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TNF inhibits malaria hepatic stages *in vitro* via synthesis of IL-6

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Abstract

We examined the capacity of murine recombinant tumor necrosis factor (rmTNF) to induce an inhibitory effect at the hepatic stage on malaria induced by *Plasmodium yoelii* sporozoites. When injected three times, 1.0 μg of rmTNF was found to protect 78% of mice against a sporozoite challenge. In contrast, whatever the dose and the schedule of administration, no inhibition was observed when purified hepatocyte cultures were infected with *P. yoelii*. The addition of non-parenchymal hepatic cells to hepatocyte cultures restored the capacity of TNF to modulate hepatic stage development, leading to up to 44% inhibition. Antibodies to interleukin 6 reversed the anti-parasite activity in the co-culture system.

Introduction

Tumor necrosis factor alpha/cachectin (TNF), initially described for its ability to induce hemorrhagic necrosis *in vivo* (1,2), is a cytokine with various activities, including striking anti-viral (3), anti-bacterial (4) and anti-parasitic effects (5). In malaria, it has been shown that TNF administration *in vivo* protects mice challenged with parasitized erythrocytes of *P. chabaudi* (6) and reduces the development of hepatic forms of *P. berghei* (7). Although a previous report indicates that TNF reduced development of *P. berghei* in a Hep. G2 hepatoma cell line (7), preliminary data obtained in our laboratory using fresh hepatocytes did not confirm this effect. The aim of the present study was to understand the reasons for such discrepancies and to clarify the role of TNF, at the hepatic level, in sporozoite induced malaria.

Methods

Animals

Three-month-old BALB/c, C3H/HeJ, and C57B1/6 mice were purchased from Charles River Breeding Laboratories, Saint-Aubin les Elbeuf, France.

Cytokines and anti-cytokine antibodies

Murine recombinant TNF alpha (rmTNF) was a kind gift from Dr B. Allet, Glaxo IMB, Garouge, Switzerland. Rabbit anti-mouse TNF IgG was prepared as previously described (8). 6B4, a rat IgG1 anti-mouse interleukin 6 (IL-6) (9) and murine recombinant IL-6 (rmIL-6) were a gift from Dr J. Van Snick, Ludwig Institute, Brussels, Belgium.

Cytokine assays

Supernatants from hepatocyte cultures were assayed for IL-6 in a bioassay using the 7TD1 cell line as described (10). Secretion of IFN-gamma in supernatants after stimulation with rmTNF was measured with an enzyme-linked immunoassay (ELISA) using a murine monoclonal antibody to IFN-gamma (11). Interleukin 1 (IL-1) was assayed by the standard thymocyte co-mitogen assay as previously described (12,13).

Parasites

Sporozoites of the 17X strain of *Plasmodium yoelii yoelii* were obtained from infected salivary glands of *Anopheles stephensi* mosquitoes, 16 to 21 days after an infective mouse blood meal. After aseptic dissection, salivary glands were homogenized in a glass grinder and diluted in culture medium or sterile phosphate-buffered saline (PBS) (14).

In vivo experiments

Balb/c mice were divided into four groups. In the first group, each mouse received 1.0 μg of rmTNF intravenously (i.v.). After 24 h, each animal was injected with 3500 *P. yoelii* sporozoites i.v. Each mouse in group 2 received 0.5 μg rmTNF i.v. 24 h before, during,

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and 24 h after sporozoite inoculation. The third group was treated as the second group but the rmTNF dose was 1.0 μg. In the control group rmTNF was replaced by 0.2 ml of PBS and the regimen was as for group 2. Blood smears, taken daily from the third to the seventeenth day after sporozoite inoculation, were stained with Giemsa and examined for erythrocytic stages of *P. yoelii*.

Culture of hepatic stages of malaria parasites

Monoculture of hepatocytes. Rodent hepatocyte monolayers (BALB/c, C3H/HeJ, C57BL/6) were isolated by collagenase perfusion of liver fragments as previously described (15). Briefly, 60 000 cells were cultured in eight-chamber plastic Lab-Teck slides (Miles Lab. Inc., USA) in minimal essential medium supplemented with 10% fetal bovine serum and incubated with 5% CO_2 at 37°C for 24 h before use in experiments.

Co-culture of hepatocytes and non-parenchymal cells (NPC). NPC were obtained as already described (16). Briefly, livers from mice (BALB/c, C57BL/6) were perfused with HEPES buffer followed by perfusion with 0.05% collagenase (Collagenase H, Boehringer Mannheim, Germany). Then the distended and blanched liver was teased and suspended in 30 ml collagenase solution and maintained for 45 min at 37°C under magnetic agitation. The suspension was then centrifuged at 300 *g* for 10 min, the supernatant discarded, and the pellet resuspended in 5 ml HEPES. The liver cell suspension was overlaid with 7 ml 30% w/v metrizamide (Nicomed, Oslo, Norway), dissolved in HEPES without NaCl. The liver cell metrizamide gradient was centrifuged at 1400 *g* for 20 min. NPC were collected from the top layer, washed twice in HEPES and resuspended in tissue-culture medium. Sixty thousand viable NPC were added to primary hepatocyte cultures 3 h after the culture was set up (ratio 1:1).

Treatment of monocultures and co-cultures. Both culture types were incubated with 0.5, 1.0 or 2.5 μg/ml rmTNF. Control cultures were maintained without rmTNF. Forty thousand *P. yoelii* sporozoites were added 24 h after rmTNF addition. Three and 24 h after sporozoite inoculation culture supernatants were taken for cytokine assays and replaced either with fresh medium or with medium containing rmTNF. Forty-eight hours after sporozoite inoculation, supernatants were taken again for cytokine assays and cultures were stopped and stained with Giemsa to find schizonts. In addition, other cultures of both types were incubated with 10 IU/ml rmIL-6 for 24 h before sporozoites were inoculated; these cultures were stopped 48 h later.

Neutralization of IL-6 by an anti-IL-6 mAb. Using the same protocol as described in the previous paragraph, anti-murine IL-6 mAB (1/100) was added to cultures either at the time of cytokine incubation or 30 minutes before sporozoite inoculation. The cultures were then maintained as described above.

Results

In vivo *experiments*

Injection of 1.0 μg rmTNF the day before, at the time of, and the day after inoculation of 3500 sporozoites prevented occurrence of parasitemia in 78% of the mice. This was a consistent finding,

Table 1. Effect of *in vivo* TNF administration on *P. yoelii* sporozoite infection in BALB/c mice

Treatment rmTNF (μg/mouse)			Protection (number of mice)	Delay[a]
−24 h	+3 h	+24 h		
0.5	0.5	0.5	0/5	1 day ± 0.8
1	–	–	1/10	4 days ± 1.5
1	1	1	16/20	5 days ± 1.0
0	0	0	2/20	

[a]Delay represents the specific mean ± SE in the appearance of parasitemia in non-protected mice, compared to controls injected with PBS.

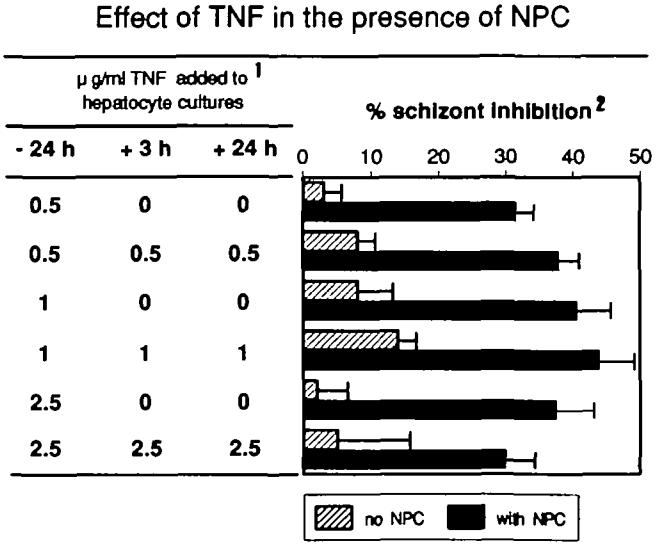

Fig. 1. Effect of TNF in the presence of NPC. [1]rmTNF was added at different times (indicated in hours) after sporozoite inoculation. [2]Percentage inhibition was estimated by counting numbers of 48-h schizonts in cultures with or without rmTNF. Results are expressed as means ± SEM of six individual cultures. The controls were either hepatocytes without NPC (Schizont number: 65 ± 3) or hepatocyte with NPC (Schizont number: 70 ± 6).

mice being aparasitaemic 17 days after infection. In the remaining 20% of treated but non-protected mice, the rise in parasitemia was delayed by a mean of 5 days compared to PBS treated mice. A single injection of rmTNF (1.0 μg/mouse) 24 h before sporozoite inoculation was much less protective (Table 1). A lower dose of rmTNF (0.5 μg/mouse), even when administered three times (the day before, on the day of, and 24 h after sporozoite inoculation) had no protective effect.

In vitro *experiments*

Effects of TNF on parasite development in vitro. Using BALB/c hepatocyte monocultures (Fig. 1), no significant parasite inhibition could be observed when cultures were incubated with 0.5 μg/ml rmTNF 24 h before sporozoite inoculation. This effect was not improved by repeating the TNF administration (24 h before, during, and 24 h after infection), nor by increasing the dose to 2.5 μg/ml. Similar results were obtained with primary cultures from

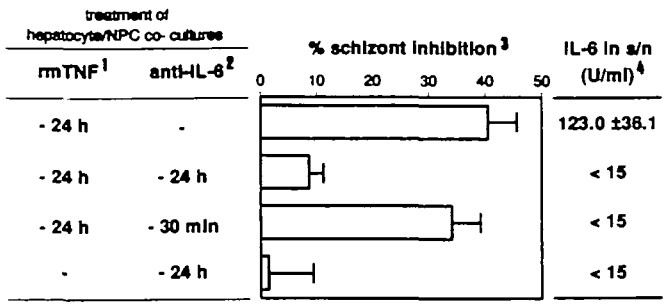

Fig. 2. IL-6 levels in culture supernatants. [1]1.0 μg/ml, final concentration [2]1:100, final dilution. [3]Results of six individual cultures, expressed as means ± SEM, compared to infected hepatocyte cultures without TNF. The schizont number in untreated control cultures was 86 ± 7. [4]Assayed 3 h after sporozoite inoculation.

Table 2. Effects of *in vitro* IL-6 incubation on the pre-erythrocytic parasite development of *P. yoelii* in Balb/c hepatocyte cultures, in the presence and absence of non-parenchymal cells (NPC)

Treatment		% Inhibition[c]	
rmIL-6[a]	anti-IL-6[b]	no NPC	with NPC
-24 h	-	42 2±3 3	86.5±7.0
-24 h	-24 h	5.2±4 5	7.3±4.0
-24 h	-30 min	37.6±6.4	79.0±7.5
-	-24 h	4.0±3 8	2.5±6.5
-	-30 min	3.2±4.0	6.8±2 0

[a]10 IU/ml, final concentration.
[b]1/100, final dilution
[c]Percentage inhibition of schizont development of six individual cultures; expressed as means ± SEM, compared to infected non-treated hepatocyte cultures. The schizont number in control cultures was without NPC 75±4, and with NPC 69±5.

C3H/HeJ and C57/BL6 mice (data not shown). The addition of NPC to hepatocyte cultures (ratio 1:1), in the presence of 0.5 μg TNF resulted in 31% schizont inhibition (Fig. 1). The degree of inhibition was not improved by repeating the addition of TNF (24 h before, during, and 24 h after inoculation) nor by increasing the dose to 2.5 μg/ml. Identical results were obtained using co-cultures from C57/BL6 and C3H/HeJ mice (data not shown). However, incubation of lower doses of rmTNF were less effective than those shown in Fig. 1 (data not shown).

Effects of TNF-induced IL-6 secretion and its neutralization in culture supernatants. Purified murine hepatocytes were cultured alone or in the presence of NPC (co-culture). Both culture types were incubated with 1.0 μg/ml rmTNF 24 h before sporozoite inoculation and IL-6 levels were measured in supernatants (Fig. 2). It was found that IL-6 levels were high in co-cultures 3 h after sporozoite inoculation (mean: 123 U/ml, Fig. 2) and rose to a mean of 490 U/ml after 48 h, whereas IL-6 was not detectable in monocultures. Addition of anti-IL-6 mAb to co-cultures at the time of rmTNF incubation virtually abolished the inhibitory effect induced by TNF. In contrast, the addition of anti-IL-6 mAb 30 min before sporozoite inoculation did not modify the rmTNF effects (36±5.6% inhibition; Fig. 2), despite the fact that IL-6 remained undetectable after 3 h. Neither IFN-γ nor IL-1 could be detected in primary or in co-culture supernatants at 3, 24 and 48 h after sporozoite inoculation, incubated or not with rmTNF.

Effects of murine recombinant IL-6 and its neutralization by an anti-IL-6 mAb. Incubation of rmIL-6 inhibited parasite development up to 42±3.3% in primary cultures, while 86±7.0% inhibition was reached in co-cultures. With an anti-murine IL-6 mAb (1/100) nearly 90% of the rmIL-6 (10 IU/ml) induced inhibition could be neutralized in both primary and co-cultures (Table 2).

Discussion

The *in vitro* studies presented here were performed in order to define the site and mode of action of TNF. It is shown that when primary hepatocyte cultures are used instead of tumor cell lines, TNF fails to induce a protective effect, whatever the dose and the schedule of administration and whatever the strain of mice. These results are in contrast to a recent study, where TNF was found to inhibit development of *P. berghei* in a Hep. G2 cell line (7). We have shown that acute-phase proteins (APPs) secreted after cytokine stimulation (17–19) inhibit parasite development *in vitro* and *in vivo* (20–22). Therefore, the fact that TNF induces different APPs in tumor cell lines and in normal rat hepatocytes (23,24) might explain the differences observed in these two experimental models. Since a TNF dose of 1 μg/mouse clearly protects *in vivo* without inducing necrosis (25) but has no effect on hepatocytes *in vitro*, we questioned whether an *in vitro* model containing only hepatocytes was valid. We found in these experiments that the addition of NPC to hepatocytes restored the capacity of TNF to modulate parasite hepatic stage development (Fig. 1). In our co-culture assay, NPC essentially consist of pit cells (large granular lymphocytes), Kupffer cells, T-cells, natural killer cells, endothelial cells from sinusoids, epithelial cells, fibroblasts and Ito cells. Some of these cells are known to produce one or several of the following cytokines: IFN-γ, IL-1, and IL-6 (26–28), all of which are known to interfere with the hepatic stages (29–32). We recently demonstrated that IL-6 induces various effector mechanisms including oxygen radicals (31) and nitrites (32).

In these co-cultures, markedly elevated IL-6 concentrations were found upon addition of TNF, whereas IL-1 and IFN-γ remained undetectable. To confirm that IL-6 was directly involved in the TNF-induced parasite inhibition, anti-IL-6 mAb was added to mono- and co-cultures of hepatocytes. Anti-IL-6 mAb dramatically decreased the TNF-induced inhibition when added 24 h before sporozoite inoculation. The preservation of TNF effects when addition of anti-IL-6 mAb was delayed until 30 min before sporozoite inoculation showed that the release of IL-6 and the subsequent modification inside the hepatocytes takes place in the very early phase of culture after TNF introduction. This is consistent with the observation of high IL-6 concentrations in 24 and 48 h culture supernatants (data not shown), even when initial IL-6 production had been neutralized by the addition of anti-IL-6 mAb. The specific inhibitory effect of IL-6 on pre-erythrocytic stages of parasite development was furthermore demonstrated by incubating hepatocyte cultures directly with rmIL-6 (Table 2). These results strongly suggest that IL-6 is a crucial mediator of the observed TNF effect. The higher inhibitory effect of IL-6 on

the parasite development observed in the presence of NPC suggests a stimulation in NPC of additional factors such as yet to be identified cytokines or substances that act synergistically with IL-6 (33).

TNF has also been demonstrated to be involved in L-arginine-dependent cytotoxic effector mechanisms in macrophages (34) which can have parasiticidal effects (32,35,36).

In malaria the production of various cytokines, and particularly of TNF, is increased during the blood stage (37,38), and these cytokines might modulate infection by liver stages. Furthermore, it has been shown that cytotoxic T cells are involved in the protection induced by immunization with irradiated sporozoites (39,40). We recently demonstrated that immunization with peptide corresponding to the non-repetitive part of the circumsporozoite of *P. yoelii*, induced CD4+ and CD8+ T cells effective in the destruction of cultured hepatic schizonts (41; Rénia *et al.*, in preparation). Moreover, these T cells are known to produce cytokines which include TNF and IL-6 (42). Taken together, these data further illustrate the interdependence of different stages of development in malaria parasites.

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Abbreviations

APP	acute-phase protein
IFN-γ	interferon gamma
IL-1	interleukin 1
IL-6	interleukin 6
mAb	monoclonal antibody
NPC	non-parenchymal cell
rmTNF	murine recombinant TNF

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