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Effect of recombinant tumour necrosis factor on acute infection in mice with *Toxoplasma gondii* or *Trypanosoma cruzi*

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SUMMARY

Recombinant tumor necrosis factor (rTNF) has been shown to protect mice against lethal bacterial infections. We previously reported that in *in vitro* experiments with mouse peritoneal macrophages, rTNF inhibited intracellular multiplication of *Trypanosoma cruzi* but not of *Toxoplasma gondii*. These disparate results led us to study the effect of rTNF on the *in vivo* infection with these parasites. Daily administration of 0·5 and 5·0 μg rTNF resulted in a dose-dependent, significantly decreased time to death ($p < 0.05$) in mice infected with lethal doses of *T. cruzi*. The same effect was found in mice infected with *T. gondii* and given a daily dose of 5·0 μg rTNF. Lower doses of rTNF did not significantly affect time to death of mice infected with either parasite.

INTRODUCTION

Tumour necrosis factor (TNF) is produced by activated macrophages (Matthews, Riley & Neale, 1980). Although the biological functions of TNF *in vivo* have not yet been clearly defined, studies by Cerami and co-workers (Beutler *et al.*, 1985a; Beutler, Milsark & Cerami, 1985b) have demonstrated that TNF is identical to cachectin, is induced by endotoxin treatment or by infection with parasites or bacteria, and probably acts as a hormone-like intermediary between reticuloendothelial cells and other cells of the body. These studies followed the early observation (Rouzer & Cerami, 1980) that rabbits infected with *Trypanosoma brucei* developed cachexia and suppression of lipoprotein lipase levels, later shown to be an indicator of TNF bioactivity (Beutler *et al.*, 1985a). In contrast to this observation, sera containing TNF have been reported to inactivate malarial parasites *in vitro* (Haidaris *et al.*, 1983; Taverne, Dockrell & Playfair, 1981) and to protect against *Klebsiella*, *listeria* (Parant, 1980) and *Plasmodium* (Clark *et al.*, 1981; Taverne, Depledge & Playfair, 1982) *in vivo* in mice. These reports suggested that TNF may play a biotherapeutic role in parasitic diseases and may also antagonize and/or contribute to the pathogenesis of infection. The anti-microbial properties of recombinant TNF (rTNF) and its precise role in host defence against microbial pathogens are even less defined than those for unpurified TNF. Recombinant TNF was recently reported to have a protective effect in mice infected with *Legionella pneumophila* (Blanchard *et al.*, 1988) and *Mycobacterium avium* complex (Bermudez *et al.*, 1987). *In vitro*, rTNF inhibits intracellular growth of *Trypanosoma cruzi*, but not *Toxoplasma gondii* in murine macrophages (De Titto, Catterall & Remington, 1986). Studies were designed to determine the effect of *in vivo* administration of recombinant TNF on the course of infection in mice exposed to these two protozoan pathogens. It was found that rTNF administered *in vivo* induced significantly more rapid mortality in both inbred and outbred mice infected with either *T. cruzi* or *T. gondii*, while control mice treated with rTNF alone showed no apparent effects. These results were obtained with rTNF from the same source and at similar doses as those reported by others to protect mice from lethal bacterial infections (Blanchard *et al.*, 1988; Bermudez *et al.*, 1987).

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MATERIALS AND METHODS

Mice

Six- to 8-week-old female Swiss Webster and BALB/c mice were used in mortality experiments. Six- to 8-week-old Swiss Webster mice were used to produce tachyzoites of *T. gondii*. Four-week-old male Swiss Webster mice were used to produce trypomastigotes of *T. cruzi*. All mice used in these experiments were obtained from Simonsen Laboratories (Gilroy, CA).

Micro-organisms

Tachyzoites of the C56 and RH strains of *T. gondii* were prepared according to described procedures (Hauser, Sharma & Remington, 1982; Wilson, Tsai & Remington, 1980). For purposes of infecting mice, trypomastigotes of the Y strain of

T. cruzi were purified from blood of acutely infected mice at the peak of parasitemia (De Titto, Catterall & Remington, 1986). Briefly, blood was collected in heparin (10 U/ml) 7 days after infection, diluted 1:2 with 6% dextran (T70; Pharmacia, Uppsala, Sweden) in Hanks' balanced salt solution (HBSS; Gibco, Santa Clara, CA), incubated 45 min at 25°, and centrifuged at 60 *g* for 20 min. The supernatant, free of red blood cells, was removed to a separate tube, then the parasites were collected by centrifugation at 600 *g* for 20 min, washed once with HBSS, and counted in a Neubauer haemacytometer. For the purpose of infecting macrophage monolayers, culture trypomastigotes were obtained from infected L929 fibroblasts maintained in RPMI-1640 (Gibco) containing 10% fetal calf serum (Kanbara & Nakabayash, 1983).

Microbicidal assays

Unelicited, resident murine peritoneal macrophages were obtained as described elsewhere (Wilson, Tsai & Remington, 1980). Monolayers of macrophages were established by culturing 6×10^4 cells in RPMI containing 10% fetal calf serum and 40 μg/ml gentamicin in 8-chambered Lab-Tek slides (Lab-Tek Industries, Naperville, IL). After 2 hr of incubation, non-adherent cells were removed by washing with warm saline. Culture trypomastigotes of *T. cruzi* were adhered to the monolayers at a 3:1 parasite:cell ratio and incubated for 18 hr. Tachyzoites of the RH strain of *T. gondii* were added to separate monolayers at a 1·5:1 parasite:cell ratio and incubated 1 hr with and without 1×10^4 U rTNF/ml in the described medium. Extracellular parasites were then removed by exhaustively rinsing with HBSS. Immediately thereafter, cells were fixed with absolute methanol and stained with Giemsa stain. The remaining *T. cruzi*-infected monolayers were further incubated 24, 36, or 60 hr in RPMI containing 10% fetal calf serum and 1×10^4 U rTNF/ml, washed, fixed and stained. A parallel experiment was performed in which *T. cruzi*-infected macrophage monolayers were incubated in the same medium containing 1×10^4 U rTNF/ml for 16, 24, 36 and 60 hr, then washed to remove rTNF and further incubated in rTNF-free medium. For experiments with rTNF incubation periods exceeding 48 hr, medium containing rTNF at the appropriate concentration was replenished after 48 hr. At 60 hr post-infection, all monolayers were washed, fixed and stained as described above. The remaining *T. gondii*-infected monolayers were further incubated 18 hr with and without the same concentration of rTNF. In some experiments, macrophage monolayers were pre-treated with 1×10^4 U rTNF/ml for 24 hr prior to infection, then infected and incubated in the absence of rTNF. The number of infected macrophages and the number of parasites per 100 macrophages were determined by counting at least 200 cells in each monolayer. All counts were performed on duplicate wells and the mean values were calculated.

TNF administration

Recombinant murine TNF-alpha was kindly supplied by Dr H. Michael Shepard of Genentech Inc., South San Francisco, CA, and contained ≤0·125 endotoxin U/ml by Limulus Amoebocyte Lysate Assay. TNF was stored undiluted at 4° and never frozen. TNF activity and concentration were assessed at Genentech Inc. (Kirstein, Fiers & Baglioni, 1986). TNF was diluted immediately before use in normal saline that was determined to contain undetectable levels of endotoxin by Limulus Amoebocyte Lysate Assay. Mice were injected daily by the intraperitoneal route with 5·0, 0·5 and 0·05 μg rTNF starting at 24 hr prior to infection. Control mice were injected with endotoxin-free saline. Twelve mice per treatment group were infected by the intraperitoneal route with 10^3, 10^4 and 10^5 tachyzoites of the C56 strain of *T. gondii*, and 10^2 and 10^4 blood trypomastigotes of *T. cruzi* diluted in endotoxin-free saline. Mice infected with *T. gondii* were injected daily with rTNF for 10 days, and mice infected with *T. cruzi* were injected daily with rTNF until all mice were dead. Mortality was recorded daily at the same time each day. For mice infected with *T. cruzi*, parasitemias in control and treated mice were determined (Brener, 1962) on Days 5, 7, 9 and 12 after infection. For mice infected with *T. gondii*, ascitic fluids were examined within several hr of the time of death to verify the presence of tachyzoites of *T. gondii* in the peritoneal cavity.

Table 1. Effect of rTNF on *in vitro* infection of murine macrophages with *T. gondii*

Treatment	No. parasites/100 cells	
	0 hr	20 hr
Control	50*	421
rTNF −24–0 hr†	54	511
−24–20 hr	70	494
0–20 hr	76	467

* Data are the mean results of duplicate slides, and are representative of at least three experiments with identical design.

† Macrophages were treated with 1×10^4 U/ml of rTNF during the time periods indicated and infected with the RH strain of *T. gondii* at 0 hr. All slides were fixed and stained at 20 hr post-infection.

Statistics

Statistical analyses were performed by using Student's *t*-test and Mann-Whitney *U*-test.

RESULTS

Effect of rTNF on microbicidal assays

The intracellular multiplication of *T. gondii* in macrophages was not impaired by the presence of 10^4 U rTNF/ml in the medium prior to, during, or following infection (Table 1). In contrast, intracellular multiplication of *T. cruzi* was inhibited by rTNF; whereas the numbers of parasites/100 macrophages increased by 106% in 60 hr in control monolayers, this value was 22% in monolayers incubated with rTNF (Table 2, $P < 0.05$). Pre-treatment of macrophage monolayers for 24 hr prior to infection had no effect on intracellular multiplication of either

Table 2. Effect of rTNF on *in vitro* infection of murine macrophages with *T. cruzi*

Hours post-infection	No. parasites/100 cells (% increase) Experimental*	Control
0	186†	189
28	222 (20)	265 (40)
60	231 (22)	391 (106)
76	292 (55)	520 (175)

* Macrophages were treated with 1×10^4 U/ml of rTNF from the time of infection until the end of the indicated infection period with replenishment of the medium and rTNF at 48 hr post-infection.

† Data are the mean result of duplicate slides and are representative of three experiments of identical design.

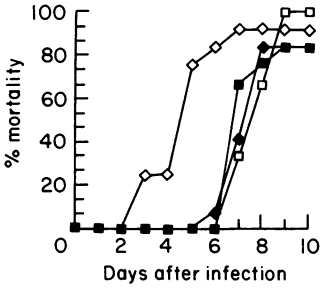

Figure 1. Effect of *in vivo* administration of rTNF on time to death and overall mortality of BALB/c mice infected with 1×10^5 tachyzoites of the C56 strain of *T. gondii*. TNF was injected daily by the intraperitoneal route into 12 mice per treatment group from 24 hr prior to 8 days after infection. (□) Endotoxin-free saline; (◆) 0·05 μg rTNF/day; (■) 0·5 μg rTNF/day; (◇) 5·0 μg rTNF/day. Data shown are representative of 6 experiments including three different infecting doses of *T. gondii* and the BALB/c and Swiss Webster mouse strains.

parasite. These results are in agreement with those previously reported (De Titto, Catterall & Remington, 1986) with rTNF from a different source.

Effect of rTNF on mortality and time to death of mice infected with *T. gondii* and *T. cruzi*

In both the BALB/c and Swiss Webster mouse strains and at all infecting doses of *T. gondii* and *T. cruzi* tested, 5 μg rTNF/day significantly shortened the time to death (representative data shown in Figs 1 and 2). Five days after infection with *T. gondii*, mortality was 75% in mice treated with 5·0 μg rTNF/day and 0% in untreated mice ($P < 0{\cdot}01$; Fig. 1). Similarly, at 22 days post-infection with *T. cruzi*, 100% mortality was observed in mice treated with 5·0 μg TNF/day compared with 50% mortality in untreated mice ($P < 0{\cdot}02$; Fig. 2). Mice that were infected with *T. cruzi* and given 0·5 μg rTNF/day also exhibited significantly shorter times to death ($P < 0{\cdot}05$; Fig. 2); this occurred in a dose-dependent fashion. Mice that received a dose

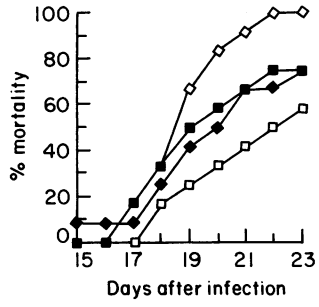

Figure 2. Effect of *in vivo* administration of rTNF on time to death and overall mortality of Swiss Webster mice infected with 1×10^2 blood trypomastigotes of *T. cruzi*. TNF was injected daily by the intraperitoneal route into 12 mice per treatment group from 24 hr prior to infection until death. (□) Endotoxin-free saline; (◆) 0·05 μg rTNF/day; (■) 0·5 μg rTNF/day; (◇) 5·0 μg rTNF/day. Data are representative of four experiments including two different infecting doses of *T. cruzi* and the BALB/c and Swiss Webster mouse strains.

Table 3. Effect of *in vivo* administration of rTNF on parasitemia (parasites/ml blood) in Swiss Webster mice infected with 1×10^4 blood trypomastigotes of *T. cruzi*

Days post-infection	μg rTNF/day 0	0·05	5·0
7	4830±274*	2415±279	0
9	18,917±4676	19,320±871	9418±3871
12	14,973±6950	16,663±6731	23,908±7755

* Data are the mean ± SEM of duplicate counts of blood from each of three different mice per treatment group.

of 0·05 μg rTNF/day did not significantly differ in their time to death or overall mortality from mice that received control saline. Uninfected BALB/c and Swiss Webster mice that received 5·0 μg rTNF/day did not exhibit any apparent toxic effects or differences from uninfected mice that received control saline.

Effect of rTNF on parasitemia

The first observation of blood trypomastigotes was in Swiss Webster control and rTNF-treated mice at 7 days after infection with *T. cruzi* (Table 3). No blood parasites were seen at 5 days after infection. At 7 days after infection, an apparently dose-dependent inhibition of parasitemia was effected by rTNF (Table 3). At 12 days post-infection, however, differences in numbers of blood parasites between control and rTNF-treated mice were no longer evident. This transient inhibitory effect of rTNF on parasitemia was observed in experiments performed with both BALB/c and Swiss Webster mice.

DISCUSSION

These results demonstrate that *in vivo* administration of rTNF exacerbates infection of mice with two different protozoan pathogens. This effect was dependent on infection and not on toxicity of the rTNF since uninfected mice that received the same doses of rTNF alone showed no ill effects. Exacerbation of infection was dependent on the dose of rTNF administered. Of the doses tested, 0·5 and 5·0 μg rTNF/day in *T. cruzi*-infected mice and 5·0 μg rTNF/day in *T. gondii*-infected mice produced a statistically significant exacerbation of infection.

Although infusion of high doses of rTNF alone can induce acute shock in experimental animals (Tracey *et al.*, 1986), the highest dose of rTNF administered in this study (5·0 μg/day) was chosen because it produced no apparent untoward effects in uninfected mice. In addition, the highest dose we used is well below the lowest dose (20·0 μg/day) of murine rTNF-alpha found to produce significant weight loss in mice (Kramer *et al.*, 1988) using the same source of rTNF. Protozoan parasites contain no known LPS, but animals that have been infected with *T. brucei* undergo a wasting process thought to be mediated by TNF (Rouzer & Cerami, 1980). Of note is the fact that the effects of rTNF that we observed were not produced in uninfected mice by rTNF alone. Thus this exacerbating effect of TNF administration during parasite infection may involve a mechanism that differs from that described by Cerami *et al.* of classical TNF-induced acute endotoxic shock (Tracey, Lowry & Cerami, 1988; Tracey *et al.*, 1987). The results of Rothstein & Schreiber (1988) should be noted, however, showing that TNF interacts with bacteria that lack LPS to produce lethal shock in mice. In addition, and in agreement with our results, Clark *et al.*, (1987) found that mice infected with another parasite, *Plasmodium*, were more sensitive to TNF treatment than were uninfected animals.

T. gondii and *T. cruzi* induce high levels of interferon-gamma (IFN-γ) in the serum and ascitic fluid within 24 hr of infection (Jones, Alkan & Erb, 1986; R. E. McCabe and J. S. Remington, unpublished observations). Whether IFN-γ (or some other cytokine) induced by infection with *T. gondii* and *T. cruzi* interacts with administered rTNF to produce a synergistic toxicity or a significant suppression of resistance to infection in mice leading to more rapid mortality is unclear.

These findings strongly emphasize the inherent risks of extrapolating efficacy results of cytokine therapy from *in vitro* models of infection to the situation *in vivo*. In addition, they reveal the importance of identifying potential interactions between administered and induced cytokines that can profoundly influence the prognosis of disease.

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