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Effects of Cortisone, Cyclophosphamide, 6-mercaptopurine and 8-azaguanine on Experimental *Trypanosoma cruzi* Infection in Mice

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It is well recognized that *Trypanosoma cruzi* cultured serially *in vitro* for many years still remains infective to animals (Packchanian and Sweets, 1947). However, the infectivity of cultured form of *Trypanosoma cruzi* is much lower than that of the trypanosomal form obtained from the blood when inoculated into a susceptible animal with the same inoculum size (Rosenberg *et al.*, 1969).

On the other hand, it was found that the experimental infection with *Trypanosoma cruzi* was intensified by administration of immunosuppressive agents such as the antimetabolite, 8-azaguanine (Shoemaker and Hoffman, 1969) or corticosteroid (Pizzi *et al.*, 1952; Okumura and Décourt, 1969).

The present investigation was undertaken in order to determine whether the administration of cortisone, cyclophosphamide, 6-mercaptopurine and 8-azaguanine show an effect on the susceptibility of mice to the inoculation with the cultured organisms.

Materials and Methods

The Berenice strain of *Trypanosoma cruzi* was used in this experiment. The organism was maintained serially at 28°C in 200 ml tissue culture bottle containing 25 ml of LIT medium. The composition of LIT medium is as follows; 4.0 g of NaCl, 0.4 g of KCl, 8.0 g of Na_2HPO_4, 2.0 g of glucose, 100 ml of calf serum, 20 ml of 10 % hemoglobin solution and 750 ml of distilled water as a basic mixture. To 1,000 ml of the above described mixture, 100 ml of 5 % solution of ox liver infusion and 5 g of tryptose were added (Fernandes and Castellani, 1966). The organisms cultured one week were collected and washed two times with a phosphate buffered saline (PBS). Number of the organisms suspended in PBS was estimated by means of hemocytometer. The smears of this suspension was stained by Giemsa for estimation of a percentage of metacyclic form.

In the first series of the experiments, the susceptibility of the mice to the Berenice strain was estimated by inoculating them with various inoculum sizes of the trypanosomes. A determined number of the organism was injected intraperitoneally into male albino mice weighing 10 to 20 g. Four mice in each inoculum size group were sacrificed every three days starting from 7th day after the inoculation. The blood was drawn from the heart of the sacrificed mice using a heparinized syringe. One drop of the blood was placed on a slide glass for microscopic examination. The remainder was cultured in a modified NNN medium and examined for the presence of the parasites one month after the cultivation. Thus, the inoculum size which causes parasitemia was determined. In the next series of the experiments, cortisone, cyclophosphamide, 6-mercaptopurine or 8-azaguanine was administered to the mice inoculated with the inoculum, size of which was a little smaller than what caused parasitemia.

This article was dedicated to Prof. Haruo Kinosita, Zoological Institute, Faculty of Sciences, University of Tokyo, in the commemoration of his 60th birthday.

Cortisone (cortisone acetate, Merck and Co., Rahway, New Jersy, U.S.A.) was administered intramuscularly to the mice at a dose of 0.25 mg per 20 g of body weight, being dissolved in 0.1 ml of saline solution, three times at 48 hours' intervals starting from the day of the inoculation. Cyclophosphamide (Endoxan, Shionogi Co. Ltd., Osaka, Japan) was injected intramuscularly to the mice at a daily dose of 1 mg per 20 g of body weight, being dissolved in 0.1 ml of saline solution, for six days starting from the day of the inoculation. 6-mercaptopurine (6-MP, Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.) was administered to the mice at a dose of 50 mg per kg of body weight. 6-MP was dissolved according to the method described by Stahl *et al.* (1966). The mice received intramuscular injections of this compound daily for six consecutive days starting from the day of the inoculation. 8-azaguanine (Sigma Co., St Louis, Missouri, U.S.A.) was administered to the mice at a dose of 50 mg per kg of body weight. The method of dissolving 8-azaguanine used in this experiment is that of Shoemaker and Hoffman (1969). The mice received intramuscular injections of this compound daily for six consecutive days starting from the day of the inoculation.

The 4 mice in each group were sacrificed every week and the blood to be examined was drawn from the heart with a heparinized syringe. The estimation of parasitemia was carried out by means of microscopic examination and cultivation of the blood, as stated above.

Results

Blood of the mice inoculated with cultured *Trypanosoma cruzi* was odserved in order to examine the relationship between occurrence of the parasitemia and the inoculum size. As is shown in Table 1, parasitemia was recognized only in the mice inoculated with the larger number of the organisms when the blood was cultivated. This fact indicates that the trypanosomes in the blood, if any, are too small in number to be detected by the direct microscopic examination. Parasitemia of mice was not recognized in case of the smaller inoculum size such as less than 3×10^6 organisms. It is concluded that parasitemia in the mice inoculated with the cultured trypanosomes occurs depending upon the number of the organisms inoculated.

In the next series of the experiments, cyclophosphamide, cortisone, 6-MP or 8-azaguanine was administered to the mice which were inoculated with the organisms less than 3×10^6 in number, in order to observe the effect of the compounds on the parasitemia. The parasitemia in the mice was examined every 7 days after the inoculation. The result is shown in Table 2. In the blood of control mice the trypanosomes were not found by means of both direct and cultural exa-

Table 1 Parasitemia in mice following inoculation with cultured *Trypanosoma cruzi*, Berenice strain

Number of organism inoculated*	Days after inoculation															
	7				10				13				16			
	Direct		Culture**		Direct		Culture		Direct		Culture		Direct		Culture	
	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
3,000,000	0	4	4	0	0	4	4	0	0	4	4	0	0	4	2	2
300,000	0	4	0	4	0	4	0	4	0	4	0	4	0	4	0	4
30,000	0	4	0	4	0	4	0	4	0	4	0	4	0	4	0	4

* Percentage of the metacyclic form in this inoculum was 28%.
** Examination methods for detection of the trypanosomes.
\+ Trypanosomes were positive by the examinations.
− Trypanosomes were negative by the examinations.

Table 2 Effects of cyclophosphamide, cortisone, 6-MP and 8-azaguanine on the parasitemia in mice inoculated with *Trypanosoma cruzi*, Berenice strain

	Days after inoculation															
	7				14				21				28			
	Direct		Culture*		Direct		Culture		Direct		Culture		Direct		Culture	
	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Cyclophosphamide group	0	4	4	0	0	4	4	0	0	4	0	4	0	4	0	4
Cortisone group	0	4	0	4	0	4	0	4	0	4	2	2	0	4	0	4
6-MP group	0	4	4	0	0	4	4	0	0	4	0	4	0	4	0	4
8-azaguanine group	0	4	2	2	0	4	0	4	0	4	0	4	0	4	0	4
Control group	0	4	0	4	0	4	0	4	0	4	0	4	0	4	0	4

* Examination methods for detection of the trypanosomes.
\+ Trypanosomes were positive by the examinations.
− Trypanosomes were negative by the examinations.

Table 3 Effects of cyclophosphamide, cortisone, 6-MP and 8-azaguanine on the mortality of the mice inoculated with *Trypanosoma cruzi*, Berenice strain

	Number of mice	Number of organisms inoculated	Dose (mg/day)	Mortality by 2 weeks after inoculation (%)
Cyclophosphamide group	10	4×10^7	1.0	80
Cortisone group	10	4×10^7	0.25	0
6-MP group	10	4×10^7	1.0	30
8-azaguanine group	10	4×10^7	1.0	0
Control 1 (cyclophosphamide)	10	0	1.0	0
Control 2 (cortisone)	10	0	0.25	30
Control 3 (6-MP)	10	0	1.0	0
Control 4 (8-azaguanine)	10	0	1.0	0
Control 5	10	4×10^7	—	0

minations. In cases of cyclophosphamide and 6-MP-treated mice, the trypanosomes were demonstrated by the cultivation of the blood but not by the direct examination. In cases of administration of cortisone and 8-azaguanine, parasitemia was recognized in the half of the animals 21 days and 7 days after the inoculation respectively by cultural examinations. As the trypanosomes were not detected in the blood of control mice, the results indicate that the administration of cyclophosphamide and 6-MP is effective on enhancement of parasitemia of the parasites.

Mortality of the infected mice which were injected with the compounds was compared with that of the control mice ; i.e. the mice administered only the compounds or the mice inoculated with the parasites only. The result is shown in Table 3. Death of cyclophosphamide-treated mice occurred in 80 % by two weeks after the inoculation. The trypanosomes were found in the blood of the mice that died. In the control animals mentioned above, no mice died by two weeks. The effects of cortisone, 6-MP or 8-azaguanine on the mortality of the mice inoculated with the parasites were examined in the same way as in the case of cyclophosphamide. Administration of 6-MP resulted in death of 30 % of the infected mice, while no death occurred in the control. In contrast to the results obtained in the case of cyclophospha-

mide, it was found that the use of cortisone and 8-azaguanine showed little difference in mortality between the experimental and the control mice. Therefore, it is concluded that the infection was intensified markedly with cyclophosphamide, moderately with 6-MP and not with the other compounds.

Discussion

In case that the cultured forms of *Trypanosoma cruzi* are used as an inoculum, it is necessary to inoculate a large number of the organism to mice for the establishment of the infection, comparing with the case of the blood form of the trypanosomes. The result of the present study shows that cyclophosphamide and 6-MP intensify the experimental trypanosome infection in mice as evaluated both in the enhancement of parasitemia and the mortality of the mice infected. As was pointed out by Weiner *et al.* (1971), the administration of the former substance induces a immunosuppression of the host animals. The result of the present studies appears to indicate that the infection with the trypanosomes in the immunosuppressed mice is established much easier by inoculation with a small number of the cultured organisms than in the control mice. Whereas, in contrast to the expectation, the administration of the other immunosuppressive compounds, cortisone and 8-azaguanine, showed almost negligible effect on the enhancement of the infection. In addition, the effect of 6-MP also was not satisfactorily as was indicated in this experiment. The results obtained in the present experiment are different from those by previous investigators. Pizzi *et al.* (1952) demonstrated that the administration of cortisone at a dose of 0.5 mg/day for 10 days, higher dosage than that of the present experiment, produced an intensification of experimental *Trypanosoma cruzi* infection in mice. Okumura and Décourt (1969) also observed the intensification of experimental trypanosome infection in mice when the daily dosage of 0.4 mg of cortisone was administered. The administration of 8-azaguanine produced an intensified infection in mice when it is administered at a dosage of 75 mg/kg/day (Shoemaker and Hoffman, 1969). On the basis of these results, it is probable that the dosage used in the present experiments were too small to cause the sufficient immunosuppression in mice.

Summary

Effects of immunosuppressive agents on *Trypanosoma cruzi* infection were evaluated by using the experimental mice inoculated with cultured form of Berenice strain of the organisms. In order to enhance the infection with the organism, cortisone, cyclophosphamide, 6-mercaptopurine and 8-azaguanine were administered to the mice inoculated with the organism, number of which was a little too small to produce parasitemia in normal mice. The trypanosomes in the blood of cyclophosphamide and 6-MP-treated mice increased in number as compared with these of the control mice. The mortalities of cyclophosphamide and 6-MP-treated mice were 80 % and 30 % respectively by two weeks after the inoculation of the trypanosomes showing great differences from those of the mice treated by the other compounds as well as the control mice. It may be concluded that the administration of cyclophosphamide was effective enough to intensify the experimental trypanosomes infection in mice.

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Trypanosoma cruzi のマウス感染性に対する cortisone, cyclophosphamide, 6-mercaptopurine, 8-azaguanine の効果について

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Trypanosoma cruzi が *in vitro* で長期にわたつて継代培養されても実験動物に対する感染性が失われることはない．しかし感染を成立させるに必要な接種量は，感染動物の血液より得られる trypanosoma 型を接種する場合と比較して培養された虫体を接種する場合にははるかに多くの量を必要とする．今回の実験ではマウスに parasitemia を起させない程度の量を接種すると同時に cortisone, cyclophosphamide, 6-mercaptopurine, 8-azaguanine などを投与し，感染が促進されるかどうかを調べた．LIT medium で1週間培養した虫体をマウス腹腔へ3段階の希釈濃度で接種した．そして7日後より3日おきに4匹ずつのマウスの心臓より血液をとり虫体の有無を検査した．1滴の血液を鏡検する直接法ではいずれの場合にも虫体を見出すことはできなかつた．しかし残りの血液を NNN 培地で培養し，1カ月後の検査では，3×10^6 の虫体を接種したマウスの血液に虫体が検出された．次に無処置のマウスで虫体が検出されないような虫体接種量濃度でそれぞれに，cortisone を1日おきに 0.25 mg, cyclophosphamide を 1 mg/day 6日間, 6-mercaptopurine を 1 mg/day 6日間, 8-azaguanine を 1 mg/day 6日間筋肉中へ投与し，血液中の虫体検出を行なつた．この場合 cyclophosphamide および 6-MP を投与したマウスでは，血液培養による方法で虫体が検出された．培養された *Trypanosoma cruzi* を接種すると同時にこれらの薬剤を投与したマウスの接種後2週までの死亡率をみると，cyclophosphamide を投与した場合 80 %と非常に高く，6-MP を投与した場合 30 %であつた．他の cortisone, 8-azaguanine を投与した場合には死亡率は control と比較して変りなかつた．以上の結果から cyclophosphamide および 6-MP の投与が，培養虫体のマウスに対する感染性を促進させる効果があると思われる．