Chagas' Disease and its Insect Vector. Effect of Azadirachtin A on the Interaction of a Triatomine Host *(Rhodnius prolixus)* and its Parasite *(Trypanosoma cruzi)*

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The ED_{50} for moulting inhibition by injected azadirachtin A is for fourth instar larvae of all the triatomines, *Triatoma vitticepes, T. pseudomaculata, T. maculata, T. brasiliensis, T. lecticularis, T. matogrossensis, T. infestans, Rhodnius prolixus, R. neglectus, R. robustus, Panstrongylus megistus,* and *P. herrera* in the range of 10–25 ng/larva. In *Rhodnius prolixus,* the survival of *T. cruzi* was studied after treatment with the drug. If the trypomastigotes were fed in presence of 1.0 μg azadirachtin A/ml blood, the number of parasites decreased near to the limit of detection within 30 days. If the drug was applied 20 days after *T. cruzi* infection, it still completely abolished the parasite in the host's gut within the subsequent 20 days. The same holds true if the insect larvae were pretreated with azadirachtin A 20 days before the subsequent infection with *T. cruzi.* Azadirachtin A, if applied at the dose of 1 μg/ml, did not affect the hemolytic activity of the crop contents or the proteinase content of the intestine. A parallel between azadirachtin effects on the hormone balance of the host and growth inhibition of the parasite is discussed on the basis of the present results.

Chagas' disease [1] is endemic in most parts of Brazil. Its causative agent, *Trypanosoma cruzi,* is transmitted by triatomine bugs during a blood meal. The physiological relationship between the pathogenic *T. cruzi* and its blood sucking insect vectors is, like in other trypanosomiases, far from a real understanding. There are several reports on growth and metacyclogenesis of the parasite in the insect's gut. These biological events are apparently modulated by numerous factors related to the insect and/or the parasite [2–4]. One of these factors which may influence the triatomines' infection by the trypanosomes, is the physiological status of the insect.

The high biological activity of azadirachtin, an insect growth inhibitor isolated from neem *(Azadirachta indica)* seed, on the development and reproduction of insects is well established [5–9]. These effects are mainly due to the suppression of hemolymph ecdysteroid [6, 10–12], and juvenile hormone [12, 13] titers. Also an indirect effect on hormone titers through a direct influence of the drug on gut motility has been discussed [9]. Recent reports on *Rhodnius prolixus* demonstrate that azadirachtin, given by a blood meal, both inhibits development of the insect's immature stages and, in the adult, egg maturation and deposition [7, 8, 10, 11, 14].

A profound understanding of azadirachtin action on triatomines requires more information, also on distribution, clearance, and metabolism of the drug in the host insect. Such studies are in progress in our laboratories for *R. prolixus* which we are using as a model insect vector. We now report on the effect of azadirachtin A on several triatomine species. The result supports our former data which were obtained from the studies with *R. prolixus.* We further report on the severe changes in rates of survival of *T. cruzi* after azadirachtin treatment of its insect host. The data speak in favour of a host – mediated effect on survival of the parasite which is based on changes in

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the endocrine situation, and not on intestinal changes of its arthropod host.

Materials and Methods

Insect culture

Fifth-instar larvae and adult females of *R. prolixus* from our lab stock [15] were used for this study. The different triatomine species were taken at fourth instar from stock cultures maintained at Fundação Oswaldo Cruz.

Blood and feeding procedure

Groups of 4–8 animals per day were allowed to feed on citrated mouse blood through a membrane feeder [7]. Azadirachtin A [16] was diluted in 1:4 ethanol-saline and added to the blood immediately before feeding. Only fully gorged insects were used for the experiments.

Infection with T. cruzi

The *T. cruzi* clone Dm 28c was obtained and kept in the laboratory as previously described [17]. Infected mice were bleeded and the blood diluted with homologous blood to give a concentration of $6-8 \times 10^4$ trypomastigotes per ml. This parasite suspension was added into the feeding apparatus. Here too, azadirachtin was added immediately before feeding.

Parasite quantification

At different days after exposure of the bugs to infected blood, the entire intestinal tract (crop, midgut, and rectum) was removed and gently ground in 1.0 ml phosphate buffered saline (PBS; pH 7.2) using a small homogenator. The total number of parasites was determined in a Neubauer hemocytometer.

Estimation of hemolytic activity

For quantification of hemolytic activity, the procedure as already described by Azambuja *et al.* [18] was followed.

Cathepsin-like proteinase activity

The proteolytic activity of the digestive tract was measured following the procedure as described by Houseman *et al.* for cathepsin B [19] and cathepsin D [20].

Results

1. Effect of azadirachtin A on different triatomines

In order to come to clear and comparable results on the biological activity of azadirachtin, the drug was applied by injection and to all the larvae at the same physiological state, *i.e.*, into fourth-instar larvae one day after a blood meal. Inhibition of the next moult was taken as an indication of a positive effect on the hormone titers. The data collated in Table I present a fairly identical ED_{50} concentration of 10–25 ng azadirachtin per larva for a 50% inhibitory effect on moulting. This result is also a satisfactory proof for the same mode of action of the drug in all triatomines. Data obtained from studies with *R. prolixus* may therefore be generalized without much of reservations.

2. Survival of T. cruzi in azadirachtin A treated R. prolixus adults

Six to eight fifth-instar larvae were used for each day (Fig. 1). For the control group, the blood contained a defined amount of trypomastigotes (6×10^4/ml). The azadirachtin group was fed in addition to the same amount of *T. cruzi* a concentration of 1.0 μg/ml of the drug. There is a clear and relatively fast effect on growth and survival of the parasite. Their number per insect increased in the control by about one order of magnitude within three weeks after infection. However, in the azadirachtin-treated

Table I. Effective dose for 50% moulting inhibition (ED_{50}) in fourth-instar larvae of several triatomine species. One day after a blood meal on mice, they were injected 1 μl of PBS, pH 7.2, containing 1, 5, 10, 20, and, respectively, 40 ng azadirachtin A. For each concentration and species, 8–12 larvae were used.

Triatomine species	Moulting inhibition ED_{50} (ng/larva)
Triatoma vitticepes	20
Triatoma pseudomaculata	15
Triatoma maculata	18
Triatoma brasiliensis	15
Triatoma lecticularis	23
Triatoma matogrossensis	15
Triatoma infestans	20
Rhodnius prolixus	15
Rhodnius neglectus	10
Rhodnius robustus	18
Panstrongylus megistus	25
Panstrongylus herrera	22

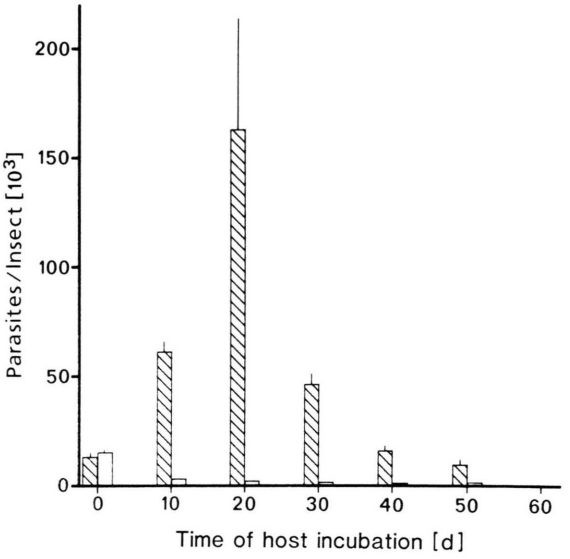

Fig. 1. Effect of azadirachtin on the course of parasitation of *R. prolixus* by *T. cruzi*. Concomitant azadirachtin uptake and infection during a blood meal on day zero. Histograms of the number of parasites present in whole gut homogenate of the host. Control (shaded) and treated (open bars) fifth-instar larvae were fed on blood containing 7×10^4 parasites/ml. Whereas the control animals moulted into adults around day 25 after feeding, there was no moult in the azadirachtin (1.0 μg/ml) treated group. Each bar represents the average from 6–8 individuals. Data are given with SD.

larvae it dropped to about one fifth of the initial concentration at day zero. After 30 days infestation of their gut *T. cruzi* had decreased to near the detection limit.

3. In vivo effect of delayed azadirachtin A application on development of T. cruzi in R. prolixus

R. prolixus fifth-instar larvae were infected with *T. cruzi* through a blood meal. They received a second blood meal twenty days later, half of them with uninfected blood, the other half with blood containing 1.0 μg azadirachtin A per ml of blood. The striking result of azadirachtin application to the infected host is shown in Fig. 2. Whereas in the now adult control insects the infection maintains a more or less constant level 20 days later, there was practically no parasite detected in the azadirachtin-treated group which also had moulted into adults.

4. In vivo effect of azadirachtin A, preceding infection of the insect host with T. cruzi

The long persistance of azadirachtin effects in the endocrine system of *R. prolixus* is well proven [7, 8, 10, 11, 14]. Does the drug, the applied dose of which completely inhibits the next (larval – adult) moult, also interfere with the parasite's growth? The experimental group, as well as the control, were infected with *T. cruzi* 20 days after the first blood meal. Only the control group moulted into adults five days after the second blood feeding, and they were normally infected 20 and respectively 40 days later. In the group which had been pretreated with azadirachtin, the number of detectable parasites had reached almost zero level (Fig. 3).

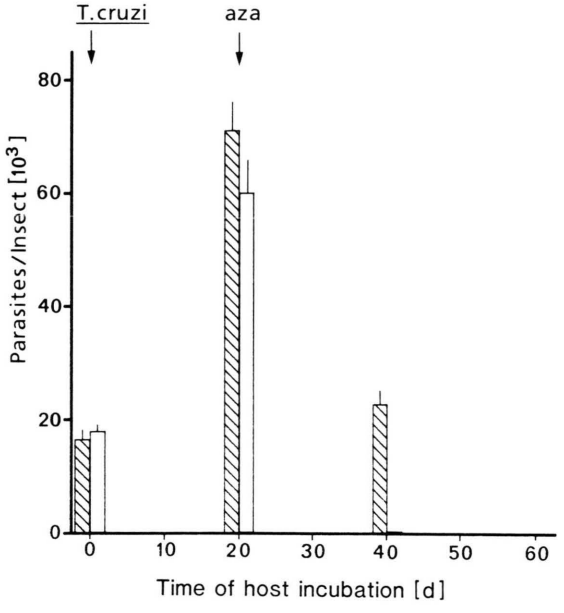

Fig. 2. Effect of delayed azadirachtin application on vitality of the parasite. Histograms of the number of trypanosomes present in whole gut homogenate of *R. prolixus*. The two groups received a blood meal on day zero (6×10^4 parasites/ml). Twenty days later they were fed another blood meal without the parasite. The control group (shaded) received blood without, the test group (open bars) with 1.0 μg azadirachtin/ml. Both groups moulted into adults 25 days after their infection at day zero. Each bar represents 6–8 individuals. Data are given with SD.

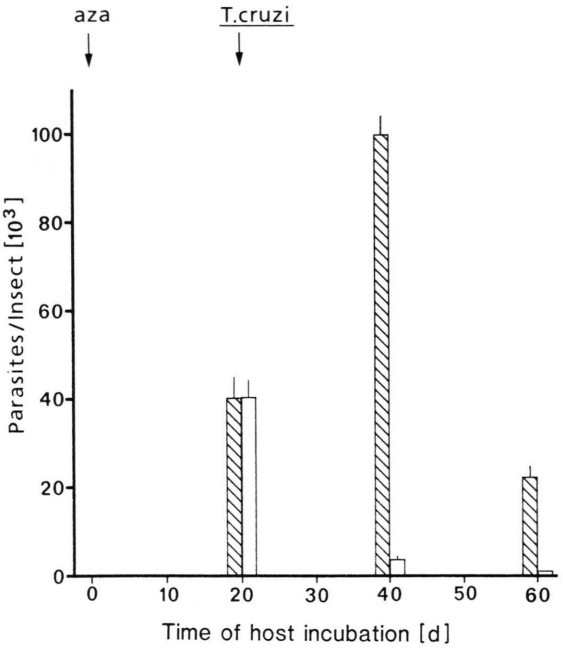

Fig. 3. Effect of a preceding azadirachtin application on survival of the parasite. Histograms of the number of trypanosomes present in whole gut homogenate of *R. prolixus*. At day zero, the control group (shaded) received blood without parasites, the experimental group (open bars) in addition 1.0 µg azadirachtin/ml blood. Infection with *T. cruzi* (8×10^4 parasites/ml) was achieved by another blood feeding of both groups on day 20. The control moulted to adults around day 25, whereas the azadirachtin-treated larvae persisted in the larval stage. Each bar represents 6–8 insects. Data are given with SD.

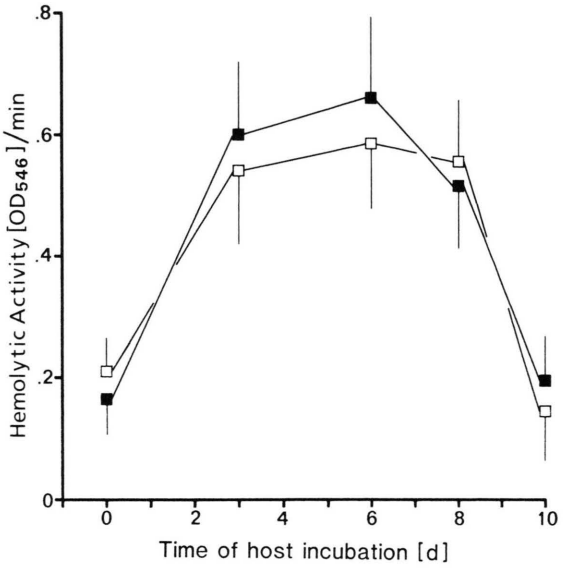

Fig. 4. Effect of azadirachtin A (1 µg/ml) fed to adult *R. prolixus* females by a blood meal on hemolytic activity of crop contents. Each point in the two curves represents the average out of four animals (± S.D.). The control (■) and the azadirachtin A (□) group were fed on day 8 after their preceding adult moult.

5. Effect of azadirachtin A on hemolytic and proteolytic enzyme activities

The data for hemolytic activity of control and azadirachtin A treated adult *R. prolixus* females are shown in Fig. 4. The lytic activity in the crop steeply increases during the first two days after blood feeding, keeps constant up to day 8, and then falls again to a level which is same as on day zero. There is no statistically significant difference in hemolytic activity between both the groups during all the ten days after feeding.

A similar behaviour is found for the characteristic proteolytic enzymes in the midgut of control and azadirachtin A treated adult *R. prolixus* females (Fig. 5). As in the crop compartiment, activity increases from a low level after the blood meal and then maintains a constant value after four (cathepsin B) and resp. two (cathepsin D) days after feeding, without a subsequent decrease in the proteolytic intestinal activity. No statistically significant difference is visible between the control and the azadirachtin A treated groups.

Discussion

No considerable advances have been made for a better understanding of the many factors which influence the development of *T. cruzi* in its insect host since the fundamental discovery of the trypanosomid basis of this human disease by Carlos Chagas in 1909 [1]. The genetic and physiological make-up of the insect vector – its age, intestinal flora, and effects of excretions – may influence growth of the parasite in the insect's intestine. A hemolytic factor has recently been described in the crop of *R. prolixus* with a max-

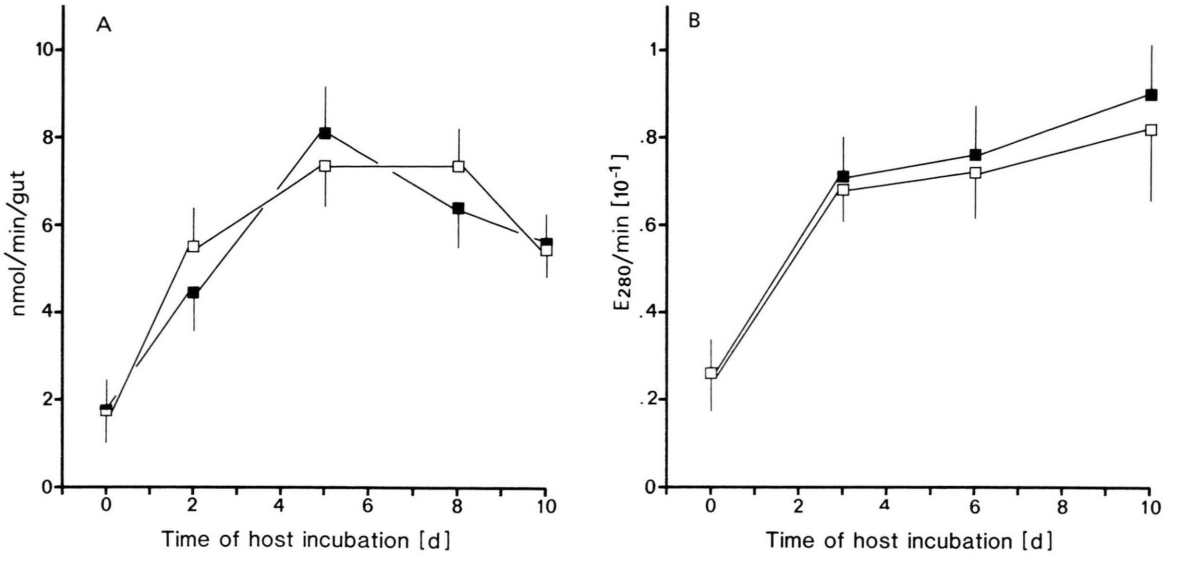

Fig. 5. Effect of azadirachtin A (1 μg/ml) fed to adult *R. prolixus* females by a blood meal on cathepsin activity of the midgut. Each point in the two curves represents the average for cathepsin B (5A) and cathepsin D (5B) out of four animals (± S.D.). Midgut from the control (■) and the azadirachtin A (□) group animals from Fig. 4 were used for this experiment.

imum activity 2–4 h after a blood meal [18]. An extensive study on the origin, distribution in gut regions, and properties of the major *R. prolixus* midgut hydrolases has just been published [21]. The trypanosomes primarily adhere to the rectal wall from where they are released with the feces. This behaviour reflects the high specificity of the parasite for its triatomine host, and within the host for the rectal wall or even specifically the rectal gland. What kinds of stimuli are responsible for this insect-parasite interaction? The answer is completely unknown. The molecular aspects of the interaction *T. cruzi* and its invertebrate host has been discussed on the basis of present knowledge [4]. An overview of the life cycle of *T. cruzi* in the insect vector has been published by Zeledon [22]. No biochemical indication for the prerequisite of the parasite's specific growth in the intestine of its blood-sucking host has been quoted by these authors.

Azadirachtin decreases or even abolishes the trypanosomid parasite *T. cruzi* from the gut of its host, *R. prolixus*. The time of drug application plays a minor role. Preincubation and therefore concomitant feeding with parasites and azadirachtin A (Fig. 1) decreases the amount of parasites as well as if the insect is treated after (Fig. 2) or before (Fig. 3) infection with the parasite. However, neither maintenance of *T. cruzi* in LIT medium in presence of azadirachtin did affect its development, nor preincubation of the parasite with the drug reduced its infectivity to mice (unpubl. results). From these results it is clear that the curative effect of azadirachtin is inevitably linked with the insect host.

Two possibilities for an explanation of this host-specific growth and differentiation of *T. cruzi* can be postulated on the basis of our present knowledge of azadirachtin mode of action. Either it acts indirectly through the extensive changes in the endocrine control of the insect's growth and development, or directly by either growth inhibition of the parasite or intoxication of the intestine. Whereas a direct toxic effect on the parasite is less probable due to our results in LIT medium and in mice, an intoxication of the gut could be discussed on the basis of results reported by Mordue *et al.* [9, 23] from experiments with *Locusta migratoria*. When the authors applied pharmacological doses of azadirachtin, up to 80 μg/g (the ED_{50} for *L. migratoria* is about 1.5 μg/g), they found an effect on the explanted gut of this insect when measuring the frequency of contractions. Our

own results, however, do not show any effects of non-toxic azadirachtin doses on such sensitive parameters of intestinal physiology like the hemolytic activity in the crop (Fig. 4) or the cathepsin B- and D-like proteolytic activity in the midgut (Fig. 5) of *R. prolixus*. Hemoglobin is the main nutritive source for the hematophagous insect. Consequently, any toxic azadirachtin effects should also be reflected by changes in the hemolytic activity of the crop. The two proteolytic enzymes are dominant in the midgut [21] and are primarily needed for hemoglobin digestion. We can conclude from our results that the gut is not intoxicated by the drug, and therefore this is not an explanation for the *T. cruzi* curing effect of azadirachtin in *R. prolixus*.

The mode of azadirachtin action is not yet understood on its molecular basis. However, recent studies on absorption, storage, organ distribution and excretion of the drug in *R. prolixus* [24] show a fairly identical influence like in *L. migratoria* [16]. Extensive studies in both species have shown a strong effect on the neuroendocrine control of hormone titers [5–8, 10–14, 16]. Only minute amounts of the molecule are retained in *L. migratoria*, most of it unchanged in the Malpighian tubules [25]. With our present data we postulate an indirect effect of the drug through an altered endocrine situation of *R. prolixus* which then affects growth and development of its parasite *T. cruzi* in a still unknown way. Studies for identifying the molecular basis of this curing factor are in progress in our laboratories.

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