EFFECTS OF 6-MERCAPTOPURINE ON CYST DEVELOPMENT IN EXPERIMENTAL TOXOPLASMOSIS*

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The protozoan parasite Toxoplasma gondii is characterized by both virulent and avirulent strains. The latter result in chronic infections, with the development of cysts in a variety of tissues after a period of some 1 to 2 weeks of infection (Lainson, 1958, 1961; Nakayama and Matsubayashi, 1961). Because the circumstances leading to the development of the cyst are not known, some controversy still exists over various hypotheses that have been advanced in explanation of the phenomenon of cyst development. One such controversial hypothesis implies that the host's immune response is prerequisite, that cysts will develop only after some form of immunity has been acquired by the host (Frenkel, 1961; Nakayama and Matsubayashi, 1961).

An approach to this problem was suggested by the growing body of literature on the antimetabolites in biological research. It has been shown that the administration of the purine analogue 6-mercaptopurine (6-MP) leads to the complete suppression of several types of immune responses, including the humoral antibody response, in experimental animals and man (Schwartz, 1963). The mode of action of 6-MP in its effects on the immune response cannot be defined with certainty as yet, but the drug is believed to act by blocking the synthesis of the purine moiety of the nucleic acids (Hitchings and Elion, 1963).

The purpose of the present study was to follow the course of infection of 2 relatively avirulent strains of T. gondii in mice whose ability to respond im-

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munologically was being inhibited by 6-MP; to note if any changes would occur either in the pathogenesis of the disease, or in the development and appearance of the cyst.

METHODS AND MATERIALS

The albino mice used in this study were obtained from a local animal supply house in Tokyo, and were 1 to 2 months of age. Cysts of the Beverley and S-273 strains of Toxoplasma were recovered from the brains of infected stock mice. Groups of stock mice were sacrificed and their brains removed and homogenized in saline in a tissue grinder. Dilution counts were performed, and the final volume adjusted to yield approximately 30 cysts per ml of brain suspension. Penicillin and streptomycin were added to final concentrations of 200 units and 1.0 mg per ml of brain suspension as an anti-bacterial precaution. The standard mouse-infecting dose was 15 cysts in a 0.5 ml aliquot of suspension, and was administered by intraperitoneal inoculation.

Trophozoites of the very virulent RH strain of Toxoplasma were obtained from a pool maintained in this laboratory by continuous passage thru mice every 4 days. Two ml of saline were introduced into the peritoneal cavity of infected mice, and then immediately withdrawn via the same syringe. The peritoneal rinses of a large number of mice were pooled, and injected into the experimental mice in doses of 0.5 ml. Trophozoite counts were not performed, but large numbers of trophozoites were present.

The 6-MP was administered to the mice at a rate of 50 mg per kg of body weight. This figure was based on results of Akiyama (1964), who obtained good inhibition of the immune response against Salmonella enteriditis in mice with this dosage level. A fresh solution of 6-MP was prepared daily by dissolving 100 mg of 6-MP hydrate (6-MP·H₂O, Nutritional Biochemicals Corporation, Cleveland, Ohio; and Mann Research Laboratories, Inc. New York, N.Y.) in 0.8 ml of 1N NaOH, and then adding saline to a final concentration of 10 mg per ml of solution. One mouse-dose was 1 mg of 6-MP in 0.1 ml of solution, injected IM every day for 18 days, starting from the first day of the Toxoplasma infection. The daily injections were alternated between the outer and the inner aspects of the thigh muscles of the hind legs of the mice.

The experimental mice were closely observed every day, and weighed every third day. Mice to be sacrificed were bled to death from the retro-orbital sinus, and the blood collected. The serum was then separated, inactivated at 57°C, and stored in a deep freeze at minus 20°C. In addition to periodic sacrifice, mice
found dying or dead were immediately autopsied, and the brain and secondary tissues such as the liver, spleen, and eyes removed. Portions of all the organs were press-smeared and examined fresh, or alcohol-fixed and giemsa-stained. Other portions of the organs were fixed for future histological examination (sectioning at 5μ, H and E stain). Peritoneal exudate was withdrawn at periodic intervals for microscopic examination and subinoculation into clean mice.

The serological studies consisted of hemagglutination and agar-gel double diffusion tests. For the hemagglutination test, the procedure of Nobuto (1965) was followed, using Bis-Diazo-Benzic (B-D-B) treated, antigen-coated pig erythrocytes. The antigen was a partly purified mouse peritoneal exudate rich in RH trophozoites. A strongly positive, infected pig serum served as a positive control for the test. The agar-gel double diffusion test was modified from the technique described by Yakulis and Heller (1959). The 2 antigens employed here were made from washed, sonic-disrupted, infected mouse peritoneal exudate containing RH trophozoites. One antigen was used directly as such, and the other used after an attempt at partial purification and concentration by ammonium sulphate precipitation of protein, redissolving the precipitate in PO₄-buffered saline (pH 7.5), and then dialyzing against PO₄-buffered saline overnight to remove the (NH₄)₂SO₄.

RESULTS

Experiment 1. The Beverley strain of T. gondii in 6MP-treated mice.

During the first few days of the experiment, the BEV-infected, 6MP-treated mice started to exhibit symptoms indicating the onset of severe disease: ruffled fur, diarrhea, huddling and shuddering, loss of weight, and an increasing lethargy. These symptoms grew progressively worse with the passage of time. Mice started dying after only 3 days, and the mortality rate rose rapidly, from 8 percent after 1 week to 90 percent by the fourth week (see Fig. 1). Examination of peritoneal exudate revealed the presence of trophozoites as late as 23 days after infection.

In contrast, the 2 groups of control mice (receiving only the 6-MP treatment or the BEV infection) remained in good condition the first week. The second week however saw a loss of weight and some deaths in the BEV controls, following the appearance of ascites in a number of the mice. By the third week, the BEV mice were regaining their lost weight and looked to be in good shape, although a few additional mice died. By the end of the fourth week, 30 percent of the BEV mice had died. Trophozoites were not found in peritoneal exudate.
after day 15. The 6-MP control mice, during the third week, strenuously objected to the last 8 or so injections of the drug, quite the opposite of the 6MP-BEV mice which remained listless and apathetic during and after their daily injections. The 6-MP control mice suffered their crisis during the fourth week, with a sudden loss of weight and heavy mortality amounting to 30 percent of the animals, but after a few days once again went on an upswing. These results can be visualized in Fig. 2.

Examination of brain smears of the 6MP-BEV mice revealed a few very
small cysts on day 9. By day 15, large numbers of cysts were being found, with a frequent observation being numbers of cysts gathered in small clusters. By day 30, cysts were being detected with ease because of their numbers and size, and the clusters, containing from 3 to 15 cysts, were now exhibiting cysts of different sizes (see Figures 5, 7, and 12).

The BEV control mice presented essentially the same sequence of events as above, but with lesser numbers of cysts and without large clusters. In all other respects, such as the size and outward appearance of the cysts, the number of parasites enclosed within, and infectivity to clean mice, there were no discernable differences between the Toxoplasma cysts seen in the 2 groups of mice.

On day 31, only 4 of the original 40 6MP-BEV mice were still alive. Two of the mice were sacrificed by exsanguination, and the remaining 2 mice were challenged with the virulent RH strain, as were the surviving 6-MP and BEV control mice. All the 6-MP control mice died within 5 days, and were swarming with RH. The 6MP-BEV mice staggered through to the sixth and the eighth day before dying, and an examination of their peritoneal exudate revealed small numbers of RH trophozoites. The BEV control mice however, went through the RH challenge apparently unaffected. On the eighth day of the RH infection, some BEV mice were tapped, and their peritoneal exudate examined. An occasional scattered trophozoite was detected, and this exudate proved lethal upon subinoculation into clean mice after 5 days of infection. Two weeks after the RH challenge, the BEV mice were still in good condition, and had not suffered any mortalities. Examination of peritoneal exudate at this time proved negative.

The results of the hemagglutination and agar-gel diffusion tests are listed in Table 1. With the antigens employed, only the hemagglutination tests resulted in the detection of positive sera, in mice that had been chronically infected from 2½ to 5 months with Toxoplasma. The agar-gel double diffusion tests were uniformly negative.

Experiment 2. The S-273 strain of T. gondii in 6MP-treated mice.

The format of this experiment was identical to the previous one, with but 1 change. The number of 6-MP injections was reduced from 18 to 11, starting from the day of infection. The results obtained closely paralleled those described for the BEV strain (see Fig. 3), but lagged behind by a period of 4 or 5 days, possibly due to the lesser virulence of the S-273 strain. The 6MP-S273 mice started falling visibly ill by day 6, and dying by day 9. At the end of 2 weeks, 45 percent of the mice had died. This figure rose to 75 percent after 3 weeks, and then to 90 percent dead by the end of the fourth week (see Fig. 4). Tropho-
Table 1
Hemagglutination and agar-gel double diffusion tests using Toxoplasma-infected mouse peritoneal exudate antigens

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Hemagglutination Serum dilutions</th>
<th>Agar-gel double diffusion Sonic-Disrupted (NH₄)₂SO₄ ppt. ag.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/16</td>
<td>1/256</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
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<tr>
<td>Normal</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BEV 3 weeks</td>
<td>-</td>
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<td>BEV 4 weeks</td>
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<td>-</td>
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<tr>
<td>BEV 5 weeks</td>
<td>+</td>
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<tr>
<td>BEV 10 weeks</td>
<td>Ⅱ</td>
<td>Ⅱ</td>
</tr>
<tr>
<td>BEV 20 weeks</td>
<td>Ⅱ</td>
<td>+</td>
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<tr>
<td>6MP-BEV 4 weeks</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pig</td>
<td></td>
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</tr>
<tr>
<td>Normal</td>
<td>-</td>
<td>-</td>
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<tr>
<td>BEV 4 weeks</td>
<td>Ⅱ</td>
<td>Ⅱ</td>
</tr>
</tbody>
</table>

Zoites were detected in peritoneal exudate up to day 17, and the first cysts observed on day 18. The cysts were very small in both size and number, and contained only a few parasites within. On day 30, the cysts were found to be somewhat larger in size, but still much smaller than expected.
than BEV cysts after the same period of infection. A few additional small clusters were observed, with the largest cluster consisting of 6 cysts.

The S-273 control mice showed no signs or symptoms of disease throughout the experiment. There were some mortalities, to a total of 12 percent dead after 4 weeks. The first cysts were observed in a mouse that died on day 23, and were sparse in number and quite small. No clusters were found.

The 6-MP control mice again suffered a distinct decline during the fourth week, but the overall mortality was lower than in the previous experiment, down from 30 percent to 18 percent dead after 4 weeks.

All the surviving mice were challenged with RH on day 34. The 6-MP control mice and a group of normal control mice were all dead by the fifth day, and swarming with RH. The 6MP-S273 mice died between day 7 and day 10, and moderate numbers of RH trophozoites were observed in their peritoneal exudate. The S-273 control mice went through to day 14, suffering neither ill effects nor deaths due to the RH infection.

DISCUSSION

It is evident that not only does cyst development occur in 6MP-inhibited mice, but that it may actually occur somewhat earlier, and in greater numbers, with a conspicuous finding being the gathering of many cysts in large clusters. These clusters of cysts constitute distinct lesions of the brain, and may be the cause of many of the severe symptoms, and even the death, of the experimental
mice. The “how and why” the clusters are formed is open to speculation. During
the first week or so of an infection, trophozoites can be isolated with ease from
the blood of infected animals (Jacobs and Jones, 1950). It appears that the
parasitemia is caused by the continuous spilling over of trophozoites from liver
and spleen foci into the blood stream, via which the trophozoites are carried to
the brain of the host. In the brain, cyst development occurs, most likely as a
normal part of the life cycle. With the onset of the immune response, the number
of parasites able to pass the blood barrier is doubtlessly curtailed, resulting in
fewer trophozoites reaching the brain. Concurrent with the clearing of the
parasitemia is the gradual eradication of trophozoites from the tissues, thus
eliminating the source of the parasitemia as well. Any suppression or retarda-
tion of the immune response likely leaves the brain of the host open to constant
invasion, as witness the presence of cysts of different sizes within many of the
clusters, indicating that trophozoites were continuously arriving and adding to
the clusters.

It is of interest to note that on a few occasions, some small clusters of 3 and
4 cysts were detected in the BEV controls as well, in mice that had fallen ill and
died between the second and fourth week of the infection. There is of course
individual variation in the response of a group of animals to any sort of disease
stimulus, and the finding of clusters of cysts in some of the untreated control
animals may represent the occasional failure of a host’s immune response in
preventing lethal numbers of parasites from reaching the brain.

Consideration was given to the possibility that the 90 percent mortality seen
in the 6MP-BEV mice may have been due in part to synergism between the
individual effects of the 6-MP and the BEV infection, both of which killed 30
percent of their respective controls. However, in the experiment with the less
virulent S-273 strain, the same 90 percent mortality was obtained in the 6MP-
S273 mice, even though the control mortality rates had dipped to only 12 percent
in the S-273 controls, and to 18 percent in the 6-MP control mice.

Evidence implicating suppression of the immune response as the mechanism
of the 6-MP action was provided by the results of the mouse-protection tests,
in which all the mice surviving the rigors of the first Toxoplasma infection and
6-MP treatment were challenged with the RH strain. Previous studies (Stahl
and Akao, 1964) have shown that prior infections with avirulent strains of
Toxoplasma will protect mice against the virulent RH strain. In the present
study, this immunity to reinfection was well demonstrated by the BEV and S-273
control mice, which easily survived the RH challenge. However, in the 6MP-
treated mice, this protection failed to materialize, although the dying mice did
manage to survive a few days longer than did the normal controls, and only modest numbers of RH trophozoites were recovered in their peritoneal exudate.

The results of the hemagglutination and agar-gel double diffusion tests failed to reveal the presence of antibodies in the 6MP-BEV mice after 30 days of infection. However, it must be emphasized that in view of the crudity of the peritoneal exudate antigens employed, and the fact that the 30-day BEV controls as well were serologically negative, the results of these tests cannot be regarded as conclusive. Indeed, until we can obtained fractionated and purified antigens of Toxoplasma for study, such questions as whether we are dealing with functional, but non-detectable antibody, or perhaps detectable, but non-functional antibody shall remain to handicap our understanding of the nature and significance of the immune response to Toxoplasma.

SUMMARY

The administration of the purine antagonist 6-MP greatly alters the course of a Toxoplasma infection in mice. The pathogenesis of the disease, in terms of morbidity and mortality, is much more severe; cyst production occurs in greater numbers, and possibly somewhat earlier than in normal mice; many cysts are found gathered in clusters; and the mice do not develop an effective immunity against a challenge with the virulent RH strain. A consideration of the pathological and serological findings in the 6MP-treated mice suggests that suppression of the immune response to Toxoplasma was achieved, and that as a result of this suppression, the various changes in the course of the infection occurred. The host immune response, therefore, does not appear to be a prerequisite to the formation and development of the Toxoplasma cyst.

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REFERENCES

PLATE I

Fig. 5. Cluster of cysts, 100x
6MP–BEV 30 days, mouse
Unstained press-smear of brain

Fig. 6. Compact cluster, 400x
6MP–BEV 30 days, mouse
Unstained press-smear of brain

Fig. 7. Cluster of young cysts
6MP–BEV 15 days
Mouse brain, 7µ, H+E, 400x

Fig. 8. Young maturing cysts
6MP–BEV 21 days
Mouse brain, 7µ, H+E, 400x

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Fig. 9. Compact cluster, 100x
6MP−BEV 30 days
Mouse brain, 7μ, H+E stain

Fig. 10. Same as Fig. 9
400x

Fig. 11. Compact cluster, 400x
6MP−BEV 30 days
Mouse brain, 7μ, H+E stain

Fig. 12. Newly formed cysts adding to a cluster
6MP−BEV 30 days
Mouse brain, 7μ, H+E, 400x