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EFFECTS OF AZADIRACHTIN IN *RHODNIUS PROLIXUS:* DATA AND HYPOTHESES

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The effects of azadirachtin A, a tetranortriterpenoid from the neem tree Azadirachta indica *J., on both development and interaction between* Trypanosoma cruzi, *the causative agent of Chagas' disease, and its vector* Rhodnius prolixus *were studied. Given through a blood meal, a dose-response relationship of azadirachtin was established using antifeedant effect and ecdysis inhibition as effective parameters. A single dose of azadirachtin A was able to block the onset of mitosis in the epidermis and ecdysteroid titers in the hemolymph, determined by radioimmune-assay, were too low for an induction of ecdysis. The survival of* T. cruzi *was also studied in* R. prolixus *treated with the drug. If the trypomastigotes were fed in presence of azadirachtin A the number of parasites drastically decreased. If the drug was applied after infection of the bug with* T. cruzi, *the parasite was still abolished from the gut. If the insect was pretreated with azadirachtin A before infection the same observation was obtained. A single dose of azadirachtin A was enough for a permanent resistance of the insect host against its reinfection with* T. cruzi *and for blocking the ecdysis for a long time. The effects of azadirachtin A on the hormonal balance of the host and growth inhibition of the parasite will be discussed on the basis of the present results.*

Key words: *Rhodnius prolixus* – azadirachtin – *Trypanosoma cruzi*

It has been suggested that the manipulation of the insect endocrine system is a promising alternative to the classical neurotoxic insecticides. Plants often contain compounds which display strong growth inhibitory and/or sterilizing capacity. Several plant compounds may act as feeding deterrents or interrupt insect endocrine production. This latter property is exhibited by a serie of compounds, azadirachtins, isolated from neem (*Azadirachta indica*) seeds, which cause dramatic changes in the endocrine system of several species of insects disrupting insect development and reproduction (Rembold, 1989).

We present here our findings of the effects of azadirachtin on neuroendocrine control in *Rhodnius prolixus* and, as consequence of the disturbance of neuroendocrine secretion the effects on development of *Trypanosoma cruzi,* the causative agent of Chagas disease, within the bug.

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BIOLOGICAL ACTIVITY

Azadirachtins given orally can interfere in biological events such as feeding, development and reproduction of *R. prolixus.* The insect avoids taking blood containing higher concentrations of azadirachtin A, which however, does not display nonspecific toxic effects. The effective dose of azadirachtin A (ED_{50}) for feeding inhibition was 25.0 μg/ml of blood (Garcia & Rembold, 1984). At lower concentrations azadirachtin A strongly inhibits larval growth and development of *R. prolixus* (Garcia & Rembold, 1984; Garcia et al., 1984a). All the 5 larval stages stopped growth when they ingested azadirachtin A at 1.0 μg/ml of blood (Garcia, unpublished data). Clearly, feeding inhibition was not the basic reason for growth inhibition. We have demonstrated clearly that the ED_{50} for moult inhibition was 625-fold lower than the ED_{50} for feeding inhibition (Garcia & Rembold, 1984; Garcia et al., 1984a). These findings emphasize that both physiological effects of azadirachtin A are distinct from each other and in particular that inhibition of ecdysis is not due to a reduced blood intake. It seems therefore appropriate

to consider that inhibition of acdysis in the azadirachtin-treated insects is related to a direct and/or indirect interference with events which are under ecdysteroid regulation. This hypothesis is supported by the observation that ecdysone administration of azadirachtin-treated larvae overcame the inhibition of ecdysis (Garcia & Rembold, 1984; Garcia et al., 1990); further evidence was that ecdysial stasis was due to a dramatic reduction of ecdysteroid titers induced by azadirachtin A (Garcia et al., 1986; 1987). The fact of the ecdysteroid titers were negligible and remained at a low level during the intermoulting cycle was reflected in the onset of epidermal cellular changes related to ecdysis (Garcia et al., 1986). The insects which did not undergo ecdysis survived for a long time as "permanent" larvae even after several blood meals. They only presented "signals" of development under an ecdysome therapy (Garcia et al., 1990). Azadirachtin A also induced sterilization in *R. prolixus*. Adult females of *Rhodnius* had reduced oocyte growth and consequently diminished egg production. In addition, a significant correlation between these effects and the titers of both vitellogenin and vitellin as well as of ecdysteroids was observed (Feder et al., 1988). There was no reversal of these effects by ecdysone application (Garcia et al., 1987).

The long persistent effect of azadirachtin A on development and reproduction of *R. prolixus* was studied. Azadirachtin A, for example, fed at concentrations of 1.0 to 5.0 μg/ml in a blood meal decreased or even abolished the moulting and egg deposition for at least 120 days after its application (Garcia et al., 1990). These effects of azadirachtin A suggests its interference with the neuroendocrine regulation of these events. We have therefore considered three hypotheses to explain the effects of azadirachtin A on *R. prolixus*. First, azadirachtin A induces permanent changes in the endocrine regulation of these physiological processes. In this respect, it is now well known that 5th-instar larvae (Garcia, unpublished data) and adult females of *R. prolixus*, rapidly excrete, as unchanged compound, dihydroazadirachtin A within 24-48 h of ingestion (Garcia et al., 1989c). The dihydroazadirachtin A was treated as a xenobiotic and was eliminated by the insect as rapidly as possible. Though dihydroazadirachtin A may actively be transported during diusesis, a constant quantity of this compound was recovered from the head and viscera of larvae and adult females two weeks after its application (Garcia, unpublished data; Garcia et al., 1989c). It is therefore possible that minute concentrations of azadirachtin A remains tightly bound to the endocrine tissues switching off neuroendocrine stimulation and/or persisting in the endocrine organs for a long time. If true, we can then study neurosecretory release in *R. prolixus* treated with azadirachtin A. It is generally accepted that this insect is passing through a "head critical period" for ecdysis in relation to its feeding time (Wigglesworth, 1934, 1940). This period depends on a factor originating from the head ("brain hormone") (Wigglesworth, 1940). The brain hormone is identical with the prothoracicotropic hormone (PTTH) due to its stimulatory effect on the prothoracic glands which secrete the moulting hormone (Gilbert et al., 1980). Recently, Knoblock & Steel (1989) showed the timing of release of PTTH in relation to the "head critical period" during larval-imaginal development through evidence obtained from head transplantation experiments in *R. prolixus*. Using the same system decribed by Knoblock & Steel (1989), we demonstrated that also in *R. prolixus*, as in *Locusta migratoria* (Subrahmanyam et al., 1989; Subrahmanyam & Rembold, 1989). the neuroendocrine system is a target for azadirachtin A. Unlike the untreated controls, heads from azadirachtin A treated donor larvae were unable to maintain the ecdysteroid titers in decapitated hosts (Garcia et al., 1990). On the basis of this finding, we suggested that azadirachtin A interferes with the PTTH release from the brain and from the corpus cardiacum of *Rhodnius* larvae. This hypothesis is also supported by the fact that if azadirachtin A was applied after the "head critical period", i.e., after the regulatory program for controlling the next moult is switched on, the insect moulted normally (Garcia et al., 1986; 1987). Further support is that after azadirachtin A application to *L. migratoria*, turnover of neurosecretory proteins is almost completely abolished and neurosecretory materials are accumulated (Subrahmanyam et al., 1989; Subrahmanyam & Rembold, 1989).

Second, the absence of active prothoracic glands or ovaries, which produce ecdysteroids in larvae and adult females of *R. prolixus*, respectively, should also explain our results using azadirachtin A. In support of this hypo-

thesis, it was shown that prothoracic glands and ovaries isolated *in vitro* produced only small amounts of ecdysteroids following azadirachtin A treatment to the living bug (Garcia et al., 1987; Feder et al., 1988). A decrease in ecdysteroid synthesis was also observed even when normal prothoracic glands and ovaries were incubated *in vitro* with azadirachtin A. The relevance of the *in vitro* inhibition of prothoracic glands and ovaries hormonal secretion compared to the overall effects *in vivo* is often difficult to establish since azadirachtin A may act simultaneously on other target systems. Also, the dose of azadirachtin A used for *in vitro* tests may not correspond to the dose utilized for *in vivo* experiments, i.e., it is possible that the effects observed in *in vitro* assays are due to a pharmacological as oppost to a physiological dose of the compound.

Finally, it is possible the effects on *R. prolixus* of azadirachtin A could be due to intoxication of the insect on the basis of our data. We have shown that there is only a minimal effect of the compound on mortality and apparently the physiological condition of the bugs were normal. Moreover, we also showed that ecdysone application in "permanent" larvae led the insects to moult; hence, ecdysone therapy showed us that the insects had an azadirachtin induced hormonal dieficiency up to 120 days after the treatment (Garcia et al., 1990). Consequently, any acute or chronic toxic effect of the compound can be eliminated as an explanation of the effect of azadirachtin A in *R. prolixus*.

AZADIRACHTIN AND *TRYPANOSOMA CRUZI* – *RHODNIUS PROLIXUS* INTERACTION

Chagas' disease, caused by the parasitic flagellate *T. cruzi*, is endemic in several parts of Brazil and South-America. The parasite is transmitted through triatomine insects, such as *R. prolixus*, following blood feeding. Ingested trypomastigotes transform into epimastigotes in the bug's crop. Later they multiply and differentiate into metacyclic trypomastigotes, the infective forms, eventually accumulating in the rectum, from where they are transmitted when the bug defecate during or after feeding (see details in Brener, 1973). One of the major obstacles in understanding *T. cruzi* dynamics, in nature, is the lack of knowledge concerning the interaction of the bug and parasite. This relationship apparently can be modulated by numerous parameters related to the insect and/or parasite (Garcia et al., 1984b). One of the factors which may influence trypanosome infections in triatomines is the physiological status of the insect. If the physiological condition influences the development of *T. cruzi* in the vector, can be investigated via the use of azadirachtin which stops growth and reproduction and might affect normal development of the parasite. We therefore applied the compound to the insect via a blood meal and at different intervals together with, or before, or after infection with *T. cruzi*. Following this experiment we observed that the number of parasites was reduced near to the limit of detection during 30 days after treatment with azadirachtin (Garcia et al., 1989a, b; Rembold & Garcia, 1989). Azadirachtin did not kill the trypanosomes directly as infected blood if treated with azadirachtin remains infectious (Garcia et al., 1989 a, b). A single dose of azadirachtin is enough for a resistance of at least 120 days of the vector against its re-infection with the trypanosomes (Garcia et al., in preparation). What kinds of azadirachtin effects are responsible for this vector-parasite disruption? Several hypotheses can be postulated. Either azadirachtin acts indirectly through the extensive change in the endocrine control of the insect's development, or directly by interfering in the gut physiology. A disturbance of digestive function seems less probable since we did not show any effects of non-toxic azadirachtin doses on such sensitive markers of gut physiology as the hemolytic activity in the crop or cathepsin B- and D-like proteinases in the midgut (Garcia et al., 1989b); indirect action through endocrine control mechanisms is more likely. In this case azadirachtin disruption of the endocrine regulation somehow would interfere with the trypanosome development in the insect's gut. Alternatively, azadirachtin would cause changes in the gut in such a way that the digestive tract is no longer an acceptable microenviroment for trypanosome survival and development. Whatever the explanation for these effects, azadirachtin will be a valuable tool to evaluate triatomine-*T. cruzi* interaction and possible other host-parasite relationships.

CONCLUSION

Although several classes of chemicals have been found to interfere with moulting in

various insects, azadirachtin has the most potent insect growth inhibitor properties associated with endocrine control. By classical surgical and head extirpation-transplantation procedures, we have shown that the primary effect of azadirachtin involves inhibition of neurohormone release during the "head critical period" of the moulting process of immature stages of *R. prolixus*. Azadirachtin, as a model for pharmacokinetic studies, reveals difference of the metabolism of xenobiotic compounds in this insect. Although it is eliminated unmetabolized during diuresis actively passing through the Malpighian tubules like other xenobiotics, azadirachtin is stored in unusually large quantities in the endocrine tissues and other organs. Its potent antimoulting activity is also associated with a long persistence of tissue bound azadirachtin in the endocrine organs.

Additional effects have been observed and are apparently associated with secondary effects resulting from the overall physiological or biochemical changes in azadirachtin-treated insects. Most notable of which is the inhibition of epidermal mitosis and ecdysteroid synthesis in the prothoracic glands and ovaries of *R. prolixus;* however, direct inhibition of ecdysteroid synthesis in these organs by azadirachtin has also been demonstrated.

Azadirachtin abolishes *T. cruzi* infection in the gut of the bug, *R. prolixus.* A parallel between its effects on the hormone balance of the host and growth inhibition of trypanosomes is suggested from these results. Another possible explanation could be that inhibition of trypanosome development in *R. prolixus* gut is not associated with endocrine disruption leading to hormone deficiency but rather with changes of the microenviroment of the gut preventing trypanosome establishment and morphogenesis.

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