HEMOLYTIC EFFECTS OF A MEMBRANE ACTIVE POLYPEPTIDE FROM CENTRAL ASIAN COBRA VENOM

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Basic polypeptides with molecular weights ranging from 6000-7000 daltons, well defined in literature as "membrane-active polypeptides," have been fractionated from many Elapid venoms. Procedure of separation of six such components from the venom of Central Asian cobra (Naja naja oxiana Eichwald) has also been reported. Though some studies dealing with the hemolytic effects of two polypeptides, viz., \( V'_6 \) and \( V''_6 \), are available in literature, the action of other components has remained obscure. The present work provides a complete account of the hemolytic properties of one more polypeptide, viz., \( V''_6 \).

Pure \( V''_6 \) and phospholipase \( A_2 \) (EC 3.1.1.4) were fractionated from \( N. naja \) according to Yukelson et al. and Sakhidev et al. respectively. Percentage of hemolysis was determined as suggested by Aloof-Hirsch et al. Incubation medium contained 0.05 ml 50% suspension of human erythrocytes and 0.15 ml reaction medium containing the material to be tested. Erythrocyte suspension was washed 3 times by physiological saline prior to its use. Measurements were made at 37°C, incubation time ranged from 1.5 hr. Degree of hemolysis has been expressed in percentage calculated on the basis of release of hemoglobin which was measured spectrophotometrically at 540 nm. Unless specifically stated experimental conditions remained same as described above.

Results obtained indicate that 200 \( \mu g \) \( V''_6 \) can bring about direct hemolysis which depends upon incubation time, concentration of the cytotoxin and the type of erythrocyte. As seen in Fig. 1a, hemolytic effect of the polypeptide reaches 6% in 3 hr. Degree of hemolysis increases with increase in \( V''_6 \) concentration from 400 \( \mu g \) to 2000 \( \mu g \) in the medium. A sharp rise in the lytic effect of the polypeptide is seen when its concentration in the medium reaches 1200 \( \mu g \) (Fig. 1b). Hemolytic effect of \( V''_6 \) on erythrocytes from different sources showed the following order: human > rat > rabbit (Fig. 1c), confirming the differences in susceptibility of different erythrocytes to the lytic effect of membrane-active polypeptide as suggested by Condrea et al. In addition to this, perhaps the differences in lipido-protein structure of erythrocyte membranes from different sources while regulating the electrostatic and hydrophobic interaction of the polypeptide ultimately decides the degree of lytic effect.

Though the direct hemolysis caused by \( V''_6 \) is less, it considerably increases in the presence of phospholipase \( A_2 \). Degree of hemolysis increased from 10% to 50% when the reaction medium contained 50 \( \mu g \) of pure phospholipase \( A_2 \) along with 1000 \( \mu g \) of \( V''_6 \) (Fig. 2a). Many reports of such synergistic

![Fig. 1. Dependence of hemolytic action of \( V''_6 \) on (a) incubation time (\( V''_6 \) 6-200 \( \mu g \)), (b) concentration of \( V''_6 \) (incubation—3 hr), (c) type of erythrocyte: 1—Human, 2—rat, 3—rabbit (\( V''_6 \) 400 \( \mu g \).](attachment:image.png)

Incubation medium (general volume 0.2 ml) contained 0.05 ml 50% erythrocyte suspension and 0.15 ml physiological saline (0.9% NaCl) containing appropriate concentration of material to be tested.
phospholipase $A_2$ were together incubated for 3 hr. This explains the absolute necessity of the interaction of the cytotoxin with the erythrocyte for developing the lytic effect of phospholipase $A_2$. Cytotoxin certainly differs from other specific agents such as albumin which may stimulate hemolytic activity of phospholipase $A_2$ because of adsorption of lys-products, which in turn brings about labilisation and lysis of the membrane\cite{4}. Calcium ions, well known in literature as activator of phospholipase $A_2$, could not replace cytotoxin in bringing about hemolysis in this experiment.

Role of calcium ions in direct and potentiated hemolysis of $V_c$ 6 and phospholipase $A_2$ has been presented in Fig. 3. In case of direct hemolysis increasing $Ca^{2+}$ concentration in the medium suppressed the hemolytic effect of $V_c$ 6 (Fig. 3a) indicating absence of any action on diverse membrane systems are available in literature including that related to other fractions of Central Asian cobra venom\cite{5}. Two alternatives, often professed and noted below, were experimentally checked to understand the mechanism of synergistic action between the cytotoxin and phospholipase. (1) Cytotoxin effectively labilises the erythrocyte membrane and facilitates the hydrolysis of phospholipids by phospholipase as a result of which hemolysis occurs. (2) Cytotoxin only facilitates phospholipase interaction with the substrates of membrane erythrocyte resulting in hydrolysis of phospholipids. The lyso-products formed then bring about hemolysis.

$V_c$ 6, for comparison, albumin and calcium ions, were added separately to erythrocyte suspensions pre-incubated with phospholipase $A_2$ for 2 hr and were further incubated for 1 hr. Suspension with $V_c$ 6 showed a considerable decrease in hemolytic activity (Fig. 2b) as compared to the one where $V_c$ 6 and

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**Fig. 2.** (a) Potentiated hemolysis. 1—$V_c$ 6, 2—$V_c$ 6 + 50 $\mu$g phospholipase $A_2$.

(b) Effect of $V_c$ 6 (500 $\mu$g), albumin (500 $\mu$g) and $Ca^{2+}$ (5 mmole) on 2 hr preincubated erythrocytes with phospholipase $A_2$ (50 $\mu$g). 1—$V_c$ 6, albumin, 3—$Ca^{2+}$. Other experimental conditions were as described in Fig. 1.

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**Fig. 3.** Effect of calcium ions on direct (a) and potentiated hemolysis (b).

(a) 1—200 $\mu$g $V_c$ 6, 2—200 $\mu$g $V_c$ 6 + 0.5 mmole $Ca^{2+}$, 3—200 $\mu$g $V_c$ 6 + 0.5 mmole $Ca^{2+}$.

(b) 1—100 $\mu$g $V_c$ 6 + 50 $\mu$g phospholipase $A_2$, 2—50 $\mu$g $V_c$ 6 + 100 $\mu$g phospholipase $A_2$, 3—100 $\mu$g $V_c$ 6 + 50 $\mu$g phospholipase $A_2$, 4—400 $\mu$g $V_c$ 6, 5—50 $\mu$g phospholipase $A_2$, 6—50 $\mu$g phospholipase $A_2$, 7—50 $\mu$g phospholipase $A_2$.

Other experimental conditions were as described in Fig. 1.
contamination in the component used. These results are in agreement with those reported in literature. The results also indicate that inhibitory role of Ca$^{2+}$ in direct hemolysis is probably related to the competition between Ca$^{2+}$ ions and cytotoxin molecules for attachment to the membrane surface. In the case of potentiated hemolysis (Fig. 3b) 100% hemolysis was noticed in 3 hr, when 400 $\mu$g V$^{2+}$6, 50 $\mu$g phospholipase A$_4$ and 5 mmole Ca$^{2+}$ were used. When the concentration of Ca$^{2+}$ was lowered to 0.5 mmole in the same combination 85% hemolysis was observed in 3 hr. In the absence of Ca$^{2+}$ 35% hemolysis was attained. V$^{2+}$6 on its own brings about only 8% hemolysis. The other combinations, such as phospholipase A$_4$, 5 mmole Ca$^{2+}$, phospholipase A$_4$ and 0.5 mmole Ca$^{2+}$ bring about negligible hemolysis (see Fig. 2b). Lankisch et al. showed that Ca$^{2+}$ in higher concentrations (45 mmole) causes hemolysis of erythrocytes. It is possible that lower concentrations of Ca$^{2+}$ without causing any hemolysis help disruption of membrane phospholipids by phospholipase A$_4$. The total lytic effect evolved is thus quite faster as seen by 100% and 85% hemolysis noticed in 3 hr (see Fig. 2b).

Figure 4 depicts effect of pH and temperature on direct hemolysis caused by V$^{2+}$6. With an increase in pH of the medium from 6.0 to 8.0 lytic action of the polypeptide also increases. At pH 8.0, 25% hemolysis was observed (Fig. 4a). Temperature dependent studies showed that with a rise in the temperature of the medium, hemolytic effect of V$^{2+}$6 also increases showing a sharp rise above 37°C. At 40°C, 30% hemolysis by the cytotoxin is observed (Fig. 4b). It was recently reported that with increase in the temperature and pH of the medium, lipids of the erythrocyte membranes undergo structural transformation leading to changes in relative position of cholesterol and phospholipids, more labilisation of the membrane and thus, probably facilitates pronounced cytotoxic effect.

If this characterisation of V$^{2+}$6 is compared with earlier studies dealing with V$^{2+}$1 and V$^{2+}$5, V$^{2+}$6 appears to be a weaker polypeptide. This relates to its low basicity as compared to V$^{2+}$1 and V$^{2+}$5, thus emphasising the role of positive charge of the toxic molecules in the mechanism of its lytic effect. In addition to this, structural differences amongst these polypeptides to a great deal decide their extent of interaction with the erythrocyte membrane and the phospholipase A$_4$ during hemolysis.

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**FORMATION OF GIANT HEPATOCYTES IN RESPONSE TO RADIATION**

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Radiation induced giant cell formation has been observed in mammalian cell culture\(^1-^8\), as well as *in vivo* studies (testes\(^3^-^4\), adrenals\(^5\)). The present report deals with radiation induced formation of giant hepatocytes in mice.

Swiss albino mice were exposed to 225, 450 and 900 R of whole-body gamma irradiation from a \(^{60}\)Co source. The animals were sacrificed on 1, 2, 3, 7 and 14 days post-irradiation. Pieces of liver were fixed in Bouin's fluid. After routine procedure the sections were cut and stained with Harris haematoxyline and eosin and observed under a light microscope.

The giant hepatocytes (cells) were observed in all the three dose groups and two types could be distinguished, (a) multinucleated giant hepatocytes, containing three or more nuclei in a larger cytoplasmic mass arranged in the form of a string or in a spherical fashion (Figs. 1 and 2) and (b) mononucleated giant hepatocytes, containing single large nucleus (Fig. 3). The multinucleated giant cells were observed on 1, 2 and 3 days post-irradiation, whereas the mononucleated cells at later intervals (7 and 14 days).

Montgomery\(^1\) stated that, (i) multinucleated giant cell formation is the result of fusion of two cells, and (ii) mononucleated giant cell is formed due to the dissociation of cell division from the cell growth. In the present case the former type of cells might have been formed by the fusion of mononucleated and/or binucleated hepatocytes, as different stages of cell fusion have been observed. It has been reported that fatty degeneration of cell membrane in the testes results in the formation of giant cells\(^4\). In addition to the method of mononucleated giant cell formation as reported earlier\(^1\), these cells in the present study may also be formed by the fusion of nuclei in the binucleated cells (Fig. 4). Giant cell formation is an irreversible.

*Fig. 1.* Liver 2 days after exposure to 900 R of gamma rays showing multinucleated giant hepatocyte (arrow) × 400.

*Fig. 2.* Liver 3 days after exposure to 225 R of gamma rays showing a multinucleated giant hepatocyte (arrow) × 400.