

## Enhanced Resistance to Acute Infection with *Trypanosoma cruzi* in Mice Treated with an Interferon Inducer

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For an exploration of the effects of interferon-inducible resistance mechanisms in acute American trypanosomiasis, the synthetic interferon inducer tilerone hydrochloride was administered to mice of the C57BL/6J strain, which is highly resistant to *Trypanosoma cruzi*, 18 to 24 h before infection with a potentially lethal dose of bloodstream trypomastigotes. Although all of the control mice died within 30 days of the acute infection, approximately 50% of the tilerone-treated animals were able to survive indefinitely ( $P < 0.05$ ). The tilerone-treated mice demonstrated significant levels of serum interferon and splenic natural killer cells at the time of infection. Macrophages isolated from the peritoneal cavities of tilerone-treated C57BL/6J mice appeared to kill significant numbers of trypanosomes during 2 to 3 days of in vitro culture, indicating that activated macrophages may contribute to the enhanced resistance to *T. cruzi* infection in these mice. Beige mice treated with tilerone did not survive *T. cruzi* infection as well as tilerone-treated heterozygotes did, suggesting a role for natural killer cells in interferon-induced resistance. These results suggest that interferon or effector mechanisms enhanced by interferon induction can play a significant role in influencing resistance to *T. cruzi* infection.

Developmental stages of *Trypanosoma cruzi*, the protozoan that causes human Chagas' disease, can be found in the blood and tissues; however, the parasite multiplies only intracellularly in the mammalian host. The acute phase of the disease is fatal in approximately 10% of hospitalized cases (14). After the acute phase, immunity to acute secondary infection develops; however, the initial infection may persist in a chronic phase, which can eventually affect the heart and visceral organs. The factors governing resistance to acute trypanosomiasis in humans are unknown. Likewise, there appears to be a wide differential in innate susceptibility to acute *T. cruzi* infection among inbred strains of mice. Animals with the C57BL genetic background are relatively resistant to the disease, as determined by development of low levels of parasitemia and a high percentage of survival, whereas A-strain mice are highly susceptible (31). Again, the factors responsible for these differences are not understood, although they do not appear to be determined by the murine major histocompatibility complex (31).

There is some evidence of correlation between levels of interferon or interferon-inducible

activities, such as natural killer (NK) cell and macrophage function, and host resistance to a wide variety of intracellular bacterial and protozoal infections (1, 4, 8, 10, 18, 24), suggesting that these activities may participate in natural resistance mechanisms. In the current study, to explore the relationship between interferon and resistance to *T. cruzi*, we used a synthetic inducer, tilerone hydrochloride, to exogenously augment interferon levels in mice of the innately resistant C57BL/6J inbred strain and subsequently challenged them with a dose of *T. cruzi* bloodstream trypomastigotes which would be lethal under normal conditions. Significantly enhanced survival was observed in the tilerone-treated mice compared with that of control animals. It was determined that this probably was not due to a direct effect of interferon on the parasite, but possibly to an augmentation of NK cell or macrophage activity in the treated animals.

### MATERIALS AND METHODS

**Animals.** Female mice of the A/J and C57BL/6J strains, 5 to 6 weeks of age, were obtained from Jackson Laboratories, Bar Harbor, Maine. At this age, all animals weighed between 12 and 18 g. Mice of the C57BL/6 line with the beige mutation (C57BL/6-*bg*<sup>l</sup>/*bg*<sup>l</sup>) and their heterozygous littermates (*bg*/*+*) were

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also obtained from Jackson Laboratories at 6 weeks of age.

**Interferon induction.** Mice were treated with the interferon inducer tilerone hydrochloride (generously supplied by William L. Albrecht, Merrell-National Laboratories, Cincinnati, Ohio). A solution of tilerone in phosphate-buffered saline (PBS) was administered at 250 mg/kg of body weight by gastric intubation. This dose has been shown to induce high titers of interferon in the organs of mice 18 to 24 h after oral administration (16). Control animals were treated with equivalent amounts of PBS only.

**Trypanosome infection.** Trypomastigotes of *T. cruzi* (Y strain) were obtained from the blood of A/J mice 5 to 7 days after infection with  $1.5 \times 10^5$  parasites. The donor mice were irradiated with 700 R from a  $^{137}\text{Ce}$  source 1 to 2 days before infection, a procedure which has been shown to permit recovery of bloodstream forms free of bound antitrypanosome antibodies (12). In some experiments, the isolated trypomastigotes were opsonized before injection by incubation for 30 min at 37°C in a 1:60 dilution of hyperimmune serum obtained from multiply infected mice known to contain specific immunoglobulin G antibodies at a titer of 1:1,600 as tested by the enzyme-linked immunosorbent assay (19).

Unless otherwise stated, mice were infected 18 to 24 h after drug treatment. C57BL/6J mice were injected intraperitoneally with  $5 \times 10^3$  or  $2 \times 10^4$  washed trypomastigotes, A/J mice received either  $10^3$  or  $10^4$  trypanosomes, and beige mice were infected with  $5 \times 10^3$  trypomastigotes. The course of the infection was followed by determination of parasitemia (7) during the first 2 weeks and by cumulative mortality. Groups of seven animals were used for each experimental regimen. Differences in the length of survival time between control and tilerone-treated groups were evaluated by the Wilcoxon two-sample test, using the two-tailed 5% significance level.

**Functional analysis of tilerone-treated mice.** (i) **Serum interferon levels.** Approximately 18 to 24 h after treatment with PBS or tilerone, mice were sacrificed by ether overdose and bled. Serum was collected and stored at -70°C for analysis of interferon levels. Interferon levels were determined by Bioassay Systems, Inc., Woburn, Mass., using mouse L929 fibroblast indicator cells and vesicular stomatitis virus, Indiana strain. Duplicate samples of serum were tested in 12 serial twofold dilutions, and endpoints were taken as that dilution resulting in 50% inhibition of the viral cytopathic effect. Interferon activity of test samples was calculated on the basis of activity of an NIH mouse interferon reference standard.

(ii) **NK cell activity.** NK cell activity was measured by the ability to lyse YAC-1 lymphoma cells in a standard 4-h  $^{51}\text{Cr}$  release assay (3). Spleens were removed 18 to 24 h after treatment with PBS or tilerone and teased into cell suspensions. The spleen cells were placed into culture with  $^{51}\text{Cr}$ -labeled YAC-1 tumor cells at effector/target ratios of 100:1. Activity was determined as the percent specific release, according to the formula:  $[(A - B)/(C - B)] \times 100$ , where *A* represents the mean counts per minute in supernatant fluids obtained from quadruplicate samples of spleen cells plus tumor cells, *B* represents background spontaneous release from tumor cells incubated alone, and *C* represents maximum release obtained by six

cycles of freezing and thawing of the tumor cells followed by the addition of distilled water.

(iii) **Macrophage activity.** The ability of macrophages from these mice to kill *T. cruzi* was evaluated as described previously (8, 11). Resident peritoneal cells were obtained from mice 2 days after treatment with PBS or tilerone by lavage with minimum essential medium containing 2 U of heparin per ml. Pooled cells from three to four animals were washed and resuspended in minimum essential medium containing 10% heat-inactivated (56°C for 45 min) fetal calf serum (Microbiological Associates, Walkersville, Md.). A suspension containing  $9 \times 10^5$  cells was layered over sterile 12-mm glass cover slips and allowed to adhere for 2 h at 37°C. Nonadherent cells were then washed off, and a suspension of  $6 \times 10^5$  bloodstream trypomastigotes was layered onto the cover slips. After 4 h of incubation, the remaining extracellular parasites were removed from the cultures by washing five times with minimum essential medium. Triplicate cultures were evaluated for intracellular parasites immediately after exposure and after incubation for 48 to 60 h by fixing them in methanol and staining them with Giemsa solution. Cells in 8 to 10 random 400× fields were examined, and the percentage of infected cells and the mean number of intracellular parasites per 100 cells were calculated.

## RESULTS

**Effect of tilerone treatment on *T. cruzi* infection.** The three experiments in Fig. 1 show that tilerone treatment significantly reduced mortality in C57BL/6J mice ( $P < 0.05$ ). Such a twofold or greater enhancement of survival was observed in a total of seven experiments in which various infectious doses of trypomastigotes were used. Opsonization of the inoculum did not affect this survival pattern. The difference in mortality was not reflected in the level of parasitemia, which was similar at 1 and 2 weeks after infection in both tilerone-treated and control mice, perhaps reflecting the predilection of the Y strain for rapid invasion of host cells.

The surviving C57BL/6J mice from experiment 1 were found to be completely resistant to acute infection when given a second inoculation with as many as  $2.5 \times 10^5$  trypomastigotes. However, culture of the blood (20) taken from the tilerone-treated mice which had survived initial infection in experiment 2 revealed that after 150 days some were still harboring parasites.

In one experiment, tilerone was also effective in treating established infections. C57BL/6J mice were treated with tilerone 4 days after infection with  $2 \times 10^4$  trypomastigotes, rather than 24 h before. Although all PBS-treated control animals were dead by 21 days after infection, 100% survival of the drug-treated mice was observed when the experiment was terminated at day 60.

In contrast to the results observed with C57BL/6 mice, tilerone failed to protect A/J

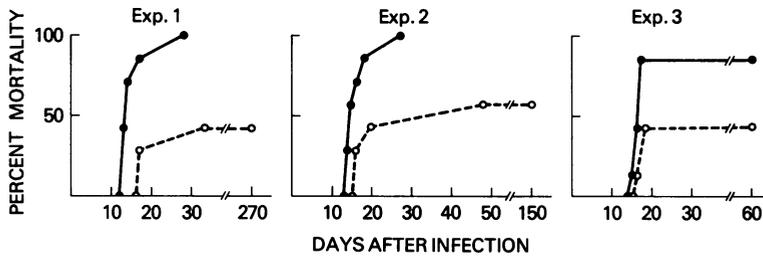


FIG. 1. Enhancement of survival in tilerone-treated C57BL/6J mice. Animals were treated with PBS (●) or tilerone (○) 18 to 24 h before infection with *T. cruzi* as described in the text. Mice were infected with  $2 \times 10^4$  and  $5 \times 10^3$  opsonized trypomastigotes in experiments 1 and 2, respectively, and with  $5 \times 10^3$  untreated parasites in experiment. 3.

mice inoculated with  $10^3$  to  $10^4$  *T. cruzi*. When A/J animals were examined concomitantly with C57BL/6 mice in the experiments depicted in Fig. 1, the control and the drug-treated mice were all dead by days 17, 21, and 27 after infection in experiments 1 to 3, respectively.

**Effect of tilerone treatment on serum interferon levels and NK cell activity.** Although the effects of tilerone on interferon and NK cell activity have been well documented previously (3, 16), it was desirable to perform a direct comparison of these activities in the two strains of mice used in the current study. Results from a representative experiment are shown in Table 1. At 18 to 24 h after tilerone treatment, serum interferon levels were elevated in both strains. Similarly, the YAC-1 cytotoxic activity of spleen cells was significantly enhanced ( $P < 0.001$ ) in both A/J and C57BL/6 mice. In agreement with the findings of others (21), the endogenous levels of NK cell activity in the spleens of A-strain mice were consistently somewhat lower than those in C57BL/6 mice tested within the same assay.

**Influence of the beige gene on tilerone-induced resistance.** In an effort to determine the role of NK cells in the system described here, we examined the effect of tilerone treatment on resistance to acute infection with *T. cruzi* in NK cell-deficient (25, 26) homozygous beige (*bg/bg*) mice and their phenotypically normal heterozygous (*bg/+*) littermates. PBS-treated control *bg/bg* animals died rapidly after infection, reaching 100% mortality within 22 days (Fig. 2). *bg/+* control mice also succumbed rapidly. By day 20, 86% of these animals were dead; however, the one remaining animal was able to survive acute infection and lived until the experiment was terminated at day 170. Again, a striking effect of tilerone treatment was observed in the heterozygous mice, of which none died until day 50 and 86% survived for the entire course of the experiment ( $P < 0.01$ ). Tilerone treatment partially protected the *bg/bg* mice. On day 22, when all of the control homozygous animals were dead, 71% of the treated mice were still alive, and

although three more animals died later after infection, two lived until the end of the experiment. Thus, although the survival rate of the tilerone-treated *bg/bg* mice was not significantly different from that of the control *bg/bg* animals, neither was it different from that of the tilerone-treated heterozygotes ( $0.05 < P < 0.06$  in both cases), indicating that the beige mutation depresses responsiveness to tilerone but does not eliminate it.

**Effect of tilerone on macrophage activity.** More parasites entered macrophages from the tilerone-treated C57BL/6J mice than from the appropriate control mice after 4 h of incubation (Table 2). Macrophages from treated A/J mice also took up greater numbers of trypomastigotes. After 2 to 3 days of cultivation, the numbers of parasites within cells from tilerone-treated C57BL/6 animals declined ( $P < 0.001$ ), suggesting that some of the trypanosomes were being killed during this period.

TABLE 1. Effect of tilerone treatment on interferon titer and NK cell activity

Mouse strain	Interferon titer <sup>a</sup>	NK cell activity <sup>b</sup>
A/J		
Control <sup>c</sup>	<8	39 ± 3
Tilerone <sup>d</sup>	4,096	57 ± 2
C57BL/6J		
Control	<8	49 ± 2
Tilerone	4,096	64 ± 2

<sup>a</sup> Interferon activity in a pool of sera from five mice was determined by inhibition of viral cytopathic effect.

<sup>b</sup> NK cell activity was determined by lysis of YAC-1 tumor cells in a 4-h assay. Data represent mean percent specific release ± standard deviation by spleen cells from five animals. Both differences are significant at  $P < 0.001$  by the two-tailed Student *t* test.

<sup>c</sup> Control animals were treated with PBS as described in the text.

<sup>d</sup> Tilerone-treated animals received a solution of the drug in PBS at 250 mg/kg of body weight.

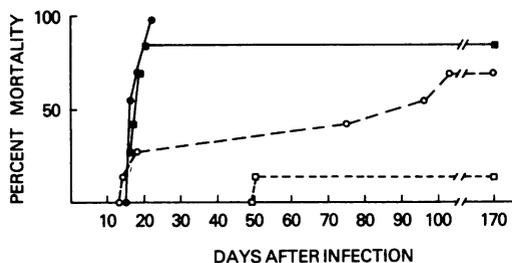


FIG. 2. Response of tilerone-treated beige mice to *T. cruzi* infection. Control *bg/+* (■) or *bg/bg* (●) mice were treated with PBS, and tilerone-treated *bg/+* (□) or *bg/bg* (○) mice received the drug 18 h before all were infected with  $5 \times 10^3$  trypomastigotes.

Interestingly, although no evidence for killing was observed, neither did the parasites appear to be multiplying appreciably within the C57BL/6 control macrophages in either experiment. At the same time, however, nests of amastigotes were observed forming within concomitantly infected control A/J cells after *in vitro* cultivation. Parasite growth, absent in C57BL/6 macrophages but present in A/J macrophages, therefore appeared to be a function of the cells rather than of the trypomastigote populations used for the infection or the experimental protocol, in contrast with the observations of others (17) that there is no difference in growth rate of parasites in cells from susceptible and resistant strains of mice when trypomastigote culture forms are used. In the one experiment in which tilerone effect was tested in cells from A/J mice, the number of intracellular parasites remained con-

stant, although there was a decline in the proportion of infected cells.

## DISCUSSION

Recently, considerable interest has been focused on the role of interferon or interferon-related effector mechanisms in resistance to infection and disease. In this study, we have shown that resistance to acute infection with the protozoan parasite *T. cruzi* can be greatly augmented by treatment with an interferon-stimulating drug.

*In vivo* induction of interferon (10) and treatment with interferon *in vitro* (24) have been shown to inhibit the intracellular growth of the parasites *Plasmodium berghei* and *Toxoplasma gondii*, respectively. Furthermore, both of these parasitic infections have been shown to stimulate interferon production in mice (2, 9, 27). Although these findings were suggestive of a regulatory activity for interferon on the parasite or the parasitized cell, the observations were made before the discovery of the effects of interferon on various cell functions aside from those related directly to antiviral activity, and no attempts were made to characterize the mechanisms involved. Likewise, an interferon-like mediator has been observed in the serum of *T. cruzi*-infected mice (28). However, in previous studies, treatment with interferon inducers either had no effect on this infection or actually increased the mortality rate (13, 15). Possible explanations for the discrepancy between these earlier negative findings and those reported here include differences in the strains of mice used in

TABLE 2. Effect of tilerone treatment on resident peritoneal macrophage activity

Mouse strain	Expt no.	Incubation time (h)	% Infected macrophages <sup>a</sup>		No. of parasites/100 macrophages <sup>b</sup>	
			Control	Tilerone	Control	Tilerone
CJ7BL/6J	1 <sup>c</sup>	4	10.3 ± 2.1	20.6 ± 4.1 <sup>d</sup>	16.5 ± 3.0	36.0 ± 8.0 <sup>d</sup>
		60	7.9 ± 3.3	3.6 ± 2.9 <sup>e</sup>	13.9 ± 5.4	7.3 ± 6.3 <sup>e</sup>
	2 <sup>f</sup>	4	5.2 ± 1.2	11.4 ± 1.9 <sup>d</sup>	6.3 ± 1.6	16.6 ± 5.1 <sup>d</sup>
		48	1.0 ± 0.6 <sup>e</sup>	1.9 ± 0.5 <sup>e</sup>	7.8 ± 2.6	5.9 ± 6.2 <sup>e</sup>
A/J	1	4	5.1 ± 1.5	9.3 ± 1.8 <sup>d</sup>	6.4 ± 2.3	14.8 ± 3.3 <sup>d</sup>
		60	5.4 ± 2.7	3.4 ± 2.8 <sup>e</sup>	29.4 ± 12.0 <sup>e</sup>	14.0 ± 9.7
	2	4	3.2 ± 0.5	ND <sup>g</sup>	4.1 ± 1.3	ND
		48	4.4 ± 1.1	ND	19.3 ± 4.9 <sup>e</sup>	ND

<sup>a</sup> Mean percentage of adherent cells ± standard deviation which contain at least one trypanosome.

<sup>b</sup> Mean number of intracellular parasites observed per 100 cells ± standard deviation.

<sup>c</sup> Eight randomly selected 400× fields were evaluated.

<sup>d</sup> Control versus tilerone, significant at  $P < 0.001$  by two-tailed Student's *t* test.

<sup>e</sup> 4 h versus 48 or 60 h, significant at  $P < 0.001$  by two-tailed Student's *t* test.

<sup>f</sup> Ten randomly selected 400× fields were evaluated.

<sup>g</sup> ND, Not done.

these experiments, as well as differences in the strain, stage, and infectious dose of the parasite. In our own experiments, the effect of tilerone was observed in the innately more resistant C57BL/6J inbred mice, but not in the susceptible A/J strain, even though the latter animals were infected with a dose of trypanosomes that was as much as 10-fold lower.

Because of the multiplicity of effects which interferon exerts on the immune system, we have been unable to determine the exact mechanism(s) through which tilerone treatment enhances resistance. The fact that enhanced survival was observed in treated C57BL/6J mice but not in treated A/J mice argues against there being any direct effect of the drug on the parasites. Nor does the effect appear to be due to a direct interaction of interferon with the parasites, since tilerone also stimulated significant levels of interferon in the serum of the unprotected A/J mice (Table 1). In vitro studies have also indicated that interferon has no direct effect on the infectivity or the multiplication of *T. cruzi* (5); however, these studies in which human interferon and primate kidney cell cultures are used may not be representative of the in vivo situation.

Enhanced NK cell (3) and macrophage (29) activities have been associated with interferon induction, and our results indicate that these cells may participate in the enhanced resistance to *T. cruzi* that we observed after tilerone treatment. When the effect of tilerone treatment was tested in beige mice (Fig. 2), the survival pattern after *T. cruzi* infection was consistent with the pattern of NK cell activity that has been reported in these animals (25, 26). Tilerone treatment resulted in a significant enhancement of survival in the heterozygous, phenotypically normal mice. In contrast, the homozygous, NK cell-deficient, beige animals responded only slightly to the drug. That some residual response was observed in the *bg/bg* mice is not surprising, since they show a moderate enhancement of NK cell antilymphoma target activity after interferon stimulation (25) and have a normal component of NK cells with activity against solid tumors (30). Our findings in the beige mouse model would therefore seem to support a role for NK cells in tilerone-induced resistance to *T. cruzi*. The observation that tilerone treatment significantly enhances NK cell activity against YAC-1 targets in A/J mice, as well as in C57BL/6J mice, is difficult to reconcile with this observation. However, it is possible that spleen cell activity toward lymphoma cell targets does not accurately reflect in vivo activity toward trypanosome targets. It is also possible that the infectious doses of trypomastigotes used for A/J mice in this study, although lower than those for C57BL/6

mice, may have led to such an overwhelming parasite burden in these very susceptible animals that enhancement of NK cell activity was insufficient to control infection.

An alternative, or possibly synergistic, role for macrophages in tilerone-induced resistance in this system is indicated by in vitro experiments in which an increased initial uptake of trypomastigote bloodstream forms was observed in cells from tilerone-treated C57BL/6 mice; these cells appeared to be killing a percentage of the parasites during in vitro culture (Table 2). These results agree with observations (22, 23) that tilerone treatment enhances the spreading and phagocytic activity of macrophages and (8) that macrophages activated by BCG infection (in which interferon may play a role [29]) are able to kill trypanosomes in vitro.

Interferon-induced resistance to *T. cruzi* infection may prove to be the result of a combination of mechanisms. Tilerone has been found to exhibit a number of immunomodulatory effects, including stimulation of humoral immunity (for a review, see reference 16), and no attempt has been made to examine the influence of this treatment on the development of specific immune responses in the current system. Tilerone treatment 4 days after infection appeared to be even more efficacious than pretreatment in one experiment, which suggests that the drug can also affect the course of established infection. Further experiments confirming this result and exploring the possible augmentation by tilerone of nonspecific resistance mechanisms arising as a consequence of infection, such as the *T. cruzi*-induced cytotoxic cells described recently by Hatcher and Kuhn (6), or of specific immune responses should prove to be valuable in the evaluation of the therapeutic use of tilerone. It is possible that more extensive study of the responses of interferon-treated C57BL/6 mice, in which innate resistance has been significantly augmented, could lead to the identification of those mechanisms responsible for controlling *T. cruzi* infection in vivo.

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