Nicarbazin in Schistosome Infections: I. Antibody Formation in Mice and Hamsters Infected with Schistosoma mansoni

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HILLYER, G. V., REYES, C. N., AND HERNÁNDEZ-ALMENAS, N. 1975. Nicarbazin in schistosome infections: I. Antibody formation in mice and hamsters infected with Schistosoma mansoni. Experimental Parasitology 37, 442–448. The immunoprecipitin response of mice infected with Schistosoma mansoni and treated with nicarbazin, an egg suppressive agent, is significantly lower than in untreated-infected mice. The precipitin response to cercarial extract is virtually abolished in treated mice indicating common antigenic determinants with eggs of the same species. Haemagglutinins to adult worms are also significantly diminished in treated mice. Finally, circumoval precipitins are absent in treated mice when the drug is given continuously to infected mice in order to prevent egg laying by the female adult parasite. The results suggest that a significant portion of the precipitating antibody produced in schistosome infections reactive with cercariae and adult worms, as well as eggs, is probably a secondary antibody response due to common antigenic determinants found in eggs.

INDEX DESCRIPTORS: Schistosoma mansoni; Schistosomiasis; Immunoprecipitins; Nicarbazin; Antibody. Immunity; Diffusion, double, Hamsters; Mice; Chemotherapy; Hemagglutination, indirect; Circumoval precipitin test.

INTRODUCTION

Nicarbazin is an equimolar complex of 4,4'-dinitrocarbanilide and 2-hydroxy-4, 6-dimethylpyrimidine. Campbell and Cuckler (1967) demonstrated that nicarbazin inhibited egg production of Schistosoma mansoni female adult worms in infected mice. In addition, they reported that the drug, even when fed to infected mice as 0.3% at the diet for 18 wk, did not kill the worms. This inhibition of egg production was subsequently confirmed by Pellegrino and Katz (1969) using the drug incorporated in the diet of mice infected with Schistosoma mansoni as evidenced by oogram changes. Egg laying resumed as soon as the drug was withdrawn. The drug had no egg suppressive effect, however, in hamsters infected with S. mansoni. Warren (1970) found that mice with schistosomiasis mansoni treated with nicarbazin from the fifth to the tenth week after exposure developed no signs of hepatosplenic disease.

Previous work in mice infected with S. mansoni had demonstrated that precipitating antibodies in mice developed after the onset of egg laying by the female adult parasite (Hillyer and Frick 1967). This strongly suggested that the schistosome egg plays a role in precipitating antibody formation and that some of its antigenic determinants shared with adult worm and cercarial extracts were reflected when these antigens were utilized in the in vitro tests. This report demonstrates that when nicar-
Antibodies in Nicarbazin Treated Rodents with Schistosomes

Nicarbazin is utilized to suppress egg laying by the female parasite, a significant reduction of precipitating antibody is observed as compared to untreated infected controls.

Materials and Methods

Exposure of animals. Schistosoma mansoni cercariae were obtained from pooled, infected Biomphalaria glabrata snails at the Puerto Rico Nuclear Center. Hamsters were exposed through their cheek pouches under Nembutal anesthesia. Mice were exposed either subcutaneously or intraperitoneally without anesthesia (Peters and Warren 1969). At varying intervals thereafter the animals were bled by cardiac puncture, perfused using standard techniques, and the worms counted. The mesentery veins were subsequently examined, as well as the livers, to ensure accurate counts.

Nicarbazin. Nicarbazin was obtained from the Merck Institute for Therapeutic Research, Rahway, New Jersey, through the courtesy of Dr. D. A. Ostlind. The drug was supplied at 0.3% concentration mixed in laboratory chow. Normal chow was also supplied without the drug.

Immunologic tests. Ouchterlony double diffusion tests using extracts from S. mansoni adult worms and cercariae were done as previously described (Hillyer and Frick 1967). Indirect hemagglutination tests were done utilizing a "Schistosomiasis-IHA-Reagent" kit donated by Behringwerke AG (Marburg-Lahn, Germany). We are grateful to Dr. B. Enders for making these kits available to us. The antigen in the IHA kit was S. mansoni adult worm. Circumoval precipitin tests (COP) were done utilizing lyophilized S. mansoni eggs collected from infected mouse livers. The tests were done as described by Yogore et al. (1968).

Experiment No. 1. Ten hamsters were exposed to 180 S. mansoni cercariae and divided into two groups after receiving normal chow for 4 wk. At this time, one group of five hamsters was switched to laboratory chow containing 0.3% nicarbazin. All animals were bled 4, 6, and 8 wk postexposure. All were found to have patent infections.

Experiment No. 2. This experiment was similar to No. 1 except that the hamsters were exposed to 160 S. mansoni cercariae and bled 5, 7, and 8 wk postexposure. All had patent infections.

Experiment No. 3. Fifteen Swiss albino mice were exposed to 100 S. mansoni cercariae subcutaneously. These were then divided into two groups of eight and seven mice and were fed normal chow. On Day 26 post-exposure the group of eight mice was switched to nicarbazin diet. Two of three mice were bled and autopsied on wk 6, 7, and 8.

Experiment No. 4. Thirty-four female Swiss albino mice were each exposed by intraperitoneal injection to 50 S. mansoni cercariae. These were then divided into three groups of 11 or 12 mice (Table I). Group I received a normal diet for 10 wk at which time they were bled for serum and killed to determine worm burden. Group II received a normal diet for 5 wk followed by a diet containing 0.3% nicarbazin for an additional 5 wk after which they were killed. Group III received nicarbazin diet for 5 wk followed by a normal diet for an additional 5 wk at which time they were killed.

Results

Experiment Nos. 1 and 2. All hamsters had patent infections. Fourteen were autopsied and all had S. mansoni eggs in their livers, irrespective of diet received, indicating that nicarbazin had no apparent effect in suppressing egg production by the parasite in hamsters. With one exception at 4 wk, anti-adult worm precipitins appeared by the fifth and sixth weeks of infection. Antibodies to cercariae first appeared on the fifth week in both groups of hamsters and increased in intensity through 8 wk when one to three precipitin bands were
observed. Strongest adult worm precipitins were also observed at 7–8 wk of infection when one to three precipitin bands were observed, irrespective of diet.

Experiment No. 3. Perfusion of mice which received a normal diet showed them to have an average of 18 male (±6) and 16 female (±8) adult worms for a total worm average of 34 (±14). In contrast, mice which received a nicarbazin diet had an average of 4 male (±4) and less than 1 female (±1) for a total worm average of 5 (±4). Comparisons of males, females, or totals from both groups using Student's t test showed the differences to be highly significant (P less than 0.001). The few eggs seen in the livers from two of eight mice which received a nicarbazin diet were obviously deformed. When one compared the precipitating antibody production in these mice at 8 wk of infection one observed in the nicarbazin-treated group a slight decrease in anti-adult worm precipitins and a significant qualitative and quantitative decrease in anti-cercarial precipitins (Fig. 1).

Experiment No. 4. The results of the fourth experimental group are summarized in Table I. Several features are of interest in this experiment. Mice treated with nicarbazin for 5 wk had approximately half the number of worms as mice receiving a nor-

### Table I

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Period of treatment (wk)</th>
<th>No. of mice</th>
<th>Average worms/mouse</th>
<th>IHA (reciprocal titer)</th>
<th>COP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male 16 ± 6</td>
<td>10 ± 4</td>
<td>26 ± 8</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>11</td>
<td>Female 10 ± 4</td>
<td>0</td>
<td>26 ± 8</td>
</tr>
<tr>
<td>II</td>
<td>6–10</td>
<td>12</td>
<td>Male 7 ± 4</td>
<td>2 ± 3</td>
<td>3 ± 4</td>
</tr>
<tr>
<td>III</td>
<td>0–5</td>
<td>11</td>
<td>Female 9 ± 5</td>
<td>5 ± 3</td>
<td>0</td>
</tr>
</tbody>
</table>

Effect of the Administration of Nicarbazin (0.3% in Diet) on the Worm Burden and Antibody Response of Mice Infected with Schistosoma mansoni for 10 wk.
NORMAL DIET. MICE WHICH RECEIVED NICARBAZIN DURING THE LATTER HALF OF THE EXPERIMENTAL PERIOD (GROUP II) HAD 25 OF 27 (TOTAL) FEMALE WORMS WHICH ON GROSS OBSERVATION WERE SMALLER THAN NORMAL. IN ADDITION, IT HAD A SMALL POPULATION OF WORMS WHICH COULD NOT BE SEXED AND WERE LABELED SCHISTOSOMULES. GROUP III MICE, WHICH RECEIVED NICARBAZIN FOR THE FIRST 5 WK OF INFECTION, DEMONSTRATED THAT TREATMENT WITH NICARBAZIN HAD AT LEAST A PARTIALLY REVERSIBLE EFFECT AS NO SCHISTOSOMULES OR grossLY UNdERSIZED FEMALE WORMS WERE OBSERVED AFTER THE MICE WERE FED FOR 5 ADDITIONAL WK ON NORMAL CHOW. IT SEEMED, HOWEVER, THAT SOME OF THE WORMS WERE BEING KILLED, OR DEVELOPED SO SLOWLY THAT THEY WERE NOT PRESENT IN THE MSENTERY VEINS OR LIVER AT THE TIME OF AUTOPSY. USING STUDENT'S T TEST TO COMPARE MALES, FEMALES OR TOTAL WORMS OF GROUPS I VS II OR I VS III, THE DIFFERENCES WERE FOUND TO BE HIGHLY SIGNIFICANT (P EQUALLED OR WAS LESS THAN 0.005). ADDING THE SCHISTOSOMULE POPULATION TO THE POPULATION OF MALES OR FEMALES OF GROUP II AND COMPARING THESE TOTALS WITH GROUP I STILL RESULTED IN SIGNIFICANT DIFFERENCES (P EQUALLED OR WAS SMALLER THAN 0.05).

PRECIPITATING ANTIBODY PRODUCTION ALSO WAs AFFECTED. A REPRESENTATIVE OUChTERTONY double diffusion test using pooled sera from each group of mice against adult worm and cercarial extracts of S. mansoni is seen in Fig. 2. AS COMPARED with pooled sera from mice infected for 10 wk which received a normal diet (I), the group which received nicarbazin for the first half of the infection period (III) showed that a return to normal diet resulted in an apparently normal pattern of precipitating antibody formation with strong precipitins to adult worms and cercariae. This was in marked contrast to Group II which received nicarbazin during the latter half of the infection period. In this case, there was a marked diminution of precipitating antibody to both antigenic extracts. Particularly striking was the virtual absence of precipitins to cercarial extracts in this group in spite of the fact that Groups II and III had similar worm burdens. Thus it appeared that production of eggs by the schistosome

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**Fig. 2.** Precipitins in the sera of mice infected with Schistosoma mansoni for 10 wk and fed normal chow (I), or feed containing 0.3% nicarbazin during the first 5 wk (III) or the last 5 wk (II) of infection. Upper and lower wells contained adult worm extracts (W) or cercarial extracts (C) of S. mansoni.
adult female played a major role in stimulating a secondary antibody response reflected in the precipitation patterns shown in Figs. 1 and 2. Indirect hemagglutination tests showed that the reciprocal titers to adult worm antigens were highest in the untreated group and lowest in the group treated during the latter half of the infection. COP tests, however, were positive.

Other work (Hillyer and Reyes, unpublished) has shown that when mice were treated with nicarbazin from the first day of infection until autopsy, circumoval precipitins in their sera were uniformly negative at 5, 6, 7, and 8 wk of infection. Untreated mice, in contrast, had circumoval precipitins beginning on the sixth week. In this same group IHA reciprocal titers were 8 or less in the treated mice during wk 5–8. Untreated mice, in contrast, had titers of 16 at 5 wk, 32 at 6 and 7 wk, and 64 at 8 wk of infection.

**DISCUSSION**

In an earlier report we (Hillyer and Frick 1967) found that precipitating antibody to adult worms and cercariae appeared after the onset of egg laying of the adult female schistosome and suggested that the egg played a major role in precipitating antibody production with respect to schistosome infections. Oliver-Gonzalez et al. (1955) suggested that “cercarial antibodies develop mainly as a result of immunization against the cercarial factor in the egg.” Jaimes and von Lichtenberg (1965), using immunofluorescent techniques, found that the earliest antibody rise in murine infections with *S. mansoni* was due to worm antigens. However, a steep rise in titer correlated with the period of rapid egg deposition. They suggested that, since cercariae were the antigens utilized, “this florid phase of schistosome infection can be interpreted as a secondary immunological host response to an escalatory challenge with eggs, and marks the maximal stage of host sensitization and immunological reactivity.” The observation that there is a markedly diminished antibody response to cercariae in the serum of mice treated with nicarbazin and obtained after inhibition of egg production by the female parasite supports this hypothesis. The diminished response to adult worm extracts also suggests that the egg also probably plays a significant role in the production of antibodies reactive with adult worms. The finding that IHA titers are always lower in treated-infected mice also suggests some common antigenic determinants with eggs. Thus mice which are continuously under the effect of nicarbazin have much lower titers. The group of mice (Table I) which received nicarbazin during wk 6–10, however, showed less dramatic differences with only a one-dilution difference from the group receiving nicarbazin for the first 5 wk. Eggs were seen in all groups, however, although Group II mice had fewer and these were in various stages of dissolution by their respective granulomas. Since mice of Groups II and III had comparable worm burdens it is safe to compare them as to immunological reactivity.

Examination for circumoval precipitins was also done. In this case, infected mice which were continuously treated with nicarbazin had negative COP responses from the fifth through the eighth week of infection. Untreated mice were reactive by the sixth week. The mice in experiment No. 4 (Table I) were all positive by the COP test. Although there were obvious qualitative and quantitative differences on eggs in Group II vs III no titration was done in the COP test since it is essentially a qualitative test. The results are consistent with the report of Maddison et al. (1971) who suggest that “cross-reacting antigens present in the cercarial and adult worm stages are functional in the CF, CHR, BF, and IHA tests; . . . however, that a component in the egg is essential for the stimulation of COP antibody.”

We of course do not know whether nicar-
bazin by itself is an inhibitor of antibody production and studies under way are being done precisely to determine this point. However, the fact that treated mice with no eggs in their livers have negative COP tests whereas treated mice with eggs in their livers have positive COP tests suggest that the drug in itself does not inhibit antibody formation. The results, along with the references quoted, seem to support the hypothesis that eggs play a significant role in the antibody response observed against eggs, adult worms, and cercariae, although by no means exclude antigenic stimulation by those antigens.

Our studies with hamsters infected with *S. mansoni* essentially confirm the study of Pellegrino and Katz (1969). They found that an interruption of egg laying occurred in experimentally infected mice, a temporary interruption occurred in infected monkeys, and no interruption occurred in hamsters infected with *S. mansoni*. Thus it appears that nicarbazin is metabolized differently in mice as compared to hamsters in such a manner that the schistosomicidal active component is either inactivated, degraded, or not formed in hamsters. Precipitating antibody production in treated and untreated infected hamsters was comparable, further supporting the apparent importance of the schistosome egg in inducing antibody production in the host. In addition, the results further support the hypothesis that nicarbazin itself is not an inhibitor of antibody synthesis.

In contrast to the work of Warren (1970), the results in Table I suggest that treatment of infected mice may result in the elimination of a portion of the total population of schistosome worms. This is being studied further since we have some preliminary evidence that the schistosome can recover from the effect of the drug depending on the length of the period of exposure to the drug as stated by Warren. In addition, it is possible that the drug slows the normal development of the parasite in such a way that it is not recovered in the perfusion of mesentery veins and liver. Also, presses of the liver and examination of the mesentery veins after perfusion failed to reveal additional worms.

The size of the worms of both sexes is also affected. Warren (1970) demonstrated that the worms in mice treated for 5–10 wk were one-third the size of those in the control when treatment was stopped. In addition, Lennox and Bueding (1970) demonstrated that nicarbazin significantly reduced the dry weights of schistosomes and extensively damaged the reproductive system in females. Although we did not measure the recovered worms, gross examination showed that the worms from mice treated with nicarbazin at the time of sacrifice were dramatically smaller than those from the normal control group. Current studies are under way to study this further.

Nicarbazin as an experimental model is of interest regarding the immune response of the host to infection with schistosomes. Warren (1970), for example, demonstrated clearly that hepatosplenic schistosomiasis did not develop in infected mice when treatment was initiated just prior to egg laying. However, it is not known whether the drug in itself inhibits delayed hypersensitivity or cell mediated immunity. Likewise, we have demonstrated that nicarbazin, by inhibiting egg laying by the parasite contributes, probably indirectly to the inhibition of formation of some types of precipitins and hemagglutinins to the parasite. Thus comparisons could be made between specific humoral and cell mediated responses in schistosome infections. Finally, the drug could possibly be used to study the role of the host's humoral response and its relation to protective immunity. Studies on this concept are presently in progress.

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REFERENCES


