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Elevated Environmental Temperature Enhances Immunity in Experimental Chagas' Disease

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C3H mice are highly susceptible to the Brazil strain of *Trypanosoma cruzi*. These mice usually die during the acute phase of infection and develop a profound immunosuppression to heterologous and parasite antigen. In this study, we confirmed earlier reports that infected mice maintained at elevated environmental temperature (36°C) are significantly more resistant to *T. cruzi* than are mice kept at 20 to 24°C. To determine whether the benefits of increased environmental temperature were due to alterations in the host immune system, the production of antibody to heterologous antigen and the development of parasite-specific T-helper cells were examined in noninfected and *T. cruzi*-infected mice. Mice were immunized with either sheep erythrocytes (SRBC) or trinitrophenyl groups (TNP) conjugated to fixed culture forms of *T. cruzi*, and the splenic direct plaque-forming cell (DPFC) responses to SRBC and to TNP-conjugated SRBC were determined. The DPFC response to SRBC from infected mice maintained at elevated environmental temperature was much higher than the suppressed response of infected mice held at room temperature and slightly higher than the response of age-matched noninfected control mice. Likewise, maintaining infected mice at 36°C significantly enhanced the parasite-specific responses of T-helper cells, as reflected by anti-TNP DPFC responses of mice immunized with TNP-conjugated TC.

The immune response of mice infected with *Trypanosoma cruzi* (the causative agent of Chagas' disease) has been extensively studied, and it appears that essentially all effector mechanisms of the immune system participate to some degree in resistance to this protozoan parasite (14, 17, 28). Although these various effector mechanisms are induced to respond to *T. cruzi*, a significant nonspecific suppression of both humoral and cell-mediated responses also develops in infected mice (3, 6, 17, 27). Suppression of parasite-specific responses has also been demonstrated and is thought to reduce the resistance of an infected host to *T. cruzi*. Tarleton and Kuhn (27) used *T. cruzi* conjugated to the hapten trinitrophenyl (TNP; the conjugated preparation is designated TNP-TC) in the Mishell-Dutton culture system to measure the direct plaque-forming cell (DPFC) response of noninfected and *T. cruzi*-infected mice to TNP-TC. The antihapten B-cell responses thus measured reflected the activity of carrier-specific T-helper cells to *T. cruzi*. The response of spleen cells from infected mice to TNP-TC was not found to be significantly higher (and was often lower) than that of normal spleen cells. Because the T-helper cells of *T. cruzi*-infected mice are presumably primed as a result of sensitization during infection, a much greater anti-TNP-TC response might have been expected from spleen cells of these mice than from those of noninfected controls. The observation that the response appeared minimal suggested that parasite-specific suppression of T-helper-cell activity could be occurring during infection. Indeed, the addition of exogenous interleukin 2 (IL-2), or the replacement of resident macrophages with normal macrophages in in vitro assays, increased the responsiveness of spleen cells from infected mice to TNP-TC, which suggested suboptimal activity of anti-*T. cruzi* T-helper cells in infected mice (27). From these observations, it seems that the initial priming to *T. cruzi* during infection may not be sufficient to expand the parasite-specific T-helper-cell population and that perhaps the same suppressor mechanisms which down-regulate reactions to heterologous antigens might also be suppressing the response of *T. cruzi*-specific T-helper cells (27).

It has been known for some time that changes in environmental temperature can influence the resistance of an infected host to a variety of infectious organisms (2, 9, 15, 16, 20–22, 24; A. Trejos, M. A. de Urquilla, and A. R. Paredes, Abstr. Prog. Protzool., 155, p. 144, 1965). In particular, several investigators have demonstrated an increased resistance to *T. cruzi* infection when infected mice are maintained at elevated environmental temperature (35 to 37°C). This increased resistance is reflected in decreased parasitemias and increased longevity (2, 9, 15, 16, 20; Trejos et al., Abstr. Prog. Protozool., 1965). However, the effect of increased environmental temperature on those aspects of the immune response which are important for resistance to *T. cruzi* has not been determined. In this study, the effects of elevated environmental temperature, not only on parasitemia and longevity but also on antibody responses to heterologous antigens and on the activity of *T. cruzi*-specific T-helper cells, have been examined. It was found that infected mice maintained at 36°C had much higher nonspecific and parasite-specific immune responses than did infected mice maintained at 20 to 24°C.

MATERIALS AND METHODS

Mice. Female C3HeB/FeJ mice (Jackson Laboratory, Bar Harbor, Maine) were 10 to 12 weeks old at the time of infection. Mice were kept for 4 weeks at room temperature (20 to 24°C) before use. After being infected, groups of mice were either maintained at room temperature or, for preliminary studies, transferred to a walk-in constant-temperature room, where they were maintained at 36 ± 1°C (relative humidity, 30 to 35%). During subsequent experiments, mice were maintained in an environmental chamber (Lab-Line Instruments, Inc., Melrose Park, Ill.) at 36 ± 0.25°C. Control groups of noninfected mice were also maintained at either

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room temperature or 36 ± 0.25°C for the duration of the experiments.

Infection. Mice were infected intraperitoneally with 10^3 blood-form trypomastigotes of a Brazil strain of *T. cruzi*. Maintenance and characteristics of the parasite have been described elsewhere (18).

Immunization. Mice were injected intraperitoneally with either 0.2 ml of a 10% solution of sheep erythrocytes (SRBC; Colorado Serum Co., Denver, Colo.) or 4 × 10^7 trinitrophenylated *T. cruzi* 4 days before use in the Jerne plaque assay (3).

Preparation of TNP-TC. Epimastigotes of *T. cruzi* maintained in liver infusion tryptose medium were used to prepare TNP-TC (27). Once harvested (23), epimastigotes were first washed three times in Dulbecco phosphate-buffered saline (DPBS; GIBCO Laboratories, Grand Island, N.Y.) and fixed in 4% neutral buffered Formalin. Next, the fixed parasites were washed three times in DPBS and labeled with trinitrobenzene sulfonic (TNP) acid (Sigma Chemical Co., St. Louis, Mo.) as described previously (3). TNP-labeled parasites were washed in DPBS and stored at 4°C.

Preparation of TNP-SRBC. A 1-ml amount of packed SRBC was added dropwise to 7.0 ml of 0.28 M cacodylate buffer containing 20.0 mg of TNP. The SRBC suspension was then mixed for 10 min at room temperature and washed at least four times in DPBS containing 1% glucose (11).

Jerne plaque assay. Spleen cells from mice immunized with SRBC were assayed on day 4 postimmunization for the direct plaque-forming cell (DPFC) response to SRBC, using the slide modification of the Jerne plaque assay (13). Spleens were removed from the mice and teased apart with forceps in the presence of Hanks balanced salt solution. To further disperse the cells, the suspension was drawn into a 3-ml syringe through a 23-gauge needle and discharged through a 26-gauge needle. Spleen cells were then centrifuged, suspended in DPBS, and depleted of erythrocytes by hypotonic shock. Remaining spleen cells were centrifuged and resuspended in Hanks balanced salt solution. For each mouse, nine slide preparations of spleen cells and SRBC were made, three each at cell dilutions of 1:20, 1:40, and 1:80 in Hanks balanced salt solution. Slides were incubated with guinea pig complement (diluted 1:30 in DPBS) for 1.5 to 2.0 h, glutaraldehyde fixed, and dried, and the number of plaques was counted.

An adaptation of this Jerne plaque assay was used to determine the effect of increased ambient temperature on parasite-specific immune responses. TNP-TC was used as the immunizing antigen, and TNP-SRBC was used for the detection of plaque-forming cells. Antibody responses thus measured reflect both B-cell anti-TNP and T-helper-cell responses to *T. cruzi* (27).

Parasitemias. Parasitemia levels were monitored in all infected mice at regular intervals during the course of infection. To measure parasitemia, 4 μl of blood was taken from the tail, placed on a slide, and covered with an 18-mm circular cover slip. The number of parasites present in 100 fields of view was determined, and the number of parasites per milliliter of blood was calculated.

Statistical analysis. All DPFC data were normalized by a $\log_2$ transformation (10) and analyzed by Bartlett's test of homogeneity of variances, followed by a two-factor analysis of variance (25). Differences were considered significant at $P \leq 0.05$.

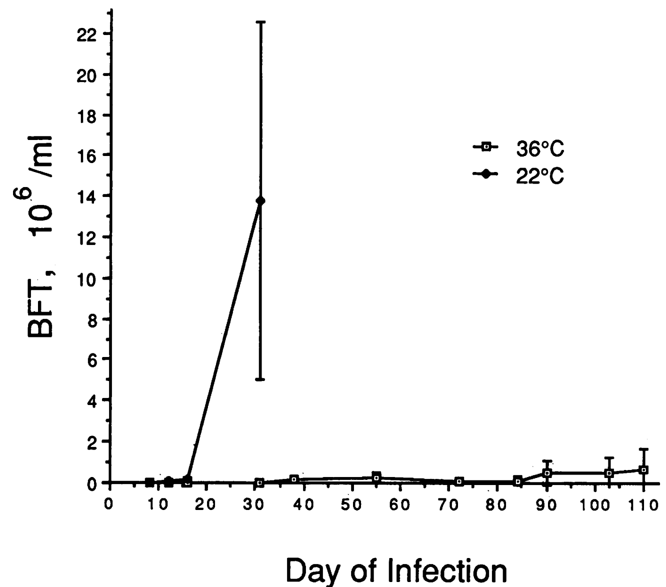

FIG. 1. Effect of environmental temperature on parasitemia in *T. cruzi*-infected mice maintained at either 22 to 24 or 36°C. Data are presented as mean ± standard deviation of blood-form trypomastigotes (BFT) per milliliter of blood (five mice per group).

RESULTS

Effect of increased environmental temperature on parasitemia. Parasitemias were measured throughout the course of infection to determine the effect of increased environmental temperature on levels of parasites in the peripheral blood (Fig. 1). Blood-form trypomastigotes began to appear in the peripheral blood on day 8 of infection in mice maintained at room temperature; however, parasites were not seen in the blood of mice maintained at 36°C until day 16 of infection. The mean parasitemia of mice maintained at 36°C never reached more than 7.1 × 10^5 parasites per ml of blood. In sharp contrast, the group of mice maintained at room temperature reached a mean parasitemia of 1.4 × 10^7 parasites per ml of blood on day 31, just before death. Two mice in this group developed parasitemias of greater than 2.3 × 10^7 parasites per ml of blood. Other experiments of shorter duration (30 to 35 days) have confirmed these results (data not shown).

Effect of increased environmental temperature on longevity. Mice maintained at room temperature and infected with 10^3 blood-form trypomastigotes died between days 30 and 38 of infection, with a mean survival time of 34 days postinfection. All infected mice, however, survived throughout the course of experiments while maintained at 36°C and had very low parasitemias.

Rectal temperature of experimental mice. A YSI telethermometer and rectal probe (Yellow Springs Instrument Co., Yellow Springs, Ohio) were used to monitor the body temperatures of mice. All C3H mice had the same circadian rhythm with respect to rectal temperature (data not shown); the lowest body temperatures were recorded in the middle of the afternoon, and the highest generally occurred at 3:00 a.m. (during periods of high activity). In addition to these daily changes, the temperatures of mice maintained at room temperature varied with ambient temperature. However, there were no differences in body temperatures of infected or noninfected mice held at the same ambient temperature (22 or 36°C). For mice held at room temperature, the mean of

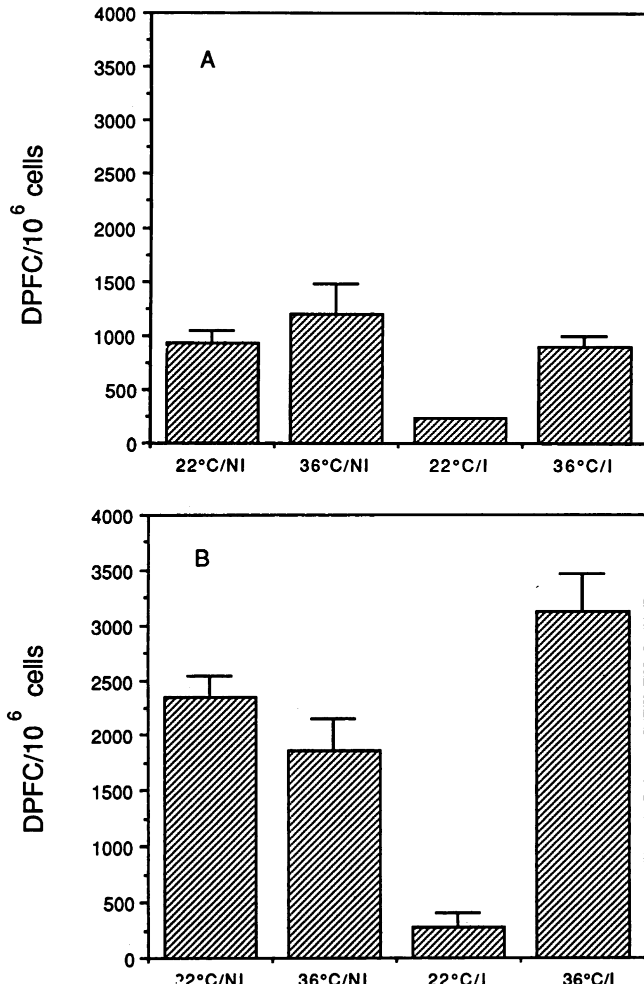

FIG. 2. In vivo anti-SRBC responses. Numbers of DPFC per 10^6 spleen cells were measured on days 25 (A) and 35 (B) of infection for *T. cruzi*-infected mice (I) and for age-matched noninfected controls (NI) maintained at either 22 or 36°C. Data are presented as mean ± standard error (three mice per group, triplicate samples from each mouse).

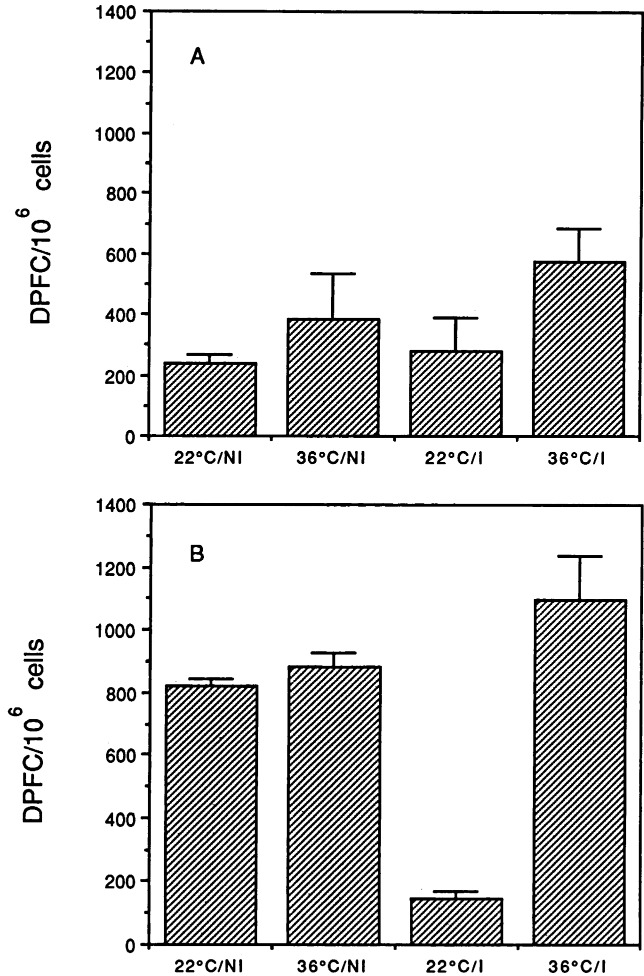

FIG. 3. In vivo anti-TNP responses. Mice were immunized with TNP-TC and assayed against TNP-SRBC. Numbers of DPFC per 10^6 spleen cells were measured on days 25 (A) and 34 (B) of infection for *T. cruzi*-infected mice (I) and for age-matched noninfected controls (NI) maintained at room temperature or 36°C. Data are presented as mean ± standard error (three mice per group, triplicate samples from each mouse).

daily low temperature was 33.7°C, and the mean daily high temperature was 35.7°C. In contrast, the mean of daily low temperature for mice held at 36°C was 37.4°C, whereas the mean daily high temperature was 38.8°C.

Antibody responses to SRBC. The effect of increased environmental temperature on the capacity of infected mice to produce antibody to SRBC was measured on days 25 and 35 of infection and compared with the responses of age-matched noninfected controls. Day 25 of infection was chosen because at room temperature it is at this time that the acute phase of the infection is rapidly progressing, and day 35 represents the time of highest parasitemia just before death. On day 25, both elevated ambient temperature and infection were shown to have a significant effect on DPFC responses ($P \leq 0.005$). Infected mice maintained at 36°C had a significantly greater anti-SRBC DPFC response than did infected mice maintained at 22°C; noninfected mice at both temperatures had a significantly greater response overall than did infected mice (Fig. 2A). Whereas infected mice maintained at 36°C for 25 days had significantly greater DPFC responses than did infected mice maintained at 22°C, noninfected mice often gave a greater response when maintained at the lower temperature.

An examination of the DPFC response to SRBC on day 35 of infection was of particular interest because by day 35 infected mice held at room temperature experience rapid increases in parasitemia as well as severe immunosuppression to heterologous antigens (6). As expected, on day 35 infected mice held at room temperature showed about 89% suppression of the DPFC response to SRBC (Fig. 2B). A significantly different response was obtained from infected mice maintained at 36°C. Anti-SRBC DPFC responses of these mice were 11-fold greater than those of infected mice maintained at room temperature. Noninfected, age-matched controls maintained at either 36°C or room temperature also produced elevated DPFC responses to SRBC, but the responses were not quite as high as the response obtained from infected mice held at 36°C. Age-matched, noninfected, and nonimmunized controls were also assayed with each experiment, and essentially no DPFC responses were observed (data not shown).

Anti-TNP responses. To determine the effect of increased

ambient temperature on parasite-specific immune responses, the DPFC responses to TNP from mice immunized with TNP-TC and from age-matched noninfected controls, maintained at either room temperature or 36°C, were measured on day 25 of infection (Fig. 3A). No significant differences were found in the DPFC responses between the two groups at this time.

On day 34 of infection, when nonspecific immunosuppression is maximal (6), there also appeared to be parasite-specific suppression of the T-helper-cell response to *T. cruzi* from infected mice held at room temperature (Fig. 3B). In contrast, mice maintained at 36°C had a much stronger (seven- to eightfold) DPFC response to TNP, which suggested that parasite-specific T-helper-cell activity was significantly enhanced. A strong anti-TNP DPFC response was also obtained from age-matched noninfected mice immunized with TNP-TC and held at either 36°C or room temperature (approximately sixfold stronger than that of infected mice maintained at room temperature). There were essentially no DPFC responses from age-matched, noninfected, and nonimmunized controls, nor were there any anti-SRBC DPFC responses from mice immunized with TNP-TC (data not shown). These data indicate that increased temperature significantly affects the DPFC response to TNP (and thus the response of *T. cruzi*-specific T-helper cells) and that the effects of the different temperature conditions depend on whether the mice are infected. All differences were significant at $P \leq 0.001$.

DISCUSSION

The therapeutic effect of increased environmental temperature on *T. cruzi* infection in mammals has been described previously by several investigators (2, 9, 15, 16, 20; Trejos et al., Abstr. Prog. Protozool., 1965); however, the mechanism(s) responsible for this phenomenon has not been examined. In these earlier studies, mice or rats held at higher temperatures (35 to 37°C) experienced less intense infections with *T. cruzi*, as indicated by decreased parasitemia, increased longevity, and in some cases an absence of parasites in cardiac tissue (2; Trejos et al., Abstr. Prog. Protozool., 1965). Such reports were confirmed in an extensive study by Marinkelle and Rodriguez (20). In at least one report, favorable results were described from the use of fever therapy to treat humans with Chagas' disease (1). Most of the patients experienced a rapid improvement in condition after intravenous administration of a pyrogenic vaccine, and treated patients were found to be negative for circulating parasites as early as 4 days after initiation of treatment (1).

The results of experiments reported herein provide further evidence for the therapeutic effects of increased environmental temperature on *T. cruzi* infection. C3H mice infected with *T. cruzi* and maintained at 36°C experienced both lower parasitemia levels and greater longevity than did infected mice held at room temperature. The peak mean parasitemia of infected mice held at room temperature was 19- to 20-fold greater than that of infected mice held at 36°C. In addition, all infected mice maintained at room temperature died by day 38, whereas infected mice maintained at 36°C survived throughout the course of the experiments.

When examining the effects of increased environmental temperature on immune responses to parasite infections, it is difficult to distinguish between direct effects of temperature on the parasite and actual enhancement of the immune response. Although it is not likely that body temperatures that are lethal to *T. cruzi* were reached by the mice used in this study, direct effects of increased temperature on the parasite should not be discounted. In one study, it was concluded that the decreased parasitemia observed in *T. cruzi*-infected mice maintained at 37°C was probably due to effects of temperature on the parasite (9). Whereas incubation temperature may have an effect on the parasite itself, evidence also exists to support the hypothesis that elevated environmental temperature can enhance immune responsiveness. Otieno (22) demonstrated that the positive effects of elevated environmental temperature on the course of *Trypanosoma brucei* infection in mice could be prevented by treating the mice with either sublethal irradiation or cyclophosphamide before infection.

Enhancement of immune responsiveness has also been observed by Jampel et al. (12), who reported that increased incubation temperature enhances the immune response of murine spleen cells when challenged with SRBC in vitro. T-helper cells, which have been shown to function suboptimally in *T. cruzi*-infected mice (27), are believed to be the target of this temperature-induced enhancement of immune responsiveness (12).

The immunosuppression observed in *T. cruzi*-infected mice has been experimentally overcome by addition of exogenous IL-2 (4, 26) and by repeated antigenic stimulation (5). Our study provides evidence for temperature-induced enhancement of T-cell-dependent responses to heterologous antigens in vivo as well as to parasite antigens in *T. cruzi*-infected mice. On day 35 of infection, the DPFC response to SRBC from mice held at 36°C was much higher than that of infected mice held at room temperature and was in fact slightly higher than the DPFC response of age-matched noninfected controls.

Previous studies have made use of the hapten-carrier complex TNP-TC to investigate suppression of parasite-specific T-cell responses in *T. cruzi*-infected mice (27). *T. cruzi*-specific T-helper-cell activity appeared suppressed in these mice, and it was suggested that mechanisms which control immunosuppression to heterologous antigens might also be reducing parasite-specific immune responses. In our study, TNP-TC was used to investigate the effects of elevated environmental temperature on parasite-specific responses in vivo. The results showed that parasite-specific responses of T-helper cells to *T. cruzi* are suppressed. On day 34 of infection, the DPFC response of infected mice held at room temperature was much lower than that of age-matched noninfected controls. The DPFC response to TNP-TC from infected mice kept at 36°C, however, was slightly higher than that of age-matched noninfected controls and significantly higher than that of *T. cruzi*-infected mice held at room temperature. These results indicate that maintaining mice at an elevated environmental temperature may abrogate parasite-specific suppression of T-helper-cell responses.

The mechanisms by which immunosuppression occurs in *T. cruzi*-infected mice are not understood; therefore, the mechanisms by which immunosuppression may be overcome are also an enigma. It does appear that this immunosuppression is mediated by suppressor macrophages (6) and is apparently due, at least in part, to a deficiency in the production of IL-2 (27). It is not likely that any single aspect of the immune system is responsible for overcoming the suppression which has been observed. However, it is possible that the reduction of suppression seen in *T. cruzi*-infected mice that have been maintained at elevated environmental temperature could involve the monokine IL-1, which among many other functions is responsible for the induction of the fever response (7). Previous studies (8, 12, 19) have indi-

cated that the T-cell response in vitro to IL-1 is greatly enhanced at increased incubation temperatures (4- to 10-fold at 39°C) (8). In one such study, Lederman et al. (19) examined the IL-1-driven secretion of IL-2 by the murine T-cell lymphoma LBRM-33-1A5 at various incubation temperatures. The rate of IL-2 production was found to increase linearly with increasing temperature (33 to 41°C) during the first 8 h of incubation, which indicated that the response of IL-2-secreting T cells is temperature dependent.

Continuing studies are focused on determining the mechanisms by which immunosuppression occurs during infection as well as the possible mechanisms by which elevated environmental temperature enhances immune responsiveness in infected mice.

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