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Tumour necrosis factor (TNFα) in leishmaniasis

I. TNFα MEDIATES HOST PROTECTION AGAINST CUTANEOUS LEISHMANIASIS

F. Y. LIEW, C. PARKINSON, S. MILLOTT, A. SEVERN & M. CARRIER
Department of Experimental Immunology, Wellcome Biotech, Beckenham, Kent

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SUMMARY

Genetically resistant CBA mice developed significantly larger lesions to *Leishmania major* infection when they were injected with rabbit anti-tumour necrosis factor (TNF)-specific antibodies compared to control mice injected with normal rabbit immunoglobulin. BALB/c mice recovered from a previous infection following prophylactic sublethal irradiation also developed exacerbated lesions when treated with the anti-TNF antibody. Injection of TNF into the lesion of infected CBA mice significantly reduced the lesion development. Furthermore, TNF activates macrophages to kill *Leishmania in vitro*. These data demonstrate that TNF plays an important role in mediating host-protection against cutaneous leishmaniasis.

INTRODUCTION

It is generally accepted that cell-mediated immunity plays a pivotal role in the host resistance against the protozoa parasite *Leishmania* spp. (Howard, 1985). The major effector cells are Th1-equivalent cells, which secrete interferon-gamma (IFN-γ) upon specific antigen stimulation (Liew, 1989; Heinzel *et al.*, 1989). IFN-γ has been shown to be a major inducer of macrophages for the elimination of this intracellular parasite (Mauel & Behin, 1982). In susceptible mice, Th2-equivalent cells are preferentially activated and they secrete IL-3, IL-4 and IL-5. These lymphokines are able to inhibit the leishmanicidal-inducing activity of IFN-γ (Liew *et al.*, 1989). Hence, lymphokines appear to play important modulating roles in leishmaniasis. However, so far there is little information on the possible role of tumour necrosis factor (TNFα) in leishmaniasis.

TNFα (Cachectin) is a protein produced mainly by macrophages, with a wide range of biological activities and may be important in inflammatory processes. In infectious diseases, TNF is essential to host resistance against *Listeria monocytogenes* infection (Havell, 1987; Nakane, Minagawa & Kato, 1988), *Trypanosoma cruzi* infection (Wirth & Kierszenbaum, 1988) and *Plasmodium chabaudi adami* (Clark *et al.*, 1987) infection in mice. In contrast, TNF is associated with the fatal outcome of cerebral malaria infection (Grau *et al.*, 1989) and with the fatal symptoms of bacterial meningitis in humans (Waage *et al.*, 1989; Leist *et al.*, 1988) and in mice (Leist *et al.*, 1988). It is reported here that TNF appears to play an important role in host resistance against cutaneous leishmaniasis.

Abbreviations: TNFα, tumour necrosis factor alpha.

Correspondence: Dr F. Y. Liew, Dept. of Experimental Immunobiology, Wellcome Biotech, Langley Court, Beckenham, Kent BR3 3BS, U.K.

MATERIALS AND METHODS

Mice

CBA/T6T6 and BALB/c mice, aged 8–10 weeks, were obtained from the colonies at Wellcome Biotech, Beckenham, Kent.

Parasites

The maintenance, cultivation and isolation of the promastigote stage of the parasite *Leishmania major* (LV39) have been described in detail elsewhere (Liew, Howard & Hale, 1984). For animal infection, groups of mice were injected s.c. (in 0·05 ml phosphate-buffered saline; PBS) in the left hind footpad with 1×10^7 (CBA) or 1×10^6 (BALB/c) stationary-stage promastigotes. Some BALB/c mice were exposed to 550 rads gamma-irradiation (cesium source at 50 rads/min), 4 hr before infection, in the shaved rump, with 2×10^7 promastigotes. These mice subsequently recovered completely from the infection. The lesion that developed in the footpads was measured with a dial-calliper and expressed as footpad thickness increase (in $\times 10^{-1}$ mm). The footpad swelling has been found to correlate with parasite load in the footpad and in the draining lymph nodes (Heinzel *et al.*, 1989; F. Y. Liew and C. Parkinson, unpublished data).

Materials

Recombinant murine TNFα was obtained from Genzyme (Koch-Light Ltd, Herts), and anti-TNF antibody was raised in rabbit by s.c. injection of 10 μg TNF in Freund's complete adjuvant (FCA) and boosting with 10 μg TNF in FCA 2 weeks prior to serum collection. The antibody as well as normal rabbit Ig was partially purified by precipitation with 50% ammonium sulphate. The TNF neutralizing activity and specificity were tested before use. Recombinant IL-1β was produced in *Escheri-*

chia coli at Wellcome using standard molecular biological and expression technology. It was tested against naturally derived IL-1β to ensure full specific activity.

TNF assay

TNF activity was determined by the L929 lytic assay (Ruff & Gifford, 1980) and expressed in reference to a standard preparation of recombinant murine TNF. One unit equals the amount required to induce 50% lysis of L929 fibroblast. Lytic activity of 640 units of recombinant TNFα was completely inhibited by 2 mg of the rabbit anti-TNF antibodies. Normal rabbit Ig has no TNF neutralizing activity.

Injection of TNF into infected mice

Groups of CBA mice were infected in the footpad with 1×10^7 *L. major* promastigotes and injected daily (starting from Day 6 post-infection) with 1000 units of murine recombinant TNF (in 20 μl PBS) into the lesion. Control mice were similarly infected and injected daily with PBS or 1000 units of IL-1β. The lesions that developed were measured daily for 14 days.

Leishmanicidal assay

This was carried out as described by Pham & Mauel (1987). Peritoneal exudate cells were collected in culture medium [RPMI-1640 plus 10% fetal calf serum (FCS), L-glutamine, penicillin, streptomycin] from CBA mice injected i.p. 5 days previously with 3 ml of a 2% sterile, hydrolysed starch solution (BDH Chemicals, Poole, Dorset). The cells were plated at 1×10^5 cells/0·1 ml culture medium/well in 96-well flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) and were incubated at 37° and 5% CO_2 for 24 hr. Non-adherent cells were removed and the adherent cells washed three times with pre-warmed medium. To each well was added 175 μl of medium containing 10 ng/ml of lipopolysaccharide (LPS; Sigma) and various amounts of TNF. Cultures were then incubated at 37° and 5% CO_2 for a further 2 hr, followed by the addition into each well of 1×10^5 *L. major* promastigotes in 25 μl of culture medium containing 10 ng/ml of LPS. The mixture was cultured for 72 hr at 37° and 5% CO_2. The cultures were then washed extensively (> 3 times) with pre-warmed medium. To each well was added 100 μl of 0·01% sodium dodecyl sulphate solution in serum-free medium at 37° for 30–40 min. Schneider's medium supplemented with 30% FCS and L-glutamine was added (100 μl/well), and the cultures incubated at 28° for a further 72 hr. The cultures were then pulsed with 1 μCi/well of [^{3}H]thymidine ([^{3}H]TdR; 26 Ci/nmol; Radiochemical Center, Amersham, Bucks) and the uptake of radioactivity by the parasites after 18 hr of further culturing was measured by a liquid scintillation counter (LKB, Betaplate 1205, Bromma, Sweden). The leishmanicidal activity was expressed as reduction in radioactivity uptake (absolute c.p.m.). Each point represents mean of three to six replicate cultures $\pm$1 SEM.

Statistical analysis

All experiments were repeated two to three times. Significance of the results was analysed by Student's *t*-test; $P < 0.05$ is considered significant.

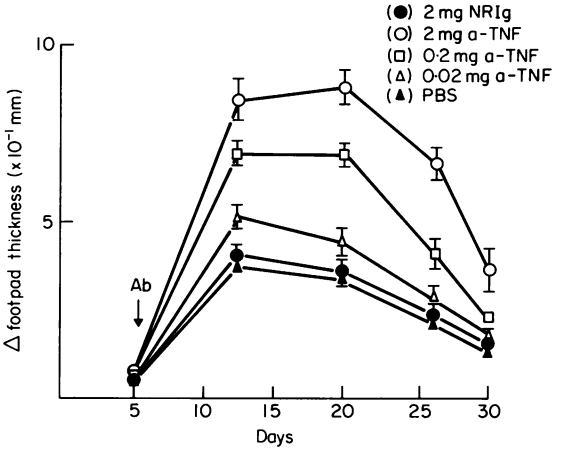

Figure 1. Effects of anti-TNF antibodies on *L. major* infection in CBA mice. Groups of CBA mice were infected in the footpad with 10^7 promastigotes and injected once i.v. on Day 6 with various doses of rabbit anti-TNF antibody or 2 mg of normal rabbit immunoglobulin as indicated. The lesions that developed were measured up to 30 days. Vertical bars = 1 SEM, $n = 5$.

RESULTS

Effects of anti-TNF antibody on *L. major* infection in CBA mice

Groups of CBA mice were infected in the footpad with 10^7 *L. major* promastigotes. Six days later, they were injected i.v. with graded doses of a rabbit anti-TNF antibody preparation or 2 mg of normal rabbit immunoglobulin (NRIg). The lesions that developed were measured at regular intervals. Mice infected with *L. major* and injected with NRIg developed lesions indistinguishable from those of control mice that were similarly infected but injected with PBS (Fig. 1). In contrast, infected mice receiving the anti-TNF antibody developed significantly larger lesions compared to controls.

Effects of anti-TNF antibody on *L. major* infection in BALB/c mice

CBA mice are genetically resistant to *L. major*. In contrast, BALB/c mice are highly susceptible to the parasite. Normally susceptible BALB/c mice that developed fatal leishmaniasis became resistant to *L. major* if they were given whole body sublethal gamma-irradiation (550 rads) shortly before infection (Howard, Hale & Liew, 1980). The recovered mice were resistant to the lethal effects of reinfection, with the development of limited lesions that healed spontaneously. When these recovered and reinfected BALB/c mice were injected with anti-TNF antibody, they developed significantly larger lesions compared to controls injected with NRIg (Fig. 2).

Effect of TNF on lesion development

CBA mice infected in the footpads with 10^7 promastigotes were injected with 1000 U/day of TNF into the lesion, starting from Day 6 after infection. Control mice were injected daily with PBS or 1000 U/day of IL-1β. Table 1 shows that mice injected with TNF developed significantly smaller lesions compared to controls. It is of interest to note that mice receiving IL-1β developed larger lesions on Day 14. The mechanism of this disease enhancement is currently being investigated.

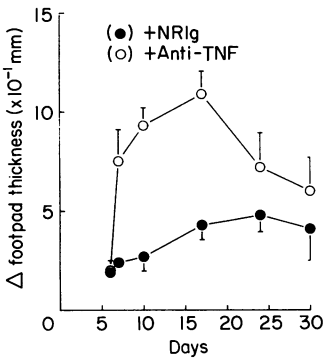

Figure 2. Effects of anti-TNF antibody on *L. major*-infected, recovered BALB/c mice. BALB/c mice that had recovered from a previous infection (2×10^7 promastigotes on Day −180) following prophylactic gamma-irradiation (550 rads, Day −180) were infected in the footpad with 10^6 promastigotes. Six days later, they were injected once i.v. with 2 mg of rabbit anti-TNF antibody or normal rabbit immunoglobulin. The lesions that developed were measured for up to 30 days. Vertical bars = 1 SEM, $n = 5$.

Table 1. Effect of TNFα on lesion development

Treatment	Footpad lesion size ($\times 10^{-1}$ mm) Day 8	Day 14
PBS	4·0±0·4	6·0±0·3
IL-1β (10^3 U/day)	4·9±0·4	8·4±0·9
TNF (10^3 U/day)	2·5±0·5	3·7±1·1

CBA mice were infected in the footpads with 10^7 promastigotes and injected daily with 10^3U of TNFα or IL-1β into the lesion, starting from Day 6 post-infection. Figures underlined are significantly different ($P < 0{\cdot}01$) from control (PBS group). Mean±SEM, $n = 5$.

Table 2. TNF induces macrophage leishmanicidal activity*

TNF in culture (U/ml)	[^{3}H]TdR incorporation (c.p.m.)	P†
4000	2960±350	<0·01
400	4620±130	<0·05
40	5100±350	<0·05
4	5700±580	NS
—	7400±250	—

*For details of leishmanicidal assay, see the Materials and Methods. Results shown are representative of four experiments.

†P values compared to control without TNF (last line), figures = mean c.p.m. ± 1 SEM, $n = 3$.

Effects of TNF on macrophage leishmanicidal activity

The ability of TNF to activate macrophages to kill intracellular *L. major* was analysed in a standard leishmanicidal assay. Table 2 demonstrates that TNF can significantly induce peritoneal macrophage to eliminate *L. major* infection *in vitro*. TNF is not directly toxic to parasites and LPS is necessary for effective leishmanicidal activity induced by TNF (data not shown).

DISCUSSION

TNFα, or Cachectin, was originally discovered in the sera of endotoxin-treated mice and rabbits after infection with bacillus Calmette–Guérin by its destructive effect on tumour cells *in vitro*. Subsequently, its *in vivo* role in infectious diseases also became evident (reviewed by Playfair, 1988). TNF shows an anti-viral effect in several cell lines against a number of animal viruses, such as vesicular stomatitis virus, encephalocarditis virus, adenovirus type 2 or herpes simplex virus-2 (Mestan *et al.*, 1986; Wong & Goeddel, 1986). In murine listeriosis, TNF appears to be essential for host resistance. During sublethal infection with *L. monocytogenes*, injection with anti-TNF antibody resulted in acceleration of listeriosis (Havell, 1987; Nakane *et al.*, 1988). However, the host protective effect of TNF in bacterial infection is by no means a general phenomenon, since TNF in the cerebrospinal fluid is strongly associated with the fatal outcome of bacterial meningitis (Leist *et al.*, 1988; Waage *et al.*, 1989). In parasitic infection, administration of recombinant human TNF released from intraperitoneal osmotic pumps could effectively suppress the *Plasmodium chabaudi adami* infection in CBA mice (Clark *et al.*, 1987). In contrast, a single injection of rabbit antibody to TNF on Day 4 or 7 fully protects CBA mice infected with *P. berghei* from cerebral malaria without modifying the parasitaemia (Grau *et al.*, 1987). In experimental *Trypanosoma cruzi* infections, treatment of macrophages with recombinant TNF plus lipopolysaccharide resulted in a significant reduction in the number of intracellular organisms compared with mock-treated macrophages (Wirth & Kierszenbaum, 1988). Here, it is documented that TNFα is host protective in experimental cutaneous leishmaniasis.

The protective effect of TNF observed here is likely to be manifested by its ability to activate macrophage leishmanicidal activity (Table 2). The precise mechanism of action here is unclear. TNF has been shown to synergise with IFN-γ to inhibit virus replication (Wong & Goeddel, 1986; Feduchi, Alonso & Carrasco, 1989). A combination of IFN-γ and TNF results in a synergistic enhancement of DR mRNA and protein induction in U937 and THP-1 human monocytic cell lines (Arenzana-Seisdedos *et al.*, 1988). TNF is not able to directly induce DR gene expression but rather amplifies ongoing expression of this gene. Both TNF and IFN-γ play a critical role in the ability of the severe combined immunodeficiency (SCID) mutant mice to contain *Listeria monocytogenes* infection (Bancroft *et al.*, 1989).

It is interesting to note that TNF is not detected in appreciable amounts in the serum or the supernatants of spleen cell cultures of susceptible mice with progressive leishmaniasis (F. Y. Liew, C. Parkinson, S. Millott, A. Severn and M. Carrier, manuscript in preparation). Earlier studies demonstrated that the outcome of leishmaniasis is determined by the balance of Th1 and Th2 cells (Liew, 1989). Th1 cells produce IFN-γ, which activates macrophages to kill the intracellular parasites, whereas the Th2 cells produce IL-3 and IL-4, which inhibit the IFN-γ-

induced macrophage-leishmanicidal activity (Liew *et al.*, 1989). Mice with progressive leishmaniasis contain excessive Th2 cells, as evident by high levels of IL-4 mRNA (Heinzel *et al.*, 1989) in the spleen and lymph node cell populations. T-cell clones (Scott *et al.*, 1988) from these mice also produce excessive levels of IL-4. It has also recently been reported that IL-4 can effectively inhibit the ability of monocytes to produce TNFα *in vitro* (Hart *et al.*, 1989). It is therefore conceivable that in highly susceptible BALB/c mice with progressive leishmaniasis, the production of the host-protective TNF is inhibited by IL-4 secreted by the Th2 cells. This mechanism thus represents an additional means by which Th2 cells might contribute to disease exacerbation in leishmaniasis. Details of such a mechanism and the evidence supporting it will be reported in subsequent communications.

Note added in proof

Recently R. G. Titus, B. Sherry and A. Cerami also reported that TNF plays a protective role in experimental murine cutaneous leishmaniasis (*J. exp. Med.* **170,** 2097, 1989).

REFERENCES

Arenzana-Seisdedos F., Mogensen S.C., Vullier F., Fiers W. & Virelizier J.-L. (1988) Autocrine secretion of tumour necrosis factor under the influence of interferon-gamma amplifies HLA-DR gene induction in human monocytes. *Proc. natl. Acad. Sci. U.S.A.* **85,** 6087.

Bancroft G.J., Sheehan K.C.F., Schreiber R.D. & Unanue E.R. (1989) Tumour necrosis factor is involved in the T-cell-independent pathway of macrophage activation in *Scid* mice. *J. Immunol.* **143,** 127.

Clark I.A., Hunt N.H., Butcher G.A. & Cowden W.B. (1987) Inhibition of murine malaria (*Plasmodium chabaudi*) *in vivo* by recombinant IFN-gamma or tumor necrosis factor and its enhancement by butylated hydroxyanisole. *J. Immunol.* **139,** 3493.

Feduchi E., Alonso M.A. & Carrosco L. (1989) Human gamma interferon and tumour necrosis factor exert a synergistic blockage on the replication of herpes simplex virus. *J. Virol.* **63,** 1354.

Grau G.E., Fajardo L.F., Piquet P.-F., Allet B., Lambert P.H. & Vassalli, P. (1987) Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. *Science*, **237,** 1210.

Hart P.H., Vitti G.F., Burgess D.R., Whitty G.A., Piccoli D.S. & Hamilton J.A. (1989) Potential anti-inflammatory effects of interleukin-4: suppression of human monocyte tumor necrosis factor-α, interleukin-1 and prostaglandin-E2. *Proc. natl. Acad. Sci. U.S.A.* **86,** 3803.

Havell E.A. (1987) Production of tumor necrosis factor during murine listeriosis. *J. Immunol.* **139,** 4225.

Heinzel F.P., Sadick M.D., Holaday B.J., Coffman R.L. & Locksley R.M. (1989) Reciprocal expression of interferon-gamma or interleukin-4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T-cell subsets. *J. exp. Med.* **169,** 59.

Howard J.G. (1985) Host immunity to leishmaniasis In: *Leishmaniasis* (eds K. P. Chang and R. S. Bray), p. 140. Elsevier Science Publishers BV, Amsterdam.

Howard J.G., Hale C. & Liew F.Y. (1980) Immunological regulation of experimental cutaneous leishmaniasis. III. Nature and significance of specific suppression of cell-mediated immunity in mice highly susceptible to *Leishmania tropica*. *J. exp. Med.* **152,** 594.

Leist T.P., Frei K., Kam-Hansen S., Zinkernagel R.M. & Fontana A. (1988) Tumor necrosis factor in cerebrospinal fluid during bacterial, but not viral, meningitis. Evaluation in murine model infections and in patients. *J. exp. Med.* **167,** 1743.

Liew F.Y. (1989) Functional heterogeneity of $CD4^+$ T-cells in leishmaniasis. *Immunol. Today*, **10,** 40.

Liew F.Y., Howard J.G. & Hale C. (1984) Prophylactic immunisation against experimental leishmaniasis. III. Protection against fatal *Leishmania tropica* infection induced by irradiated promastigotes involves $Lyt\text{-}1^+2^-$ T-cells that do not mediate cutaneous DTH. *J. Immunol.* **132,** 456.

Liew F.Y., Millott S., Li Y., Lelchuk R., Chan W.L. & Ziltener H. (1989) Macrophage activation by interferon-gamma from host-protective T-cells is inhibited by interleukin (IL)-3 and IL-4 produced by disease-promoting T-cells in leishmaniasis. *Eur. J. Immunol.* **19,** 1227.

Mauel J. & Behin R. (1982) Leishmaniasis In: *Immunology of Parasitic Infections* (eds S. Cohen and K. S. Warren), p. 299. Blackwell Scientific Publications.

Mestan J., Digel W., Mittnacht S., Hillen H., Blohm D., Moller A., Jacobsen H. & Kirchner H. (1986) Antiviral effects of recombinant tumor necrosis factor *in vitro*. *Nature* (*Lond.*), **323,** 816.

Nakane A., Minagawa T. & Kato K. (1988) Endogenous tumor necrosis factor (Cachectin) is essential to host resistance against *Listeria* monocytogenes infection. *Infect. Immun.* **56,** 2563.

Pham T. & Mauel J. (1987) Studies on intracellular killing of *leishmania major* and lysis of host macrophages by immune lymphoid cells *in vitro*. *Parasite Immunol.* **9,** 721.

Playfair J.H.L. (1988) The multiple roles of tumour necrosis factor. *Ann. inst. Pasteur Immunol.* **139,** 281.

Ruff M.R. & Gifford G.E. (1980) Purification and physico-chemical characterisation of rabbit tumor necrosis factor. *J. Immunol.* **125,** 1671.

Scott P., Natovitz P., Coffman R.L., Pearce E., & Sher A. (1988) Immunoregulation of cutaneous leishmaniasis. T-cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J. exp. Med.* **168,** 1675.

Waage A., Brandtzaeg P., Halstensen A., Kierulf P. & Espevik T. (1989) The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between Interleukin-6, Interleukin-1 and fatal outcome. *J. exp. Med.* **169,** 333.

Wirth J.J. & Kierszenbaum F. (1988) Recombinant tumor necrosis factor enhances macrophage destruction of *Trypanosoma cruzi* in the presence of bacterial endotoxin. *J. Immunol.* **141,** 286.

Wong G.H.W. & Goeddel D.V. (1986) Tumour necrosis factor alpha and beta inhibit virus replication and synergize with interferons. *Nature* (*Lond.*), **323,** 819.