control. Each control blood specimen was treated with a volume of saline equal to the volume of venom solution used in the corresponding experimental trial.

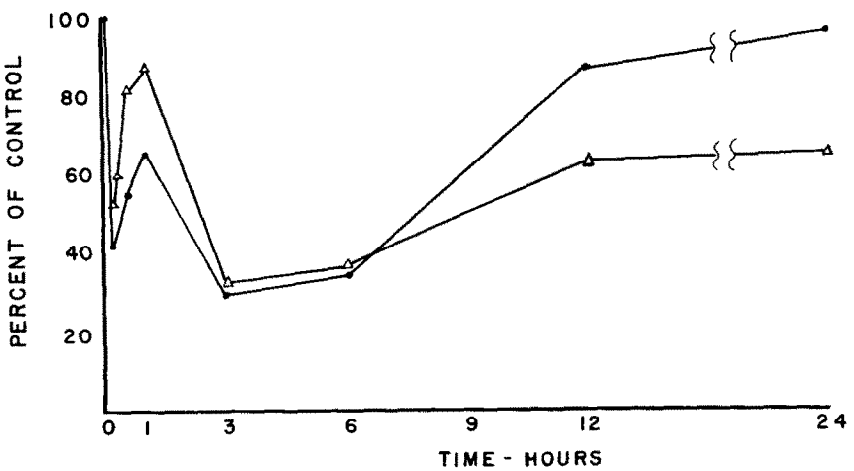

FIG. 1. Effect of administration of *Crotalus atrox* venom upon basophil leukocytes of seven rabbits. Dose 1 mg/kg i.v.; ●—● absolute basophil counts; △—△ relative basophil counts.

RESULTS

Experiments with whole snake venom

Intravenous administration of 1 mg of *Crotalus atrox* venom per kg of body weight to seven rabbits produced the alterations in absolute and relative basophil leukocyte counts noted in Fig. 1. The mean absolute basophil count prior to venom administration was 757/mm³. Five minutes following the administration, the mean count had fallen to 331/mm³ (difference significant to $P < 0{\cdot}05$). After a restoration toward

normal, a second decline occurred, to 223/mm³ at 3 hr and 267/mm³ at 6 hr (differences from controls significant to $P < 0{\cdot}01$). The absolute and relative basophil counts then showed a steady return toward initial control values. There was little divergence between the relative and absolute basophil counts during the first 6 hr. The total white blood cell count was depressed slightly during the first 3 hr, recovered to its control value at 6 hr, then exhibited a moderate elevation from 12 to 24 hr. This leukocytosis accounts for the divergence of the absolute and relative basophil counts during this period. The blood was again normal at 48 hr and remained so for the following 4 weeks during which observations were made on all of these animals. This dose of venom is very toxic, and approximately 30 per cent mortality occurs. Initial blood samples from the animals which died are not included in the reported data.

In order to determine if snake venom was acting directly in producing the observed depression of basophils, a group of three *in vitro* experiments was performed on freshly drawn rabbit blood. These samples were incubated with 0·005 mg of *Crotalus atrox* venom per ml. It can be seen in Fig. 2 that the exposure of blood *in vitro* to this

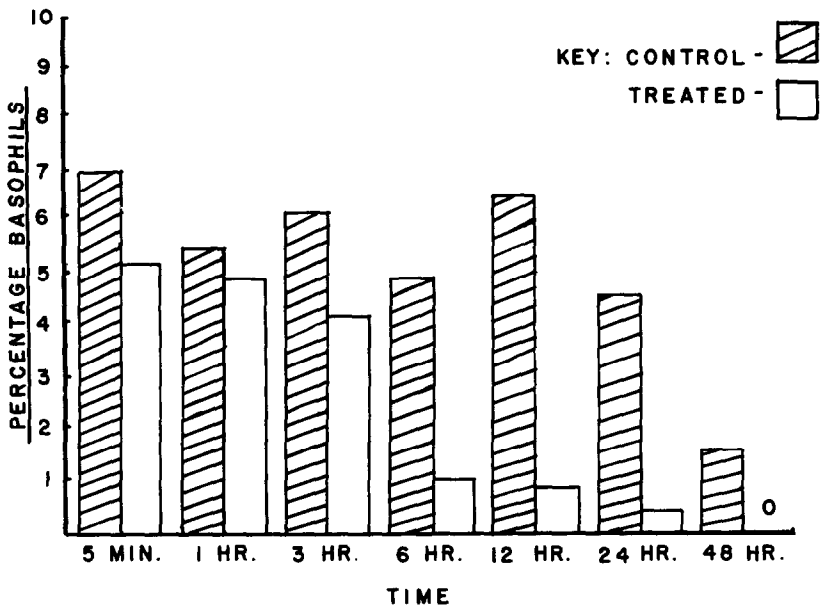

FIG. 2. Effect of incubating rabbit blood with 0·005 mg of *Crotalus atrox* venom per ml. Each column is the mean of three experiments.

venom produced a loss of basophils rather slowly, with the greatest drop occurring between 3 and 6 hr. Saline controls demonstrated a preservation of approximately 75–80 per cent of the initial basophils for 24 hr, while the venom-treated blood samples contained approximately 15 per cent after 6 hr, even less thereafter. The total white blood cell counts in the trials *in vitro* do not change significantly for 12 hr, then gradually decline.

Experiments with venom fractions or modified venom

Since snake venoms are known to contain a variety of lytic enzymes besides lecithinase A,[12] some attempts were made using modified or fractionated *Crotalus atrox* venom to determine if the effects observed could reasonably be attributed to the lecithinase or to one of the other components.

A fractionation of the venom by a pyridine–acetic acid method,[9] resulted in a white precipitate (fraction I) and a yellow supernate (fraction II). These were lyophilized and tested for lecithinase activity. The bulk of the activity was found in fraction I, but fraction II also contained detectable activity. The administration of each of these fractions to groups of rabbits produced the effects demonstrated in Fig. 3. It can be seen that fraction I contains most of the basophil-destroying activity of the whole venom, while fraction II is much less active.

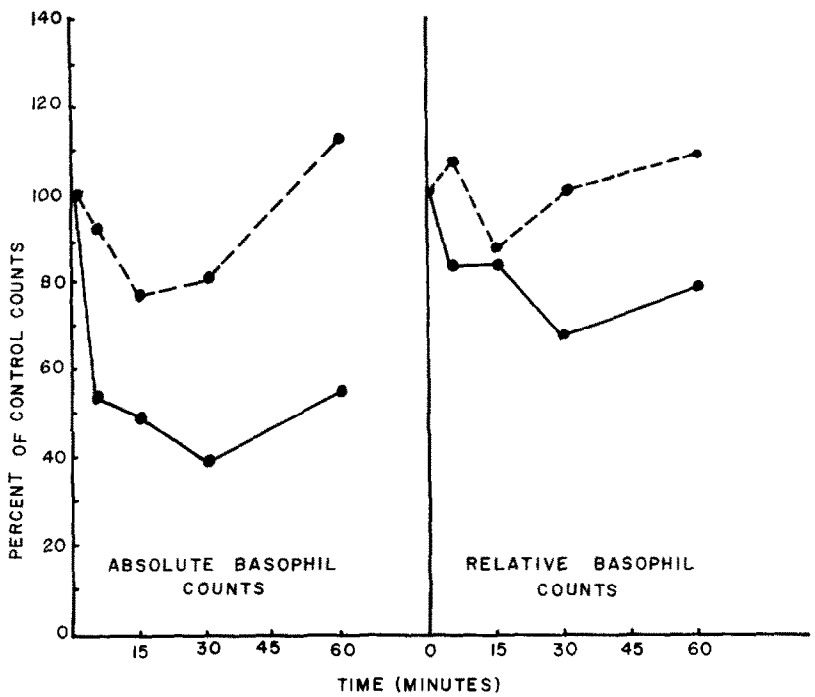

FIG. 3. Effect of *Crotalus atrox* venom fractions upon rabbit basophil leukocytes. ●—● fraction I, 1–3 mg/kg intravenously; nine experiments. ●– – –● fraction II, 1–1·3 mg/kg intravenously; six experiments.

Within the dosage range of 1–3 mg/kg, the response of basophil depletion to fraction I was approximately proportional to the dose. The phenomenon was qualitatively the same throughout this dosage range. In Fig. 3, results from nine experiments within this dose range are combined.

For simplicity of graphical representation, only the first 60 min of rabbit response to these fractions are shown. Blood samples were, however, followed serially for days in most cases. It can be seen that the maximum basopenia occurred 30 min after administration. No delayed response, such as that with whole venom, occurred with

fraction I. The only notable response during the period after 1 hr was a bone marrow stimulation resulting in leukocytosis with relatively normal percentage of basophils. This occurred with both fractions, being most pronounced with fraction I.

Fraction II showed little effect upon basophil leukocytes, and little on total leukocyte counts. This can be seen in Fig. 3 from the results of the treatment of six rabbits with 1–1·3 mg/kg of fraction II.

The lethal dose for fraction I prepared by the pyridine–acetic acid method was not established, but it is greater than 5 mg/kg. Occasional deaths from fraction II were experienced at all dose levels above 1 mg/kg. Records of animals that subsequently died are not included in Fig. 3.

Since the pyridine–acetic acid method does not result in a complete separation of the lecithinase activity, a study was done using venom solutions boiled for 5 min at a pH of 5·5–6·0 (the pH of fresh venom solutions in saline) and at a pH of 8·5–9·0.[13] The whole fresh venom contained both lecithinase and proteolytic activities. When boiled at the acid pH, a dense precipitate formed quickly. After centrifugation, the supernate demonstrated almost undiminished lecithinase activity, while the proteolytic

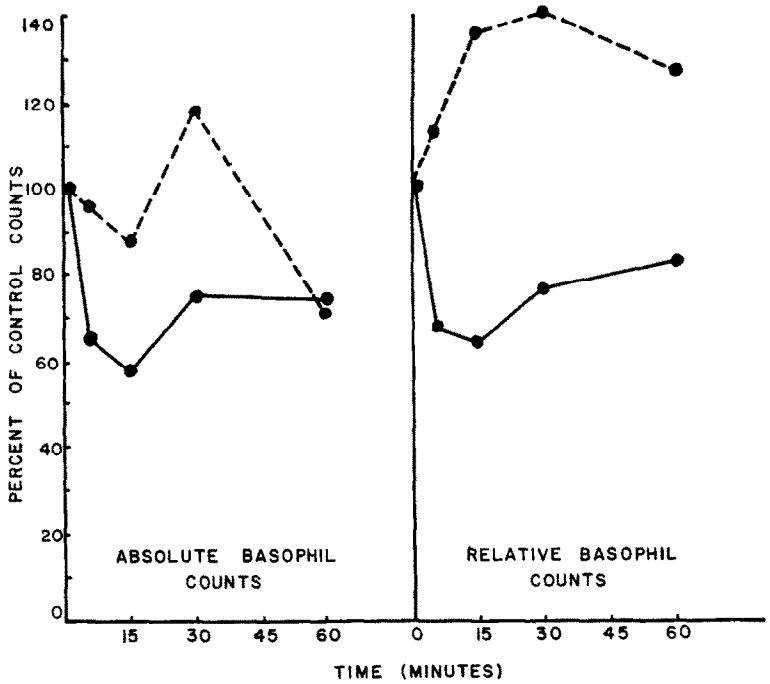

FIG. 4. Effect of boiled *Crotalus atrox* venom upon rabbit basophil leukocytes. ●—● boiled pH 5·5–6, six experiments. ●– – –● boiled pH 8·5–9, six experiments.

activity was completely absent. When boiled at the alkaline pH, no precipitate formed, and the clear solution showed neither lecithinase nor proteolytic activity.

The intravenous administration of 3 mg/kg, using two groups of six rabbits each, and venom boiled under the two conditions described above, produces changes in basophil counts shown in Fig. 4. It can be clearly seen that the "acid-boiled" venom

produced a rapid basopenic response, while the "alkaline-boiled" produced no early fall in basophils. In the following hours, there was some delayed fall in basophils from the alkaline-boiled fraction, while marked leukocytosis occurred following administration of the acid-boiled venom.

DISCUSSION

One of the interesting aspects of the basophil curves following the administration of *Crotalus atrox* venom is the sharp early fall in the basophil count, followed by a restoration toward normal, and a secondary slower decline reaching a minimum at from 3 to 6 hr. In our earlier studies[5] on the effects of 48/80, we had considered the possibility that a rapid basopenic response might be a de-staining phenomenon. Almost certainly, this cannot be the case with snake venom. Thus, venom incubated with blood *in vitro* did not cause a rapid fall of basophil count. The dose of venom used was approximately equivalent to the dose used in the *in vivo*-study, if one assumes that the active substance in this venom is distributed to the extracellular fluid in the intact animal. Certainly, if a physicochemical de-staining process could occur at all, it would be apparent in the *in vitro*-study.

On the other hand, though the time of response differs in the experiments carried out *in vivo* and *in vitro*, it is clear in both cases that the basophil leukocyte is extremely sensitive to the presence of *Crotalus atrox* venom. This is shown by the decline in relative and absolute basophil counts at times when the number of total white blood cells had not significantly changed. This indicates a much greater resistance to the effects of venom on the part of the other white cell types of blood. The reason for the greater sensitivity of basophils to the lecithinase of snake venom is not clear, since it is presumed that phospholipids are structural elements in the membranes of most cells.

If one can rule out de-staining as a cause of the unusual shape of the basophil depletion curve, several other interesting possibilities present themselves to explain the biphasic response:

(1) One of the phases of basophil depletion may be due to an endocrine response to the administration of snake venom (in support of this possibility is the suggested relationship of adrenal and pituitary hormone action to basophil depletion[14, 15]).

(2) The initial rapid drop in basophil count may be the result of the interaction of snake venom or lecithinase with some non-endocrine tissue component outside of the blood (since the rapid response is seen predominantly with the lecithinase-containing fractions or preparations, it seems likely that such a tissue component should be a phospholipid).

(3) The initial rapid drop in basophil count *in vivo* may be due to a cellular alteration produced by snake venom or lecithinase of a type which would not affect the metachromatic staining characteristics of these cells, but which would alter them sufficiently so that *in vivo* they would be removed from the circulation by some sequestration mechanism. This possibility may be amenable to evaluation by the study of basophils in supravital preparations with time-lapse recording. This would explain the slow rate of basophil disappearance which we observe *in vitro*.

The study of the effects of boiled and fractionated venom implicates lecithinase as being responsible particularly for the rapid phase of the basophil depletion curve. If it is correct[16] that only lecithinase A survives boiling in acid pH, it follows that this

enzyme accounts for little of the toxicity of whole *Crotalus atrox* venom, but accounts for the major share of the rapid basophil-depleting activity seen *in vivo*. It seems likely that other mechanisms must account for the secondary fall of basophils. Adrenal cortical activation may be a possibility. Further studies with venom fractions are called for to establish this point.

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