Changes induced in *Biomphalaria glabrata* (Say, 1818) following trials for artificial stimulation of its internal defense system

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Biomphalaria glabrata *can react through different pathways to* Schistosoma mansoni *miracidium penetration, according to the degree of resistance/susceptibility presented by different snail strains, which is a genetically determined character, resistance being the dominant feature. However, it has been observed that previous susceptible snail strain may change its reactive behavior along the course of infection, exhibiting later a pattern of cercarial shedding and histopatopathological picture compatible with high resistance. Such observation suggests the possibility of* B. glabrata *to develop a sort of adaptive immunity face a schistosome infection. To explore on this aspect, the present investigation looked for the behavior of* S. mansoni *infection in* B. glabrata *previously subjected to different means of artificial stimulation of its internal defense system. Snails previously inoculated with irradiated miracídia (Group I); treated with* S. mansoni *antigens (Group II) or with a non-related parasite antigen (Group III) were challenged with 20 viable* S. mansoni *miracidia, and later looked for cercarial shedding and histopathologic changes at different times from exposition. Nodules of hemocyte accumulations were found at the site of antigen injection. These nodules resembled solid granulomas, and were larger and more frequent in snails injected with* S. mansoni *products as compared to those injected with* Capillaria hepatica*. However, the presence of such granulomas did not avoid the* S. mansoni *challenge infection from developing in a similar way as that seen in controls. The data are indicative that hemocytes are able to proliferate locally when stimulated, such capacity also remaining localized, not being shared by the population of hemocytes located elsewhere within the snail body.*

Key words: hemocytes - *Biomphalaria glabrata* - *Schistosoma mansoni* - antigens

Previous attempts have been made to change the natural reactivity of *Biomphalaria glabrata* toward invasion by the trematode *Schistosoma mansoni*. Lie et al. (1983) observed that snails previously exposed to irradiated miracidia presented a lower infection rate (23%) when later challenged with non-irradiated miracidia, as compared with intact controls (73%). Sire et al. (1998) used instead infection with one single live miracidium and observed that this was enough to induce several degrees of alterations in the parasites derived from a challenge infection. Others have used different experimental models to investigate the development of resistance in *B. glabrata*. Sullivan et al. (1982) subjected *B. glabrata* to irradiated *Ribeiroia marini* miracidia and observed the development of resistance to *Echinostoma paraensei*. Lemos and Andrade (2001) investigated the reason for a finding already known by others, that cercarial elimination presents a progressively decreasing curve with passing time. Their data disclosed that histological changes similarly and progressively varied from no-reaction at the beginning of cercarial elimination, to diffuse hemocyte proliferation, with formation of encapsulating reactions around sporocysts and developing cercariae, at the final period of observation. This strongly suggested that a kind of adaptative immunity developed in infected and previously susceptible snails.

To find out whether this type of reactivity could be artificially induced, we executed a series of experiments so designed in order to stimulate the internal defense system of a highly susceptible *B. glabrata* strain.

MATERIALS AND METHODS

Snails - Adult *B. glabrata* snails from the Feira de Santana (FS) strain, measuring 11 to 13 mm in diameter were maintained under controlled conditions of room temperature (around 26ºC), with free access to appropriated feed. This strain has been maintained in the Laboratory for more than 20 years.

Experimental groups and procedures

Group 1 - Thirty snails were inoculated with ± 20 ml of distilled water containing 15 *S. mansoni* miracidia, which had been previously irradiated with 4000 rads from a Cesio 137 irradiator, IBL 937C, type H, from Cis Bio International, Gif-sur-Yvette, France. Injection was made at the exposed cephalo-podal region Ten days later the snails were challenged with exposition to 20 freshly eliminated non-irradiated *S. mansoni* miracidia. The snails were killed 35 and 42 days later.

A control for this group was represented by another 30 snails in which the irradiated miracidia were replaced by non-irradiated, recently eliminated ones. All the other steps were exactly the same as for the above Group.

Group 2 - Thirty snails were inoculated at the cephalo-podal region with 20 ml of a *S. mansoni* whole worm antigen in PBS, which contained 0.5 mg/ml protein concentra-

tion. Inoculation was made twice, with a 6-day interval, the challenge infection being made three days later with exposition to 20 recently eliminated miracidia. The snails were killed at the 35th, 42nd, and 49th days following the challenge infection.

Group 3 -Thirty snails submitted to all the procedures as the preceding group, in which the sole difference was that a *Capillaria hepatica* soluble antigen replaced the *S. mansoni* antigen.

A control group for the two above groups was represented by 30 snails, in which all the procedures were the same, with the difference that PBS was used instead of the antigenic preparations.

Antigen preparation - Adult worms, either *S. mansoni* or *C. hepatica*, were homogeinized in 2 ml of cold sterile PBS, pH 7.4, during 1 h. After centrifugation at 1400 rpm/min during 45 min, the supernatant were separated and maintained in a freezer at –20ºC for later measurement of protein concentration by the BCA method.

Histopathology - Previously menthol anesthetized snails were taken out of the crushed shells and fixed in Bouin fluid for 4 h, then transferred to 70% alcohol for several days. After dehydration in absolute alcohol and clearing in xylol, whole organisms were paraffin embedded and cut at 5-10 µ. Microtome sections were stained with hematoxylin and eosin.

Counting of hemolymph cells - The snail shell was perforated with a 26-G ½ insulin needle at the hepato-pancreas level. The emerging lymph was aspirated, placed in siliconized tubes (Vacuum II Labnew®) and maintained at 4ºC. Cell count was performed in a mixture of 10 µl hemolymph with a 5% neutral red solution, and examined in Neubauer chamber.

Morphormetry - Areas from histological sections stained with hematoxylin and eosin were recorded and measured under a light microscope (Zeiss Axioskop 2, Germany) provided with a TV camera (JVC TK-128OU, Japan) and a Zeiss software Axiovision 2.0. The area was calculated in μm^2, under a 10×10 magnification.

Statistical analysis - A Kruskal-Wallis non-parametric test was used for comparison of three or more independent groups, followed by a Dunn post-test. A $p < 0.05$ was considered as significant within a same group at different points of analysis or for comparison of different points from different groups, i.e., 35, 42, and 49 days post miracidial exposition.

RESULTS

Cercarial elimination - Results are depicted on Figs 1 and 2. The snails inoculated with irradiated miracidia shed less cercariae at both checking points than their respective controls. This also occurred with the snails previously sensitized with *S. mansoni* antigen, but at the 35 days post-exposure only. On the other hand, the snails sensitized with *C. hepatica* antigen exhibited a mild increase in cercarial elimination. However, none of these findings mentioned above reached statistical significance (Fig. 1A).

Lymph-cell counting - Two cell types were found in hemolymph: a dominant one, represented by large cells with excentric nucleus, abundant cytoplasm with peripheral projections, adherent to Neubauer glass chamber, and stained with neutral red; another type, much less frequent, represented by small cells, with round and small nucleus, and scanty cytoplasm. These types were not separated during counting. There was a slight increase of the cell number 42 days after cercarial exposition in snails previously injected with irradiated miracidia. Similar mild differences were noted in other groups, but in none of them such differences reached statistical significance (Fig. 1B).

Histological findings - At the point of antigenic inoculation, both of *S. mansoni* and *C. hepatica* soluble antigens, there was noted the presence of multiple nodules of hemocyte accumulations. That occurred in 92.3% of the snails inoculated with *S. mansoni* material (Figs 2A,B). The number of nodules varied from 1 to 38 per snail and they measured from 6195,07 μm^2 to 137.625,73 μm^2. These nodules remained localized at the cephalo-podal region and were similarly observed in non-infected controls and in infected snails at 35, 42, and 49 days post exposition. The presence of these hemocytic nodules did not interfered with the development of sporocysts and cercariae from the challenging infection, which was the same as that seen in the controls (Fig. 2C). The infection resulted in a disseminated proliferation of the parasite, and their forms were found without reaction in almost all snail tissues, including the areas with the hemocytic nodules (Fig. 2B).

The snail treated with *C. hepatica* antigen presented the same type of nodules at the site of injection, but less frequently than in the previous group. They occurred in 31% of the injected snails. The size of the nodules also was smaller, reaching 5250,24 μm^2 up to 11.530,76 μm^2, and were also less numerous (1 to 3 nodules per snail) in comparison with the group treated with *S. mansoni* antigen. The snails injected with PBS (control group) did not present hemocytic proliferation at the site of injection.

The snails solely injected with irradiated miracidia did not develop parasitic structures, but exhibited hemocytic nodules at the site of inoculation. These nodules were less localized that those seen in the previous groups, and a few of them were found in renal tissue, digestive glands and ovo-testis (Fig. 2D).

There were 1 to 8 nodules present in 80% of the snails, counted after the challenged infection. When these snails were submitted to infection, with live non-irradiated miracidia, the infection developed in them as disseminated and severe as that seen in the controls. This control group which consisted of snails inoculated at the cephalo-podal region with live non-irradiated miracidia, also presented hemocyte nodules at the point of inoculation, but with the challenging exposition progressing to disseminated infection.

DISCUSSION

Although the present attempts to stimulate the *B. glabrata* internal defense system did result in local proliferative hemocyte reactions having a granulomatous char-
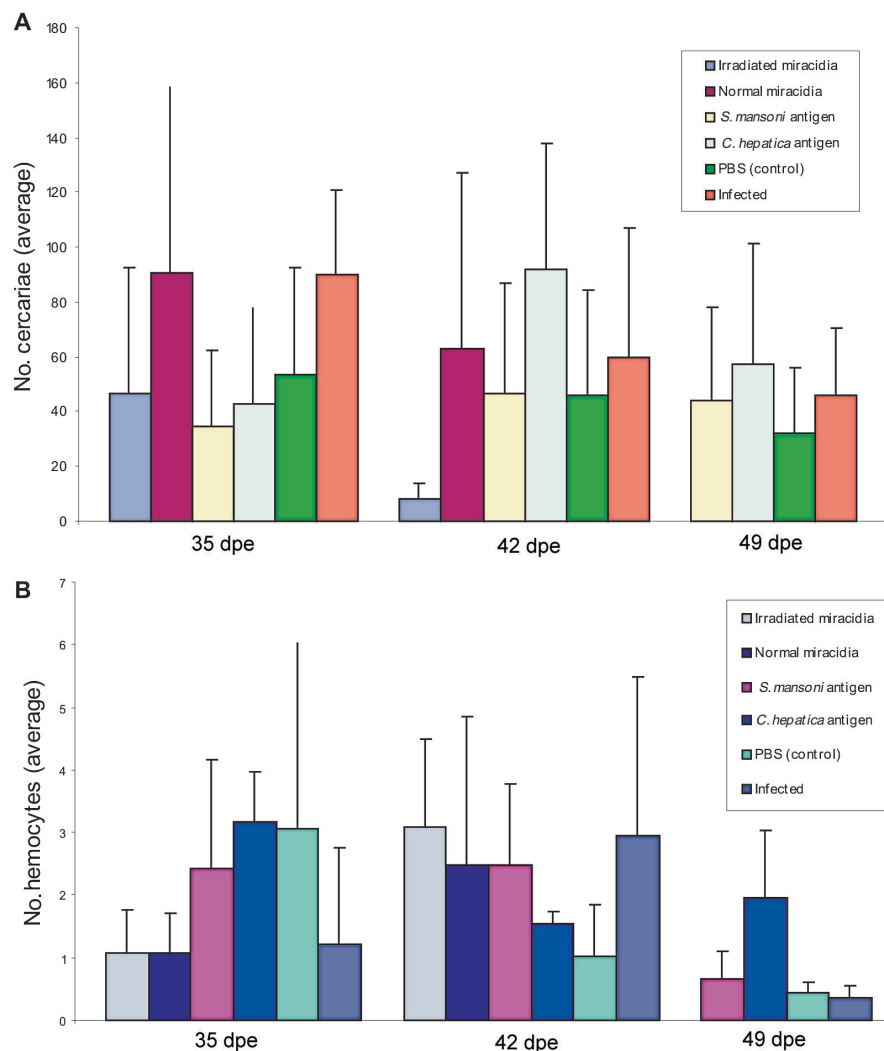
Fig. 1A: total number of cercariae (average and standart deviation) eliminated per group of snails at different periods of time after infection (p > 0.05); B: total number of hemocytes (average and standart deviation) observed in groups of five snails at different periods of time - days post-exposure (dpe) (p > 0.05).

acter, such local reactions were not correlated with any evidence of an enhancement of resistance toward a challenging S. *mansoni* infection. This dissociation between the histopathologic picture and the biological behavior seems rather interesting. It suggests that the defense cells of *B. glabrata* can respond to local stimulation without the production of factors, soluble or otherwise, capable of stimulating other hemocytes locate elsewhere in the snail tissues. The local response may also reflect the slowness of the snail circulation, which may help prevent the dissemination of the stimulus caused by the antigenic materials in other areas of the snail body, regardless the fact that the antigen was not particulate, but soluble.

The findings also clarify the fact that the hemocytes can take origin from anywhere within the snail body, whenever an appropriate focal stimulus occurs (Pan 1958, 1963), instead of having a central origin from a specific organ, the APO or "amebocyte forming organ" (Sullivan & Spence 1994, Sullivan et al. 2004).

Of course there is close association between a histological picture of hemocyte proliferation, with formation of encapsulating granulomatous-like structures around degrading parasitic forms, and the degree of resistance measured by cercarial elimination (Godoy et al. 1977, Lemos & Andrade 2001).

Another interesting detail refers to the presence of a certain degree of specificity disclosed by the local reactions produced by the injections of antigens into *B. glabrata*. The reactive nodules were larger and more numerous with material derived from *S. mansoni* as compared to *C. hepatica*. According to Tripp (1961) the tissue response in *B. glabrata* is non-specific, since it can be induced by inert material. However, Bayne et al. (1984) demonstrated the existence of specific sites for attachment of *S. mansoni* sporocyst antigens on the hemocyte surface, both in susceptible and resistant snails

When injections with irradiate miracidia were utilized, some nodules appeared formed around cellular debris,
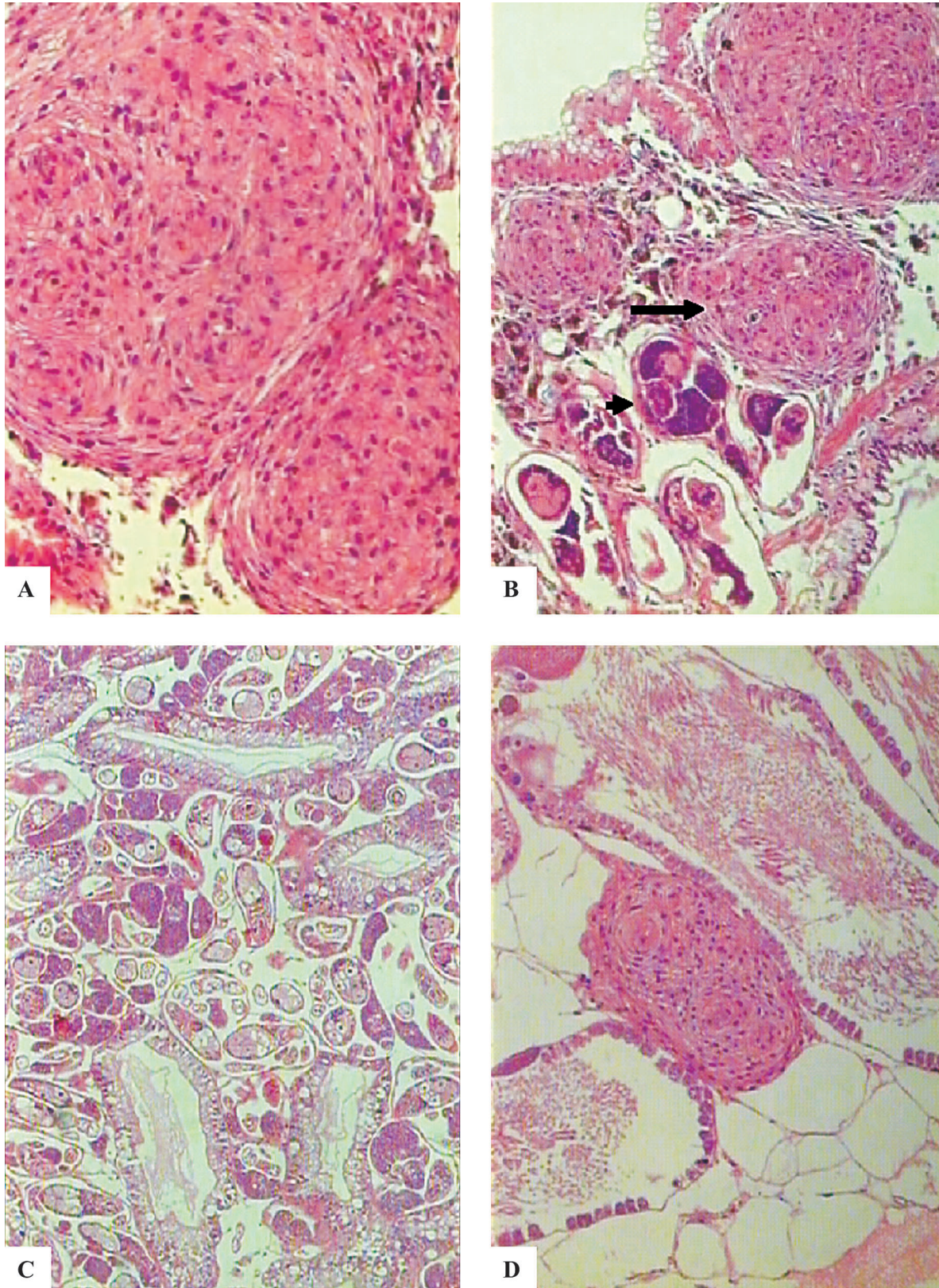
Fig. 2A: large accumulations of hemocytes forming well circumscribed nodules at the site where soluble *Schistosoma mansoni* antigen has been injected. × 400; B: multiplying forms of *S. mansoni* appear without any signs of tissue reaction in spite of the presence of the antigen-induced hemocyte nodules in close proximity (arrows). × 200; C: numerous proliferating forms of *S. mansoni* can be seen within the interstitial tissue of the digestive glands. Note absence of reaction in a snail previously injected with *S. mansoni* extracts. × 100; D: isolated hemocytic nodule found at the ovo-testis region, away from the site of inoculation which was at the cephalo-podal area. × 200. All pictures taken from slides stained with hematoxilyn & eosin.

probably originated from dead or dying miracidia. Michelson and Dubois (1981) observed that encapsulating reactions around miracidia only appeared when the parasite was already dead, since the live one is not recognized as non-self. In our preparations with irradiated miracidia, nodular hemocyte reactions were observed, what can be taken as signs of miracidial destruction. But, this did not result in protection enhancement. The snail strain used in the present experiments was a very susceptible one for the local *S. mansoni* stock. Such combination resulted in severe snail infection. It is not known how these variables interfered with the present findings. Probably further attempts with less virulent parasite strains and less susceptible snails would be worthwhile to dissect factors involved in protection after "immune" stimulation.

Further investigations are required to clarify how these very primitive organisms are able to mount a defense reaction against invading parasites. It is hoped that present findings would be of help in the planning of new studies along these lines.

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