BAT SEMINAL VESICLE PROTEIN: ITS CHARACTERIZATION AND PHYSIOLOGICAL PROPERTIES

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Summary. The seminal vesicle of the bat (*Myotis lucifugus*) contained a protein toxic to mice and rabbits but not to bats. This protein was precipitated with ammonium sulphate, non-dialysable, inactivated by papain and relatively heat stable. The lethal action was caused by hypotension due to a general relaxant effect on smooth muscle. Epinephrine only momentarily elevated blood pressure. The toxin had no effect on electrical transmission along the motor-nerve axon and across the neuromuscular junction. Haematocrit values increased significantly after bat seminal vesicle was injected, while a highly significant ($P > 0.01$) decrease in the proportion of circulating lymphocytes and a highly significant ($P > 0.01$) increase in proportion of circulating heterophils occurred. Isolated mouse duodenum and uterine preparations showed a diminution in contraction frequency and a decrease in muscle tone in the presence of the toxin. This was reversible by washing the toxin from the system.

The hypothesis was proposed that this seminal vesicle protein enters the female bat during copulation, blocks sperm transport, and alters the phagocytic system, thus allowing bat spermatozoa to remain in the female reproductive tract over an extended period of time.

INTRODUCTION

Hunter, Barker, Johnson, Fahning & Schultz (1971) found that bat seminal vesicle (BSV) was highly toxic to rabbits on parenteral injection. Toxic substances are rarely found in secretions of warm blooded animals (Russell & Saunders, 1967). One such rare substance is cobayin, a constituent of guinea-pig seminal plasma (Weil, Roberts & Dube, 1964). It is highly toxic on parenteral administration to the rabbit and, to a lesser degree, to the mouse, rat and dog, but not to the guinea-pig. The submaxillary gland of the short-tailed shrew (*Blarina brevicauda*) also possesses a substance which is toxic when injected intraperitoneally into mice (Pearson, 1942; Ellis & Krayner, 1955).

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The present report deals with the characterization and physiological effects of the toxic material from bat seminal vesicles.

MATERIALS AND METHODS

Collection of tissues

Collection and preparation of little brown bat (*Myotis lucifugus*) tissues was described by Hunter *et al.* (1971). Protein content was determined by Folin-Ciocalteau. Each suspension was frozen (-20°C) until used.

Test animals

New Zealand White rabbits, 2.3 to 2.9 kg, Swiss White mice, 15 to 25 g, and *Myotis lucifugus* bats were used to test for toxic activity.

Physical properties of toxin

Bat seminal vesicle extract (1.32% protein) was treated as follows:

- (a) heated to 100°C for 5 min in a water bath;
- (b) placed in Visking tubing and dialysed against 10 vol of 0.85% sodium chloride solution at 4°C for 24 hr;
- (c) diluted 1:10 with 0.1 M-sodium phosphate buffer (pH 7.0) containing 0.01 M-cysteine HCl and 2 mM-EDTA before adding 95 units of papain/mg BSV protein and incubating at 37°C for 17 hr;
- (d) saturated with ammonium sulphate, centrifuged at 10,000 g for 10 min and separated into precipitate and supernatant fluid. The precipitate was then suspended in water equal to the original volume.

The treated material was injected intraperitoneally (0.5 ml) into each of two Swiss White mice weighing 15 to 25 g. Deaths occurring 6 to 24 hr after injection were recorded.

Mode of action

Sixteen mice were decapitated and sections (8 mm) of uterine horn and duodenum were suspended in a smooth muscle chamber containing 99 ml of mammalian Krebs-Ringer bicarbonate solution at 37.5°C and aerated with 95% O_2 and 5% CO_2 . Contractions were recorded with an isotonic linear core myograph. Bat seminal vesicle (1 ml) or fractions of it, were added to the muscle chamber and the response recorded.

Sephadex G-200 fractions of BSV, whose preparation was described in an earlier publication (Hunter *et al.*, 1971), were also added to the muscle chamber and the response recorded.

Four mice were given doses (5.3 mg/kg, i.p.) of BSV protein which were known to kill within 4 hr. Two mice were decapitated 1 hr after injection. A gastrocnemius muscle and its sciatic nerve were removed. Electrical stimulation was applied to the motor-nerve axon and the response of the muscle recorded. The other two mice were anaesthetized with 100 mg pentobarbital/kg. A gastrocnemius muscle and sciatic nerve were isolated *in situ*. Electrical stimulation (1 V for 2 msec duration) was applied every 60 sec to the sciatic nerve.

The gastrocnemius muscle response was recorded until the mouse died from the effects of BSV toxin.

Three rabbits were anaesthetized with sodium pentobarbital (23 mg/kg, i.v.). The left femoral artery was cannulated and attached to a Bourdon type pressure transducer assembly. Blood pressure was recorded for 5 min and then 0.5 ml of BSV (0.66 mg protein) was injected into the marginal ear vein and the response recorded.

Bat seminal vesicle extract (0.5 ml containing 0.66 mg protein) was injected i.v. into each of six rabbits. Phosphate-buffered saline was injected similarly into six control rabbits. Blood samples (0.2 ml) were taken before injection and at 15, 30, 60, 120 and 360 min after injection for micro-haematocrit and differential white blood cell determinations. Differential counts of 100 cells were made on smears stained with Wright's stain.

RESULTS

Properties of toxin

The intramuscular injection of BSV into rabbits resulted in death within 24 hr, while other bat tissues produced no ill effects (Table 1). Rabbits showed

TABLE 1
TOXICITY OF VARIOUS BAT REPRODUCTIVE ORGANS
IN RABBITS

<i>Organ*</i>	<i>No. of observations</i>	<i>% Mortality†</i>
Testis	12	0
Epididymis	12	0
Ampulla	12	0
Prostate	12	0
Seminal vesicle	6	100

* 4% w/v suspension and 0.5 ml administered intramuscularly.

† Rabbits not dead by 24 hr survived with no ill effects.

signs of inactivity and stiffness within 15 min. Only a painful stimulus would cause them to move. Ears drooped on rabbits after 1 to 2 hr and abdomens felt swollen. Fur was raised on mice and they shivered and felt cold to the touch. Animals urinated within 2 min after injection and not again. External signs of diarrhoea were seen only in mice. Breathing became laboured and animals died within 30 min to 24 hr after injection, depending on dose and weight of animal. At necropsy, the only gross pathological changes were seen in the small and large intestines which were extremely swollen due to fluid accumulation. Contents present in the stomach at the time of injection evidently remained there until the animal died.

Bat seminal vesicle was toxic to rabbits and mice when injected i.m. or i.p. (Table 2). Deposition of BSV into the vagina of mice was also lethal, but incorporation into the water they drank was not harmful. Bat seminal vesicle was not harmful to bats when administered by way of the vagina or intraperitoneally.

Experiments employing ammonium sulphate precipitation, absorption at extinction 278, staining with amido black 10B after separation by Sephadex G-200 chromatography and agar gel electrophoresis (Hunter *et al.*, 1971) led to the conclusion that the toxic material was a protein. This toxic protein was non-dialysable, destroyed by papain and resistant to heating to 100°C for 5 min (Table 3).

TABLE 2
TOXICITY OF BSV AFTER VARIOUS ROUTES OF ADMINISTRATION IN DIFFERENT ANIMALS

Route of administration	Animal	No. of observations	% Mortality*
Intramuscular	Rabbit	6	100
Intravenous	Rabbit	6	100
Intraperitoneal	Mouse	24	100
Oral	Mouse	4	0
Inter-vaginal	Mouse	6	100
Intraperitoneal	Bat	12	0
Inter-vaginal	Bat	4	0

* Animals not dead by 24 hr survived with no ill effects.

TABLE 3
TOXICITY OF BSV* IN MICE AFTER VARIOUS TREATMENTS

Treatment	No. of observations	% Mortality†
BSV control	3	100
Heat, 100° C for 5 min	3	100
Dialysis, within bag	3	100
Dialysis, diffuse	3	0
Papain digestion	3	0

* 0.26 mg protein/kg body wt.

† Mice not dead by 24 hr survived with no ill effects.

Mode of action

The addition of as little as 0.07 mg BSV protein to isolated mouse duodenum and uterine preparations markedly inhibited smooth muscle activity (Text-fig. 1A and 1D). Bat seminal vesicle was equally effective in inhibiting activity during pro-oestrus, oestrus, metoestrus or di-oestrus. The smooth muscle responded to BSV with a diminution in the frequency of contractions and a relaxation in muscle tone which was sustained until reversed (Text-fig. 1B), either by washing BSV from the bath or by introducing a smooth muscle stimulant (oxytocin).

The addition of Sephadex peak B containing the toxic protein of approximately 44,600 molecular weight (Hunter *et al.*, 1971), reduced both uterine and duodenal activity in the same way as whole BSV (Text-fig. 1C, E).

Bat seminal vesicle toxin had no effect on electrical transmission along the motor-nerve axon as the gastrocnemius muscle contracted normally when the attached nerve was electrically stimulated, even when mice were close to death

TEXT-FIG. 1. Effect of BSV on contractility of various muscles from mice. (A) Relaxant effect of BSV on isolated non-gravid myometrium; (B) stimulatory effect of 1 unit oxytocin on a non-gravid myometrium isolated from mouse injected i.p. 2 hr earlier with 2.6 mg BSV protein/kg body wt; (C) relaxant effect of Sephadex G-200 fraction B of BSV on isolated non-gravid myometrium; (D) relaxant effect of BSV on isolated duodenal segments; (E) relaxant effect of Sephadex G-200 fraction B of BSV on isolated duodenal segments; (F) response of the gastrocnemius muscle when the sciatic nerve was stimulated *in situ* with 1.0 V of 2.0 msec duration every 60 sec. BSV (5.3 mg BSV protein/kg body wt) was injected i.p. into mouse 112 min before death.

(Text-fig. 1F). When duodenum and uterine segments were removed from these mice and placed in a smooth muscle chamber, the initial motility (amplitude) was approximately 5% of that seen in control mice. However, after 15 to 30 min, the duodenum and uterus returned to near normal activity. This suggested that the inhibition by BSV was reversible.

Within 20 sec after i.v. injection of BSV (0.2 mg protein/kg body wt), the blood pressure dropped to about 36% of normal and remained there until death (Text-fig. 2). Injecting epinephrine temporarily raised blood pressure, but it returned to the depressed level within 2 min.

After BSV (0.2 mg protein/kg body wt) was injected i.v., haematocrit values

TEXT-FIG. 2. Effect of BSV (0.2 mg/kg body wt) followed 5 min later by epinephrine (0.4 mg/kg) on mean blood pressure in rabbits. The drop in blood pressure was significant ($P > 0.01$). Time under bars represents period during which mean blood pressure was calculated.

increased significantly ($P > 0.05$) over the next 4 hr (Text-fig. 4). Control rabbits showed a lowered haematocrit over this same period which was a normal response to repeated bleeding.

After injection of BSV i.v., the proportion of circulating lymphocytes fell significantly ($P > 0.01$) over the next 6 hr (Text-fig. 3) while the proportion of circulating heterophils rose significantly ($P > 0.01$) above baseline levels. Basophils and eosinophils remained unchanged during this time. The proportion of circulating leucocytes remained approximately constant over the 5-hr period in control rabbits.

DISCUSSION

The symptoms associated with BSV toxicity, the hypotensive effects of BSV on the cardiovascular system, and the apparent lack of effect on nerve and voluntary muscle, pointed to a specific inhibitory effect on all smooth muscles of the body. No evidence was observed for red blood cell destruction. The increased

TEXT-FIG. 3. Effect of BSV on differential white blood cell values in rabbits. Values are means $\pm$ 1 S.E. Sham-injected controls are illustrated in the top figure.

TEXT-FIG. 4. Effect of BSV (x) on haematocrit values in rabbits. Values are means $\pm$ 1 S.E. Sham-injected controls (●).

haematocrit values suggested vaso-concentration, since much serous fluid rapidly accumulated in the gut. Although total leucocyte counts were not run, on the basis of leucocyte density in blood smears stained with Wright's stain,

approximately twice as many leucocytes were found per unit area 4 to 6 hr after injection of BSV. Associated with this was a marked infiltration of heterophils into the blood stream. Since the proportion of lymphocytes fell significantly during this period, either their production remained constant or they infiltrated to other tissues of the body.

The bat, *Myotis lucifugus*, breeds in the autumn and ovulation occurs in the spring (Wimsatt, 1960). We have observed spermatozoa free in the lumen of the female bat during hibernation. The relation of the toxic proteinaceous principle to reproduction in the bat remains obscure. Our attempts to obtain semen to date have been unsuccessful. Therefore, we have no evidence that this toxic protein reaches the female tract together with spermatozoa during mating. Nor have we shown that it is secreted by the seminal vesicles. If present in seminal plasma, this protein, either bound to spermatozoa or free, could be transported throughout the female reproductive tract. Since this toxic protein had a dramatic effect on uterine smooth muscle, it could block sperm transport and thus allow spermatozoa to remain in the uterus and not be evacuated through the Fallopian tube. Because the effect of BSV on isolated uterine preparations was reversible, proteolytic enzymes induced through growth of the spring follicle might remove the toxic protein and allow normal reproductive processes to proceed. BSV also affected both the number and distribution of leucocytes. Guthrie (1933) noted large accumulations of small leucocytes within the tubes, uterus and vagina of the hibernating bat. Guthrie observed few large leucocytes but, when seen, they always contained ingested sperm heads. Hence, BSV protein through its effect on the leucocyte system might prevent spermatozoa deposited in the autumn of the year from being phagocytosed.

These ideas would explain why spermatozoa could remain in the female over a 4-month period, but do not explain how the spermatozoa remain viable during this period.

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