Cantharidin: Potent Feeding Deterrent to Insects

Abstract. *Cantharidin, the well-known terpenoid compound from the blood of blister beetles (and active principle of Spanish fly), is a feeding deterrent to insects, effective at a concentration of 10^{-5} molar.*

Beetles of the family Meloidae have long been known for their peculiar characteristic of "reflex bleeding" when disturbed. Typically the response consists of emission of blood from the "knee" joints only (Fig. 1A), but droplets may also emerge sporadically from other parts of the body. Even gentle manipulation can elicit the discharge, which is a true autohemorrhage, not contingent upon infliction of injury. Contact with the blood can raise blisters on human skin, a reaction attributable to cantharidin, the active principle in the blood of these so-called "blister beetles." Spanish fly, the medicinal preparation once widely used as a topical vesicant (and misused as a purported aphrodisiac), is made from the pulverized corpses of meloid beetles (*1*). Cantharidin also acts as a powerful systemic poison to higher vertebrates—0.5 mg/kg is reportedly lethal to humans (*2*)—and reflex bleeding is generally believed to protect meloid beetles against at least some vertebrates (*1, 3, 4*). Other predators, including insects, are also said to be deterred by meloid blood (*4*), but whether cantharidin is the protective factor involved remained unknown. We have found cantharidin to be a highly effective feeding deterrent to certain predaceous insects.

Tests with individual meloid beetles showed that these insects can exercise considerable control over the extent and localization of the hemorrhagic discharge. General bleeding from all knee joints usually occurred only when a beetle was handled or otherwise subjected to generalized stimulation (Fig. 1A). If an "assault" was restricted to a single leg, say by pinching it with forceps, only that particular leg tended to bleed (Fig. 1B). Persistent stimulation of the same leg sometimes caused the response to spread to other legs, but usually only to those of the same side. Stimulation of localized sites on the body itself also tended to elicit a response from only the nearest leg or legs, as was shown by prodding beetles with a warm spatulate probe. As a result of the beetle's movements, the discharged droplets commonly spread from their sites of emission to other parts of the body. The results were essentially identical with all species of Meloidae tested (*Cysteodemus wislizeni, Epicauta albida, E. brunnea, E. (Macrobasis) immaculata, E. lemniscata, E. pestifera, Megetra punctata*).

The ability to bleed selectively from individual legs was in itself suggestive of adaptive specialization for defense against small rather than large predators. Tests with ants revealed the effectiveness of the mechanism. Individual beetles (*Epicauta brunnea*) that were released near an ant colony (*Pogonomyrmex occidentalis*) were promptly attacked by ants, which typically seized them by the legs and antennae. Restricted discharge from individual legs of beetles was repeatedly noticed, and generalized emission from most or all legs occurred only when a beetle was placed directly beside the colony entrance and overwhelmed by a swarm of ants. Massive assaults occasionally caused the beetles to cease moving and "feign death," sometimes even before being induced to bleed. Such fake corpses were usually dragged away by the ants and abandoned at a distance from the nest, where the beetles became active again and escaped. Ants contaminated by blood always scurried away from the beetles, and during their escape showed frequent cleansing activities, including a characteristic wiping of their bodies in the soil (*5*). All beetles survived without noticeable injury, and none (surprisingly) appeared to have been stung by the ants. Fresh corpses of beetles placed amid ants were also dragged off and abandoned, although squashed mealworms (larvae of *Tenebrio molitor*) placed beside them as controls proved acceptable. Tests were also done with caged carabid beetles (*Calosoma prominens*) but these provenly voracious predators refused to attack Meloidae (*E. brunnea*). However, hand-held *Calosoma* could be induced to bite into meloid beetles held directly between their mandibles, and when these *Calosoma* were subsequently released on sand they ploughed their mouthparts in the substrate in an obvious effort to cleanse themselves (Fig. 1C). Droplets of meloid blood administered to the mouthparts of *Calosoma* induced the same response.

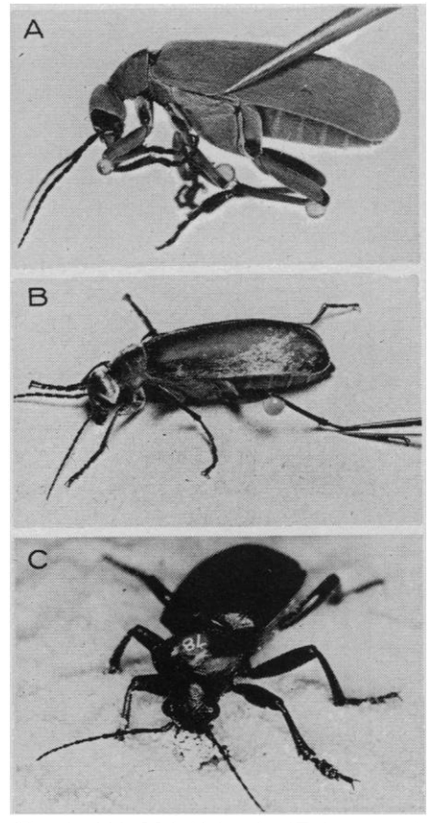

Fig. 1. (A) Meloid beetle (*Epicauta* sp.) responding to pinching of its body by "reflex bleeding" from the femorotibial joints of several legs. (B) Beetle (*Epicauta immaculata*) subjected to stimulation of single leg, bleeding from that leg only. (C) Predaceous beetle (*Calosoma prominens*) ploughing its head in the substrate, following contamination of its mouthparts with meloid blood.

Additional experiments were designed to evaluate the deterrency of cantharidin itself. Feeding tests with another species of ant (*Formica exsectoides*) showed that cantharidin can effectively reduce the acceptability of a sugar solution. The liquid samples were presented to the ants in open-ended capillary tubes, at a customary feeding site on the foraging platform of a laboratory colony of the species. The tubes were maintained at a tilt, so that downward flow automatically replenished the amounts withdrawn by the ants from the lower accessible ends of the tubes. All tubes were of calibrated capacities, and drinking rates were determined by measuring their rates of depletion (*6*).

Cantharidin solutions were made up in 0.1*M* glucose, at five concentrations ranging from 0.77×10^{-5} to $7.65M \times 10^{-5}M$. In any one feeding session the ants were offered a choice between one cantharidin solution and 0.1*M* glucose. Twenty tubes were offered simultaneously, closely spaced in a row, ten filled with the given cantharidin solution and the other ten with the sugar solution

(the tubes were interspersed at random). A session lasted 90 minutes, and involved visitation by an estimated 50 to 75 ants. Sessions were spaced at daily intervals, and each of the five cantharidin solutions was tested in two sessions. Two control sessions were carried out in which 0.1*M* glucose was offered in all 20 tubes (*7*). The sequence of sessions was randomized. The results, based on the measured drinking rates from 20 tubes per sample, are given in Fig. 2. Cantharidin evidently had a significant depressant effect on the drinking rate, even at the minimal tested concentration of $0.77 \times 10^{-5}M$ ($P < .001$; Student's *t*-test).

A second bioassay was based on the demonstrated mouth-cleansing response of *Calosoma*. Individual beetles, handheld, were stimulated orally by inserting a brush dipped in test sample between their mandibles, causing them to bite into the brush. They were then released on sand in an enclosure, and a record made of whether or not they cleansed their mouthparts by wiping them in the substrate. Test samples were prepared by dissolving cantharidin (five concentrations, ranging from 0.66×10^{-1} to $0.66 \times 10^{-5}M$) in a lipid-water emulsion (*8*). The emulsion itself provided the control (0*M* cantharidin). Twenty-five beetles were used in the assay. The group was tested in daily sessions, with one sample per session and one test per beetle (sequence of sessions was randomized). Some samples were tested in two sessions. Between tests the beetles had access to water and food (mealworms) and none showed noticeable ill effects from exposure to the samples. The results, shown in Fig. 3, present incidence of cleaning (percentage of 25 beetles responding per session) as a function of cantharidin content of the sample. *Calosoma* is obviously orally sensitive to cantharidin, and even the sample of lowest concentration tested ($0.66 \times 10^{-5}M$) was significantly more effective in eliciting cleansing than the control ($P < .05$; chi-square test with Yates's correction).

Table 1. Cantharidin content of the blood in 11 species of Meloidae. Numbers represent single assays from individual beetles.

Species	Cantharidin ($\times 10^{-3}M$)
Epicauta amaicha	30, 34, 75, 85
E. aspera	0.6
E. brunnea	102
E. corvina	7, 29
E. lauta	41, 54
E. ochrea	28
E. pennsylvanica	2, 6
Nemognatha lurida	22
Pyrota palpalis	15, 25
Zonitis dunniana	1, 3
Z. punctipennis	1, 2, 20, 25

Assays were made of the concentration of cantharidin in the blood of various species of Meloidae. Blood samples of known volumes were taken from 22 beetles of 11 species by squeezing them gently in forceps and trapping the emergent blood droplets in calibrated capillary tubes. Cantharidin content was determined by gas chromatographic comparison with known concentrations of authentic cantharidin (*9*). As is evident from the results (Table 1), cantharidin content of meloid blood is a parameter of some variability. Significantly, however, the detected concentrations exceeded by a factor of 100 or more the minimal concentrations that proved deterrent to *Formica* and *Calosoma*.

The finding that cantharidin has deterrent potential vis-à-vis insects has obvious applied implications. The compound is relatively stable and nonvolatile, and is effective on insects at concentrations matching some of the lowest insect taste thresholds recorded (*10*). Whether less toxic molecular variants of cantharidin might be comparably deterrent to insects and hence potentially useful as control agents should be investigated. How precisely cantharidin affects insects, whether by distastefulness, "irritation," or some other orally discernible quality, remains unknown. It also appears that some insects are insensitive to cantharidin. Certain asilid flies and mirid bugs have been reported to take Meloidae in nature (*3, 11*) and we have found preying mantids (*Stagmomantis* sp.), reduviid bugs (*Apiomerus* sp.), and ant lion larvae to feed on the beetles (*Epicauta* spp.) in the laboratory.

One wonders about the basis of discrimination of vertebrates against Meloidae. The usual assumption is that vertebrates are affected by the toxicity of cantharidin, and are somehow able to associate the delayed syndromes caused by ingestion of the beetles with the memory of having eaten them. The possibility that they can also taste or otherwise detect cantharidin orally, and therefore discriminate against Meloidae on the basis of unpalatability, remains to be tested. We refrained from tasting cantharidin ourselves because of reluctance to risk the "érections douloureuses et prolonguées" reportedly (*12*) elicited by cantharidin in man.

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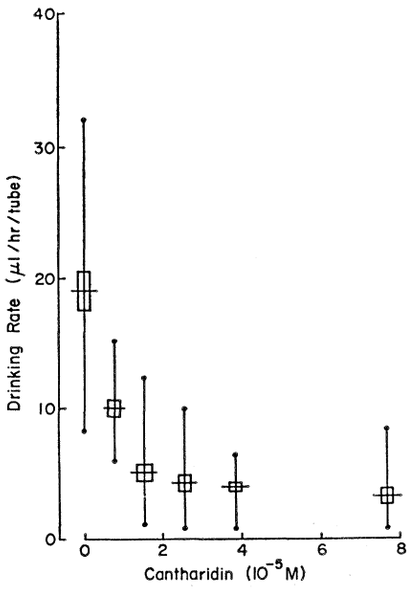

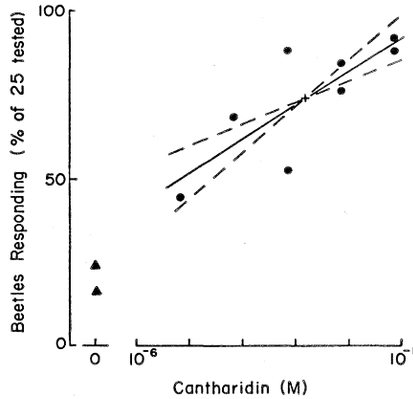

Fig. 2 (left). Effect of dissolved cantharidin on the acceptability of sugar water (0.1*M* glucose) to ants (*Formica exsectoides*). Horizontal lines indicate mean drinking rates, vertical lines give ranges, and bars indicate 1 standard error on each side of mean. Fig. 3 (right). Oral cleansing by beetles (*Calosoma prominens*) plotted as a function of the cantharidin content of a lipid-water emulsion used as oral contaminant. Controls (triangles) show response to the emulsion itself. Regression line and 95 percent confidence interval for its slope are indicated.

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6. The tubes were calibrated on the basis of their measured (ocular micrometer) inner diameters. Consumption rates were calculated from measurements of meniscus movement (corrected for evaporative loss of fluid).
7. Statistically the 20 tubes in the controls signified choice between 0.1*M* glucose and 0*M* cantharidin. Drinking rates were measured only from those 10 tubes that were randomly designated to correspond to 0*M* cantharidin.
8. The emulsion consisted of cholesterol, Tween 20, olive oil, and glass distilled water in the ratio of 1:3:3:20 by weight. Cantharidin was dissolved in the hot lipid phase before addition of water.
9. Blood samples were treated with concentrated HCl, and the hydrolysate was taken up in hot acetone, dried in a stream of nitrogen, and dissolved in chloroform for injection into the gas chromatograph. Two columns were used for each sample: 3 percent Silicone DC 560 (F60) on Chromosorb P 60/80 mesh, 6 feet by ⅛ inch (operated isothermally at 200°C), and 8 percent SE30 and 4 percent NPGS on Chromosorb W 08/100 mesh, 8 feet by ⅛ inch (programmed at 4°C per minute, 140° to 180°C). Operating conditions for both columns were identical: injector and flame ionization detector temperatures 250° and 275°C; air, hydrogen, and nitrogen flows 140, 14, and 28 ml/min, respectively. Correspondence of the designated peaks to cantharidin was unequivocally confirmed by gas chromatography–mass spectrometry (authentic cantharidin supplied by Inland Alkaloid Co.).
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13. We thank D. S. Robson for advice on statistical procedure, J. Hribar and J. Duxbury for mass spectrometric analyses, R. B. Selander for live Meloidae, F. G. Werner and W. R. Enns for identification of Meloidae, and W. L. Brown, Jr., for identification of ants. Study supported by NIH grant AI-02908 (T.E.) and NIH fellowship 5-FO1-GM40210 (J.E.C.). Paper No. 37 of the series *Defense Mechanisms of Arthropods.*

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B-Cell Alloantigens Determined by the H-2 Linked Ir Region Are Associated with Mixed Lymphocyte Culture Stimulation

Abstract. *Cell surface antigens, controlled by genes located in the Ir region of the murine major histocompatibility complex, are shown serologically to be expressed preferentially on bone marrow–derived lymphocytes. These antigens may play a major role as stimulators in mixed lymphocyte cultures.*

Recent developments in several different fields of immunology have focused attention on H-2, the murine major histocompatibility complex (MHC). Analysis of this region has led to the identification of four closely linked subregions in the linear order: H-2K, Ir, Ss-Slp, and H-2D. These are conventionally written from left to right with respect to the centromere of the ninth linkage group (*1*) (Fig. 1a). H-2K and H-2D genes code for serologically detectable antigens on cell membranes which seem to be important in graft rejection (*1*). To the right of H-2K lies the Ir region, which appears to control the ability of particular strains to generate an immune response to a large range of synthetic and naturally occurring antigens (*2*). Ir has been further subdivided into Ir-IgA and Ir-IgG loci which control the ability of mice to make antibody against myeloma proteins of these two classes (*3*). Ss governs the concentration of a serum protein of unknown function, and Slp, which as yet has not been genetically separated from Ss, controls production of an alloantigen of this protein expressed only in males (*4*).

Through the use of congenic resistant mouse strains, which are genetically identical except for their MHC, it has been shown that differences in the products of the MHC are necessary and sufficient to cause stimulation in mixed lymphocyte culture (MLC) (*5*). In this test, lymphocytes from mice of two different strains are placed into culture together. If the cells of one strain recognize cell surface antigens of the other as "foreign" they are stimulated to proliferate. By preventing proliferation of one population with mitomycin C, a "one-way MLC" can be obtained (*5*).

Several mouse strains have been developed in which the MHC contains a known crossover between H-2K and H-2D regions. Use of these recombinant strains in MLC testing has revealed that differences in the Ir region give the strongest MLC stimulation and that differences in the H-2K or H-2D regions with identity in the Ir region give weak stimulation (*5*). This has led to the hypothesis of multiple MLC loci of varying strength spread throughout the MHC (*6*). In examination of intra H-2 recombinants by MLC, an unusual result has been repeatedly observed with the congenic resistant strains B10.A(1R), B10.A(2R), and B10.A(4R) (abbreviated 1R, 2R, and 4R) (Fig. 1a). The strains 1R and 2R are identical with respect to known markers. In tests of cells from these three strains, 4R cells give a low but consistently significant response to 1R and 2R cells, but 1R and 2R cells do not respond above background when stimulated by 4R cells (*5*, *6*). The same result has also been seen when the 2R and 4R strains are examined for graft-versus-host reactivity by a Simonsen spleen weight gain assay (*7*): 4R cells produce significant graft-versus-host reactivity when injected into 2R mice, but no graft-versus-host reactivity is seen in the reverse combination.

In this report, we propose a genetic model to explain these "one-way" MLC and graft-versus-host reactivity results. The model, illustrated in Fig. 1b, invokes the existence of a distinct MLC locus located in Ir and of a deletion of this locus during the formation of the 4R recombinant MHC. If, during gametogenesis in the H-$2^{a/b}$ hybrid, an improper pairing of the MHC chromatids took place, then the crossover shown in solid line would not include either the H-2^a or the H-2^b MLC locus, resulting in a deletion. Such improper pairing may occur in regions containing a series of genes coding for similar functions (*8*). The Ir region has been thought to consist of a series of loci coding for membrane receptors (*2*), so it is conceivable that these loci would have regions of homology leading to improper pairing. Assuming a single crossover event, the postulated MLC locus must lie between Ir-1 and Ir-IgG, given the knowledge that 2R and 4R have the same Ir-1 but differ at Ir-IgG (*3*).

In order to test this model, congenic B10 anti-B10.A and B10.A anti-B10 antiserums were raised by the use of reciprocal skin grafts followed by weekly intraperitoneal injections of 20×10^6 lymphoid cells. These antiserums were absorbed with a number of 2R or 4R lymphoid cells sufficient to completely remove cytotoxic activity to the absorbing cell type (8×10^8 cells per milliliter of antiserum) and were