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Selective Lysis of Virus-Infected Cells by Cobra Snake Cytotoxins: A Sendai Virus, Human Erythrocytes, and Cytotoxin Model

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By using a Sendai virus–human erythrocyte model, this work found that virus-infected cells were 10-fold more susceptible to lysis in two of five examined cobra venoms. Four cytotoxins were isolated from the venom of the cobra *Naja nigricollis* that also showed 10-fold higher cytotoxicity toward virus-infected cells than to untreated cells. As selective destruction of virus-infected cells is of immense importance in clinical practice, this work demonstrates the potential of cobra cytotoxins to serve as leading compounds for the generation of derivatives or fractions with high cytotoxic specificity toward virus-infected cells. © 1999 Academic Press

In the past three decades numerous agents have been proposed for the treatment of viral diseases. However, current antiviral agents are of demonstrable benefit only in a limited number of viral infections, and all have significant potential for toxicity and for drug resistance development. Furthermore, as in the case of HIV infected patients, the antiviral drugs do not eliminate infected cells, but rather inhibit viremia by interacting with viral key enzymes. It is, therefore, of immense interest to search for new antiviral agents that may have a different mode and site of action.

Sendai virus is an enveloped animal virus, which belongs to the parainfluenza family. The steps of attachment and fusion of the virions with the cell are intermediated by the viral hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins (1). Incubation of the virions with human erythrocytes at 4°C enables viral adsorption to the cell membrane, but no fusion occurs. Fusion occurs only after raising the temperatures above 20°C (2, 3), a step that causes hemolysis of the erythrocytes.

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Snake venoms are complex mixtures of toxins and enzymes that show different activities on biological systems, such as cytotoxicity, hemorrhage, bradykinin-releasing activity, thrombin-like activity, hemolysis, cardiovascular and hypotensive effects, tissue necrosis, and neurotoxic effects (for review see Ref. 4). We have previously shown that some snake venoms could inactivate Sendai virus (5), and isolated the active 25-kDa protease from the venom of the viper *Echis coloratus,* termed Echinibin-1 (6). This factor abolished the hemagglutinating and hemolytic activities of Sendai virus by preventing the adsorption of the virions to the cells, and the virus *in vivo* infectivity of mice (6).

Cytotoxins are among the most prominent characteristics of cobra venoms. They are basic polypeptides with a chain length of 60–70 amino acids and a common folding pattern. They resemble the short snake neurotoxins in their structure, but they are more hydrophobic, and differ from the neurotoxins in their pharmacological properties by exhibiting general cytolysis (7). Although it is clear that their primary target is the cell membrane, their exact mode of action still remains to be elucidated. In previous studies, this lab screened the cytotoxicity of various snake venoms and found the strongest activity in the venom of *Naja nigricollis nigricollis* (8) from which four cytotoxins were isolated and characterized; the main cytotoxic agent was cytotoxin P4, a 10-kDa protein (9). One of the most interesting features of these cytotoxins was their preferential activity on cancer cells (9) by specifically binding to the cell membrane (10).

Elimination of free virus and virus-infected cells is an important goal in antiviral treatment. The present study is a first attempt in evaluating the potential of snake cytotoxins as antiviral agents. By using the model of the interaction of Sendai virus with human erythrocytes, this work demonstrates the potential use of cobra snake cytotoxins for preferential destruction of virus-infected cells.

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TABLE 1

Proteolytic and Antiviral Activities of Various Elapid (Cobra) Snake Venoms

Snake venom	Proteolytic activity		Percent of inactivation of Sendai virus hemolytic activity[c]
	Azocoll[a]	BAEEase[b]	
Naja atra	0.63	0.6	90
Naja nigricollis	0.0	0.0	78
Ophiophagus hannah	0.04	0.0	0
Naja melanoleuca	0.07	0.0	0
Walterinnesia aegyptia	0.08	14.4	0

Note. Proteolytic activities of the venoms and hemolytic activity of the virus were examined as described under Materials and Methods.
[a] AZOCOLase activity is expressed as OD (540 nm)/h/10 μg venom/ml.
[b] BAEEase activity is expressed as OD (540 nm)/h/10 μg venom/ml.
[c] 70 HAU of Sendai virus was pretreated with 10 μg of the various venoms in 50 μl. The percent of inactivation was determined by comparing the hemolysis caused by the treated and nontreated virus. As control erythrocytes were incubated with the various venoms only.

MATERIALS AND METHODS

Venoms

Naja nigricollis and *Naja atra* venoms were obtained from Latoxan Laboratories (Rosans, France). All other venoms were purchased from Sigma Chemical Co. (St. Louis, MO).

Virus

Sendai virus (Z strain) was injected into the allantoic sac of 10- to 11-day-old chicken embryo, harvested after 48 h from the allantoic fluid, and isolated by established procedures (11). The virus was resuspended in 160 mM NaCl/20 mM Tricine, pH 7.4, and stored at −70°C. Virus hemagglutinating activity was measured in hemagglutinating units (HAU) as described by Peretz *et al.* (11). One milliliter of the isolated virions contained about 60,000 HAU.

Blood

Fresh, human blood was obtained from a blood bank and stored for up to one month at 4°C. Prior to use, erythrocytes were washed with phosphate buffer saline, pH 7.2 (PBS), and were diluted to 2% (v/v) with the same buffer (10^8 cells per milliliter).

Estimation and Concentration of Proteins

Protein content was estimated by measuring the absorbance at 280 nm in a Gilford UV spectrophotometer, using a value of $A_{280}^{1\%} = 14$.

Interaction of Sendai Virions with Human Erythrocytes (Hemolysis Assay)

Human erythrocytes were washed and diluted to 2% (v/v) with cold PBS. One milliliter of washed erythrocytes (10^8 cells) was incubated with 70 HAU of Sendai virions for 15 min at 4°C and then washed twice with cold PBS. The pellets were resuspended in 1 ml PBS and incubated for another 60 min at 37°C in a shaking bath (90 strokes/min), followed by centrifugation at 1000*g*, and the hemolysis was determined by measuring the absorbency of the supernatant at 540 nm. The hemolysis obtained by resuspending the pelleted erythrocytes in 1 ml of distilled water was considered 100% hemolysis.

Determination of Antiviral Activity

The effect of the venoms and their various fractions on the viral activity was tested in three series of experiments. All experiments were done in duplicates, and at least in two separate sets.

(a) Virus inactivation experiments. 70 HAU of Sendai virions were incubated with various concentrations of the venoms or their fractions in 50 μl PBS for 1 h at 37°C. Then 10^8 washed human blood erythrocytes were added and a hemolysis assay was carried out as described above. As controls, erythrocytes were incubated with the venoms only or with 70 HAU of Sendai virions in PBS.

(b) Pretreatment of the erythrocytes with venom. Duplicate samples of 10^8 washed human blood erythrocytes were incubated with various concentrations of the examined venoms or the venom fractions in 1 ml PBS for 1 h at 37°C. The cells were then washed twice with PBS before 70 HAU Sendai virions were added to each erythrocyte sample and the hemolysis was tested as described above. As controls, the erythrocytes were incubated with PBS without venom prior to the addition of the virus.

(c) Infection of the erythrocytes with Sendai virus. Duplicate samples of 10^8 washed human blood erythrocytes were incubated with 70 HAU of Sendai virions for 15 min at 4°C. Excess virus was removed by washing twice with cold PBS and the pellets were resuspended in 1 ml of cold PBS. Various concentrations of the examined venoms or venom fractions were then added and the mixtures were incubated for another 60 min at 37°C in a shaking bath and centrifuged. The hemolysis was determined by measuring the absorbency of the supernatant at 540 nm.

Cytotoxicity

The cytotoxicity was assayed as described by Chaim-Matyas and Ovadia (8).

Phospholipase Activity

Phospholipase (PLA) activity was assayed with egg yolk by using the pH-stat method as described by Simon *et al.* (12).

Proteolytic Activity

Proteolytic activity was tested on three different substrates: azocoll, benzoyl arginine ethyl ester (BAEE), and gelatin. Aliquots containing various amounts of the whole venom or the venom fractions were incubated separately with 10 mg of azocoll (insoluble dye–protein complex), in 2 ml of 0.1 M phosphate buffer, pH 7.2, for 1–3

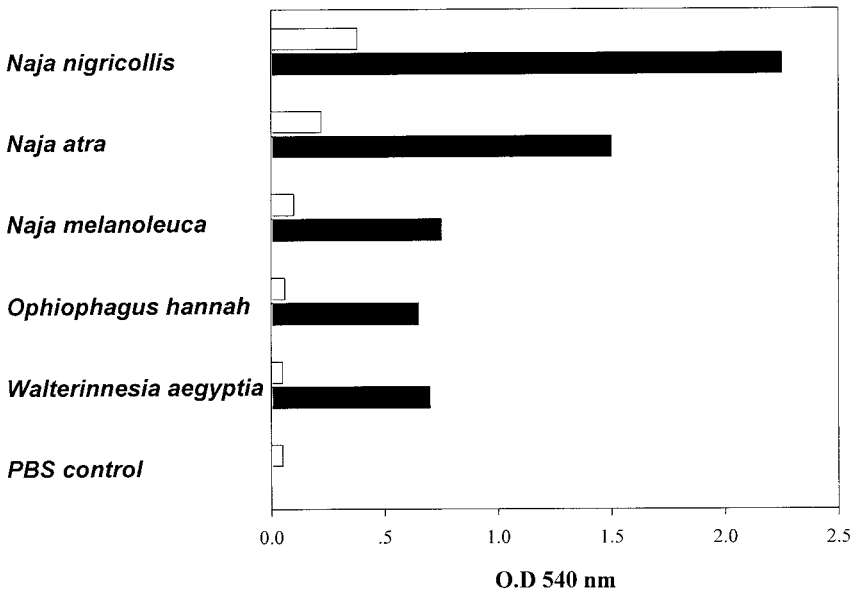

FIG. 1. Human erythrocytes (10^8) were preinfected with 70 HAU Sendai virions as described under Materials and Methods under Pretreatment of the erythrocytes with Sendai virus. After washing the excess virus, the cells were resuspended in 1 ml cold PBS and then 4 μg of each examined venom were added and an hemolysis assay was carried out. As control noninfected erythrocytes were incubated with 4 μg/ml of the examined venoms and with PBS. 100% hemolysis was achieved by addition of water to a similar sample of erythrocytes resulted in an OD (540 nm) of 2.25.

h at 37°C in a shaking bath (120 strokes per minute). Proteolytic activity was estimated by measuring the absorbency of the clear supernatant at 520 nm after centrifugation of the mixtures. BAEE-ase activity was examined by adding 5- to 25-μl aliquots of the whole venom or the venom fractions to 1 ml of 0.1 M glycine buffer, pH 9.5, containing 0.5 mM of BAEE, followed by immediate continued measurement of the absorbency at 254 nm. Proteolytic activity on gelatin films was examined as follows: a drop containing 0.2 mg of the venoms or the purified cytotoxins was applied to an undeveloped black and white film and incubated for 2 h at 37°C in a moist chamber. The film was then washed thoroughly with tap water. A transparent spot on an opaque background indicated gelatin digestion.

RESULTS

The effect of five cobra snake venoms on the capacity of Sendai virus to cause hemolysis of human erythrocytes was examined in three different settings as described under Materials and Methods: (a) Sendai virions pretreated with one of the venoms, were added to the washed erythrocytes; (b) untreated virions were added to erythrocytes which were pre-incubated with the venoms; and (c) examined venoms were added to erythrocytes pre-infected with Sendai virions.

(a) Inhibition of Sendai Virus by Cobra Venoms

Preincubation of Sendai virus with 200 μg/ml of *Naja atra* or *Naja nigricollis* venoms reduced the hemolytic activity of the virus on erythrocytes by 90 or 80%, respectively. The other three examined venoms did not affect the viral hemolytic activity (Table 1). There was no correlation between the proteolytic activity of the venoms and their ability to inhibit the viral activity (Table 1).

(b) Pretreatment of the Erythrocytes with Cobra Venoms

Incubation of 10^8 washed human blood erythrocytes with 20 μg/ml of the examined venoms or the venom fractions for 1 h at 37°C before the addition of the virions did not alter the susceptibility of the cells to hemolysis by the virus.

(c) Hemolysis of Preinfected Erythrocytes by Cobra Venoms

Erythrocytes which were pre-infected at 4°C with Sendai virions (at 4°C virions absorb to the erythrocytes membrane but fusion and, consequently, hemolysis does not occur), were 10-fold more susceptible to hemolysis by the venoms of *Naja atra* and *Naja nigricollis*. Four μg/ml of the *Naja nigricollis* venom were sufficient to cause total hemolysis of the pre-infected cells, while 45 μg/ml were needed to induce the same effect on untreated erythrocytes, indicating preferential cytotoxicity toward the infected red blood cells. The venom of *Naja atra* showed a similar but somewhat weaker effect. All other examined venoms did not exhibit this synergistic effect (Fig. 1).

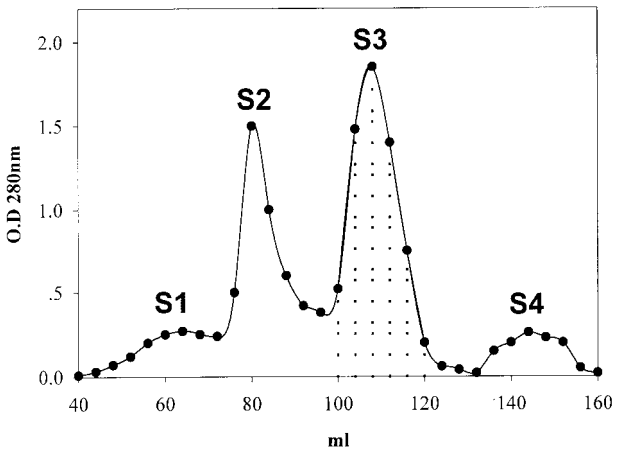

FIG. 2. Gel filtration of *Naja nigricollis* venom on Sephadex G-50. One hundred milligrams of venom dissolved in 0.01 M phosphate buffer, pH 7.4, was reduced with 2-mercaptoethanol, centrifuged and the supernatant was applied to a 1.4 × 120-cm column of Sephadex G-50. The column was eluted with the same buffer by a flow rate of 4 ml/h/cm^2. The antiviral and the cytotoxic activities were found in the S3 fraction (100–118 ml).

Purification of the Antiviral Components

The active antiviral components were purified from the venom of *Naja nigricollis*, because this venom showed the strongest preferential cytotoxicity toward infected erythrocytes. The purification included three steps as previously described (9): (i) Reduction of the venom by 2-mercaptoethanol at 30°C for 1 h followed by centrifugation at 10,000*g* for 30 min. (ii) The supernatant was subjected to gel filtration on Sephadex G-50 (Fig. 2). Of the four fractions eluted from the column, only S3 inactivated the virions and also exhibited the synergistic hemolysis of the cells pre-infected with the virions (Table 2). S3 also possesses the cytotoxic activity, whereas phospholipase activity is found in S2 (9), a fraction that does not inactivate the virus. As written in the Introduction, Sendai virus causes hemolysis only at temperatures above 20°C, at low temperatures the virus attaches the cell but fusion does not occur. At 4°C the S3 fraction did not cause hemolysis of the infected cells, whereas at 37°C the hemolysis of the infected cells considerably increased as the concentration of the fraction increased (Fig. 3). Pool S3, eluted from the column of G-50, was subjected to CM-Sepharose column chromatography and the fractions were eluted by salt gradient. Six peaks were eluted, including the cytotoxic factors P3 to P6 (Fig. 4). Fifty-five percent of the cytotoxic activity was found in factor P4, as previously reported (9). Only the cytotoxic factors P3-P6 neutralized the viral activity and induced strong hemolysis of erythrocytes pre-infected with Sendai virions (Table 2 and Figs. 5 and 6). These cytotoxins were stable at pH values of 1.5–10.5 and at temperatures up to 90°C, and maintained their antiviral properties. These activities could be inhibited by divalent ions such as Sr^{2+} and Mg^{2+} (data no shown), as was also previously found for the cytotoxicity of these factors (9). The cytotoxic fractions P3–P6 had no measurable proteolytic activity on azocoll, BAEE or gelatin, or any phospholipase A activity. The selective activity of the purified cytotoxins on infected erythrocytes is even more prominent when compared with other membrane-active component, such as phospholipase A, also isolated from the venom of *Naja nigricollis*, which did not demonstrate any antiviral effect (Table 2).

DISCUSSION

Although the immune system is capable of recognizing and destroying virus-infected cells through natural killer cells (13, 14) and by means of lymphocyte-produced cytokines (15, 16), in many viral infections the immune response can not eliminate all virus-infected cells. Such is the case of the human immunodeficiency virus (HIV) infection (17). Discontinuing the use of anti HIV-1 reverse transcriptase and protease cocktail of inhibitors, even after no detection of HIV

TABLE 2

Inactivation of Sendai Virus and Susceptibility of Erythrocytes Preinfected with Sendai Virions to *Naja nigricollis* Venom Fractions[a]

Purification step	Fraction	% Inactivation of the virus[b]	% Hemolysis of preinfected cells[c]
PBS[d]	—	0	33
PLA[e]	—	5	30
Whole venom	—	65	100
Sephadex G-50	S1	0	32
	S2	0	44
	S3	95	100
	S4	0	53
CM-Sepharose of S3	P1	0	30
	P2	0	33
	P3	95	55
	P4	96	100
	P5	92	93
	P6	90	100

Note. Samples of erythrocytes incubated with the venom or with the venom fractions only (without the virions) resulted in negligible hemolysis.

[a] The fractions examined are shown in Figs. 2 and 4. In all cases 6-μg whole venom or isolated fractions were added to the virions or to the virus-pre-infected cells.

[b] The inactivation of Sendai virions was examined as described under Materials and Methods under "Virus inactivation experiments." The percent inactivation was calculated as the hemolysis caused by virions pretreated with venom, divided by the hemolysis caused by virions treated by PBS and multiplied by 100.

[c] The percent hemolysis was determined as described under Materials and Methods under "Pretreatment of the erythrocytes with Sendai virus."

[d] Phosphate-buffered saline (PBS).

[e] Isolated phospholipase A2 from the venom of *Naja nigricollis.*

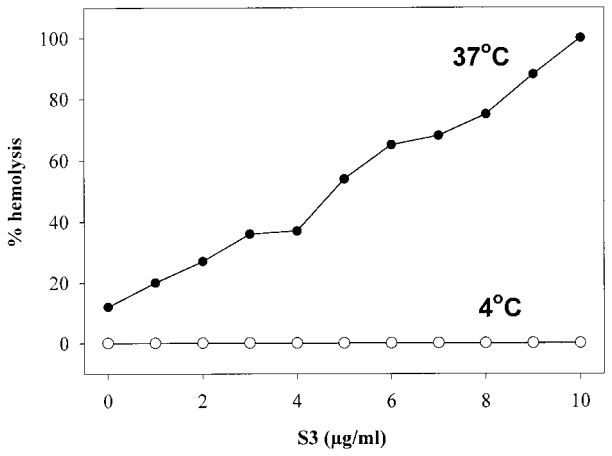

FIG. 3. Erythrocytes were preinfected with Sendai virions at 4°C as described under Materials and Methods. Aliquots of 10^8 cells were resuspended in 1-ml cold PBS containing 0.7 μg of the S3 fraction. Half of the samples were then incubated at 4°C for 30 min, while the other half were incubated at 37°C for 30 min. The mixtures were then centrifuged and the absorbency of the supernatant was measured at 540 nm. The same concentrations of the S3 fraction did not cause hemolysis of noninfected erythrocytes under the same conditions.

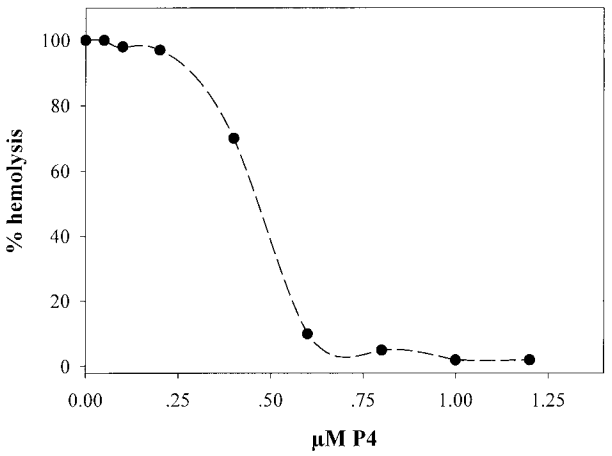

FIG. 5. Sendai virions (70 HAU) were incubated for 1 h with P4 before the hemolysis assay was carried out as described under Materials and Methods. As control, the erythrocytes were incubated with P4 only.

viremia for more than a year, results again in high viral load. This indicates the presence of infected cells capable of producing HIV virus that are not eliminated by the anti-retroviral drugs and the recuperating immune system. Destroying such infected cells should be therefore a major priority in the development of new antiviral strategies.

Recently recombinant vesicular stomatitis virus and rabies virus were sophisticatedly engineered to specifically bind and destroy only human infected HIV-1 cells, that display HIV-1 glycoproteins on their membranes (18, 19). Similarly, the use of exogenous cytotoxins, capable of selectively destroying virus-infected cells, may be used for the same purpose. As elapid (cobra) venoms are rich with very potent cytotoxic components, which disrupt cell membranes, it was interesting to find out whether: (a) cobra venoms can also interact with enveloped viruses and inhibit their activity, especially because the virus envelope contain components of the host cell membrane; and (b) the interaction of cells with an enveloped virus could increase the susceptibility of the cells to cobra venom cytotoxins. This work has therefore examined the antiviral activ-

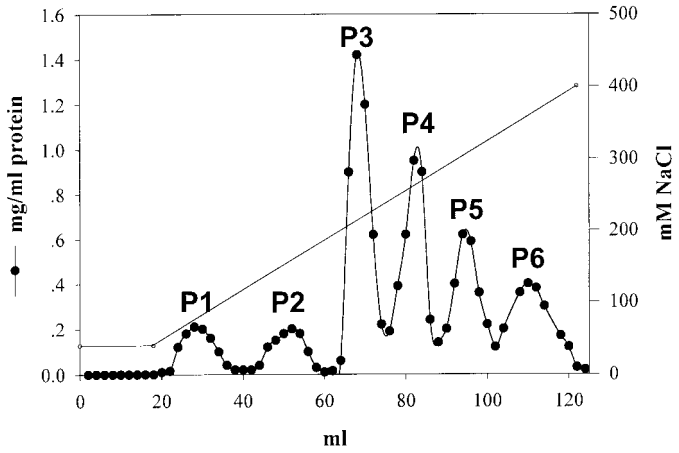

FIG. 4. Ion-exchange chromatography on CM-Sepharose. The antiviral fraction (S3) from the previous gel filtration stage was applied to 10-ml column of CM-Sepharose equilibrated with 0.01 M phosphate buffer, pH 7.2. After washing with the same buffer the column was eluted by a salt gradient as indicated. P3–P6 had antiviral activity (Table 2). These fractions also proved to be purified cytotoxins (9).

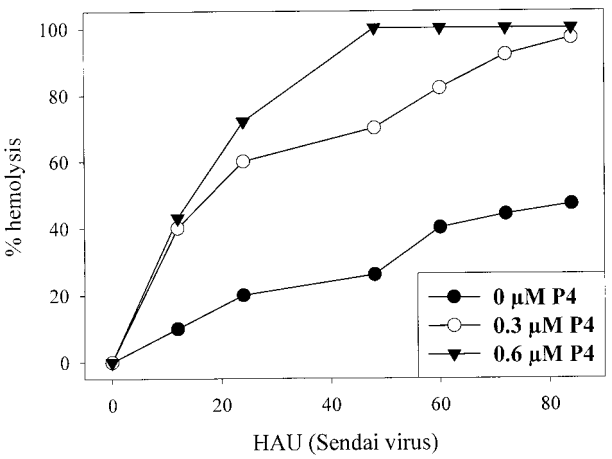

FIG. 6. Erythrocytes were infected with Sendai virions at 4°C for 15 min. Then, the cells were washed twice and resuspended in 1 ml PBS containing various concentrations of P4. The mixtures were incubated at 37°C for 30 min and the hemolysis was determined as described under Materials and Methods.

ity of five cobra venoms, which are rich with cytotoxins, by using the model of Sendai virus-human erythrocytes interaction.

Unlike the viperid Echinibin-1 (6) and Echinibin-2 (manuscript in preparation), which are not cytotoxic and their antiviral activity correlates with proteolytic activity, there was no correlation between the proteolytic activity of the examined cobra venoms and their capability to inhibit the hemolytic activity of Sendai virus on human erythrocytes. Venoms with considerable proteolytic activity, such as that of *Walterinnezia aegyptia*, did not exhibit antiviral activity, whereas the venom of *Naja nigricollis* showed considerable antiviral activity despite its non-measurable proteolytic activity. Accordingly, all four cytotoxins isolated from the venom of the snake *Naja nigricollis*, had potent antiviral activity, without any measurable proteolytic activity on azocoll, BAEE or gelatin, nor they demonstrated any phospholipase A activity on egg yolk. This is the first example of nonproteolytic snake venom component with an antiviral activity.

All four isolated cytotoxins from the venom of *Naja nigricollis* inhibited the free virus. More important, they demonstrated selective hemolysis of erythrocytes pre-infected with Sendai virions. Better understanding of how the viral glycoproteins in the cell membrane enhance the susceptibility of the cells to the snake cytotoxins, may shed light on their mode of action and may help in the development of new antiviral agents and strategies. This may also help to develop more specific compounds, such as derivatives or fragments of the snake cytotoxins, which will loose their non-specific cytotoxicity, but will be able to destroy virus-infected cells. Various derivatives and fragments of cytotoxin P4 are being examined. Future work should also study the effect(s) of the snake cytotoxins on other viruses with clinical significance and on other cell types infected by viruses.

REFERENCES

1. Kingsbury, D. W. (1990) *in* Paramyxoviridae and Their Replication (Fields, B. N., and Knipe, D. M., Eds.), pp. 945–962, Raven Press, New York.
2. Areoti, B., and Henis, Y. I. (1988) *Biochemistry* **27,** 5654–5661.
3. Lyles, D. S., and Landsberger, F. R. (1979) *Biochemistry* **18,** 5088–5095.
4. Lee, C. Y. (1977) *Snake Venoms*, Springer-Verlag, Berlin.
5. Borkow, G., and Ovadia, M. (1992) *Life Sci.* **51,** 1261–1267.
6. Borkow, G., and Ovadia, M. (1994) *Antiviral Res.* **23,** 161–176.
7. Dufton, M. J., and Hider, R. C. (1988) *Pharmacol. Ther.* **36,** 1–40.
8. Chaim-Matyas, A., and Ovadia, M. (1987) *Life Sci.* **40,** 1601–1607.
9. Chaim-Matyas, A., Borkow, G., and Ovadia, M. (1991) *Biochem. Int.* **24,** 415–421.
10. Borkow, G., Chaim-Matyas, A., and Ovadia, M. (1992) *FEMS Microb. Immunol.* **105,** 139–146.
11. Peretz, H., Tolster, Z., Laster, Y., and Loyter, A. (1974) *J. Cell Biol.* **63,** 1–11.
12. Simon, T., Bdolah, A., and Kochva, E. (1980) *Toxicon* **18,** 249–259.
13. Herberman, R. B., and Ortaldo, J. R. (1981) *Science* **214,** 24–30.
14. Malnati, M. S., Lusso, P., Ciccone, E., Moretta, A., Morreta, L., and Long, E. O. (1993) *J. Exp. Med.* **178,** 961–969.
15. Aderka, D., Hahn, T., and Wallach, D. (1985) *Antiviral Res.* **S1,** 141–148.
16. Wallach, D. (1986) *in* Interferon 7 (Gresser, I., Ed.), pp. 89–123, Academic Press, London.
17. Coffin, J. M. (1995) *Science* **267,** 483–489.
18. Mebastion, T., Finke, S., Weiland, F., and Conzelmann, K. K. (1997) *Cell* **90,** 847–847.
19. Schnell, M. J., Johnson, J. K., Buonocore, L., and Rose, J. K. (1997) *Cell* **90,** 849–857.