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CYTOTOXICITY OF BEE VENOM AND SOME OF ITS CHROMATOGRAPHIC FRACTIONS FOR MOUSE BONE MARROW STEM CELLS

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ABSTRACT

Mouse bone marrow cells were incubated (37° for 30 minutes) in the presence of bee venom, and then assayed for their ability to form hemopoietic splenic colonies when transfused into lethally X-irradiated recipient mice. It was found that at honey bee venom concentrations as low as 0.5 μg/ml (0.5 part per million by weight) the colony-forming ability of the marrow cells was annulled. Three constituents of bee venom, i.e., melittin, phospholipase A, and a surface-active fraction, separated and isolated by methods of Sephadex gel filtration chromatography were also evaluated for cytotoxicity on bone marrow cells. A marked decrease in yield of splenic colonies was observed with melittin and with the surfactant fraction at concentrations of 1 μg/ml, and the phospholipase A fraction was inhibitory at 4 μg/ml. It was found also that the synthetic cationic detergent 'Cyncal' (a quaternary amine) was cytotoxic for mouse bone marrow cells at 2 parts per million, whereas the non-ionic detergent, 'Triton X-100', did not inactivate the marrow stem cells at 10 parts per million. The basis for this potent biological effect of bee venom at the cellular level is briefly discussed; it is suggested that the cellular toxicity is mediated via effects at the cell surface.

SUMMARY

The Problem:

The venom of the honey bee contains a number of substances of potential pharmacological and biological interest. For example, these constituents exhibit antibacterial properties, are highly surface-active, and have been shown to increase the resistance of mice to lethal X radiation. However the effect and mode of action of bee venom and its constituent fractions at the cellular level is not known. In the present study we have investigated the action of bee venom and its constituent fractions on mouse bone marrow cells, specifically the stem cells which have the unique capacity to give rise to homopoietic colonies in the spleens of irradiated recipient mice.

The Findings:

Mouse bone marrow cells were incubated *in vitro* (37 C for minutes) in the presence of whole bee venom, and then assayed for their ability to form hemopoietic splenic colonies when transfused into lethally X-irradiated recipient mice. It was found that at bee venom concentrations as low as 0.5 μg/ml (0.5 part per million by weight) the colony-forming ability of the marrow cells was annulled. Three constituents of bee venom, i.e., melittin, phospholipase A, and a surface-active fraction, separated and isolated by methods of Sephadex gel filtration chromatography were also evaluated for cytotoxicity on bone marrow cells. A marked decrease in yield of splenic colonies was observed with melittin and with the surfactant fraction at concentrations of 1 μg/ml, and the phospholipase A fraction was inhibitory at [illegible] μg/ml. It was found also that the synthetic cationic detergent 'Cyncal' (a quaternary amine) was cytotoxic for mouse bone marrow cells at 2 parts per million, whereas the non-ionic detergent, 'Triton X-100', did not inactivate the marrow stem cells at 10 parts per million. The basis for this potent biological effect of bee venom at the cellular level is briefly discussed; it is suggested that the cellular toxicity is mediated via effects at the cell surface.

The relationship of the present findings with respect to the physiological and pharmacological effects of parenterally administered bee venom *in vivo* is as yet unknown. How, for example, does subcutaneously injected bee venom or melittin affect the bone marrow stem

cell population systemically? What is the relationship of these cellular effects to the observed increased radioresistance of mice following the injection of bee venom? Can the injection of the highly cytotoxic constituents of bee venom prolong the survival of mice bearing transplanted lymphoma? Experiments currently in progress are aimed towards resolving some of these questions.

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INTRODUCTION

The venom of the honey bee (Apis Mellifera) comprises a complex of substances of potential biologic and pharmacologic interest. Among these constituents can be listed the enzymes, phospholipase A (1) and hyaluronidase (2), the neurotoxic polypeptide, apamine (3), the hemolytic polypeptide melittin (4) which also exhibits antibacterial properties (5), and other fractions, as yet not characterized chemically, some of which possess surfactant activity (6). At the whole animal level, we have observed an increased resistance of mice to X-radiation lethality following the subcutaneous injection of bee venom one day prior to radiation exposure (7). It is of interest that melittin has been recently shown to be partially formylated at the glycine N-terminus (8); according to these authors, "this is the first instance that a formylated amino acid has been found in a polypeptide synthesized by a multicellular organism". Formylated amino acids can act as chain initiators in protein biosynthesis (9).

In the course of studies on the mode of action of bee venom and its constituent fractions at the cellular level, we observed a marked cytotoxic effect on mouse bone marrow stem cells, i.e., hemopoietic colony-forming cells—the subject of the present report.

MATERIALS AND METHODS

Assay for bone marrow stem cells The method of assay for cytotoxic activity of bee venom on bone marrow cells is based on the spleen colony technique of McCulloch and Till (10). In brief, bone marrow is removed from the femurs of freshly sacrificed mice; the cells are then suspended in ice-cold TC-199 medium or in buffered saline, the nucleated cells counted and appropriately diluted to a concentration of 1×10^8 cells per ml. Known amounts of bee venom or of its fractions are added to stoppered glass vials containing 10 ml of the cell suspension; the vials are then incubated at 37.5C for 30 minutes, following which, known numbers of cells (usually 10^5 cells) are injected intravenously into groups of 5 to 10 heavily X-irradiated (900 R) mice of the same genetic strain. Eight to nine days later, the recipient irradiated mice are sacrificed, and their spleens extirpated and fixed in AAF (acetic-alcohol-formalin) solution. Counts of the number of discrete nodules or surface colonies per spleen are made under 10X magnification, and the results for each treatment group are expressed as the mean (and range) of colony-forming units (CFU) per 10^5 marrow cells injected. It has been established by means of chromosome marker techniques that each individual spleen colony elicited by these techniques is, in fact, a clone of hemopoietic cells—the progeny of

a single bone marrow stem cell (11) and derived therefrom by processes of proliferation, self-renewal, and differentiation. The mice used in these assays were male (C57L X A)F_1 hybrids, 10 to 14 weeks of age. The X-radiation source was a Westinghouse Therapy Unit with the radiation factors employed as follows: 250 kvp; 15 ma; HVL, 1.5 mm Cu; filter 0.5 mm Cu plus 1 mm Al; dose-rate measured in air, 28 rad per minute.

Bee venom: Venom was collected by C. Mraz in Middleburg, Vermont by the method of Benton, Morse and Stewart (12).

Fractionation of the venom was carried out by gel filtration on Sephadex chromatographic columns. A detailed description of the separation and isolation of the venom components, namely melittin, phospholipase, and a surfactant fraction, is given in a recent report by Shipman and Cole (13).

For all the bee venom fractions studied, the absorbance at 280 mμ per unit concentration was found to be equal. Therefore, in preparing solutions of the respective fractions for cytotoxic essay, the following factor was used: 0.643 X absorbance at 280 mμ = mg/ml.

Synthetic detergents - cationic detergent Cyncal 14, a quaternary amine containing C_{12}, C_{14}, and C_{18} groups (obtained from Klix Chemical Co.); anionic detergent, F-90, a biodegradable linear alkyl sulfonate containing an average of 13 carbons (Klix Chemical Co., So. San Francisco, Calif); nonionic detergent, Triton X-100, a nonylphenylpolyethylated ethanol (Rohm and Haas, Philadelphia, Pa.).

The detergents were prepared initially as 1% (by weight) solutions in saline, and then were appropriately diluted for addition to the bone marrow cell suspensions.

Rattlesnake venom: Venom of Crotalus viridis, subspecies Oregonis, was collected at the Steinhardt Aquarium, San Francisco. Since this venom was found to exhibit an absorption spectrum almost identical to that of bee venom, the same factor (above) was used to calculate venom concentration from optical density at 280 mμ.

RESULTS

In the first series of experiments, unfractionated bee venom was added to bone marrow cell suspensions containing 1 X 10^6 cells/ml; following 37 C incubation for 30 minutes, aliquots containing 10^5 nucleated cells were tested for colony-forming ability by injecting them into

X-irradiated recipient mice. The results, summarized in Table I, show that bee venom at concentrations as low as 0.5 μg/ml was cytotoxic for the colony-forming cells present in normal bone marrow. That is, under these conditions the number of CFU per 10^5 bone marrow cells injected was markedly suppressed. However, at the level of 0.1 μg/ml bee venom did not reduce the colony-forming ability of the injected cells. This quantitative aspect of the action of bee venom on bone marrow cells was observed also when the concentration of the marrow cells in the suspension was increased, i.e., 10×10^5 cells/ml instead of 1×10^5 cells/ml. Under these conditions, a bee venom concentration of 0.5 μg/ml did not significantly reduce the number of measurable CFU. The results of a single experiment with rattlesnake venom (Crotalus virides) added to mouse bone marrow cells in vitro are also given in Table I. At the snake venom concentrations employed (5 μg/ml and 16 μg/ml), significant suppression of marrow CFU was observed.

Cytotoxicity of bee venom fractions on marrow stem cells Three constituents of bee venom, separated and isolated by methods of Sephadex gel filtration chromatography (13) were evaluated for cytotoxicity on bone marrow cells. These are: melittin, the enzyme phospholipase A, and a highly surface-active fraction designated "X". A marked decrease in yield of splenic colonies was observed (Table II) at concentrations of 1 μg/ml of melittin and of the surfactant fraction "X". The phospholipase A fraction was inhibitory at 4 μg/ml but not at 1.2 μg/ml.

In the last experiment listed in Table II whole bee venom was separated into two fractions, one containing essentially a mixture of all of the surface-active constituents, the other comprising the non-surfactant substances present. The separation procedure is one newly devised by the authors (14), carried out by an air-bubbling technique. It is evident from the data that only the surfactant fraction elicited a marked decrease in the number of bone marrow colony forming cells, i.e., was highly cytotoxic for these cells.

Effect of synthetic detergents on marrow cells The fact that the surface-active constituents of bee venom, as well as phospholipase A, were found to be cytotoxic for the marrow stem cells directed our attention to the effects of chemical detergents on these cells. Such detergents, being of known chemical composition, offer the additional advantage of potential theoretical insights into their biological effects, on the basis of their charge characteristics. As shown in Table III, incubation of mouse bone marrow cells with the anionic detergent F90 at 10 μg/ml or with the cationic detergent at a concentration of 2 μg/ml, resulted in a marked suppression in the capacity of the cells to give rise to splenic colonies in the irradiated recipient mice.

TABLE I

CYTOTOXICITY OF BEE VENOM (UNFRACTIONATED) FOR

MOUSE BONE MARROW CELLS

MARROW CELL TREATMENT	CONCENTRATION OF ADDED SUBSTANCE (μg/ml)	CFU/10^5 MARROW CELLS	
		mean	range
bee venom	0.6	0	--
bee venom	1.2	0	--
none	---	20	14-27
bee venom	0.1	15	14-27
" "	0.5	< 0.2	0-1
none	---	19	10-23
bee venom	0.1	19	16-23
" "	0.5	< 0.6	0-1
none	---	18	13-22
bee venom	1.0	0	--
" "	3.0	0	--
none	---	10	8-12
crotalus venom	16	< 0.5	0-2
" "	5	2	1-4
none	---	13	9-16

TABLE II

CYTOTOXICITY OF BEE VENOM CONSTITUENT FRACTIONS

FOR MOUSE BONE MARROW CELLS

MARROW CELL TREATMENT	CONCENTRATION OF ADDED SUBSTANCE (μg/ml)	CFU/10^5 MARROW CELLS mean	range
melittin	0.5	13	7-17
"	2.5	0	---
none	---	22	18-28
melittin	2.0	0	---
"	1.0	0	---
none	---	20	14-27
phospholipase-A	1.2	17	12-23
"	4	1	0-3
none	---	20	17-22
surfactant fraction "X"	1	0	---
" " "	2	0	---
" " "	10	0	---
none	---	8	6-9
mixed surfactant fraction	1.2	0	---
" " "	7	0	---
non-surfactant fraction	2	12	8-15
" " "	6	10	5-15
none	--	10	8-13

TABLE III

EFFECT OF CHEMICAL DETERGENTS ON BONE MARROW STEM CELL ACTIVITY

TREATMENT		CFU/10^5 MARROW CELLS	
TYPE OF DETERGENT ADDED	CONCENTRATION (µg/ml)	mean	range
non-ionic (triton X-100)	2	10	8-12
" "	10	11	8-16
cationic (cyncal 14)	2	0.2	0-1
" "	10	0	---
anionic (F-90)	2	12	8-15
" "	10	0	---
none	---	12	8-15
non-ionic (triton X-100)	2	15	12-17
" "	10	17	15-20
cationic (cyncal 14)	2	0	---
" "	10	0	---
anionic (F-90)	2	18	15-22
" "	10	0	---
none	---	18	15-23

cationic detergent - 'cyncal'; anionic detergent - 'F-90';
non-ionic detergent - 'Triton X-100'.

Prevention of bee venom cytotoxicity by mouse serum In attempting to investigate the effect of bee venom on immunological reactivity of mouse blood lymphocytes, the cells were isolated and prepared as the "buffy coat" suspension, containing about 50% serum. Under these conditions the addition of bee venom at concentrations up to 10 μg/ml followed by 37 C incubation for 30 minutes did not inactivate these cells. This unexpected result suggested to us the possibility that the presence of serum in the cell suspension might interfere with the cytotoxicity of venom. Therefore, a series of experiments were carried out, in which mouse serum was deliberately added to the saline suspensions of bone marrow cells, and the cytotoxicity of bee venom assayed as usual. The data of two sets of experiments are summarized in Table IV. It is evident that the presence of normal mouse serum—whether de-complemented by heat inactivation or not—protected the marrow colony-forming cells from the cytotoxic effect of bee venom. The data of the second experiment suggest that the 10% serum concentration was less protective than was the 50% serum. Such a quantitative effect would imply that a relatively minor constituent of mouse serum may be the effective protective factor. Such a substance could be envisioned to act either by effectively coating the cell surface of the stem cells, thereby preventing access of the cytotoxic venom compound(s), or by forming an inactive complex with it. We plan to investigate the protective activity of serum fractions in this context.

DISCUSSION

The foregoing results show clearly that bee venom and some of its isolated constituents at concentrations of the order of 1 μg/ml are toxic to bone marrow colony forming cells. This cytotoxicity was observed after *in vitro* incubation (37 C for 30 minutes) of the marrow cells at a cell concentration of 1×10^6 per ml in the presence of added venom, followed by transfusion of the treated cells into X-irradiated recipient test animals. When the cell concentration of the suspension was increased to 10×10^6 cells/ml, no detectable decrement in the number of CFU could be observed. This result suggests that the cytotoxic compounds are 'consumed' in their interaction with the marrow cells; and/or that a certain optimal ratio must obtain *vis a' vis* concentration of the compounds and total bone marrow cellular surface area, in order for cytotoxicity to take place. Indeed, it seems likely that the cytotoxic effect of the surfactant substances in bee venom, including melittin, is mediated by mechanisms involving the cell surface. Thus, in view of the known net negative charge of the cell plasma membrane, it is not unexpected that cell surfaces react with positively charged polmers (15). Further, labelled polycations have been shown to attach to different cell surfaces (16).

TABLE IV

EFFECT OF ADDITION OF MOUSE BLOOD SERUM ON CYTOTOXICITY OF BEE VENOM FOR MARROW STEM CELLS

ADDITIONS TO MARROW CELLS	CFU/10^5 MARROW CELLS	
	mean	range
bee venom only (1.7 µg/ml)	0	---
bee venom & 50% serum	13	6-15
bee venom & 10% serum	12	8-17
saline (controls)	13	7-18
bee venom only (1.7 µg/ml)	0	---
bee venom & serum (50%)	13	10-19
bee venom & inactiv. serum* (50%)	14	9-18
bee venom & serum (10%)	8	3-12
bee venom & inactiv. serum* (10%)	6	2-8
saline (controls)	13	9-15
serum (50%)	17	12-25
inactivated serum* (50%)	20	15-24

SERUM INACTIVATION: heated at 56° for 30 min.

Another experimental example can be cited which illustrates the possible effect of a bee venom constituent—the enzyme phospholipase—at the cell surface. Friedman and Pasten (17) have recently demonstrated the specific inhibition of virus growth in cultured chick fibroblasts treated with purified phospholipase C from Clostridium perfringens. About 40% of the cell's phospholipid content was hydrolysed by the enzyme, and the inhibitory effect on viral synthesis is interpreted in terms of the requirement of cell membrane integrity. Our own data show clearly that the synthetic cationic detergent was cytotoxic for bone marrow cells at 2 PPM, whereas the anionic detergent was not cytotoxic at 2 PPM, but did inactivate the bone marrow stem cells at 10 PPM; the nonionic detergent showed no toxicity at 2 or 10 PPM. It is considerable interest in this context therefore that melittin—a polypeptide—exhibits cationic properties, such that it precipitates on the addition of a solution of the anionic detergent, F-90, a linear alkyl sulfonate.

Although the hemolytic effect of bee venom is well known, to the best of our knowledge, the present observations are the first to show effects of bee venom or its constituents on nucleated cells at the cellular level. It is of interest furthermore that the hemopoietic colony-forming cells (i.e., stem cells) of the bone marrow appear to be particularly susceptible to this action, while no overt lytic effect of the bee venom on the nucleated bone marrow cell population is evident. This, in turn, suggests the possibility that bee venom may be highly cytotoxic for neoplastic cells—at least those of reticular tissue origin. Indeed we have recently observed, in preliminary experiments, that mouse lymphoma cells are inactivated after in vitro contact with bee venom (15 μg/ml) for 30 minutes at 37 C.

The relevance of the present findings with respect to the physiological and pharmacological effects of parenterally administered bee venom in vivo is as yet unknown. How, for example, does subcutaneously injected bee venom or melittin affect the bone marrow stem cell population systemically, in light of our observation that serum may alter or prevent the cytotoxic effect in the test tube? Can the injection of the highly cytotoxic constituents of bee venom prolong the survival of mice bearing transplanted lymphoma? Experiments currently in progress are aimed towards resolving some of these questions.

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13. ABSTRACT
Mouse bone marrow cells were incubated (37 for 30 minutes) in the presence of bee venom, and then assayed for their ability to form hemopoietic splenic colonies when transfused into lethally X-irradiated recipient mice. It was found that at honey bee venom concentrations as low as 0.5 µg/ml (0.5 part per million by weight) the colony-forming ability of the marrow cells was annulled. Three constituents of bee venom, i.e., melittin, phospholipase A, and a surface-active fraction, separated and isolated by methods of Sephadex gel filtration chromatography were also evaluated for cytotoxicity on bone marrow cells. A marked decrease in yield of splenic colonies was observed with melittin and with the surfactant fraction at concentrations of 1µg/ml, and the phospholipase A fraction was inhibitory at 4 µg/ml. It was found also that the synthetic cationic detergent 'Cyncal' (a quaternary amine) was cytotoxic for mouse bone marrow cells at 2 parts per million, whereas the non-ionic detergent, 'Triton X-100', did not inactivate the marrow stem cells at 10 parts per million. The basis for this potent biological effect of bee venom at the cellular level is briefly discussed; it is suggested that the cellular toxicity is mediated via effects at the cell surface.

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