Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *87*, Suppl. V, 69-72, 1992

EFFECTS OF AZADIRACHTIN ON *RHODNIUS PROLIXUS:* IMMUNITY AND *TRYPANOSOMA* INTERACTION

PATRICIA DE AZAMBUJA & ELOI S. GARCIA

Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, Av. Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brasil

The effects of azadirachtin, a tetranortriterpenoid from the neem tree Azadirachta indica *J., on both immunity and* Trypanosoma cruzi *interaction within* Rhodnius prolixus *and other triatomines, were presented. Given through a blood meal, azadirachtin affected the immune reactivity as shown by a significant reduction in numbers of hemocytes and consequently nodule formation following challenge with* Enterobacter cloacae β*12, reduction in ability to produce antibacterial activities in the hemolymph when injected with bacteria, and decreased ability to destroy the infection caused by inoculation of* E. cloacae *cells. A single dose of azadirachtin was able to block the development of* T. cruzi *in* R. prolixus *if given through the meal at different intervals, together with, before or after parasite infection. Similarly, these results were observed with diferent triatomine species and different strains of* T. cruzi. *Azadirachtin induced a permanent resistance of the vector against reinfection with* T. cruzi. *The significance of these data is discussed in relation to the general mode of azadirachtin action in insects.*

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

This paper reviews the recent knowledge on the effects of azadirachtin on *Rhodnius prolixus,* and partly points out those areas where more research is needed. In fact, it often seems that our research on the azadirachtin has simply led to the feeling that its mode of action is extraordinarily complex and remains poorly understood.

Azadirachtin, a tetranortriterpenoid isolated from the indian neem tree, *Azadirachta indica* A Juss (Meliaceae), and its closely related tree, *Melia azedarach,* has been recognized as a compound with antifeedant and growth disruption properties to insects and nematods in general (Warthen, 1979; Jacobson, 1986; Rembold, 1989) and *Rhodnius prolixus,* in particular (Garcia et al., 1984; 1986; 1987). Azadirachtin seems strongly to interfere with the prothoracicotropic hormone release from the brain of *Rhodnius* larvae, and consequently affect the production of ecdysteroids by the prothoracic glands causing ecdysial stasis (Garcia et al., 1990). Therefore, azadirachtin is considered a bioracional control agent for insects due to its high efficiency against insect pests, and its apparent non-toxicity to vertebrates.

Azadirachtin has a complex structure which was initially proposed by Zanno et al. (1975). Recently, azadirachtin structure has been determined in several laboratories using X-ray diffraction and nuclear magnetic resonance (Kraus et al., 1985). Rembold et al. (1984) demonstrated that several azadirachtins (A-D) occur in the neem seed but azadirachtin A is the predominant neem compound bearing growth-disruption effects upon insects.

AZADIRACHTIN AND IMMUNITY

The efficiency to remove microorganisms and parasites from the circulation is one of the factors which contributes to the enormous evolutionary success of insects. Usually, they use a variety of mechanisms which can recognize and eliminate bacterial and metozoan parasites (Boman & Hultmark, 1981; Ratcliffe et al., 1985). Therefore, insects exhibit cellular (hemocytes involved in phagocytosis, nodule formation or encapsulation), and humoral (antibacterial polypeptides) mechanisms, as

These studies were financed by the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq), by the Fundação Oswaldo Cruz from Brazil, and by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR).

well as non-self recognition (agglutinins and prophenoloxidases) and cell cooperation to counteract the diverse spectrum of would-be pathogens (Ratcliffe & Rowley, 1979; Pendland et al., 1988).

Regarding earlier investigations on humoral immunity in *R. prolixus* it has been shown that inoculation of bacteria induced antibacterial and lysozyme activities in cell-free hemolymph (Azambuja et al., 1985; 1986; 1989; Azambuja & Garcia, 1987).

Azambuja et al. (1991) have shown that 5th-instar larvae of *R. prolixus* had a constant number of hemocytes during two weeks after feeding. The inoculation of *Enterobacter cloacae* cells immediately causes a sharp increase in total hemocyte numbers (THC) up to day 7 which is followed by a progressive decline of hemocyte numbers. These authors have clearly shown that the ingestion of azadirachtin prevented the increase in the THC. Furthermore, they have observed that the primary infection obtained by inoculating *E. cloacae* was not destroyed by the defense reactions in the azadirachtin-treated insects. Conversely, in controls the rate of bacterial elimination from the hemolymph was constant and the bacteria were almost completely cleared by day 10 after feeding. The elimination of bacteria was coincident with the maximal number of hemocytes in the hemolymph. Therefore, they strongly suggest that the hemocytes were diminished after azadirachtin treatment so that the inoculated bacteria were still present in insects receiving this compound (Azambuja et al., 1991).

We have also demonstrated that the elimination of *E. cloacae* from the hemolymph is not due to the induction of antibacterial factors since bacteria were resistant to these factors. Taken these facts together, we have suggested that the number of viable *E. cloacae* from the insect hemolymph decreased likely due to the formation of nodules scattered through the hemolymph. Actually, nodule formation was recognized within one day after feeding, and it was formed from groups of hemocytes, packed with phagocytosed bacteria, adhering together. Small nodules floated freely in the hemolymph and larger melanized hemocytic aggregates could be seen attached to the fat bodies and muscles of dissected insects. Control insects inoculated with *E. cloacae* had a higher number of nodule formation when compared with azadirachtin-treated ones. Therefore, azadirachtin greatly reduced the ability of the bacteria infection to initiate hemocyte aggregates in the hemolymph (Azambuja et al., 1991).

The observations raise questions regarding the mechanism(s) of action of azadirachtin on *Rhodnius*. Only few aspects of the control of humoral and cell-mediated insect immunity are understood and, for an adequate interpretation of these results, the relationship between the neuroendocrine and immune system needs profound investigation. The basic idea regarding the involvement of the endocrine system in the hemocyte regulation in *R. prolixus* comes from the work by Jones (1967) using ligature experiments. He showed that ecdysteroids can regulate the number of hemocytes as well as the volume of hemolymph and induce hemocyte differentiation. Similarly, in the last larval instar of *Locusta* the prothoracic glands stimulate production and differentiation of hemocytes (Hoffmann, 1970). It remains to be shown the role of ecdysteroids and brain neurosecretion on the regulation of the immune system of *Rhodnius*.

INHIBITION OF TRYPANOSOME-VECTOR INTERACTION

Trypanosoma cruzi, a flagellated protozoan, is the causative agent for Chagas' disease, which is endemic in most parts of Latin America. Classically, this parasite is transmitted by triatomine insect vectors (for review see Garcia & Azambuja, 1991). *T. cruzi* comprises a pool of parasite populations circulating among humans, insect vectors, sylvatic reservoirs and domestic animals. This parasites display different morphological and functional forms related to its complex life cycle. It alternates between vertebrate and invertebrate hosts and also alternates between dividing stages. There are noninfective epimastigotes in the insect vector midgut and amastigotes in mammalian cells, and nonreplicative but infective, metacyclic trypomastigotes in the insect vector and bloodstream trypomastigotes in mammals (see details in Brener, 1973; Zeledon, 1987; Garcia & Azambuja, 1991).

Since we have previously established that azadirachtin induced short- and long-term inhibition of the moulting (Garcia et al., 1984), it should be of interest to infect with *T. cruzi*, insects treated or not with azadirachtin. When we have done this we found that azadirachtin

might affect normal development of *T. cruzi* in the insect vector (Garcia, 1988). This compound, given through the bloodmeal at different intervals, together with, before or after infection with *T. cruzi,* drastically reduced the number of trypanosomes in the gut of the insect vector (Garcia et al., 1989a, b; Rembold & Garcia, 1989). Since azadirachtin (i) did not kill the parasites directly neither azadirachtin-treated infected blood remained infected nor the compound altered the development of the parasites in LIT medium, and did not interfere with the trypanosome infection in mammalian hosts (Garcia et al., 1989a), it has been suggested that azadirachtin may act on the parasite-vector interaction through the insect.

Having demonstrated that azadirachtin influences the development of *T. cruzi,* in 5th-instar larvae of *R. prolixus,* we have also shown that the ED_{50} to inhibit the parasite development in the gut was 0.25 µg/ml bloodmeal and that a single dose of azadirachtin was enough to both inhibit the growth of the insect vector and impair resistance against its reinfection with trypanosomes for several months (Gonzalez & Garcia, 1992). This explains why the elimination of *T. cruzi* through urine and faeces of *R. prolixus* was completely blocked, and that the insect was not longer an efficient vector for Chagas disease after azadirachtin treatment (Gonzalez & Garcia, 1992). These results were confirmed in other triatomine vector species infected with different strains of *T. cruzi.* In fact, an unique dose of azadirachtin (1.0 µg/ml of bloodmeal) was also able to inhibit the development of two different *T cruzi* strains in *Triatoma infestans* and *Dipetelogaster maximus* (Gonzalez & Garcia, 1992). The results have strongly suggest that azadirachtin has the same effect in all triatomine species.

Two hypotheses have been postulated to explain the effect of azadirachtin on the *T. cruzi* development within its insect vector. Firstly, the compound may act indirectly through the extensive changes in the neuroendocrine system which can effect the gut environment. Secondly, azadirachtin may directly affect the gut physiology. Both hypotheses assume that azadirachtin causes changes in the gut in such way that digestive tract is no longer an acceptable microenvironment for trypanosome survival and establishment of infection in the insect vector.

CONCLUDING REMARKS

In this paper, whenever possible, the effects of azadirachtin on *R. prolixus* was discussed in terms of the results concerning both the development of *T. cruzi* and immunity of this hematophagous insect, and its mode of action upon the endocrine system. Emphasis was given in the effect of this compound on interaction of different strains of *T. cruzi* within different species of vectors and on the cellular and humoral reactions of *R. prolixus.* However, the literature on these topics is scanty and fragmentary, and the studies in our laboratory are still only at the beginning.

The state of the art regarding the action of azadirachtin in *R. prolixus* is, in great part, defined by the fact that only few laboratories in the world work with this compound in a purified form. Yet, it is obvious that further studies are necessary, mainly in respect of how azadirachtin blocks the release of neurohormones and how it induces the ecdysone deficiency causing long-term effects on several physiological events of *R. prolixus.* Therefore, several questions remain unanswered to challenge azadirachtin research in the forthcoming years.

ACKNOWLEDGEMENTS

To Dr W. Savino (FIOCRUZ) for helpful review of the manuscript.

REFERENCES

AZAMBUJA, P.; FREITAS, C. C. & GARCIA, E. S., 1985. Induction of antibacterial activity in the hemolymph of *Rhodnius prolixus. An. Acad. bras. Cienc., 57:* 133-134.

AZAMBUJA, P.; FREITAS, C. C. & GARCIA, E. S., 1986. Evidence and partial characterization of an inducible antibacterial factor in the haemolymph of *Rhodnius prolixus. J. Insect Physiol., 32:* 807-817.

AZAMBUJA, P. & GARCIA, E. S., 1987. Characterization of inducible lysozyme activity in the hemolymph of *Rhodnius prolixus. Braz. J. Med. Biol. Res., 20:* 539-548.

AZAMBUJA, P.; GARCIA, E. S.; RATCLIFFE, N. A. & WARTHEN, J. D. Jr., 1991. Immune-depression in *Rhodnius prolixus* induced by the growth inhibitor, azadirachtin. *J. Insect Physiol., 37:* 771-777.

AZAMBUJA, P.; MELLO, C. B. & GARCIA, E. S., 1989. Immunity to *Rhodnius prolixus:* inducible peptides against bacteria and trypanosomes, p. 270-276. In D. Borovski & A. Spielman (eds), *Host Regulated Developmental Mechanisms in Vector Arthropods,* Florida University Press, Vero Beach.

BOMAN, H. G. & HULTMARK, D., 1981. Cell-free

immunity in *Cecropia* pupae. *Trends Biochem. Sci., 6:* 306-309.

BRENER, Z., 1973. Biology of *Trypanosoma cruzi*. *Ann. Rev. Microbiol., 27:* 347-382.

GARCIA, E. S., 1988. Effects of precocene and azadirachtin on the development of *Trypanosoma cruzi* in *Rhodnius prolixus*. *Mem. Inst. Oswaldo Cruz, 83* (Suppl. I): 578-579.

GARCIA, E. S. & AZAMBUJA, P., 1991. Development and interactions of *Trypanosoma cruzi* within the insect vector. *Parasitol. Today, 7:* 240-244.

GARCIA, E. S.; AZAMBUJA, P.; FORSTER, H. & REMBOLD, H., 1984. Feeding and molt inhibition by azadirachtin A, B, and 7-acetyl azadirachtin in *Rhodnius prolixus* nymphs. *Z. Naturf., 39c:* 1155-1158.

GARCIA, E. S.; FEDER, D.; LIMA GOMES, J. E. P. & AZAMBUJA, P., 1987. Effects of precocene and azadirachtin in *Rhodnius prolixus*. *Mem. Inst. Oswaldo Cruz, 87* (Suppl. III): 67-73.

GARCIA, E. S.; GONZALEZ, M. S.; AZAMBUJA, P. & REMBOLD, H., 1989a. Chagas' disease and its insect vector. Effect of azadirachtin A on the interaction of a triatomine host (*Rhodnius prolixus*) and its parasite (*Trypanosoma cruzi*). *Z. Naturf., 44c:* 317-322.

GARCIA, E. S.; GONZALEZ, M. S. & REMBOLD, H., 1989b. Chagas' disease and its vector. Curing effect of azadirachtin A in the triatomine host, *Rhodnius prolixus*, from its parasite, *Trypanosoma cruzi*, p. 363-370. In D. Borovsky & A. Spielman (eds), *Host Regulated Developmental Mechanisms in Vector Arthropods*, Florida University Press, Vero Beach, Fl.

GARCIA, E. S.; LUZ, N.; AZAMBUJA, P. & REMBOLD, H., 1990. Azadirachtin depresses the release of prothoracicotropic hormone in *Rhodnius prolixus* larvae: evidence from head transplantations. *J. Insect Physiol., 36:* 679-682.

GARCIA, E. S.; UHL, M. & REMBOLD, H., 1986. Azadirachtin, a chemical probe for the study of moulting processes in *Rhodnius prolixus*. *Z. Naturf., 41c:* 771-775.

GONZALEZ, M. S. & GARCIA, E. S., 1992. Effect of azadirachtin on the development of *Trypanosoma cruzi* in the insect vector: Long-term and comparative studies. *J. Invertebr. Pathol., 60:* 201-205.

HOFFMANN, J. C., 1970. Endocrine regulation of the production and differentiation of haemocytes in an Orthopteran insect: *Locusta migratoria*. *Gen. comp. Endocr., 15:* 198-219.

JACOBSON, M., 1986. The neem tree: Natural resistance par excellence. *Am. Chem. Soc. Symp. Ser., 296:* 220-232.

JONES, J. C., 1967. Effects of repeated haemolymph withdrawals and of ligaturing the head on differential haemocyte counts of *Rhodnius prolixus*. *J. Insect Physiol., 13:* 1351-1360.

KRAUS, W.; BOKEL, M.; KLENT, A. & PEOHNL, R., 1985. The structure of azadirachtin and 22,23-dehydro23β-methoxy azadirachtin. *Tetrahedron Lett., 26:* 6435-6438.

PENDLANT, J. C.; HEATH, M. A. & BOUCIAS, D. G., 1988. Function of a galactose-binding lectin from *Spodoptera exigua* larval haemolymph: Opsonization of blastospores from entomogenous hymphomycetes. *J. Insect Physiol., 34:* 533-540.

RATCLIFFE, H. G. & ROWLER, A. F., 1979. Role of hemocytes in defense against biological agents, p. 331-414. In A. P. Gupta *Insect Haemocytes, Development, Forms, Functions, and Techniques*, Cambridge University Press, Cambridge, Ma.

RATCLIFFE, N. A.; ROWLER, A. F.; FITZGERALD, S. W. & RODES, C., 1985. Invertebrate immunity: basic concepts and recent advances. *Int. Rev. Cytol., 97:* 183-350.

REMBOLD, H., 1989. The azadirachtins – their potential for insect control. *Econ. Med. Plant Res., 3:* 903-907.

REMBOLD, H.; FORSTER, H.; CZOPPELT, CH., RAO, P. J. & SIEBER, K. P., 1984. The azadirachtins, a group of insect growth regulators from the neem tree, p. 153-162. In H. Schmutterer & K. R. S. Ascher (eds), *Natural pesticides from the neem tree (azadirachta indica A. Juss) and other tropical plants*, Eschborn: GTZ.

REMBOLD, H. & GARCIA, E. S., 1989. Azadirachtin inhibits *Trypanosoma cruzi* infection of triatomine insect host, *Rhodnius prolixus*. *Naturwisseschaften, 76:* 77-78.

WARTHEN, J. D. Jr., 1979. *Azadirachta indica:* A source of insect feeding inhibitors and growth regulators. *U. S. Dept. Agric. Res. Results*, ARR-NE-4.

ZANNO, P. R.; MIURA, I.; NAKANISHI, K. & ELDER, D. L., 1975. Structure of insect phagorepellent azadirachtin. Applications of PRFT CWD Carbon-13 nuclear magnetic resonance. *J. Am. Chem. Soc., 97:* 1975-1977.

ZELEDON, R., 1987. The development of trypanosoma cruzi within the vector, p. 59-75. In R. R. Brenner & A. M. Stoka (eds), *Chagas Disease Vectors (Anatomical and Physiological Aspects, Vol. II)*, CRC Press.