Anticholinesterase-like activity by oriental hornet *(Vespa orientalis)* venom and venom sac extract

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Summary. Oriental hornet venom or venom sac extract produces pharmacological and toxicological effects typical of anticholinesterase agents. The effects produced in animals can be counteracted by atropine and heparin.

The Oriental hornet is widespread in the Mediterranean basin and Southeast Asia[1]. It is a social insect whose colonies are founded in the spring. The worker population increases during the summer months when most of the stinging occurs. The hornet is a pest of the honeybee[2] and occasionally stings man also. Human reactions to these stings are: a) allergic, i.e., strong hypersensitivity reactions in allergic persons stemming from a single sting, or b) toxic, due to systemic intoxication produced by multiple stings. Human fatalities may occur in areas where the hornet population is dense[3]. However, although many reports have been published on the toxicological manifestations of vespine stings[4,5], the underlying toxic mechanism has not yet been clearly defined. The aim of the present paper is to bring evidence that the intoxication and mortality induced by hornet venom or venom sac extract (VSE) are not unlike those produced by the well-known anticholinesterase (anti-ChE) agents. From among the numerous reported symptoms attributable the hornet venom, the following may be singled out:

1. Symptoms referable to stimulation of the parasympathetically innervated effectors (muscarinic effects): bronchoconstriction (in guinea-pigs), increased salivation and other glandular secretions, and bradycardia (in mice, cats and dogs)[6-8].
2. Symptoms referable to stimulation of the skeletal muscle (nicotinic effects): muscular twitching, increased fatigability, weakness of skeletal muscles, paresis, flaccid paralysis (in mice and cats). In nerve muscle preparation, the responses to indirect stimulation diminished earlier and more severely than those directly induced, although the ability to sustain tetanus was maintained. Post-tetanic potentiation after cessation of repetitive stimulation occurred[8].
3. Effects on the central nervous system (CNS), such as restlessness (in dogs), dyspnea, stupor, loss of righting reflexes and coma (in mice), depression of the phrenic nerve activity, desynchronization of the EEG waves and respiratory arrest in cats[7]. The neurotoxic effects as manifested by isoelectric EEG waves and by the central depression of the respiration appeared prior to the cardiovascular ones.
4. Various other effects produced by hornet venom or VSE: a block in axonal conduction (cats), disruption of the blood-brain barrier (mice, guinea-pigs, cats), hyperglycemia (cats), effects on the striated muscle mitochondria (guinea-pigs and mice), acute tubular necrosis of the kidney (mice), dilatation of the transverse tubular system and hyperplasia and hypertrophy of sartorius muscle filament (frog)[6-13]. Most of the above-mentioned effects resemble those occurring in animals or man after intoxication with anti-ChE agents[14,15]. Unfortunately, these effects of vespine venom have in man or laboratory animals received only partial attention, probably because they are usually masked by numerous other toxic symptoms associated with acute pain, local edema and erythema, hemorrhage, irritation and heat. Most of these as well as many other toxic effects occur very soon after the stinging and may distract attention from effects resembling those produced by anti-ChE agents. What further complicates the interpretation of hornet sting reactions is the fact that in laboratory experiments only venom extracted from hornets older than 1-2 days is toxic[16], and also that the venom is a very complex solution containing pharmacological compounds which produce antagonistic effects[17]. The results obtained depend also on the route of injection. For instance, the i.v. injection of venom solution into cat or dog produces an immediate *fall* in the mean blood pressure, but injection of venom solution into the vertebral artery produces a marked *elevation* in the mean blood pressure which lasts for several minutes and is followed by a drop in blood pressure (see figure 1). Due to these complications, it is possible that time will reveal many other anti-ChE-like effects produced by hornet venom or VSE.

Materials, methods and results. Preparation of cats for VSE or venom injection and recording of results were done as reported earlier[7]. Extraction of venom or VSE was as reported by Ishay[11]. Acetylcholinesterase activity was measured in heparinized blood according to the method described by Fleisher and Pope[18]. Venom solution and VSE were separated on gel-electrophoresis before and after dialysis, as well as before and after precipitation in heparin. Blood was obtained from cats following their injection i.v. with venom. The blood was withdrawn from the femoral vein in heparinized tubes.

Injection of lethal doses of venom (2-3 mg/kg) i.v. into 3 cats resulted in the following effects: almost immediately following the injection, the cats vomited (food or gastric juices and saliva). Salivation increased and gradually led to the secretion of a dense saliva from the mouth of the cat. Concurrently, generalized convulsions of all the body parts are apparent and underneath the skin fasciculation of the skeletal muscles is evident. Immediately after the venom injection, the cats urinate and about 5 min later, they defecate. Subsequently there is intermittent diarrhea. The pupils of the eye, which were open prior to the venom injection, contract to pinpoint dimensions (figure 2). On several occasions, the contraction was asymmetric: only one pupil was contracted while the other one was less contracted or even not contracted at all. The nictitating membrane was usually asymmetrically relaxed. The cats wobble drunkenly, moving in circles, displaying difficulty in remaining erect, occasionally collapsing as if under partial anesthesia, moving about aimlessly and finally sitting down in some corner and resting there for many h. Within 20-24 h, the following is observed: The cats fail to feed and develop gradual paralysis, the paralysis always beginning at the tail and hind legs and only subsequently involving the forelegs and the trunk. The pupils are still contracted and do not change diameter from dark to light or vice versa. The respiration is noisy and spaced, the hair erect and saliva drooling from the mouth. Diarrhea persists throughout the period. Injection of atropine at a dose of 1-2 mg/kg causes considerable improvement, resulting in reduced excretion, dilatation of the pupils and improved locomotion. Later on, there is relapse, with the paralysis progressing also to the forelimbs and death as a result of respiration block occurring on day 3 or 4 post-injection.

Injection of sublethal doses of venom (1 mg/kg) or VSE (5 mg/kg) (was testes on 3 cats) results in the development

of similar effects which, however, are dramatically counteracted by the administration of atropine. Injection of venom or VSE into the caudal vein of mice also results in urination and diarrhea shortly after, as well as in paralysis which occurs first in the hindlegs and only later in the forelegs and the rest of the body.

When heparin is added to the venom solution or VSE in the proportion of 50 mg heparin to 10 mg venom or VSE, a white precipitate forms immediately. Further addition of heparin does nor result in additional precipitation. Injection of venom or VSE following its saturation with heparin results in hardly any significant behavioral change. Injection of the heparin in equal amount about 30 min after injection of a lethal dose of venom, coupled with the injection of 1 mg/kg of a atropine, leads to marked amelioration of the afore-described effects of venom injection (this was tested on 3 cats) and after 1 h the cats revert to the behavior prior to venom injection, while the markedly contracted pupils dilate, diarrhea and salivation stop and mortality ceases.

Examination of the ChE level in the blood yielded the following results: 24 h after receiving a lethal dose of VSE, the ChE levels (tested in 3 cats) were only 55.6–64.4% of the control levels. There was thus a drop of 35.6–44.4% as compared to the control.

The venom solution or VSE which is retained within the dialysis bag produces the same effects as does complete venom (as tested in 10 mice). The material which passes out of the dialysis bag does not show any clear toxic effects. Venom solution or VSE which has undergone treatment with heparin displays, upon gel-electrophoresis, fewer low mol.wt ($< 10{,}000$) and high mol.wt fractions than the intact venom solution.

Discussion. The venom of hornets is usually intended as a defense against aggressors (ordinarily other insects or arthropods) as well as for obtaining prey. Honeybees which are normally preyed upon at the hive entrance, are stung immediately after capture by the hornet and are immediately paralyzed[2]. This probably facilitates the introduction of the prey into the hornet nest where the flesh of honeybees (and other insects) serves as food for the larvae, which need animal proteins as part of the diet. In return for these meat morsels, the larvae contribute a drop of saliva rich in various sugars[19]. This process of food-exchange between different members of social insect societies has been named trophallaxis by Wheeler[20] and is considered a mainstay of social activities in insect societies.

Insects introduced as food into the nest are slightly masticated by the predatory hornet workers. The predators do not bring in the entire prey, but rather first remove its head and abbomen. The remainder, namely, the thorax, is first chewed by the workers and then offered to the larvae in very small morsels. At this stage, the meat apparently does not contain venom because the larvae that feed on it are not harmed. Subsequently the fed larvae retaliate with drops of saliva containing products of the digested meat as well as various metabolites[19]. From all the above, it is evident that the venom used to paralyze the prey bee subsequently has no harmful effect on either the adults or the larvae when ingested orally. Possibly the larvae have developed tolerance to the venom in the meat offered them and it subsequently transmits to the adults the saliva drops which contain nutrients that had undergone detoxification in the larval body.

It was summarized earlier[17] that *V. orientalis* workers and queens are preyed upon in India by 4 species of birds: *Merops viridis* L., *M. superciliosus* L., *Dendrocitta rufa* (Latham) and *Caprimulgus macrurus* Horsf. They have probably, developed tolerance to the hornet venom since they consume large amounts of these insects. Preliminary attempts to feed hornet larvae with droplets of hornet venom were fatal to the larvae. But feeding them with a diluted solution of venom at a concentration of 1:20 of the original one was not harmful.

In bees, as in other insects tested, acetylcholine does not serve as a mediator in the peripheral synapses of muscle neural junctions, but does serve as a mediator in the synapses of the CNS. The enzyme which degrades it, namely, acetylcholinesterase, is also concentrated mainly in the brain and nerves[21,22]. It appears that hornet venom acts as an immediate central paralyzing agent in that it affects the acetylcholinesterase in the system.

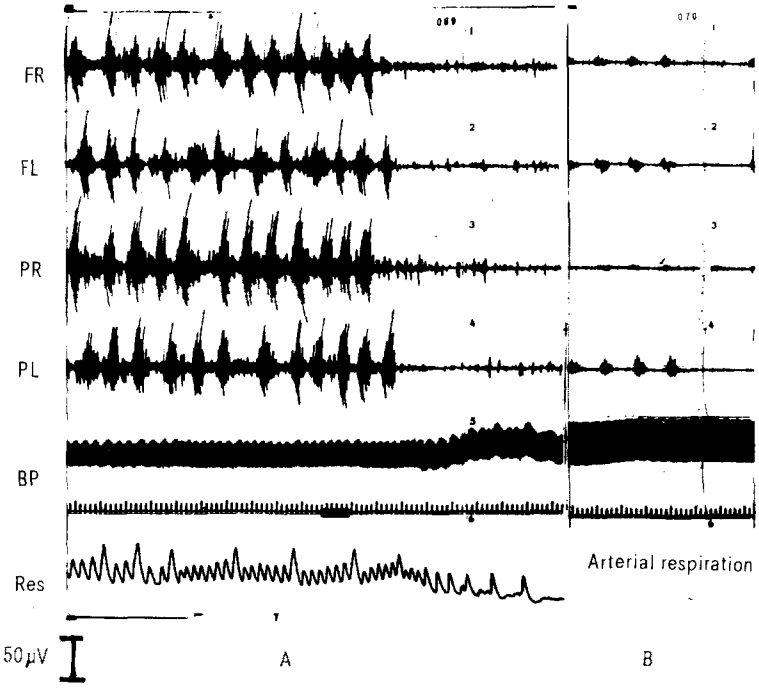

Fig. 1. Injection of 300 µg/kg of hornet venom sac extract through the vertebral artery of a cat. a: The EEG was recorded by 2 frontal (frontal right = FR, frontal left = FL) and by 2 parietal leads (parietal right = PR and parietal left = PL). Time base calibration is 1 sec/division. BP = blood pressure; Res = spontaneous respiration. 5 sec after the injection of the venom sac extract, a complete bilateral depression of the EEG occurred. 30 sec later the spontaneous respiration ceased, and at the same time a marked elevation of the mean blood pressure occurred. b: 30 sec later there was complete and irreversible depression of the EEG and spontaneous respiration, while the mean blood pressure was elevated and apparently constant. Similar injection of acetylcholine (100 µg), serotonin (100 µg), histamine (100 µg) and adrenaline (100 µg) did not produce significant changes in the spontaneous respiration or EEG.

In the past, we have attempted to ascertain in what portion of the venom apparatus of the hornet is the factor produced responsible for paralyzing bees. So far we have had no satisfactory answer because we find that both venom and venom sac extract, as well as the acidic glands and the basic gland, all contain bee-paralyzing material[23].

The venom and also each component of the venom apparatus have an acid pH, but their enzymatic activity extends over a fairly wide range - from pH 4 to pH 8[24]. For insects it has been shown that nearly all cholinesterases have a pH optimum on the alkaline side. The pH optimum ranges around 7.6-9[25,26]. Hornet venom, despite its acidity, contains very basic fractions which are precipitated by heparin. These basic fractions comprise the main toxic elements in bee venom[27], but in bees these are made up to relatively low mol.wt polypeptides, whereas in the hornet there are both basic fractions which pass through a dialysis bag as well as larger-sized ones. Injection of heparin, into an animal that has received a dose of venom, produces a significant improvement of its condition, probably because the heparin precipitates in vivo (as it does in vitro) the basic fractions in the venom which are apparently the most toxic.

I would hazard the recommendation that heparin be administered to persons that had received multiple hornet stings and are beginning to present toxic responses. Administration of the heparin would of course be feasible only in persons whose state of health permits doing so. In this connection we may mention that hornet venom acts as an anticoagulant both by causing mast cell degranulation (Rothchild and Ishay, unpublished observations), which explains why i.p. envenomation results in severe heparinemia with prolonged clotting time, as well as by inhibiting in vitro the formation of thromboplastin and thrombin[28].

It is very likely that the mast cell degranulation, which is produced immediately following hornet sting, is an expression of one of the body's natural defence mechanisms, in that the endogenous heparin which is released thereby acts to precipitate out the toxic fractions of the venom.

Various materials have been isolated or described from hornet venom. Among these there are volatile substances, mainly volatile ketones that serve as alarm substance[29], catecholamines and 5HT[30], histamine and acetylcholine, and kinins[6]. Acetylcholine has been detected in the venom of *V. orientalis* and in that of another species, *V. crabro*, and at the highest concentration existing in living tissue[31]. The activity of the following enzymes has also been reported in the venom: phospholipase(s)[24], hyaluronidase[32], di- and polysaccharidases[33] and acid, alkaline and neutral DNases[34].

Various investigations have generally tended to attribute the different toxic activities of hornet venom to the low mol.wt fractions in the venom. However, as far as we could judge, neither the biogenic amines nor the kinins in the venom possess any toxic activities characteristic of anti-ChE agents. Neither do I believe that the enzymes whose activity has been described so far are directly connected with the earlier-described toxic phenomena. But since the substance(s) responsible for such toxic effects are of antigenic character, because it is possible to immunize animals against hornet venom[35], are thermolabile and can be precipitated by heparin, they are probably basic proteins. Their exact properties are to be elucidated.

To sum up: The clinical symptoms which appear in animals following their injection with *V. orientalis* venom, and which can be at least partly counteracted by atropine (see also Geller et al.[36]), are the muscarinic phenomena like bradycardia and the changes described as bronchoconstriction and all that is connected with the peripheral respiratory tract, gastrointestinal changes and the secretions of various glands and also changes which cause diminution of the pupil diameter and also the phenomena defined as nicotinic which entail the paralysis of striated muscle, including respiratory muscle; the changes in sympathetic ganglia which cause elevation of blood pressure and those of the CNS which entail breakdown of the blood-brain barrier, disruption of electric activity in the brain, cessation of the central breathing, the drastic drop in blood pressure; and the significant drop in the activity of the ChE enzyme -

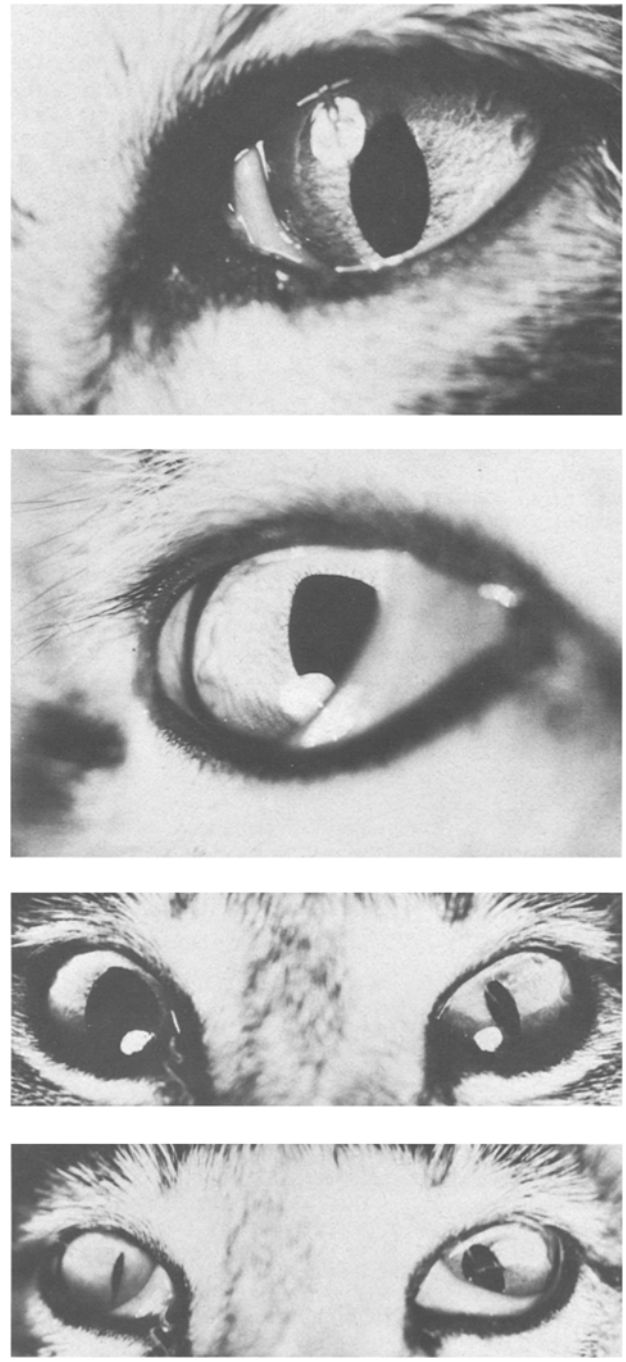

Fig. 2. Changes of the pupil constriction in a cat after injection of VSE. a: Pupils of a cat anesthetized with pentobarbital (25 mg/kg, i.v.). b: 30 sec after injection of 2 mg/kg VSE. c: After 90 sec. Note that shortly after administration of venom, asymmetrical constriction of the pupils occurred; first one is dilated while the other is constricted. Later the reverse occurred.

all these point to the fact that hornet venom acts similarly to anti-ChE agents. Such activities of the venom are reversible, because the injection of heparin (in addition to atropine) produces considerable improvement of the condition, probably freeing the receptors or the affected tissue from the venom, which probably has a higher affinity for heparin.

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Ethanol reduces Ca^{2+} concentrations in arterial and venous smooth muscle

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Summary. The present results, using isolated rat aortic strips and portal vein segments, demonstrate that ethanol (170–430 mM) significantly inhibits calcium uptake in these 2 different types of vascular smooth muscle.

Ethanol has been shown to inhibit spontaneous mechanical activity of isolated intestinal[2], uterine[3] and vascular smooth muscle[4]. Besides its direct action on blood vessels, ethanol was also demonstrated to attenuate arterial, arteriolar, venular and venous smooth muscle responsiveness to several vasoactive substances[4–6]. These direct and indirect effects of ethanol have been hypothesized to be brought about via its antagonistic or inhibitory effects on calcium ion flux in smooth muscle[2,4–6]. However, no direct evidence is, as yet, available for the latter tenet. The present study indicates that ethanol can, indeed, affect exchangeable and membrane-bound calcium in arterial and venous smooth muscle.

Methods. Thoracic aortas and portal veins, obtained from male Wistar rats (300–400 g), were cut helically and longitudinally, respectively, and set up isometrically, in vitro, as described previously[4]. The vascular tissues were equilibrated for 2 h in muscle chambers containing Krebs-Ringer bicarbonate solution (NKR), the composition of which has been reported previously[4]. Tissues bathed in NKR solution were aerated continuously with a 95% O_2–5% CO_2 mixture, and kept at 37 °C (pH 7.4–7.5). After the initial 2-h incubation period, the tissues were exposed to ^{45}Ca containing medium (0.004 μCi/ml) in the presence or absence of 170 and 430 mM ethanol for 30 min. These concentrations of ethanol were chosen since they markedly attenuate spontaneous mechanical activity and drug-induced contractions in aorta and portal vein[4–6]. At the end of 30 min, each tissue was rinsed in ice-cold NKR for 10 sec (conventional method) or for 2 or 5 min, respectively, in 50 mM La^{+++}, Ca^{++}-free medium (lanthanum method, as modified by Godfraind[7]), the composition of which is as follows (mmoles/l): NaCl, 118; KCl, 5.9; $MgSO_4$, 1.2; glucose, 10; Tris hydroxymethylaminomethane, 5.0; and $LaCl_3$, 50. The final pH of this solution was 7.15. The tissues were then blotted on ash-free filter paper, weighed and digested with the addition of 1 ml Nuclear Chicago Solubilizer (Amersham Searle Corp.) at 50 °C for 5 h. Following acidification and addition of scintillation fluid (POPOP, 6 g; PPO, 75 mg; Toluene, 1 l) ^{45}Ca was measured with a Searle Mark III Liquid Scintillation Counter. The counts were corrected for quenching and machine efficiency. The results were converted to ^{45}Ca tissue content according to the formula:

$$^{45}\text{Ca(mmole/kg wet wt)} = \frac{\text{cpm in muscle}}{\text{wet wt (kg)}} \times \frac{\text{mmole Ca/l medium}}{\text{cpm/l medium}}$$