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Production of tumour necrosis factor during murine cutaneous leishmaniasis

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Summary We have assessed the role of tumour necrosis factor-α (TNF) during cutaneous leishmaniasis and demonstrated that significant levels of TNF were released by spleen cells from infected mice after *in vitro* restimulation with *Leishmania major* promastigotes. Spleen cells from both genetically resistant and genetically susceptible mice were equally capable of producing TNF. After challenge with bacterial endotoxin, TNF activity could also be demonstrated in the serum of *L. major*-infected mice and the titres correlated with the course of cutaneous disease in susceptible and resistant mice. TNF did not exert a direct leishmanicidal effect *in vitro*. Furthermore, our study indicated that macrophages are the source of *L. major*-induced TNF activity and that its elicitation is dependent on the presence of T cells. These findings suggest that TNF acts in concert with other cytokines produced during *L. major* infection and that its role depends on the composition of T cell subsets and cytokines present.

Keywords: tumour necrosis factor-α, cytokines, *Leishmania major*, immunoregulation, mice

Introduction

Tumour necrosis factor-α (TNF, cachectin) is a multifunctional cytokine that is produced predominantly by macrophages and exerts diverse effects on a wide variety of cells including tumour cells, lymphocytes, fibroblasts and endothelial cells (Beutler & Cerami 1989). TNF has been shown to mediate inflammation caused by endotoxin of Gram-negative bacteria (Carswell *et al.* 1975) and to influence macrophage interaction with protozoan parasites (DeTitto, Catterall & Remington 1986, Kongshavn & Ghadirian 1988, Wirth & Kierszenbaum 1988) and intracellular bacteria (Bermudez & Young 1988). The role of TNF has been analysed in a variety of infectious diseases and conflicting

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results have been obtained from these studies. TNF levels are frequently increased in patients with kala-azar (Scuderi *et al.* 1986) or with severe *Plasmodium falciparum* infections and recent experimental work has supported the view that TNF may account for the pathological features of murine cerebral malaria (Clark *et al.* 1987, Grau *et al.* 1987). In the serum of patients with meningococcal septicaemia, high TNF levels correlated with a negative outcome of disease (Waage, Halstensen & Espevik 1987). On the other hand, plasma levels of TNF were low in lepromatous leprosy as compared to tuberculoid leprosy patients (Silva & Foss 1989) and a protective role of TNF has been reported for murine infections with Bacillus Calmette-Guérin (BCG; Kindler *et al.* 1989), *Listeria monocytogenes* (Havell 1987, Nakane, Minagawa & Kato 1988) and *Escherichia coli* (Cross *et al.* 1989).

Leishmania major, the cause of cutaneous leishmaniasis, is a digenetic protozoan parasite alternating between the promastigote form in the sandfly vector and the obligatory intracellular amastigote form that resides in phagolysosomes of the mammalian host's macrophages. The infected macrophage is supposed to be the target of the immune response mediated by *L. major*-specific T cells (Louis & Milon 1987). The course of disease in mice depends on the strain of animals used. Mice of genetically resistant inbred strains (e.g., C57BL/6) can control the infection with lesions healing completely, whereas genetically susceptible mice (e.g., BALB/c) develop progressive disease with lethal outcome. T cell-deficient nude mice of all genotypes are highly susceptible (Handman, Ceredig & Mitchell 1979). Since macrophages are an important effector component in host resistance to *L. major*, we considered it of interest to investigate the production of TNF in *L. major*-infected healer and non-healer mice and to correlate the level of TNF release with the state of disease. In the studies to be presented, we show that infection with *L. major* primed mice for release of TNF *in vivo*, in response to endotoxin, as well as *in vitro*, in response to either endotoxin or *L. major* promastigotes. In addition, it was found that the *L. major*-induced TNF activity of macrophages was dependent on the presence of T cells.

Materials and methods

MICE

Female mice of the inbred strains BALB/c and C57BL/6 were used at an age of 5–12 weeks. Female athymic BALB/c nu/nu (nude) mice were 5–7 weeks of age at the commencement of experiments. All mice were purchased from Charles River Breeding Laboratories (Sulzfeld, FRG) and, during experimentation, were maintained under conventional conditions in an isolation facility.

CULTURE MEDIUM

RPMI 1640 medium (GIBCO, Karlsruhe, FRG) was supplemented with L-glutamine (2 mM), Hepes buffer (10 mM), $NaHCO_3$ (7·5%), 5×10^{-5} M 2-mercaptoethanol, penicillin (100 μg/ml), gentamycin (160 μg/ml) and 10% fetal calf serum (Seromed, Berlin, FRG) selected for low endotoxin content (4·2 ng/ml as determined by the supplier).

PARASITES, INFECTION OF MICE AND ASSESSMENT OF LESIONS

The origin and propagation of the *L. major* isolate has been described in detail elsewhere (Solbach, Forberg & Röllinghoff 1986a). The cloned virulent line used for this study was confirmed to be *L. major* by isoenzyme analysis (Dr D.Evans, London School of Hygiene and Tropical Medicine, London, UK) and was maintained by passage in BALB/c mice. Promastigotes were grown *in vitro* in blood agar cultures (Solbach *et al.* 1986b). Stationary-phase promastigotes were washed in phosphate-buffered saline (PBS) and 2×10^6 organisms were injected in a volume of 50 μl intradermally (i.d.) on the dorsum of the mouse close to the base of the tail. Lesion scores were determined at regular intervals according to the following system: 0 = no lesion or healed scar; 1 = small swelling (up to 5 mm in average diameter); 2 = large swelling (more than 5 mm in average diameter) or open lesion of less than 5 mm in average diameter; 3 = open lesion of 5–10 mm in average diameter; 4 = open lesion greater than 10 mm in diameter and/or obvious metastases.

INDUCTION OF TNF PRODUCTION *IN VIVO*

At various times after infection with *L. major* or 10 days after infection with 2×10^7 BCG (Connaught Laboratories, Willowdale, Canada), groups of 3 mice were intravenously (i.v.) injected with 10 μg LPS from *E. coli* serotype 055:B5 (Difco, Detroit, USA) in a volume of 0·1 ml. After 2 h, mice were bled and serum was collected for subsequent determination of TNF activity.

INDUCTION OF TNF PRODUCTION *IN VITRO*

T cells were separated from whole spleen and lymph node cell suspensions by passage through a nylon wool followed by a Sephadex G10 (Pharmacia, Uppsala, Sweden) column. For preparation of macrophages, spleens were cut into small pieces and were digested in a solution containing 1·5 mg/ml pronase (Boehringer, Mannheim, FRG) and 100 μg/ml DNase (Boehringer) under continuous rotation (50 rpm, 37°C, 30 min) in 90-mm tissue culture dishes (Greiner, Nürtingen, FRG), as described elsewhere (Hockertz *et al.* 1986). After erythrocyte lysis and washing (in the presence of DNase), 4–5×10^7 cells in 15 ml culture medium were incubated in a tissue culture dish for 24 h (37°, 5% CO_2). Subsequently, the non-adherent cells were carefully removed from the monolayer of adherent cells by repeated washing with warm (37°C) culture medium. The dishes were put on ice for 1 h to allow the collection of the adherent population which was virtually free of lymphocytes as judged by subsequent staining with fluoresceinated anti-Thy-1 and anti-immunoglobulin antibodies. More than 95% of the adherent cells were shown to be macrophages by May-Grünwald–Giemsa and non-specific esterase staining.

For induction of TNF production, unselected spleen cells or purified T cells or macrophages were obtained from untreated or *L. major*-infected mice. 5×10^6 spleen cells or 2×10^6 T cells were placed into 2-ml cultures (Nunc, 143982; Wiesbaden, FRG) and 1×10^5 macrophages were cultured in flat-bottom microtitre plates (Nunc, 167008) in a volume of 0·2 ml. The cells were stimulated with the mitogens LPS (10 μg/ml) or concanavalin A (Con A; 2·5 μg/ml; Serva, Heidelberg, FRG) or with live *L. major* promastigotes (1×10^6/ml) collected during the stationary phase of growth. After 6 h (mitogen stimulation) or 24 h (*L. major* stimulation) of culture, supernatants were collected, replicate samples pooled, centrifuged and stored at −20°C

ASSESSMENT OF TNF ACTIVITY

For the detection of biologically active TNF and lymphotoxin (LT, TNF-β) in sera and culture supernatants, cells of the fibrosarcoma line WEHI 164, which are highly sensitive to the cytolytic effects of both cytokines, were used as target cells (Okuno *et al.* 1986). In the presence or absence of a polyvalent antiserum specific for murine TNF (100 neutralizing units (U); Genzyme, Boston, USA), samples were tested for cytotoxic activity by incubating serial dilutions (50% initial concentration) with 2×10^4 WEHI 164 cells and actinomycin D (0·5 μg/ml; Sigma, Deisenhofen, FRG) in a volume of 0·2 ml in flat-bottom microtitre plates. For quantification of viable cells, the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay was used (Tada *et al.* 1986). After 24 h incubation, MTT (Sigma) was added (10 μl of MTT solution of 5 mg/ml in PBS), and 5 h later, cultures were supplemented with 100 μl of 10% sodium dodecyl sulphate (SDS) in 0·01 N HCl and incubated overnight at 37°C. The absorbance was measured with a microplate reader (MR 700; Dynatech, Denkendorf, FRG), using a test wavelength of 550 nm and a reference wavelength of 670 nm. In this assay, the optical density (OD) values are inversely related to the degree of cytotoxic activity mediated by TNF. A standard titration of recombinant murine TNF (rTNF, kindly provided by Dr G.R.Adolf, Ernst-Boehringer-Institut, Vienna, Austria) at dilutions ranging from 2 U/ml to 0·016 U/ml was set up with each assay (1 U = 38·5 pg rTNF as determined by the supplier in a bioassay with murine tumour cells). The data were subjected to probit analysis, compared to the standard curve, and the activity of each sample was expressed in units of TNF. The detection threshold of the colorimetric assay for TNF activity was 0·5 U/ml.

Results

EFFECT OF TNF ON GROWTH OF *L. MAJOR IN VITRO*

Previous studies have shown that rTNF is able to inhibit the growth of trypanosomes *in vitro* even at very low concentrations (Kongshavn & Ghadirian 1988). To determine whether TNF also affects the *in vitro* proliferation of *L. major*, rTNF at concentrations ranging from 10^1 to 5×10^3 U/ml was added to promastigote cultures and the parasite content was determined 3 days later using the colorimetric MTT assay (Table 1). It was found that the growth of *L. major* promastigotes was not altered by any of the TNF concentrations tested. Thus, TNF does not seem to exert a direct leishmanicidal effect.

TNF ACTIVITY IN SERA OF *L. MAJOR*-INFECTED MICE

We determined the TNF level in serum of either genetically susceptible or genetically resistant mice infected with *L. major* in order to correlate the capacity to produce TNF with the course of cutaneous disease. At various intervals after i.d. infection with *L. major*, susceptible BALB/c and resistant C57BL/6 mice were challenged i.v. with LPS. Sera were collected 2 h later and pooled for determination of the TNF activity of individual experimental groups. The results in Figure 1 show that TNF activity could be detected in sera of both BALB/c and C57BL/6 mice infected with *L. major*. Serum TNF titres of

Table 1. Effect of rTNF on the growth of *L. major* promastigotes *in vitro**

rTNF (U/ml)	OD values mean ± s.d.
5000	0·376 ± 0·033
1250	0·368 ± 0·031
310	0·377 ± 0·024
80	0·370 ± 0·013
10	0·322 ± 0·029
0	0·353 ± 0·013

* Blood agar cultures containing *L. major* promastigotes (1×10^5 in 0·1 ml) and various concentrations of rTNF were incubated at 26°C for 3 days. Subsequently, 0·1 ml of parasite suspension were collected from each culture and were transferred to fresh microculture wells in order to quantify the living promastigotes by using the colorimetric MTT assay. Each value represents the mean ± s.d. of triplicate cultures.

OD: optical density.

BALB/c mice increased with progressing infection, whereas those of C57BL/6 mice peaked at a much lower level. In a control experiment with BCG-infected C57BL/6 mice challenged with LPS, high titres of TNF could be demonstrated showing that mice of this strain are well capable of releasing this factor. The results obtained with BCG-treated mice are in agreement with other reports showing that C57BL/6 mice are high-responders and BALB/c mice are low-responders with respect to endotoxin-induced TNF production after BCG priming (Haranaka, Satomi & Sakurai 1984). LPS-induced production of TNF was dependent on prior infection with *L. major* or BCG since no significant amounts of TNF could be detected in sera of uninfected mice (data not shown). In the absence of LPS stimulation, *L. major*-infected mice of either strain failed to elicit TNF activity (data not shown). Athymic nude mice infected with *L. major* elicited only low titres of LPS-induced serum TNF (80–300 U/ml) even though they develop progressive disease. This TNF activity was in the same order of magnitude as that detected in sera of uninfected nude mice challenged with LPS (data not shown).

In order to discriminate between the cytotoxic factors TNF and LT, both of which have the capacity to lyse WEHI 164 cells *in vitro*, we added an antiserum specific for TNF to the *in vitro* assay used for determination of TNF titres. The TNF activity detected in the serum samples was completely neutralized in the presence of this antiserum (data not shown). These results show that TNF alone but not LT accounts for the LPS-induced cytotoxic factor in the serum of *L. major*-infected mice.

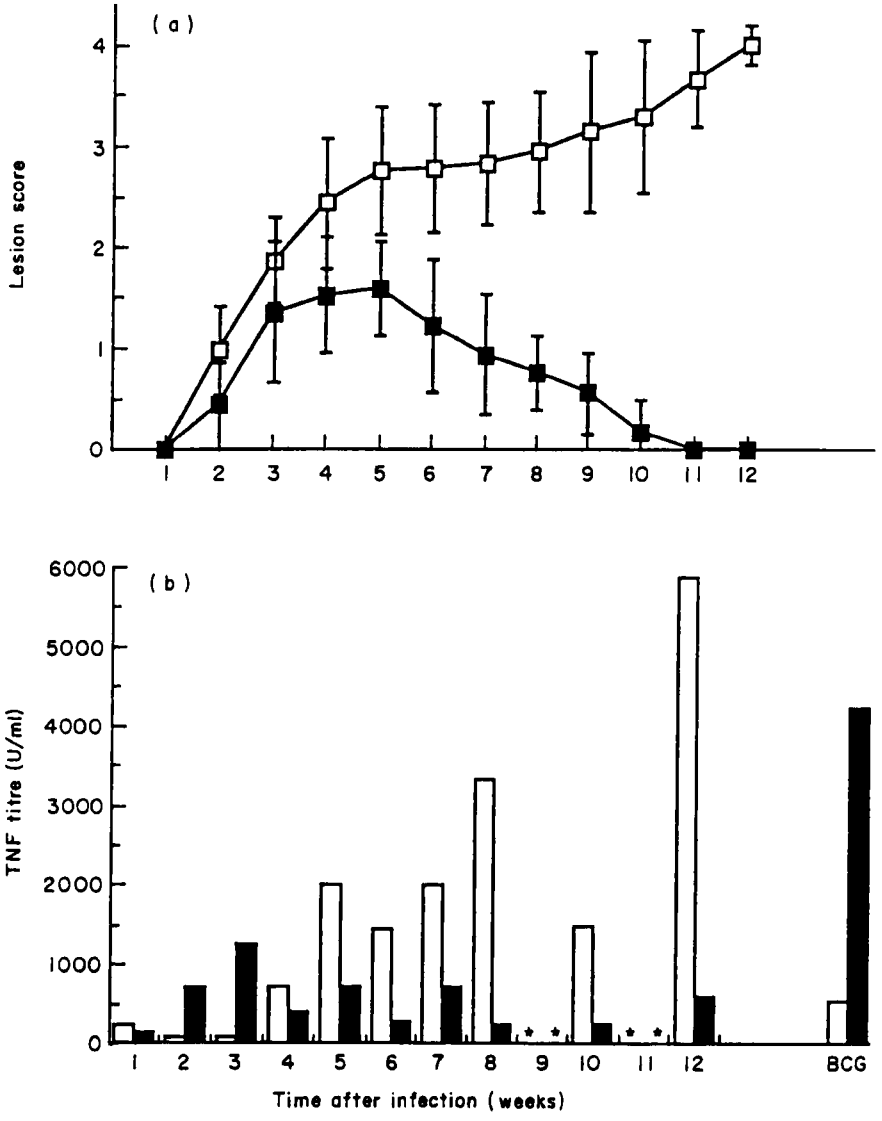

Figure 1. Course of lesion development (a) and LPS-induced TNF activity (b) in sera of BALB/c (□, open bars) and C57BL/6 (■, closed bars) mice infected with 2×10^6 *L. major* promastigotes. At various intervals after i.d. infection, cutaneous lesion scores were determined (a) and mice were injected with 10 μg LPS i.v. (b). In a control experiment, mice were infected with 2×10^7 BCG and were challenged with LPS 10 days later. Blood was collected 2 h after administration of LPS, and serum TNF titres were determined according to the cytotoxicity against WEHI 164 cells in the colorimetric MTT assay. Three mice were used for each time point. Arithmetic means of the cutaneous lesion scores (as defined in Materials and methods) are given with the respective standard errors (a). The standard errors in the TNF assays were consistently below 5% (b). * Not tested.

TNF PRODUCTION OF CELLS FROM *L. MAJOR*-INFECTED MICE IN RESPONSE TO MITOGEN STIMULATION *IN VITRO*

At various times of infection with *L. major*, unseparated spleen cells or purified spleen macrophages from BALB/c and C57BL/6 mice were stimulated with LPS *in vitro*. After 6 h, which was found to be the time of maximal LPS-induced TNF release, culture supernatants were collected and assayed for TNF activity. As it has been observed for other infectious diseases (Havell 1987, Tarleton 1988), the TNF levels detected after *in vitro* stimulation were generally much lower than those found in the serum of infected mice challenged with LPS. However, they were significantly above background and reproducible in numerous experiments. In cultures of whole spleen cells, the TNF response was more pronounced for BALB/c than for C57BL/6 mice infected with *L. major* and increased with severeness of disease (Figure 2a). These results confirm the data obtained with serum samples from infected mice. On the other hand, purified macrophages from infected BALB/c and C57BL/6 mice did not differ consistently in their ability to release LPS-induced TNF (Figure 2b). This suggests that the enhanced capacity of TNF production of *L. major*-infected BALB/c mice is caused by the increased proportion of macrophages in these animals (Modabber 1987; C.Bogdan and W.Solbach, unpublished observations). As it was the case for the serum samples, all the TNF activity in culture supernatants was totally abrogated by anti-TNF antiserum (data not shown).

T cells purified from the spleens and lymph nodes of *L. major*-infected mice failed to release TNF upon *in vitro* stimulation with the mitogen Con A (Figure 2c). Their functional integrity was confirmed by the ability to produce interleukin 2 (IL-2; data not shown), as it was expected from mitogen-stimulated T cells obtained from primed mice. These results provided evidence for macrophages being the predominant source of TNF in mice with cutaneous leishmaniasis.

TNF PRODUCTION OF CELLS FROM *L. MAJOR*-INFECTED MICE IN RESPONSE TO *IN VITRO* RESTIMULATION WITH *L. MAJOR*

In view of the finding that infection with *L. major* can prime mice for LPS-induced TNF production both *in vivo* and *in vitro*, studies were carried out to determine the capacity of these animals to release TNF in response to *in vitro* restimulation with *L. major*. In analogy to the *in vitro* assay system described above, spleen cells or macrophages were prepared at various intervals after infection and were cultured with live *L. major* promastigotes *in vitro*. Culture supernatants collected after 24 h of incubation were found to contain a maximum of *L. major*-induced TNF activity. The results presented in Figure 3 show that spleen cells from both infected BALB/c and C57BL/6 mice were able to release TNF in response to *L. major* alone (in the absence of mitogen). We could not detect consistent differences in the TNF levels produced by those mice. However, no TNF activity could be detected in cultures of purified macrophages (data not shown) even though the same preparations of macrophages were very well able to produce TNF upon LPS challenge (see above, Figure 2b). Therefore, we conclude that the elicitation of TNF activity in response to *L. major* infection is dependent on the presence of T cells.

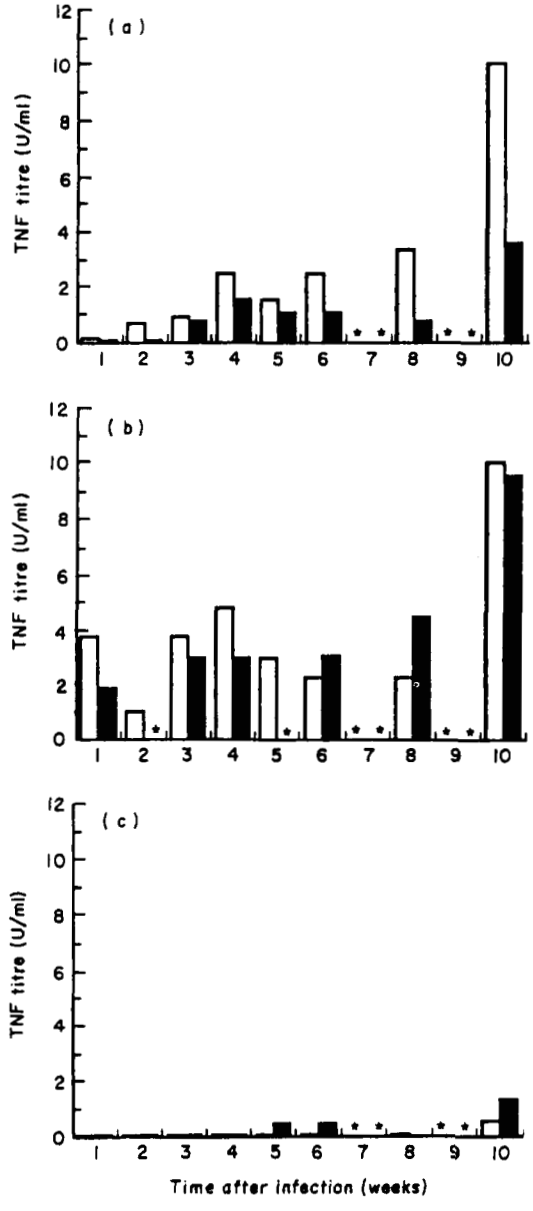

Figure 2. TNF activity of cells from infected BALB/c (open bars) and C57BL/6 (closed bars) mice in response to mitogen stimulation *in vitro*. At various times after i.d. infection with 2×10^6 *L. major* promastigotes, mice were killed and 5×10^6 unselected spleen cells in 2-ml cultures (a) or 1×10^5 purified macrophages in cultures of 0·2 ml (b) were stimulated with 10 μg/ml of LPS. Cultures (2 ml) of 2×10^6 purified T cells from spleens and lymph nodes (c) were stimulated with 2·5 μg/ml of Con A. After 6 h incubation, supernatants were collected and assayed for TNF activity in the MTT assay. The s.d. were consistently below 5%. * Not tested.

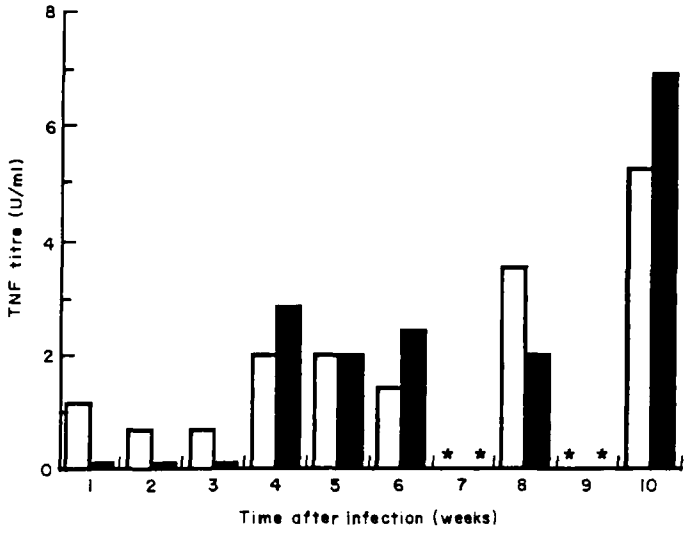

Figure 3. TNF activity of cells from infected BALB/c (open bars) and C57BL/6 (closed bars) mice after *in vitro* restimulation with *L. major*. At various intervals after i.d. infection of mice with 2×10^6 *L. major* promastigotes, 5×10^6 unselected spleen cells in 2-ml cultures were stimulated with *L. major* promastigotes (1×10^6/ml). After 24 h incubation, supernatants were collected and assayed for TNF activity in the MTT assay. The s.d. were consistently below 5%. * Not tested.

Discussion

Significant serum levels of TNF are generally induced by an initial priming step and a subsequent secondary stimulus, usually bacterial LPS (Carswell *et al.* 1975, Beutler & Cerami 1989). Upon challenge with LPS, serum TNF activity could be detected in both genetically susceptible BALB/c and genetically resistant C57BL/6 mice infected with *L. major*. However, the TNF titres were markedly higher in BALB/c than in C57BL/6 mice and increased with progression of disease in susceptible mice. Thus, the LPS-induced TNF activity in the serum appeared to correlate with severeness of cutaneous leishmaniasis. On the other hand, the TNF activity in sera from athymic BALB/c nude mice challenged with LPS was not significantly increased after infection with *L. major*, even though these animals are susceptible to the disease. However, previous reports have already pointed out that nude mice differ from euthymic mice in their ability to produce TNF (Haranaka *et al.* 1984). Even in chronically infected BALB/c mice, no serum TNF activity could be detected in the absence of an LPS stimulus. Similar findings have been reported for other bacterial and protozoal infections (Nakane *et al.* 1988, Tarleton 1988). However, our observation that spleen cells from infected mice are able to release TNF after *in vitro* restimulation with live *L. major* promastigotes strongly suggests that infection with *L. major* results in the production of TNF *in vivo* in the absence of an LPS challenge. The *L. major*-induced TNF release may be restricted to the sites of infection and may therefore not be detectable in the serum, as it has been observed in mice infected with BCG (Kindler *et al.* 1989). Alternatively, the levels of TNF secreted under natural conditions may be lower than those induced by LPS and the rapid clearance of TNF from

the circulation (Beutler, Milsark & Cerami 1985) may thus prevent its detection in the serum of *L. major*-infected mice.

While this paper was in review, a report was published that suggested a beneficial effect of TNF on leishmaniasis (Titus, Sherry & Cerami 1989). The TNF was proposed to originate from T cells in infected mice and to inhibit parasite replication in macrophages. In the present study, however, we showed that TNF activity was detected exclusively in mitogen-stimulated cultures containing purified macrophages but not in those of purified T cells. Therefore, the TNF activity elicited by *L. major*-infected mice is likely to be produced by macrophages. Furthermore, we demonstrated that TNF alone, in the absence of interferon-γ (IFN-γ) or LPS, failed to activate macrophages for killing of *L. major* amastigotes (Bogdan *et al.* 1990).

After *in vitro* stimulation with live *L. major* promastigotes, TNF activity could only be detected in cultures of unseparated spleen cells but not in those of purified macrophages from infected mice. These results favour the notion that the presence of T cells is required for the elaboration of TNF activity by macrophages in response to *L. major* infection, an interpretation which is supported by our finding that athymic nude mice infected with *L. major* elicited only low titres of serum TNF. Some T cell products such as IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) or IFN-γ are known to stimulate the differentiation of macrophages and the release of TNF (Collart *et al.* 1986, Heidenreich *et al.* 1989). The T cell dependence of the expression of TNF activity during cutaneous leishmaniasis is reminiscent of the situation in mice with cerebral malaria where T cells of the L3T4 subpopulation have been implicated in the induction of high levels of serum TNF (Grau *et al.* 1987).

The present study has shown that the levels of LPS-induced TNF production are different in susceptible and resistant mice infected with *L. major*, due to differences in the number of macrophages present, whereas the *L. major*-stimulated TNF activity is equally expressed in both susceptible and resistant mice. So, what role may TNF play in the pathogenesis of cutaneous leishmaniasis? Since the immune response to *L. major* is known to be mediated by different subsets of T cells and various T-cell-derived lymphokines have been shown to influence the outcome of murine disease (Greil *et al.* 1988, Lelchuk, Graveley & Liew 1988, Heinzel *et al.* 1989), the effect of TNF is likely to be connected with the availability of other cytokines. Interactions of TNF with various cytokines have been documented in a number of experimental systems. TNF augments the production of GM-CSF by a variety of cell types (Munker *et al.* 1986) and it stimulates the release of IL-1 from macrophages (Dinarello *et al.* 1986). TNF and IFN-γ act synergistically to induce macrophage killing of tumour cells and schistosomula of *Schistosoma mansoni* (Esparza *et al.* 1987). In view of these interactions, it seems conceivable that TNF exerts different effects in susceptible and resistant mice infected with *L. major*. This notion is supported by *in vitro* studies with macrophages stimulated with various combinations of recombinant cytokines. In combination with IFN-γ, a lymphokine produced in *L. major*-infected mice of resistant strains, TNF induced the elimination of parasites in infected macrophages, whereas it promoted parasite burden in the presence of IL-4 which is mainly expressed in susceptible mice (Bogdan *et al.* 1990). Thus, TNF may have both beneficial and deleterious effects on the infection with *L. major*, as it has been observed for viral meningitis (Doherty, Allan & Clark 1989) and malaria (Clark *et al.* 1987, Grau *et al.* 1987, Taverne *et al.* 1987). More studies on the synergistic actions of cytokines are required in order to elucidate the cascade of events resulting in either resistance or susceptibility to cutaneous leishmaniasis.

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