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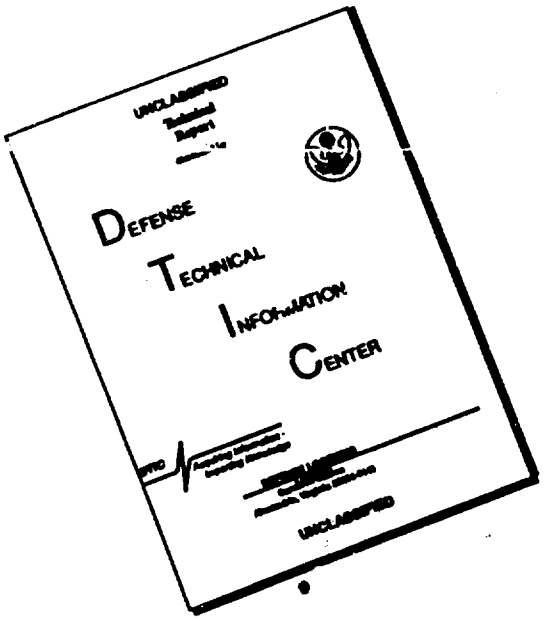

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IDENTIFICATION OF CARDIOTOXIN WITH COBRAMINE B, DLF, TOXIN δ AND COBRA VENOM CYTOTOXIN (U)

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Cardiotoxin, Cobramine B, the direct hemolytic factor (DLF) and cobra venom cytotoxin are all the most basic polypeptides isolated from cobra venom. Cardiotoxin affects various kinds of cells, causing irreversible depolarization of cell membrane. It causes contracture of skeletal muscle, systolic arrest of the heart, contraction of smooth muscle, block of axonal conduction, local irritation, etc. Cardiotoxin has a weak direct hemolytic activity on washed erythrocytes of guinea pig, dog and cat, and shows cytopathic effects on stable tumor cell cultures(HeLa, KB). Both the contracture-inducing and direct hemolytic activities of cardiotoxin can be potentiated by phospholipase A, and prevented by polyanions (gangliosides, RNA and heparin), just as the inhibition of iodide accumulation in thyroid slices by cobramine B. Moreover, the amino acid composition and molecular weight of cardiotoxin are almost identical with those of cobramine B. From these results, it is concluded that cobramine B, DLF, and possibly cobra venom cytotoxin are all identical with cardiotoxin. However, since the amino acid composition and the molecular weight of DLF are somewhat different from those of cardiotoxin, it is possible that DLF from different venoms may vary slightly in its amino acid composition. (Author)

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	ROLE	WT	ROLE	WT	ROLE	WT
Cardiotoxin						
Cobra						
Venon						
Amino acid						
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Polyanions						
Guinea-pig						
Dogs						
Cats						
Rabbits						
Taiwan						

Paper III-7

IDENTIFICATION OF CARDIOTOXIN WITH COBRAMINE B, DLF, TOXIN *γ* AND COBRA VENOM CYTOTOXIN*

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CARDIOTOXIN (Sarkar, 1947; Lee *et al.*, 1968), cobramine B (Larsen and Wolff, 1968a), the direct hemolytic factor (DLF) (Aloof-Hirsch *et al.*, 1968), toxin γ (Izard *et al.*, 1969a) and cobra venom cytoxin (Braganca *et al.*, 1967) are all the most basic polypeptides isolated from cobra venom of the same or different species. The possibility was first suggested by Meldrum (1965) that DLF may be identical with cardiotoxin. Slotta and Vick (1969) have found that the most basic polypeptide isolated from *Naja naja* venom by chromatography on CM-Sephadex column comprises the total, rather low, direct lytic activity and also the total, very strong, cardiotoxic activity of cobra venom. They suggested, therefore, that this polypeptide should be named 'cardiotoxin' rather than DLF. Similarly, cobramine B isolated from the same venom has been shown to possess a weak hemolytic as well as a marked cardiotoxic activity (Larsen and Wolff, 1967, 1968b).

On the other hand, toxin γ isolated from the venom of *Naja nigricollis* (Izard *et al.*, 1969a) has been claimed to be devoid of any lytic effect on human erythrocytes even in the presence of phospholipase A, despite its marked cardiotoxic effect (Izard *et al.*, 1969b). Similarly, the cytotoxic protein, selectively destructive to Yoshida sarcoma cells, isolated from *Naja*

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naja venom has also been found to be devoid of direct lytic effect on human and rat erythrocytes (Braganca *et al.*, 1967). Thus, it remains to be clarified whether these basic polypeptides separated from cobra venom are all identical, or at least some of them are different substances.

Qualitative and quantitative pharmacological comparison as well as chemical analyses of these basic polypeptides isolated by different workers could have answered this question unequivocally. However, since we have not succeeded to obtain samples of these toxins, the results obtained with cardiotoxin isolated from *Naja naja atra* venom will be compared with those described in the literature.

DIRECT LYTIC EFFECT OF CARDIOTOXIN

A spectrum of sensitivity of the erythrocytes of various animal species to the direct lytic action of Formosan cobra venom and its fractions is shown in Table 1. The erythrocytes of guinea-pig and dog are most sensitive, while those of cat, human and rabbit are much less sensitive. The erythrocytes of goat, rat, mouse and chicken are resistant to the hemolytic action of the venom. These findings are, in general, in good accordance with those reported by Condrea *et al.* (1964) on the venom of Ringhals (*Hemachatus haemachatus*). Among 12 fractions separated from Formosan cobra venom by CM-Sephadex column chromatography (Lee *et al.*, 1968), only the three cardiotoxic fractions (X–XII) display direct hemolytic action on the erythrocytes of the sensitive species.

As shown in Figure 1, the direct lytic action of cardiotoxin (Fr. XII) is potentiated by phospholipase A, confirming the findings of various authors (Condrea *et al.*, 1964; Willie and Vogt, 1965; Chang and Lee, 1966; Slotta and Vick, 1969) that DLF acts synergistically with phospholipase A.

Wolff *et al.* (1968) have demonstrated that inhibition of iodide accumulation in thyroid slices by cobramine B is prevented by polyanions (heparin, RNA, gangliosides, and suramine). As shown in Table 2, the direct lytic action of cardiotoxin is also inhibited by heparin, RNA and gangliosides. The polyanions presumably form soluble complexes with cardiotoxin, except in occasional experiments when a faint precipitate is formed. The formation of a precipitate by the DLF protein and heparin or dextran sulfate added in suitable proportions has been observed previously (Condrea *et al.*, 1964).

TABLE 1 Direct lytic activity of *Naja naja atra* venom and its cardiotoxic fractions on erythrocytes from various animal species

Species	% Hemolysis				
	Whole venom	Fraction No.			
		I–IX	X	XI	XII
Guinea-pig	86.0	0	44.4	46.0	47.6
Dog	51.6	0	28.4	31.8	41.2
Cat	12.0	0	6.2	7.1	9.0
Human	4.2	0	7.2	4.8	13.0
Rabbit	1.2	0	0.9	0.8	3.7
Rat	0	0	0	0	0
Mouse	0	0	0	0	0
Goat	0	0	0	0	0
Chick	0	0	0	0	0

Experimental conditions:

Final concentration of red cell suspension: 1%
Final concentration of test substances: 200 γ/ml
2 hr. incubation in phosphate-buffered saline (pH 7.4) at 37 °C.

INHIBITION OF CONTRACTURE-INDUCING ACTION OF CARDIOTOXIN BY POLYANIONS

Cardiotoxin produces a marked contracture of the chick biventer cervicis muscle (Lee *et al.*, 1968). This action of cardiotoxin is also prevented by pretreatment with heparin, RNA or gangliosides (Figure 2).

CYTOPATHIC EFFECT OF CARDIOTOXIN

It has been reported that cobra venom exerts cytopathic effects on animal cells in culture (Levaditi and Mutermilch, 1913; Ishii, 1929). As shown in

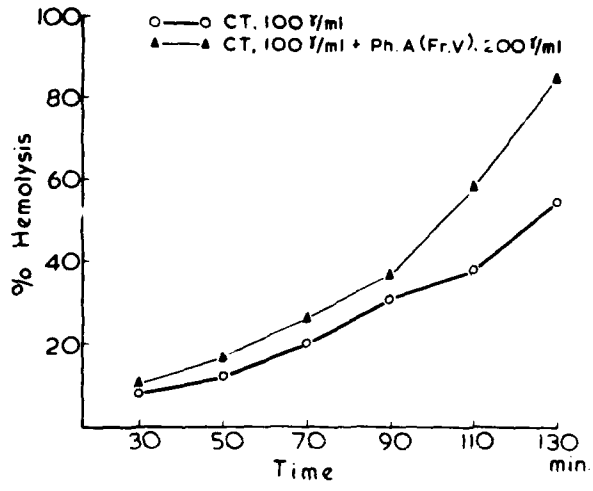

FIGURE 1. Potentiation of hemolytic action of cardiotoxin by phospholipase A (Ph A)'

TABLE 2 Effect of polyanions on direct lytic activity of cardiotoxin on guinea-pig erythrocytes

Polyanions	Concentration mg/ml	Hemolysis % ± S.E.
Control*		32.0 ± 3.7
Heparin	0.2	7.0 ± 1.5
	0.8	4.8 ± 0.3
	6.0	2.5 ± 1.0
RNA	0.25	21.1 ± 0.5
	0.5	17.2 ± 1.5
	2.5	2.5 ± 1.0
Gangliosides	0.2	17.8 ± 0.5
	0.5	2.0 ± 1.5

*Final concentration of cardiotoxin 100 γ/ml

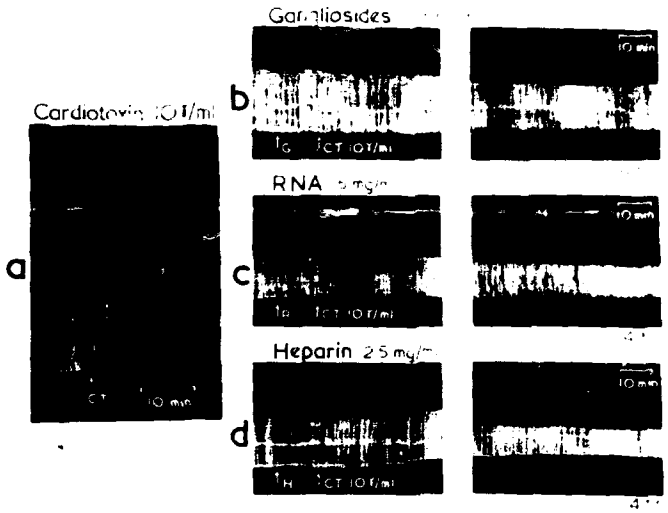

FIGURE 2. The chick's biventer cervicis muscle. Supramaximal indirect stimulation of 0.5 msec duration was applied at a rate of six per minute. (a) Contracture induced by cardiotoxin (CT) 10γ/ml; (b) Prevention of contracture by pre-treatment with gangliosides 0.15 mg/ml; (c) with RNA 5 mg/ml, and (d) with heparin 2.5 mg/ml

Table 3 and Figure 3, cytopathic effects on stable tumor cell cultures (HeLa and KB) are found in three cardiotoxic fractions (Frs. X XII) but not in the neurotoxin (Fr. VIII) or phospholipase A fraction (Fr. V). Phospholipase C from *Cl. welchii* is also devoid of any cytopathic effect in a concentration as high as 500 γ /ml. As shown in Table 4, the cytopathic effect of cardiotoxin on HeLa and KB cells is markedly inhibited by heparin. RNA is also effective while gangliosides are without effect. At present, no explanation can be given for such differences. It has not been shown whether or not the destructive action of cytotoxin on Yoshida sarcoma cells can be prevented by these polyanions.

TABLE 3 Cytopathic effect of whole and fractionated cobra venom

Venom fraction	Final Concen. (γ/ml)	HeLa Cells		KB Cells	
		24 hr.	48 hr.	24 hr.	48 hr.
Whole Venom	50	++	+++	++	++~+++
	30	++	+++	+	+
	20	+	++	±	+
	10	±	+~++	-	-
	5	-	±	-	-
Fr. XII (Cardiotoxin)	50	++~+++	+++	++	++~+++
	30	++	++	+	+
	20	+	++	-	
	10	±	+	-	
	5	-	±	-	
Fr. XI	50	++	+++		
	30	+	++		
	10	±	+		
	5	-	±		
Fr. X	100	+++	+++	+	+~++
	50	+	+	-	-
	30	±	±	-	
	10	-	-	-	
Fr. VIII (Neurotoxin)	200		-	-	
Fr. V (Phospholipase A)	500	-	-	-	±
Phospholipase C from *Cl. welchii*	500		-		

( ): No change; (±): Slight morphological changes;
(+): Rounding of cells, increased cytoplasmic granules;
(++): Cell destruction with partial disruption of cell monolayer;
(+++): Complete disruption of cell monolayer.

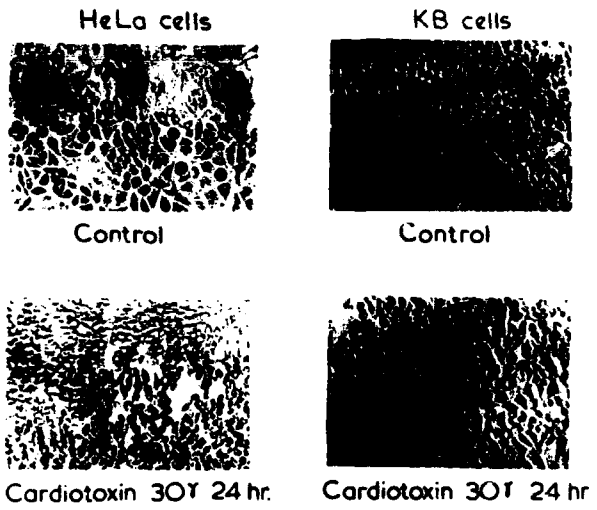

FIGURE 3. Cytopathic effect of cardiotoxin on monolayers of HeLa and KB cells

AMINO ACID COMPOSITION OF CARDIOTOXIN

Amino acid analysis, end-group analysis, and sequence studies revealed that cardiotoxin consists of 60 amino acids in a single chain cross-linked by four disulfide bridges with amino-terminal leucine and carboxy-terminal asparagine (Narita and Lee, 1970). Although Larsen and Wolff (1968a) reported that cobramine B consists of 52 amino acid residues, their analytical data on the amino acid composition of cobramine B are almost identical with those of cardiotoxin except for some minor differences (see Table 5). The amino acid composition of DLF from *Hemachatus haemachatus* venom, reported by two groups of investigators (Porath, 1966; Aloof-Hirsch *et al.*, 1968) independently, is also very similar to that of cardiotoxin (Table 6). All of these polypeptides are characterized by high lysine content (8–11 residues), surprisingly low arginine content (1–2 residues), and lack of tryptophan. Neither histidine nor glutamic acid could be found in either cardiotoxin or cobramine B, while DLF contains only one histidine and one glutamic acid residue. In view of the similarity in both their biological and chemical properties, they must be very closely related, if not entirely identical; the

TABLE 4 Effect of polyanions on the cytotoxicity of cardiotoxin

Polyanions	Final Conc. (γ/ml)	Cytotoxic effect by cardiotoxin (50 γ/ml) HeLa Cells (24 hr)	KB Cells (24 hr)
Control (Cardiotoxin only)		+++	++
Heparin	1000	–	
	500	–	+
	50	–	++
	30	+	++
	10	+	
	5	++	
	1	+++	
RNA	500	±	±
	400	+	+
	200	++	++
Gangliosides	4000	+++	++
	2000	+++	++

(–): No change; (±): Slight morphological changes;
(+): Rounding of cells, increased cytoplasmic granules;
(++): Cell destruction with partial disruption of cell monolayer;
(+++): Complete disruption of cell monolayer.

TABLE 5 Comparison of amino acid analyses of cardiotoxin (CT) and cobramine B (CB)

Amino acid	Residues per molecule					
	Average of 24, 48 & 72 hr. hydrolysates		RAE-toxin (24 hrs)	Nearest integral		From sequence analysis
	CT	CB	CT	CT	CB	CT
Lysine	7.89	7.9	7.73	8	8	9
Histidine	0	0	0	0	0	0
Arginine	1.93	1.9	2.01	2	2	2
Aspartic acid	6.34	5.4	5.78	6	5	6
Threonine	2.70	2.5	2.38	3	3	3
Serine	1.79	1.7	1.62	2	2	2
Glutamic acid	0	0	0	0	0	0
Proline	4.45	4.3	3.91	4–5	4	5
Glycine	1.87	2.0	1.76	2	2	2
Alanine	1.[illegible]	1.9	1.67	2	2	2
Half-cystine	6.98	6.3	6.58 (7.64)*	7–8	6	8
Valine	6.02	6.1	5.63	6	6	7
Methionine	1.67	1.8	1.65	2	2	2
Isoleucine	0.99	1.0	0.88	1	1	1
Leucine	5.28	5.1	4.86	5	5	6
Tyrosine	2.68	3.0	2.42	3	3	3
Phenylalanine	2.02	1.0	1.82	2	1	2
Tryptophan	0	0	0	0	0	0
Total				56–58	52	60

*CM Cystine (hydrolysate of RCM-toxin)

slight differences in the amino acid composition among these polypeptides appear to be due to different species or subspecies of the venom origin.

CONCLUDING REMARKS

Cardiotoxin, cobramine B and DLF are all the most basic polypeptides isolated from cobra venom of the same or different species. They not only share cardiotoxic, direct-hemolytic and many other biological activities but they are also very similar in their amino acid composition. Therefore, they should be regarded as 'isotoxins', if not entirely identical compounds.

TABLE 6 Amino acid composition of cardiotoxin, cobramine B and DLF

	Cardiotoxin (*N. naja atra*)	Cobramine B (*N. naja*)	DLF (*H. haemachatus*)	Peak 12 (DLF ?) (*H. haemachatus*)
Lysine	9	8	10	11
Histidine	0	0	1	1
Arginine	2	2	1	1
Aspartic acid	6	5	6	6
Threonine	3	3	3	3
Serine	2	2	3	3
Glutamic acid	0	0	1	1
Proline	5	4	5	5
Glycine	2	2	2	2
Alanine	2	2	1	1
Half-cystine	8	6	8	8
Valine	7	6	4	4
Methionine	2	2	2	3
Isoleucine	1	1	2	2
Leucine	6	5	6	7
Tyrosine	3	3	1	1
Phenylalanine	2	1	1	1
Tryptophan	0	0	0	0
Amide NH_3	4	3–4	7	4
Total	60	52	57	60
N-terminal	Leucine		Leucine	
C-terminal	Asparagine		Serine	
Molecular weight	6777	5840	6334	6707
Reference	Narita & Lee (1970)	Larsen & Wolff (1968)	Aloof-Hirsch *et al.* (1968)	Porath (1966)

Toxin γ and cytotoxin are also strongly basic polypeptides isolated from cobra venom. Both of them have been claimed to be devoid of direct-lytic effect on human and rat erythrocytes and, therefore, to be different from DLF. However, since both rat and human erythrocytes are also rather resistant to cardiotoxin, it remains to be shown that these two basic polypeptides are really different from DLF or cardiotoxin.

SUMMARY

Cardiotoxin, Cobramine B, the direct hemolytic factor (DLF) and cobra venom cytotoxin are all the most basic polypeptides isolated from cobra venom. Cardiotoxin affects various kinds of cells, causing irreversible depolarization of cell membrane. It causes contracture of skeletal muscle, systolic arrest of the heart, contraction of smooth muscle, block of axonal conduction, local irritation, etc. Cardiotoxin has a weak direct hemolytic activity on washed erythrocytes of guinea pig, dog and cat, and shows cytopathic effects on stable tumor cell cultures (HeLa, KB). Both the contracture-inducing and direct hemolytic activities of cardiotoxin can be potentiated by phospholipase A, and prevented by polyanions (gangliosides, RNA and heparin), just as the inhibition of iodide accumulation in thyroid slices by cobramine B. Moreover, the amino acid composition and molecular weight of cardiotoxin are almost identical with those of cobramine B. From these results, it is concluded that cobramine B, DLF, and possibly cobra venom cytotoxin are all identical with cardiotoxin. However, since the amino acid composition and the molecular weight of DLF are somewhat different from those of cardiotoxin, it is possible that DLF from different venoms may vary slightly in its amino acid composition.

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