The Influence of Hydrocortisone on Cellular Defence Mechanisms of *Biomphalaria glabrata*

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Since the internal defense system of mollusks consists of cellular and humoral mechanisms, we examined the role of hydrocortisone in mollusks defense cells and the influence of this steroid on the development of Schistosoma mansoni *in its intermediary host. Hydrocortisone had an immunosuppressive action in* Biomphalaria glabrata, *as reflected in the reduced number of defense cells and the altered cell physiology. Histopathological analysis showed that hydrocortisone facilitated the intramolluscan development of* S. mansoni, *by reducing the extent of the inflammatory response, seen as a greater number of viable sporocysts with no surrounding hemocytes.*

Key words: *Schistosoma mansoni* - *Biomphalaria glabrata* - hydrocortisone - sporocyst - hemocyte reaction

Hydrocortisone, like other corticosteroids, has immunosuppressive and anti-inflammatory actions, and is widely used to control inflammatory responses in infections, allergies and anaphylaxis (Stites et al. 1997). At high concentrations, hydrocortisone attenuates cellular defense reactions and delays the migration of phagocytic cells to the traumatized area by reducing vasodilatation and the subsequent vascular permeability. Corticosteroids inhibit the late manifestations of the inflammatory process, such as capillary and fibroblast proliferation, collagen deposition and wound healing (Ferri et al. 1977, Grodsky 1977).

The internal defense system of snails consists of cellular and humoral defense mechanisms (Ratcliffe 1985, Van der Knaap & Loker 1990). The hemocytes are freely circulating cells found in the hemolymph and represent the principal means of internal defense in mollusks and other invertebrates (Sima & Vetvicka 1990, Shiff 1994). These cells are brought about "Amebocytes Producing Organ" – APO (Amebocytes = Hemocytes). This organ is located at renopericardic region (Lie at al. 1975, Jeon et al. 1983, Sima & Vetvicka 1990). Hemocytes can move freely to and within tissues since mollusks have an open vascular system (Lie et al. 1987, Loker & Bayne 1988, Van der Knaap & Loker 1990).

Two main types of hemocytes (granulocytes and hyalinocytes) occur in the hemolymph of the mollusk *Biomphalaria glabrata*. The granulocytes, which are recognized by these many pseudopodia and are similar in appearance to mammal nerve cells, are responsible for phagocytosis and the immobilization of parasites by encapsulation (Muller 1985, Ratcliffe 1985). The cytoplasmic granules of these cells, which are enzymes producer, are know as real lysosomes (Cheng & Garrabant 1977, Cheng & Butler 1979, Lie et al. 1987, Ottaviani & Franchini 1988). The hyalinocytes, which are smaller than granulocytes, are spherical and have no pseudopodia. These cells have a poorly defined role in defense and there is evidence that they react to soluble antigen (Cheng & Garrabant 1977). The granulocytes and hyalinocytes have been suggested to be two different cell types (Sminia & Van der Knaap 1987, Lie et al. 1987), although others (Seta et al. 1996) believe that these cells represent different stages of development of the same cell type. Pan (1996) suggested that, under appropriate conditions, *B. glabrata* hyalinocytes can become granulocytes.

Reis et al. (1995) noted a direct correlation between the resistance to infection by *Schistosoma mansoni* and sporocyst death; this observation suggested that hemocytes were an important factor in fighting infections. Kassim and Richards (1979), Sullivan and Richards (1981), and Guaraldo et al. (1981) confirmed that sporocyst development in susceptible mollusks was slow, whereas in non-susceptible mollusks the sporocysts were surrounded by defense cells and were quickly killed. The granulomatous reaction, which provokes phagocytosis and larval killing soon after parasite penetration, is mediated by granulocytes which have a large phagocytic capacity (Pan 1965, Bayne et al. 1980, Lie et al. 1980, Guaraldo et al. 1981).

In previous work, verifying the influence of hydrocortisone on *S. mansoni* development in *B. glabrata*, we observed larger infection rate in the mollusks treated with hydrocortisone. In addition, mollusks treated with hydrocortisone released a greater number of cercariae and the time to the start of larvae elimination was shorter than that observed in untreated snails (Serrano et al. 2002). These results, as well as hydrocortisone effect in mammal defence mechanisms, principally in phagocytic cells, led us to examine whether hydrocortisone could exert an immunosuppressive action on the hemocytes of *B. glabrata* and prevent the hemocyte reaction around trematode larvae, hereby facilitating the development of the parasite.

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MATERIALS AND METHODS

Albino *B. glabrata*, 7-8 mm in diameter, from Belo Horizonte (BH). The snails were housed in the Department of Parasitology, Institute of Biology, Unicamp. The *S. mansoni* strain used was from BH (Paraense & Correa 1963).

The experimental groups used were: group I - non-infected mollusks which were not treated with hydrocortisone; group II - non-infected mollusks treated with hydrocortisone; group III - infected mollusks not treated with hydrocortisone; group IV - infected mollusks treated with hydrocortisone.

Eighty mollusks (20 per group) were used.

For the treatment with hydrocortisone, the mollusks were placed in chlorine-free water (4 mollusks per 100 ml) containing 0.3 ml of hydrocortisone solution (Solu-Cortef, 125 mg/ml). The water was changed every 24 h for four days, with new hydrocortisone added each time. On the fifth day the mollusks were placed in chlorine-free water. The treated (group IV) and not treated (group III) mollusks were infected on the second day of hydrocortisone treatment in group IV by exposing them individually to 10 miracidia at 28°C.

For a differential counts of circulating hemocytes, hemolymph was collected from all groups at 0.5, 1, 3, 5, 7, 9, 11, 24, 48 and 72 h after the end of treatment in groups II and IV. Two mollusks from each group were used at each time interval. Hemolymph was collected via the cephalopodal region with a Pasteur pipette (Michelson 1966) and the samples immediately placed in a Neubauer chamber for cell counting using phase contrast microscopy. Differential counting allowed the identification of granulocytes and hyalinocytes.

Histopathological analyses were done on mollusks from groups III and IV (n = 24 each). The snails were infected individually by exposing them to 100 miracidia of *S. mansoni*. Three mollusks from each group were then fixed in Bouin's fixative at 12, 24, 48, 72 h and 7, 14, 21 and 28 days after ending the treatment with hydrocortisone in group IV. After a 48 h fixation, the snails were removed from their shells and the body embedded in paraffin. Body sections 5 µm thick were stained with Gomori's trichromic (Guaraldo et al. 1981). The sections were examined by light microscopy and the primary sporocysts were scored for viability, for the presence of hemocyte reactions around the larvae and for the type of cells involved in the reactions. The location, viability and maturity of secondary sporocysts were also recorded.

RESULTS

The number of hemocytes in treated mollusks was always smaller than in untreated mollusks. In all groups and at all times, the number of granulocytes was greater then that of hyalinocytes (Figs 1, 2). The hemocytes in the hemolymph were differentiated into granulocytes and hyalinocytes. The granulocytes are star-shaped cells with cytoplasmic granules and are able to form pseudopodia which allow them to adhere to surfaces. In contrast, hyalinocytes are round cells which do not emit pseudopodia and are not adhesive.

There was a predominance of degenerated primary sporocysts in untreated mollusks (group III), whereas in treated mollusks (group IV) the number of viable primary sporocysts was greater (Fig. 3). Whereas 86.2% of the primary sporocysts in the tissues of group III snails were degenerated (13.8% viable), in group IV snails, 74.2% of the primary sporocysts were viable (25.8% degenerated). The presence of a pyknotic nucleus and an eosinophilic cytoplasm characterized the degenerated sporocysts, most of which were surrounded by hemocytes that isolated the larva of the surrounding circumjacent tissue. The hemocytes in this case appeared extended, with characteristics similar to fibroblasts (Fig. 5A)

Fig. 4 shows the number of hemocyte reactions around primary sporocysts in mollusks of groups III and IV. In untreated *B. glabrata*, most of the primary sporocysts

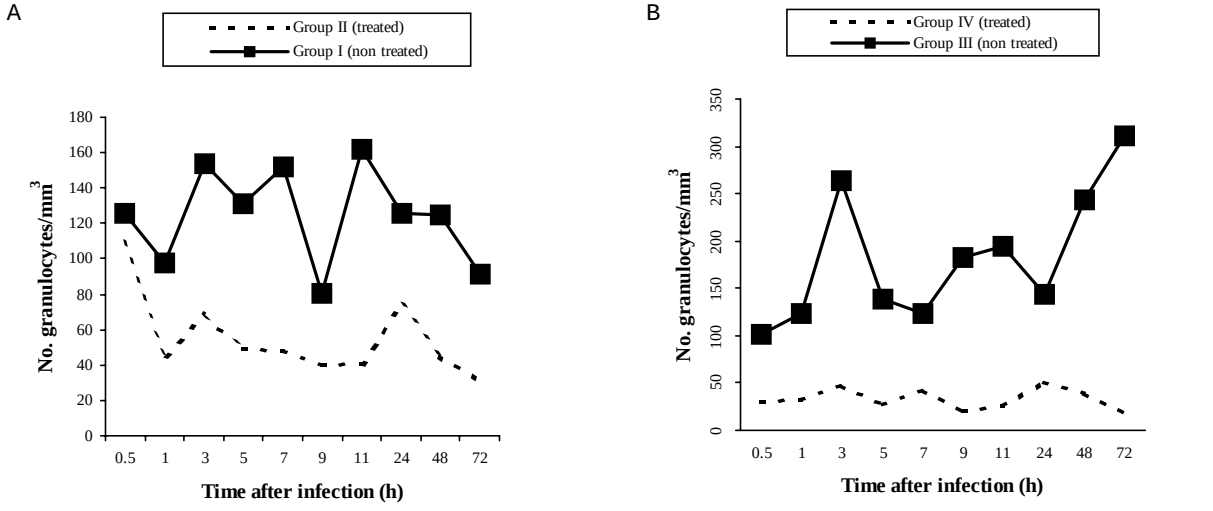

Fig. 1: average number of circulating granulocytes in A: uninfected *Biomphalaria glabrata*; B: *B. glabrata* infected with *Schistosoma mansoni*.

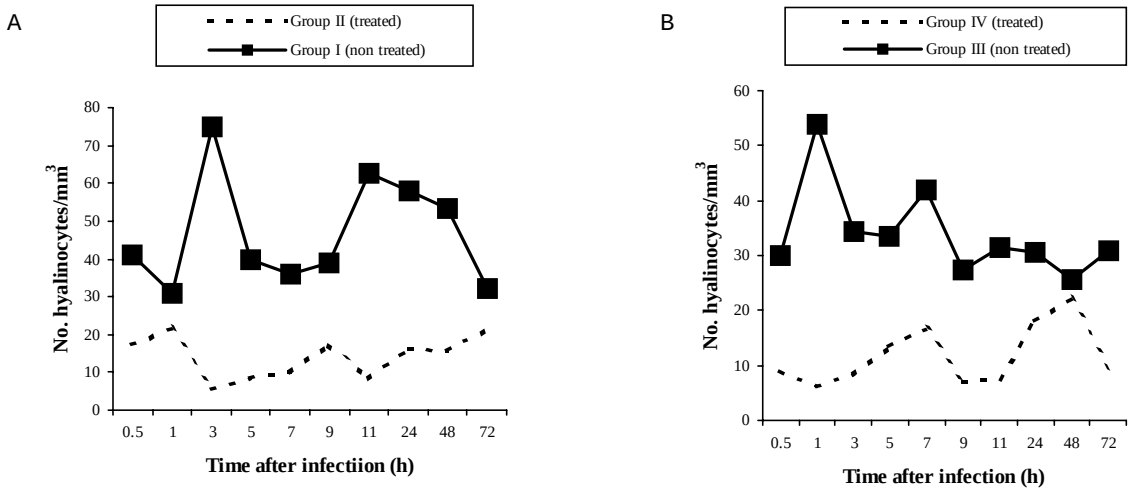

Fig. 2: average number of circulating hyalinocytes in A: uninfected *Biomphalaria* glabrata; B: *B. glabrata* infected with *Schistosoma mansoni*.

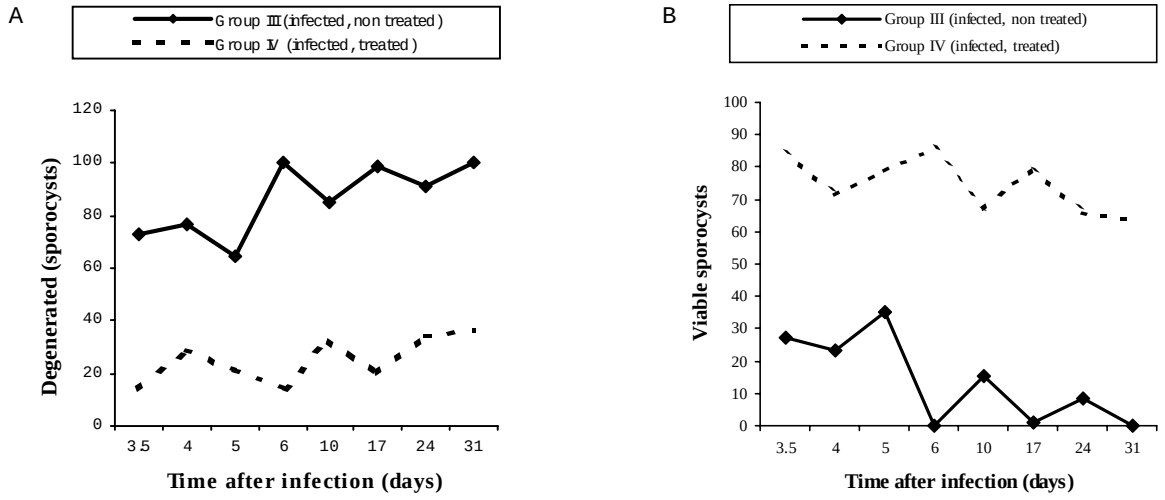

Fig. 3: average number of primary sporocysts in *Biomphalaria glabrata* exposed to 100 *Schistosoma mansoni* miracidia. A: degenerated sporocysts; B: viable sporocysts.

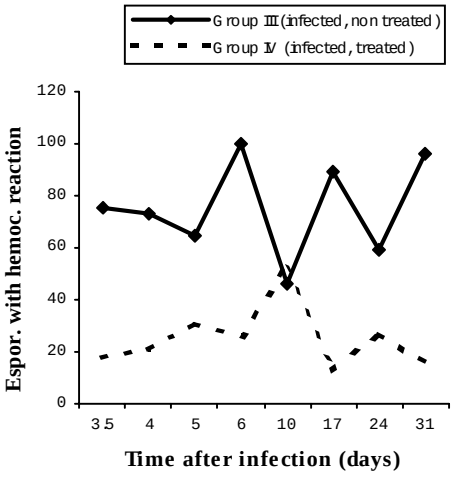

Fig. 4: average number of primary sporocysts with a surrounding reaction in *Biomphalaria glabrata* exposed to 100 *Schistosoma mansoni* miracidia.

(75.4%) were surrounded by an amebocyte reaction (Fig. 5A), whereas in treated mollusks most of the primary sporocysts (74.1%) were not involved in such a reaction. With few exceptions, the hemocytes in reactions around sporocysts were elongated, as in mammalian fibroblasts.

The first secondary sporocysts were seen in treated mollusks (group IV), and most were viable; degenerated secondary sporocysts predominated in untreated mollusks (group III). The tissue distribution of these sporocysts was similar in both groups but, quantitatively, there were more of these cells in treated mollusks.

Cercarial shedding was not examined in this experiment but, in a previous study (Serrano et al. 2002), we observed more cercarial shedding in mollusks treated with hydrocortisone. Thus in snails treated with hydrocortisone, a total of 69,519 cercariaes was obtained, whereas in untreated mollusks, only 2,514 larva were obtained. This data were significantly different ($p = 0.0001$).

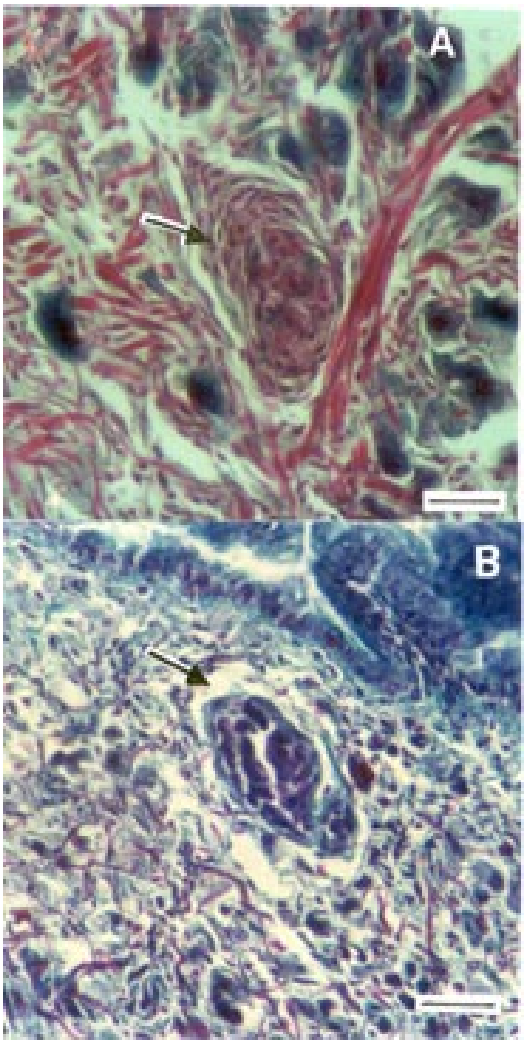

Fig. 5: cephalopodal region of *Biomphalaria glabrata* infected with *Schistosoma mansoni*. Gomori's trichromic: A: 3 days after infection; not treated with hydrocortisone. Note the presence of degenerated primary sporocyst. The arrow indicates an intense hemocyte reaction surrounding the sporocyst. Bar = 20 µm; B: 3 days after infection; treated with hydrocortisone. Note the presence of viable primary sporocyst. The arrow indicate an mid hemocyte reaction surrounding the sporocyst. Bar = 20 µm

DISCUSSION

Our results confirmed the data of Cheng (1975), Cheng and Auld (1977), and Jeong and Heyneman (1976) regarding the existence of two types of defense cells in molluscan hemolymph with granulocytes being more numerous than hyalinocytes. Hydrocortisone had an immunosuppressive effect on both cell types in *S. mansoni* infected (group IV) and non-infected (group II) mollusks. Granulocytes were the most affected by hydrocortisone and were fewer in treated mollusks (Figs 1, 2).

Joky et al. (1985) and Seta et al. (1996) observed an increase in the number of hemocytes in the first 72 h after infection with *S. mansoni*. Since in our study hydrocortisone affected the number of hemocytes, it is probable that the steroid may act on the APO (amebocyte-producing organ) of the mollusks (Jeong et al. 1983) to reduce the number of defense cells produced and/or retard their maturation. The reduction in hemocytes after treatment with hydrocortisone could also result from a direct action of the drug on these cells.

There was a significant interaction between treatment and infection in altering the number of granulocytes since group IV mollusks (infected-treated) showed the greatest reduction in the number of defense cells. Hyalinocytes were less affected by this treatment. Time had no effect on the number of cells.

Ferri et al. (1977) reported that hydrocortisone inhibited macrophage mobilization in humans. Granulocytes, like macrophages, have pseudopodia and phagocytic activity. In addition to affecting the number of defense cells, hydrocortisone also reduced the number of sporocysts with a surrounding hemocyte reaction (Fig. 4). This action may be directly on the hemocytes to affect their mobilization and the production of pro-phagocytic substances. This response could help to prevent hemocyte reactions around the trematode larvae, thereby allowing development of the parasite.

Histopathological analysis revealed similar numbers of viable and degenerated primary sporocysts. The notable characteristic of viable sporocysts is the absence of an hemocyte reaction surrounding the larva. These sporocysts contain somatic and germ cells (Cheng & Bier 1972). The somatic cells are round and of variable size, with a modestly basophilic cytoplasm and slender basophilic granulations that extend to the external membrane. The single nucleus is generally central and round and occupies about ¼ of the cell volume. The nucleolus is sometimes prominent. The nucleus of germ cells is vesicular and the cytoplasm is very rich in granulations. Both cells are surrounded by a polysaccharide membrana (Guaraldo et al. 1981).

The degenerated sporocysts (Fig. 5A) generally have a pyknotic nucleus; the cytoplasm is eosinophilic and the cytoplasmic granules tend to group together. The hemocytes concentrate in large numbers around the larva in degeneration isolating the adjacent tissue. The nucleus and cytoplasm of the hemocytes are similar to those of a fibroblast. The cytoplasm contain eosinophilic granulations that perhaps stores of secretion produts. During degeneration, the sporocysts loose their usual appearance to become eosinophilic and granulous, and form a mass surrounded by hemocytes. When degeneration is advanced, only hemocytes remain surrounding a region that gradually filled up with conjunctive tissue. However, some spaces were occupied by larvae (Guaraldo et al. 1981).

The treated group (group IV) showed a greater number of viable sporocysts with no hemocyte reaction (Fig. 3); but when this reaction occurred, it was more discrete than in group III (Fig. 5B). This attenuated response probably reflected the reduced number of defence cells and the inhibition of their ability to penetrate the traumatized site and to immobilize the larvae by encapsulation (Lie et al. 1987).

Hydrocortisone did not affect the distribution of secondary sporocysts since in both groups of mollusks these

cells occurred principally in the cephalopodal region, mantle border, ureter, hepatopancreas, ovotestis and intestine, with no significant difference between the groups.

S. mansoni developed more rapidly in treated mollusks, with mature secondary sporocysts appearing 10 days after exposure to miracidia. In the untreated group, mature secondary sporocysts were observed only 24 days after infection.

In conclusion, hydrocortisone exerts immunosuppressive and anti-inflammatory effects in *B. glabrata*. The reduction in the activity and quantity of defense cells led to a more discrete inflammatory process, with a reduced or no hemocyte reaction around the sporocysts and increased the production of viable sporocysts with no hemocyte reaction.

These data are in agreement with observation of Serrano et al. (2002) who described an increased number of cercariae released by mollusks treated with hydrocortisone.

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