Differences in Resistance to Reinfection with Low and High Inocula of *Trypanosoma cruzi* in Chagasic Mice Treated with Nifurtimox and Relation to Immune Response

PATRICIA CABEZA MECKERT, JORGE G. CHAMBÓ, AND RUBÉN P. LAGUENS*

Cátedra de Patología II, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, Calle 60 y 120, La Plata 1900, Argentina

Received 21 May 1987/Accepted 8 November 1987

Reinfection of chronic chagasic mice after treatment with nifurtimox resulted in different outcomes according to the number of parasites used for inoculation. Nifurtimox-treated chagasic animals injected with 2,500 trypomastigotes developed higher parasitemia and increased mortality compared with nontreated chagasic mice. When reinfection was done with 25 trypomastigotes, treated and nontreated animals showed similar parasitemias and mortalities, which were significantly higher in nonchagasic controls infected for the first time. Immunological studies showed that treatment with nifurtimox led to a decrease in anti-*Trypanosoma cruzi* antibodies engaged in parasite destruction, inducing either complement-dependent lysis or antibody-dependent cytotoxicity, but no difference in anti-*T. cruzi* cell-mediated immunity was found between treated and nontreated chagasic animals. It is concluded that treatment with nifurtimox leads to a loss of resistance to reinfection with a large number of trypanosomes, which is maintained with challenge with a few parasites, and that these two thresholds of premunition are probably associated with humoral and cell-mediated anti-*T. cruzi* immune responses, respectively.

Human or animal infection with *Trypanosoma cruzi* is lifelong. It is usually held that chronically infected hosts are resistant to new infections (2).

The terms premunition and concomitant immunity have been coined to define this situation. Apparently, resistance depends on a permanent antigenic stimulation due to the persistence of living parasites in the organism. Sterilization induced by treatment with trypanocidal drugs leads to a loss of premunition (3), and successful reinfection of treated animals is considered to be an index of parasitological sterilization.

In most assays performed for determination of premunition, reinfection is done with a large and usually lethal number of parasites. To our knowledge, the result of reinfection with a low number of parasites in animals parasitologically sterilized by treatment with trypanocidal drugs has not been explored. This information would be relevant for a better understanding of the human situation, since most patients treated during the acute or chronic stage of the disease remain living in areas endemic for *T. cruzi* and are therefore exposed to reinfection with a low number of parasites.

We report here the evolution of chronic chagasic mice treated with nifurtimox and reinfected with a high or low number of parasites and the effect of a trypanocidal drug on the humoral and cell-mediated anti-*T. cruzi* immune response, in order to investigate the putative mechanisms involved with the maintenance or loss of the premunition state.

MATERIALS AND METHODS

Experimental design. Chronic Chagas' disease was induced in mice as previously reported (10). Briefly, 3-month-old female BALB/c mice were infected by the intraperitoneal route with 25 trypomastigotes of the Tulahuen strain of *T.*

* Corresponding author.

cruzi. Parasitemia was determined weekly, and surviving animals which showed circulating parasites during month 1 of the postinfection (p.i.) period were used for the experiments. At 3 months p.i., mice were treated with nifurtimox (100 mg/kg [body weight]) daily for 30 days by the intragastric route (group 1). As controls, chagasic animals received distilled water (group 2) and noninfected mice of matching age and sex received nifurtimox daily (group 3) by the same route.

Two months after the end of treatment, (i) from 15 animals in each group blood was collected from the retro-orbital plexus for subinoculation and for cell and serum separation for immunological studies, (ii) 10 mice from each group were injected in the hind footpad with *T. cruzi* antigens, and (iii) 20 mice from each group were infected with either 25 or 2,500 trypomastigotes. In these animals, mortality was determined daily and parasitemia was determined weekly for 5 weeks.

Anti-*T. cruzi* antibodies. Conventional serology was performed by standard immunofluorescence (5) and hemagglutination (13) techniques. Complement-dependent lytic antibodies were investigated by the method of Krettli and Brener (8). Briefly, *T. cruzi* trypomastigotes were collected at day 7 p.i. from the peripheral blood of lethally irradiated (800 rads) mice. Parasites were incubated with fresh human serum for 45 min at 37°C and washed thoroughly with RPMI 1640 medium with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer. Mouse test serum was preheated (56°C for 30 min) and diluted 1/4 in RPMI 1640 medium-HEPES, and 10^6 trypomastigotes were incubated with 50 µl of diluted test serum for 1 h at 37°C. After 1 h, 50 µl of fresh or preheated (56°C for 30 min) human serum was incorporated, and after incubation for 45 min at 37°C the parasites were counted in a hemacytometer. Lysis was calculated as follows: % lysis = number of parasites after incubation with fresh human serum/number of parasites after incubation with preheated human serum × 100. Lysis higher than 30% was considered positive.

TABLE 1. Anti-*T. cruzi* humoral immune response

Chagasic mouse group	No. positive/total no. of mice			ADCC (% of lysis ± SD) with effector cells:		
	Hemagglutination[a]	Immunofluorescence[b]	Complement-dependent lysis[c]	Polymorphonuclear leukocytes	Blood mononuclear leukocytes	Spleen mononuclear cells
Nontreated	13/14	13/14	12/14	69 ± 8^d	58 ± 12^e	57 ± 13.5^f
Nifurtimox treated	9/11	10/13	0/13	17 ± 10.4^d	17.6 ± 13^e	13.6 ± 9.9^f

[a] Titer higher than 1/40.
[b] Titer higher than 1/32.
[c] Positive, Lysis higher than 30%.
[d] $P < 0.0005$ (Student's t test).
[e] $P < 0.05$ (Student's t test).
[f] $P < 0.025$ (Student's t test).

Anti-*T. cruzi* cell-mediated immunity. Increase in the weight of the popliteal lymph node and production of leukocyte-inhibition-migrating factor were used for studying anti-*T. cruzi* cell-mediated immunity. As the antigen, a whole homogenate of *T. cruzi* epimastigotes grown in diphasic medium and disrupted by compression-decompression was used (16). To determine the increase in weight of the popliteal lymph node, 0.04 ml of the whole homogenate containing 100 μg of protein was injected into the hind footpad. The contralateral footpad received 0.04 ml of *T. cruzi* culture medium. Three days later, the animals were killed and the popliteal lymph nodes were dissected and weighed. For investigation of leukocyte-inhibition-migrating factor production, blood from the retro-orbital plexus was collected in heparinized tubes and left to sediment in 6% dextran for 1 h at 37°C. The leukocytes were washed three times in RPMI 1640 medium with HEPES buffer, placed (2 × 10^5) in capillary tubes with one sealed end, and centrifuged. The capillary tubes, sectioned above the leukocyte layer, were placed in Falcon petri dishes with RPMI 1640-HEPES medium containing 10% fetal bovine serum and *T. cruzi* homogenate (100 μg of whole protein) and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. As a control, the same procedure was used with culture medium alone. The surface area of migrating cells was estimated with a calibrated eyepiece. Inhibition of migration was determined with the following equation: % inhibition = mean area with antigen/mean area without antigen × 100. Determinations were triplicated for each animal.

Subinoculation. A 0.2-ml sample of blood was injected intraperitoneally into suckling Swiss albino mice. Mortality was recorded daily, and parasites were sought weekly in blood by direct microscopic examination.

ADCC. Antibody-dependent cytotoxicity (ADCC) was investigated by the method of Kierszenbaum (7). *T. cruzi* trypomastigotes were collected from irradiated mice. Mononuclear and polymorphonuclear leukocytes from blood and mononuclear spleen cells were collected in a Ficoll-Hypaque gradient. Trypomastigotes and cells were incubated 2/1 with the test sera or with noninfected mouse serum diluted 1/10 in RPMI 1640-HEPES medium for 5 h at 37°C. The number of surviving parasites was determined in a hemacytometer. ADCC was calculated as follows: ADCC = 100 − number of parasites incubated with test serum/number of parasites incubated with normal serum × 100.

Statistics. Statistical analyses were performed by using the χ^2 test and Student's t test.

RESULTS

Effect of nifurtimox treatment. In the control nontreated mice chronically infected with the Tulahuen strain of *T.*

cruzi, circulating parasites could be recovered from all animals by inoculation of blood into suckling mice, although no parasites were observed by direct microscopic examination. Two months after the 30-day treatment of chagasic mice with nifurtimox, no parasites could be recovered by subinoculation into suckling mice. No mortality was observed in either the nontreated or the treated group.

Anti-*T. cruzi* antibodies demonstrable by conventional immunofluorescence and hemagglutination techniques were present in the sera of all treated and nontreated chagasic mice, but those inducing complement-dependent lysis of the parasite or ADCC activity could not be demonstrated after treatment, although they were present in the sera of most of the nontreated chagasic mice (Table 1). The investigation of anti-*T. cruzi* cell-mediated immunity did not show differences between the nontreated and nifurtimox-treated infected mice (Table 2).

Evolution of mice reinfected with 2,500 trypomastigotes. Cumulative mortality was high in the chagasic animals treated with nifurtimox, and at 5 weeks p.i. all were dead. Nontreated reinfected chagasic mice and those infected for the first time with 2,500 parasites showed similar mortalities, and at the end of the experiment half of the mice were still alive (Fig. 1). In the group of nontreated chagasic mice, about 50% of the animals showed patent microscopic parasitemia at 7 days p.i., but with the weekly samples obtained thereafter the number of animals with positive parasitemia decreased, and at 5 weeks postreinfection no parasites could be seen in the surviving animals. In the remaining two groups, all the animals showed circulating parasites during the entire experiment (Fig. 2).

The numbers of circulating parasites were different in the three groups. As can be seen in Fig. 3, in the nifurtimox-treated chagasic mice the number of parasites increased steadily until week 4 postreinfection. Meanwhile, in the nontreated and reinfected chagasic mice the number of parasites was significantly lower during weeks 1 and 2 postreinfection, when animals with circulating parasites were still present. In mice infected for the first time, the

TABLE 2. Anti-*T. cruzi* cell-mediated immune response[a]

Mouse group	LIF[b] (%)	Popliteal lymph node test[c] (mg)
Noninfected	4.16 ± 2.48^d	$+0.6 \pm 0.14^d$
Nontreated chagasic	92.5 ± 5.2	$+5.43 \pm 1.99$
Nifurtimox-treated chagasic	84.6 ± 3.91	$+8.56 \pm 4.3$

[a] Data are the means for 10 mice ± standard error of the mean.
[b] LIF, Leukocyte-inhibition-migrating factor.
[c] Difference in weight between test and contralateral lymph nodes.
[d] $P < 0.005$ compared with the remaining two groups (Student's t test).
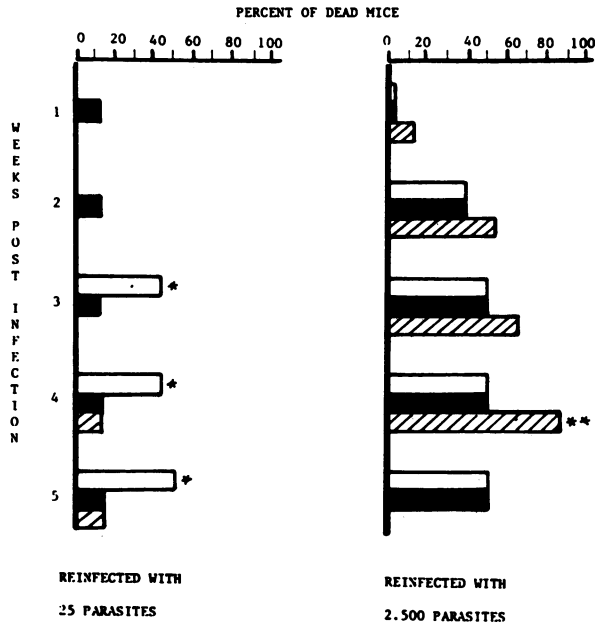
PERCENT OF DEAD MICE

FIG. 1. Percent mortality in nifurtimox-treated chagasic mice (▨) after reinfection with 25 or 2,500 trypomastigotes. □, Nonchagasic mice; ■, nontreated chagasic mice; **, $P < 0.01$ (χ^2 test); *, $P < 0.05$ (χ^2 test).

number of parasites was always higher than in the chagasic animals, although a marked weekly variation was observed.

Evolution of chagasic mice reinfected with 25 trypomastigotes. As can be seen in Fig. 2, about 75% of the animals infected the first time presented patent parasitemia during the experiment. In the chagasic animals, either treated with nifurtimox or nontreated, the number of animals with posi-

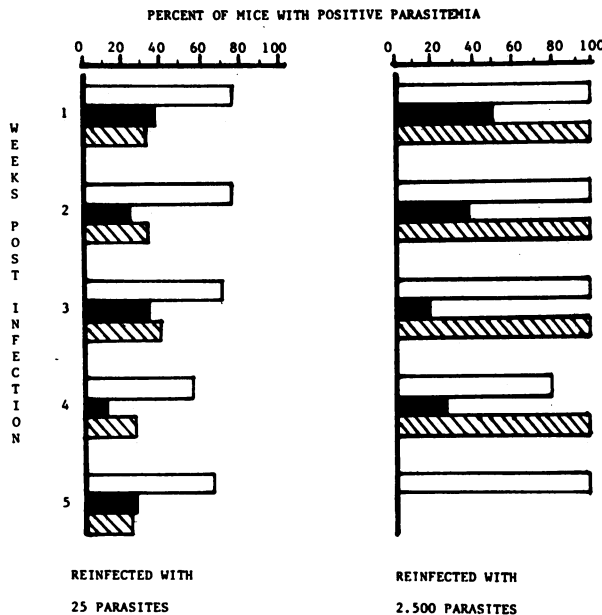PERCENT OF MICE WITH POSITIVE PARASITEMIA

FIG. 2. Percentage of mice presenting circulating parasites by direct microscopic examination. Each bar represents the percentage of 20 mice. □, Nonchagasic mice; ■, nontreated chagasic mice; ▨, nifurtimox-treated chagasic mice.
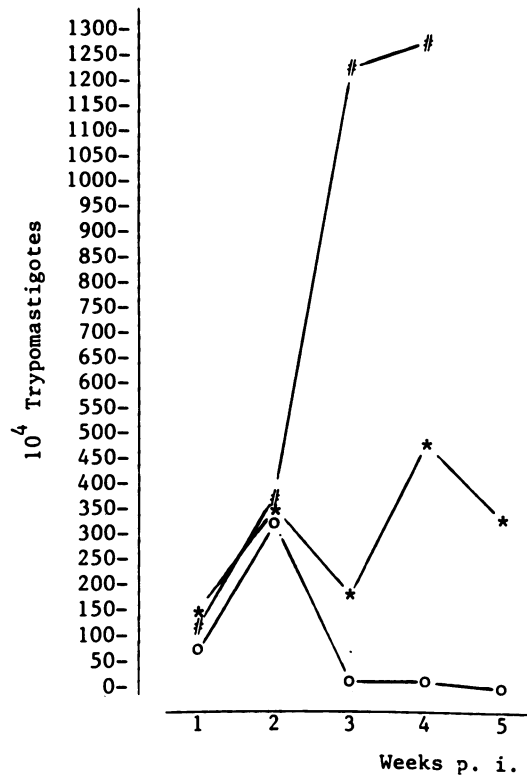
FIG. 3. Number of circulating parasites per milliliter in nifurtimox-treated chagasic mice reinfected with 2,500 trypomastigotes. O, Nontreated chagasic mice; *, mice infected for the first time; #, nifurtimox-treated chagasic mice.

tive parasitemia was significantly lower than in the previous group, as was the number of circulating parasites, which was higher in the mice infected for the first time (Fig. 4). In this group, mortality attained 60% after week 3 p.i. Meanwhile, in the chagasic mice, either nifurtimox treated or untreated, mortality was below 10% at the end of the 5-week observation time (Fig. 1).

DISCUSSION

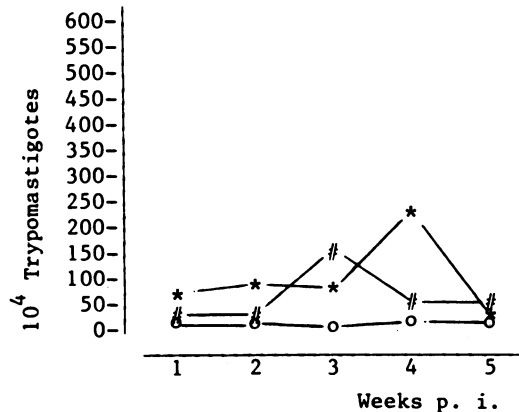Our observations show that the reinfection of nifurtimox-treated chronically chagasic mice follows a course different

FIG. 4. Number of circulating parasites per milliliter in nifurtimox-treated chagasic mice reinfected with 25 trypomastigotes. O, Nontreated chagasic mice; *, mice infected for the first time; #, nifurtimox-treated chagasic mice.

from that of nontreated animals according to the number of parasites used for reinoculation. Compared with the non-treated chronically infected animals, which were relatively resistant to reinfection with a high number of parasites, treated animals showed higher parasitemia and mortality. This indicates that they had lost their premunition state, which is associated with parasitological sterilization, according to accepted criteria (3). The fact that premunition was not completely protective, since reinfected chagasic mice presented a transient elevation of parasitemia and 50% mortality, is in agreement with previous observations from our laboratory (4).

It was surprising that the progress of the disease in nifurtimox-treated chagasic animals reinfected with 2,500 trypomastigotes was more severe than that observed in control mice infected for the first time with the same number of parasites. The possibility that treatment with nifurtimox induced an immune depression appears unlikely, since the latter animals also received the drug. Moreover, although nifurtimox can induce an immune depression, especially on cell-mediated immunity (11), the 2 months elapsed between the end of treatment and reinfection and the fact that the anti-*T. cruzi* cell-mediated immune response was similar in both the nifurtimox-treated and the nontreated chagasic mice support the opinion that the drug is not directly responsible for the exacerbated course of reinfection.

However, other explanations, such as the possibility that reinfection after nifurtimox treatment induces a secondary immune response with prevalence of antibodies not directly engaged in parasite destruction or even with a protective ability, remain to be explored.

When mice were reinfected with a low number of parasites, no difference was found between nifurtimox-treated and nontreated chagasic mice. The incidences of parasitemia and mortality were similar in both groups and were significantly lower than in the untested control mice, indicating a persistence of premunition, although the number of circulating parasites was higher in the treated than in the nontreated infected animals.

This different behavior in the evolution of reinfection with a few parasites in nifurtimox-treated chagasic mice compared with those challenged with a higher number of parasites can be tentatively correlated with the different effects of treatment on the humoral and cell-mediated anti-*T. cruzi* immune response. Antibodies inducing complement-dependent lysis and ADCC of the parasite could not be demonstrated 2 months after treatment, but a strong cell-mediated immune response was still present at that time. These observations suggest that the latter could be able to protect against a small number of parasites but would be overwhelmed with larger inocula, when anti-*T. cruzi* antibodies should be necessary to interfere successfully with the infection. Although cell-mediated anti-*T. cruzi* immunity has been repeatedly demonstrated in chronically infected humans and animals, its role in antiparasitic defense is not clear. Current opinion holds that antibodies inducing either complement-mediating lysis (9) or ADCC are the main protective mechanisms and that cellular immunity plays a minor role (18, 19). The results of our study are relevant for the human situation. Trypanocidal drugs are indicated for treatment of Chagas' disease during acute infection, because in this situation parasitological sterilization can frequently be obtained (1). In the chronic stage, although treatment is less efficient, occasional "cures" have been reported (6). Knowledge about sensitivity to reinfection in treated patients living in endemic areas, where they are constantly exposed to new infections,

should be relevant, either to determine resistance of treated humans or to consider the possibility of altering the parasite cycle by treatment of the intermediate hosts, such as dogs. In addition, the fact that our results show that apparently there are two thresholds of premunition to reinfection with *T. cruzi*, one for a low number of parasites, probably dependent on cellular immune mechanisms, and another for a high number, related to humoral immunity, should be useful for vaccine trials. Most studies on resistance after immunization with either attenuated strains (12), *T. cruzi* fragments (14), or surface glycoproteins (15, 17) have focused on the humoral response, and test challenges have been made with a large number of parasites. In our opinion, these studies should also include evaluation of anti-*T. cruzi* cell-mediated immune responses and challenge with a small number of parasites, simulating some of the conditions of human infection.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Bocca Tourres, C. L. 1969. La enfermedad de Chagas en el período agudo y su tratamiento con Bayer 2502. Bol. Chil. Parasitol. 24:25–31.
2. Brener, Z. 1962. Observacoes sobre a immunidade a superinfeccoes en camundogos experimentalmente inoculados con Trypanosoma cruzi e submetidos a tratamiento. Rev. Inst. Med. Trop. Sao Paulo 4:119–126.
3. Brener, Z. 1962. Therapeutic activity and criterion of cure on mice experimentally infected with Trypanosoma cruzi. Rev. Inst. Med. Trop. Sao Paulo 4:389–396.
4. Cabeza Meckert, P. M., and R. P. Laguens. 1981. Chronic Chagas' disease in the mouse. III. Absence of concomitant immunity after repeated infections. Medicina (Buenos Aires) 41:543–548.
5. Camargo, M. E. 1966. Fluorescent antibody test for the diagnosis of American trypanosomiasis. Technical modification employing preserved culture forms of Trypanosoma cruzi in a slide test. Rev. Inst. Med. Trop. Sao Paulo 8:227–230.
6. Cancado, J. R., and Z. Brener. 1978. Terapeutica in Trypanosoma cruzi e doenca de Chagas, p. 362–424. In Z. Brener and Z. Andrade (ed.), Trypanosoma cruzi e doenca de Chagas. Editora Guanabara Koogan, Rio de Janeiro, Brazil.
7. Kierszenbaum, F. 1979. Antibody dependent killing of bloodstream forms of Trypanosoma cruzi by human peripheral blood leukocytes. Am. J. Trop. Med. Hyg. 28:965–969.
8. Krettli, A., and Z. Brener. 1982. Resistance against Trypanosoma cruzi associated to anti-living trypomastigote antibodies. J. Immunol. 128:2009–2012.
9. Krettli, A. V., J. Romeu Cancado, and Z. Brener. 1982. Effect of specific chemotherapy on the levels of lytic antibodies in Chagas' disease. Trans. R. Soc. Trop. Med. Hyg. 76:334–340.
10. Laguens, R. P., P. M. Cabeza Meckert, M. A. Basombrio, J. G. Chambo, P. Cossio, R. M. Arana, and R. Gelpi. 1980. Infección crónica del ratón con Trypanosoma cruzi. Modelo experimental de enfermedad de Chagas. Medicina (Buenos Aires) 40:33–39.
11. Lelchuk, R., R. Cardoni, and A. Fuks. 1977. Cell mediated immunity in Chagas' disease. Alterations induced by treatment with a trypanocidal drug (nifurtimox). Clin. Exp. Immunol. 30:434–438.
12. Menezes, H. 1971. Aplicacao da vacina viva avirulenta de Trypanosoma cruzi en seres humanos (nota previa). Rev. Inst. Med. Trop. Sao Paulo 13:144–154.
13. Neal, R. A., and R. A. Miles. 1970. Indirect haemagglutination test for Chagas' disease with a simple method survey work.

Rev. Inst. Med. Trop. Sao Paulo **12**:325–328.
14. **Ruiz, A. M., M. Esteva, P. M. Cabeza Meckert, R. P. Laguens, and E. L. Segura.** 1985. Protective immunity and pathology induced by inoculation of mice with different subcellular fractions of Trypanosoma cruzi. Acta Trop. **42**:299–309.
15. **Scott, M. T., and D. Snary.** 1979. Protective immunization of mice using cell surface glycoprotein from Trypanosoma cruzi. Nature (London) **282**:73–74.
16. **Segura, E. L., E. M. Cura, I. Paulone, C. Vasquez, and J. Cerisola.** 1974. Antigenic makeup of subcellular fraction of

Trypanosoma cruzi. J. Protozool. **21**:571–574.
17. **Snary, D.** 1983. Cell surface glycoproteins of Trypanosoma cruzi. Protective immunity in mice and antibody levels in human chagasic sera. Trans. R. Soc. Trop. Med. Hyg. **77**:126–129.
18. **Trischann, T. M.** 1978. Role of cellular immunity in protection against Trypanosoma cruzi in mice. Parasite Immunol. **6**: 561–565.
19. **Trischmann, T. M., and B. R. Bloom.** 1980. Trypanosoma cruzi. Ability of T-cell enriched and depleted lymphocyte populations to passively protect mice. Exp. Parasitol. **9**:225–232.