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Tumor Necrosis Factor Alpha (TNF-α) and TNF-β and Their Receptors in Experimental Cutaneous Leishmaniasis

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Experimental infection of BALB/c mice with *Leishmania major* leads to lesions which progress without healing and visceralization, reproducing the most severe forms of human leishmaniasis, while resistant mice like CBA spontaneously resolve lesions and develop protective immunity. Given the conflicting data pertaining to the role of tumor necrosis factor alpha (TNF) in *Leishmania* infection, we analyzed the expression of TNF, tumor necrosis factor beta (lymphotoxin), and TNF receptor type I (TNF-RI) and type II (TNF-RII) genes in vivo and correlated TNF gene expression in vivo with the production of biologically active TNF by lymphoid cells in vitro. No significant difference in the expression of TNF mRNA was found between susceptible and resistant strains of mice during the course of infection. The depletion of $CD4^+$ T cells in vitro did not change the level of TNF mRNA in BALB/c lymph node cells but led to the total disappearance of TNF mRNA in CBA mice. Unprimed spleen cells did not produce detectable amounts of TNF, whereas 1 week after infection, TNF bioactivity was detected and increased in both strains of mice until 5 weeks of infection. While neutralization of TNF activity in vivo did not alter the course of infection in BALB/c mice, in CBA mice it led to an increase in lesion size and a delay in the healing process but did not interfere significantly with the outcome of infection. Finally, no significant difference in the levels of lymphotoxin, TNF-RI, or TNF RII mRNA expression was found between both strains. The information resulting from these investigations supports the notion that, in vivo, TNF is not the decisive factor responsible for the resistant versus susceptible phenotype in leishmania infection.

Tumor necrosis factor alpha (TNF) is a hormone with a broad spectrum of biological activities, produced mainly by activated macrophages and a variety of other cell types, including activated T cells, mast cells, neutrophils, and astrocytes (reviewed in reference 3). TNF exists in different forms: an inactive secreted form and biologically active membrane-associated and secreted forms (4, 12, 22). Once the protein is efficiently exported from the producing cell, it enters the circulation, where it has a very limited half-life (6 min [5]) and binds to either high-affinity 55-kDa TNF receptor type I (TNF-RI) or low-affinity 75-kDa TNF receptor type II (TNF-RII). These two different receptors bind both TNF and tumor necrosis factor beta, or lymphotoxin (LT). TNF has been shown to be the critical mediator of endotoxic shock (6) and to play a crucial role in neoplastic conditions and in a series of viral and parasitic diseases. In this last context, TNF has been reported to exert quite opposite effects in different disease models: it has been implicated in the pathogenesis of murine cerebral malaria (18), while it has beneficial effects in resistance against *Listeria monocytogenes* (20) and in the development of granulomas in bacillus Calmette-Guérin-infected mice (21).

Leishmania major, one of the causes of cutaneous leishmaniasis, is an intracellular protozoan parasite that infects mononuclear phagocytic cells in its mammalian host. The various clinical manifestations of human leishmaniasis can be mimicked in mice from various inbred strains upon infection with *L. major*. In this murine model of infection, susceptible strains such as BALB/c develop lesions which progress without healing while resistant strains like CBA spontaneously resolve lesions and furthermore develop protective immunity (2, 19). Protection not only is associated with T-cell-produced cytokines (25) but also seems to correlate with the ability to produce TNF (35). Moreover, passive immunization with antibodies directed against TNF exacerbates cutaneous lesions in resistant mice, whereas treatment of susceptible mice with recombinant TNF offers protection, as evidenced by the smaller size of lesions and decreased parasite counts (24). On the other hand, Moll et al. (29) found that primed spleen cells from infected, genetically resistant and susceptible mice were equally capable of producing TNF in vitro in response to *L. major*. TNF has no direct toxic effects on *L. major* (35); however, in combination with a variety of other cytokines, it is involved in the destruction by infected macrophages of the intracellular parasites in vitro (7). TNF in the presence of suboptimal doses of gamma interferon induced a rapid elimination of intracellular parasites (7). In contrast, TNF in combination with interleukin-4 allowed survival of intracellular parasites (7). Studies have reported that the capacity of macrophages to be activated in vitro by gamma interferon to kill intracellular *L. major* was blocked by prior treatment with interleukin-4 (23). In other studies, the latter cytokine synergized with gamma interferon to activate macrophages to kill intracellular *L. major* (8). This effect could be blocked by anti-TNF antibodies, and it was shown that this synergy induced the release of endogenous TNF by macrophages (33).

These conflicting reports led us to analyze the expression of TNF, LT, and their receptor mRNAs in vivo during the course of infection in both susceptible and resistant mice. Since it has been shown that TNF mRNA can exist in an untranslated form (4) and circulating TNF inhibitors which may interfere with the

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activity of TNF have been described (15, 26, 32), there is no assurance that the protein is actually being synthesized or that the biologically active form of the secreted protein is not neutralized by these inhibitors. Therefore, the correlation between TNF gene expression in vivo and the production of biologically active TNF in vitro by spleen and lymph node cells was investigated.

MATERIALS AND METHODS

Mice and parasites. Inbred adult BALB/c and CBA mice originally purchased from IFFA-Credo (Saint Germain-sur-Arbresle, France) and maintained at the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland, were used for these experiments. *L. major* LV39 (MRHO/SU/59/P strain) was maintained as described elsewhere (27) and taken from stationary-phase cultures when used for infection. Viable promastigotes (2 min UV inactivated) were used as the antigen for stimulation in vitro at the doses indicated in the tables and figures.

Infection of mice with *L. major*. Mice were inoculated subcutaneously (s.c.) with 2×10^6 *L. major* promastigotes into one hind footpad. The development of lesions was monitored by measuring the increase in footpad thickness compared with the thickness of the uninfected contralateral footpad.

Induction of TNF production in vitro. Spleen and popliteal lymph nodes were removed from BALB/c and CBA mice before and at different times after s.c. infection. Cells (4×10^6 to 5×10^6/ml) were stimulated in the presence or absence of 1×10^6 live *L. major* promastigotes (inactivated by 2-min irradiation with UV light) per ml in a final volume of 2 ml in 24-well Costar plates (Seromed, Berlin, Germany) at 37°C and 7% CO_2. Dulbecco modified Eagle medium supplemented with 5% heat-inactivated fecal calf serum (Seromed), L-asparagine (36 mg/liter), L-glutamine (216 mg/liter), L-arginine (200 mg/liter), 5×10^{-5} M β-mercaptoethanol, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 100 U of penicillin per ml, and 100 mg of streptomycin per ml was used as a culture medium. Twenty-four hours after stimulation of lymphoid cells with viable *L. major* promastigotes as a source of antigen, culture supernatants were collected and immediately assayed or otherwise stored at −20°C until use.

TNF activity assay. For the detection of biologically active TNF in sera and culture supernatants, we used the fibrosarcoma line WEHI 164 clone 13 assay (16) as described previously (30). Briefly, duplicate samples were tested for cytotoxicity by incubating serial dilutions with 6×10^5 WEHI 164 clone 13 cells per ml and 0.5 mg of actinomycin D (Sigma, St. Louis, Mo.) per ml in a final volume of 0.2 ml in flat-bottom microtiter plates. After 24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) was added (50 μl of 5-mg/ml MTT solution in phosphate-buffered saline) and incubated for 3 h at 37°C in a moist chamber. Plates were then centrifuged, supernatants were removed, and 100 μl of 0.04 N HCl in isopropanol was added to each well. Plates were agitated for 5 min to dissolve the blue crystals, and absorbance was measured with a multiscan enzyme-linked immunosorbent assay spectrophotometer with a 550-nm filter. A standard titration of recombinant murine TNF (kindly provided by G. A. Adolf, Ernst-Boehringer Institute, Vienna, Austria) at dilutions ranging from 653.3 to 0.3 pg/ml was set up with each assay. In some of the assays 145 μg of specific polyclonal rabbit anti-murine TNF (18) was added per well to ensure that the lytic activity was due to TNF.

Depletion of $CD4^+$ and $CD8^+$ cells in vitro. Draining popliteal lymph nodes from BALB/c and CBA mice were dissected at 3 weeks after s.c. infection. $CD4^+$ and $CD8^+$ T cells were depleted by complement-mediated lysis in vitro with cytotoxic immunoglobulin anti-CD4 (RL-172.4) (9) and/or anti-CD8 (H35-17.2) (31) antibody and rabbit complement as described previously (13). The efficacy of the treatment was documented by trypan blue exclusion. Viable cells were collected by Ficoll gradient centrifugation and used to prepare RNA and cDNA.

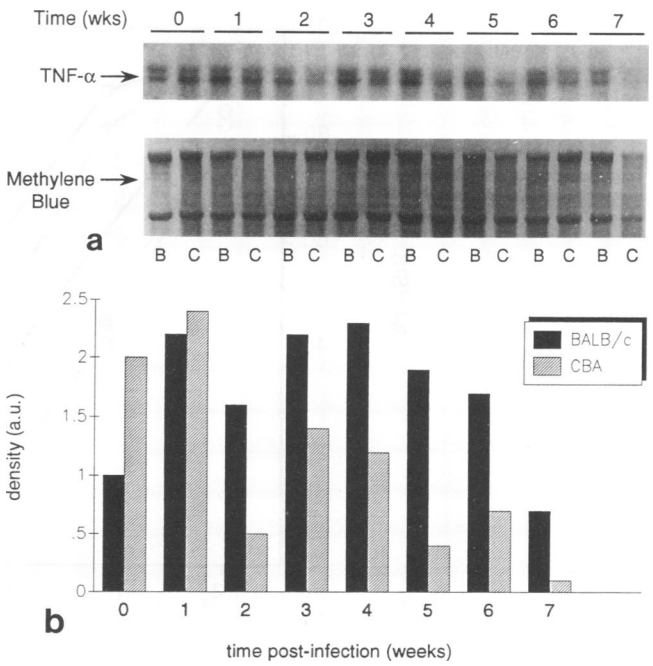

FIG. 1. TNF mRNA expression in the spleen. (a) Northern blot analysis (4 μg per lane) was performed with the spleens of uninfected (week 0) and infected susceptible BALB/c (lanes B) and resistant CBA (lanes C) mice at different times (weeks 1 to 7) after *L. major* infection. The uniformity of RNA loading was assessed after transfer by methylene blue coloration and densitometric scanning of the 18S RNA. Autoradiographic exposure was for 3 days. (b) Quantitation of mRNA was achieved by scanning the appropriate bands with a PhosphorImager system. The values obtained (arbitrary units [a.u.]) were normalized to the values obtained by densitometric scanning of the corresponding 18S RNA bands stained by methylene blue.

Northern blot analysis. Before and at various times after infection, RNA was extracted from footpads, draining lymph nodes, and spleens by the guanidinium thiocyanate method (10). Detection of TNF-specific mRNA was performed by Northern (RNA) blot analysis using the radiolabeled pSP65-TNF cRNA probe (11) after glyoxylation of the RNA, separation by agarose gel electrophoresis, and transfer to Biodyne membranes (Pall Ultrafine Filtration Corporation, Glen Love, N.Y.). Prehybridization and hybridization conditions were as described previously (11). Filters were exposed to Kodak XAR-5 films at −70°C with Dupont Cronex Lightning-Plus intensifier screens, and the intensity of specific bands was quantified by densitometry.

cDNA synthesis. Eppendorf tubes containing 5 μg of total RNA were incubated for 30 min at 37°C in a mixture of 40 mM Tris-HCl (pH 7.5), 10 mM NaCl, 6 mM $MgCl_2$, and 2.5 U of RQ1 DNase (Promega, Madison, Wis.) in order to remove any contaminating genomic DNA from the preparations. After phenol-chloroform extraction and ethanol precipitation, pellets were resuspended in water and the synthesis of the first

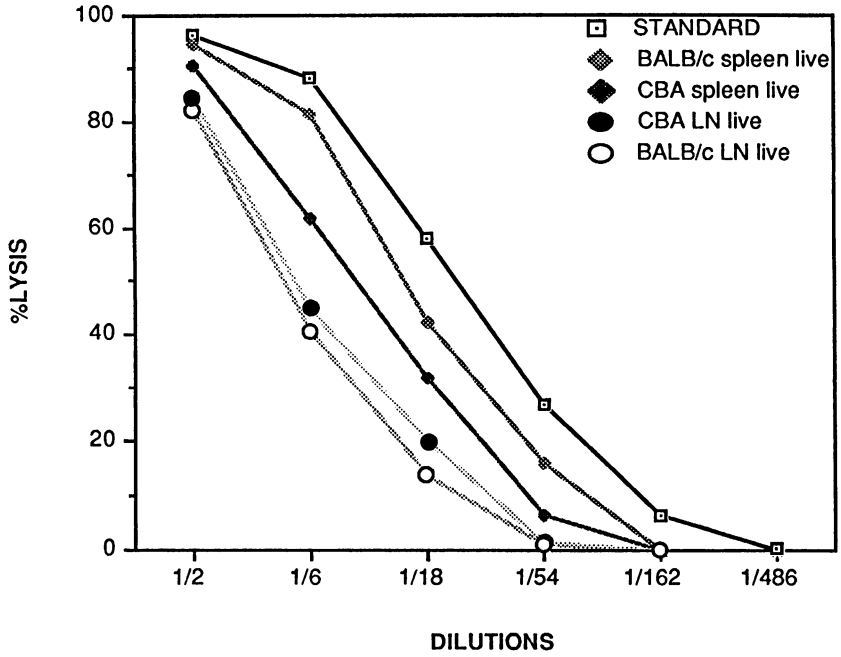

FIG. 2. TNF production by splenic and lymphoid cells from *L. major*-infected resistant and susceptible mice. Thirty-three days after s.c. infection, 5×10^6 lymphoid cells from both strains of mice were stimulated with live *L. major* promastigotes (2 min UV) as an antigen. Twenty-four hours after initiation of the cultures, supernatants were collected and serial dilutions of the supernatants were analyzed for TNF activity as described in the text. The percent lysis of the indicator WEHI-164 cells was calculated as follows: (optical density of test supernatant/optical density of viable cells) × 100. In the absence of antigenic stimulation the lytic activity of the supernatants was <5%.

strand of cDNA was performed according to the instructions delivered with the cDNA Synthesis Kit (Boehringer Mannheim AG, Rotkreuz, Switzerland), using oligo$(dT)_{15}$ and avian myeloblastosis reverse transcriptase (10 U per sample). After 1 h of incubation at 42°C, samples were diluted to a total volume of 100 μl, heat inactivated (5 min at 95°C), and kept frozen (−20°C) until use.

PCR analysis. Four microliters of cDNA (the equivalent of 200 ng of total RNA) was added to a reaction mixture containing 25 mM TAPS-HCl (pH 9.3); 50 mM KCl; 1 mM β-mercaptoethanol; 10 mM (each) dATP, dGTP, dCTP, and dTTP; 10 μCi of [α-^{32}P]dCTP (3,000 Ci mmol^{-1}; Amersham), 12.5 μg of activated salmon sperm DNA, and 2.5 U of AmpliTaq (Perkin-Elmer Cetus) in a final volume of 50 μl. Samples were amplified at 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min. Thirty-five cycles of PCR were carried out in a DNA thermal cycler (Perkin-Elmer Cetus) in the presence of a 0.2 mM concentration of each primer. The following oligonucleotides were used: TNF 1, 5′-CCCGACTACGTGCTC CTC-3′; TNF 2, 5′-GACCTGCCCGGACTCCGC-3′; LT 1, 5′-ACC CAT GGC ATC CTG AAA C-3′; LT 2, 5′-AGA CAA AGT AGA GGC CAC-3′; TNF receptor 55 kDa 1, 5′-CCG GGC CAC CTG GTC CG-3′; TNF receptor 55 kDa 2, 5′-CAA GTA GGT TCC TTT GTG-3′; TNF receptor 75 kDa 1, 5′-GAC GAA TTC ATG GAG TAG GCC TTG AGC-3′; TNF receptor 75 kDa 2, 5′-TAA GGA TCC CTG AGA CGG ACA CTC CTC-3′; GAPDH 1, 5′-TGAAGGTCGGTGT GAACGGATTTGG-3′; and GAPDH 2, 5′-ACGACATACT CAGCACCAGCATCAC-3′. One-tenth of the reaction mixture was run on a 2% agarose gel, and the appropriate band was revealed by autoradiography. The relative product amounts were quantitated by measuring the incorporated radioactivity from dried gels with a Molecular Dynamics 400A PhosphorImager. Relative quantitation was achieved by subjecting parallel samples to amplification, in subsaturating conditions, of the constitutively expressed glyceraldehyde-phosphate dehydrogenase (GAPDH) gene and the specific cytokine. The values were normalized to the values obtained by measuring the incorporated radioactivity in the GAPDH amplification products.

In vivo treatment with anti-TNF antibodies. Immunoglobulin G fractions of either rabbit antiserum to mouse TNF (18) or normal rabbit serum were prepared by protein A-Sepharose chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden). Antibodies were ultracentrifuged at 150,000 × *g* for 150 min, and only the upper deaggregated fraction was used. CBA and BALB/c mice made tolerant to rabbit immunoglobulin G fractions by the intraperitoneal injection of 1 mg of normal rabbit immunoglobulins were then infected s.c. in one hind footpad with 1×10^7 and 2×10^6 promastigotes, respectively. Two days later and weekly thereafter, mice were injected intravenously with 1 mg of monospecific polyclonal anti-TNF antibody. Infected tolerant mice served as controls. The development of lesion size was measured during the course of infection by monitoring footpad thickness.

Statistical analysis. Significance analysis between results obtained from various groups was performed by using the nonparametric Mann-Whitney U test. Probability values of <5% were considered significant.

RESULTS

TNF mRNA expression during the course of *L. major* infection. Genetically susceptible BALB/c and resistant CBA mice were infected with 2×10^6 *L. major* promastigotes s.c. in one hind footpad. At different times after infection, mice were

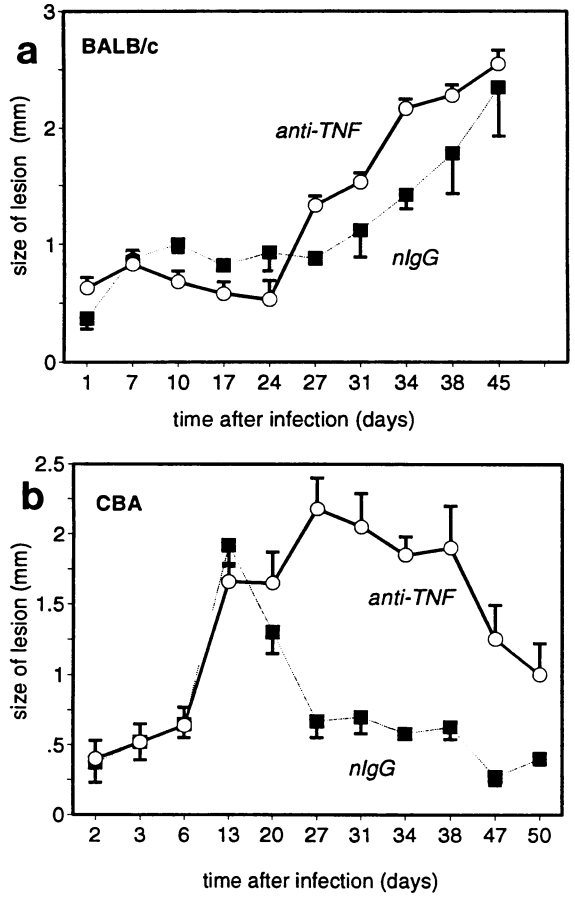

FIG. 3. Effect of anti-TNF antibody treatment on the course of lesion development in *L. major*-infected CBA and BALB/c mice. (a) BALB/c mice (n = 6) made tolerant with 10 mg of normal rabbit immunoglobulins (nIgG) (intraperitoneally) were infected s.c. in one hind footpad with 2×10^6 promastigotes. On day 2 and biweekly during the course of the experiment, mice were injected with 1 mg of anti-TNF antibody intravenously. (b) Tolerant CBA mice (n = 6) were infected s.c. with 10^7 promastigotes. Two days later and weekly thereafter, mice were injected intravenously with 1 mg of anti-TNF antibody. Infected tolerant mice served as controls. The development of lesion size was measured during the course of infection by monitoring footpad thickness for up to 50 days in CBA mice and for 45 days in BALB/c mice. Vertical bars represent mean values ± standard errors of the mean.

sacrificed and RNA was prepared from the footpad, draining lymph nodes, and spleens. TNF mRNA expression was assessed by Northern blot analysis of total RNA. A clear signal was detected in the spleens of both strains of mice during the course of infection (Fig. 1a). In normal uninfected mice the baseline level of TNF mRNA expression in the spleens of CBA mice was slightly higher than in those of BALB/c mice. One week after infection the levels of RNA expression in the spleens of both strains of mice were comparable. During the following weeks of infection the spleens of BALB/c mice expressed higher levels of TNF mRNA than did spleen cells from infected CBA mice. Quantitation of the TNF mRNA levels was performed by densitometric scanning of the Northern blot, and values obtained were normalized against the densitometric scanning of the 18S RNA. The normalized values shown in Fig. 1b demonstrate numerically that the spleens of infected BALB/c mice from 2 weeks of infection on contain higher levels of TNF mRNA. However, higher levels of mRNA do not necessarily correlate with more protein.

TNF mRNA in the lesions could be detected by Northern blot analysis 3 weeks postinfection in both strains of mice only when twice the amount of RNA was loaded (8 μg) and exposure times were prolonged to 2 weeks (data not shown). In the draining lymph node, TNF-specific signals could not be detected under these conditions. To overcome this limitation, PCR analysis was performed. The results show that there was no significant difference between both strains of mice during the course of *L. major* infection (not shown).

TNF production by spleen and lymph node cells. Total spleen and lymph node cells from *L. major*-infected resistant or susceptible mice were collected on day 33 of infection and stimulated in vitro with 10^6 live *L. major* parasites as an antigen. Twenty-four hours later, the supernatants were collected and assayed for TNF bioactivity on WEHI-164 clone 13 cells. Results showed that spleen and lymph node cells from BALB/c and CBA mice displayed comparable lytic activities (Fig. 2). No TNF bioactivity was observed in the absence of *L. major* stimulation. Addition of 145 μg of specific anti-TNF antibody strongly inhibited the lytic activity of all the tested supernatants, indicating that the activity was due to TNF.

Effect of anti-TNF antibody injections in susceptible BALB/c mice. BALB/c mice were made tolerant with normal rabbit immunoglobulins 8 days before infection with 2×10^6 *L. major* promastigotes. On day 2 of infection and once per week thereafter, mice received in one experiment 1 mg of anti-TNF immunoglobulin G fractions intravenously and, in a second experiment, the dose was doubled. Lesion size was monitored in control infected, tolerant mice and in tolerant mice injected with anti-TNF antibodies. With both doses results were comparable. Neutralization of TNF activity in vivo did not alter the course of infection in BALB/c mice in the two independent experiments (Fig. 3a).

Effect of TNF antibody injections in resistant CBA mice. The neutralization of TNF in similarly treated CBA mice, after 3 weeks of infection, led to a significant increase in lesion size and a delay in the healing process. However, it did not interfere with the overall outcome of infection in resistant mice, since after 4 weeks lesions steadily decreased in size (Fig. 3b) until the experiment was stopped on the 8th week after infection.

Expression of LT, TNF-RI, and TNF-RII mRNAs. The expression of LT, TNF-RI (55 kDa), and TNF-RII (75 kDa) mRNAs was analyzed in the lymph node cells of both strains during the course of *L. major* infection. Altogether, we found no difference in the levels of mRNA expression for LT between both strains of mice. A comparable constitutive expression of LT was found before and in the first week of infection. Thereafter, the levels of mRNA markedly decreased. Regarding TNF-RI expression, both strains showed variable levels of expression during the course of infection. The mRNA levels remained lower than the constitutively expressed levels until the 6th week of infection, and, except for the 1st week, at all time points TNF-RI mRNA levels were higher in CBA mice than in BALB/c mice. TNF-RII mRNA levels remained low in CBA and BALB/c mice until the 6th week of infection, when they rose significantly in both strains (Fig. 4).

Effect of T-cell depletion in vitro on the expression of TNF mRNA. Lymph node cells were collected from 10 BALB/c mice (2.2×10^8 cells) and 10 CBA mice (0.7×10^8 cells) on the 3rd week of infection. Cell suspensions were treated in vitro with monoclonal anti-CD4 or anti-CD8 antibodies and complement. The anti-CD4 treatment led to cell reductions of 33 and

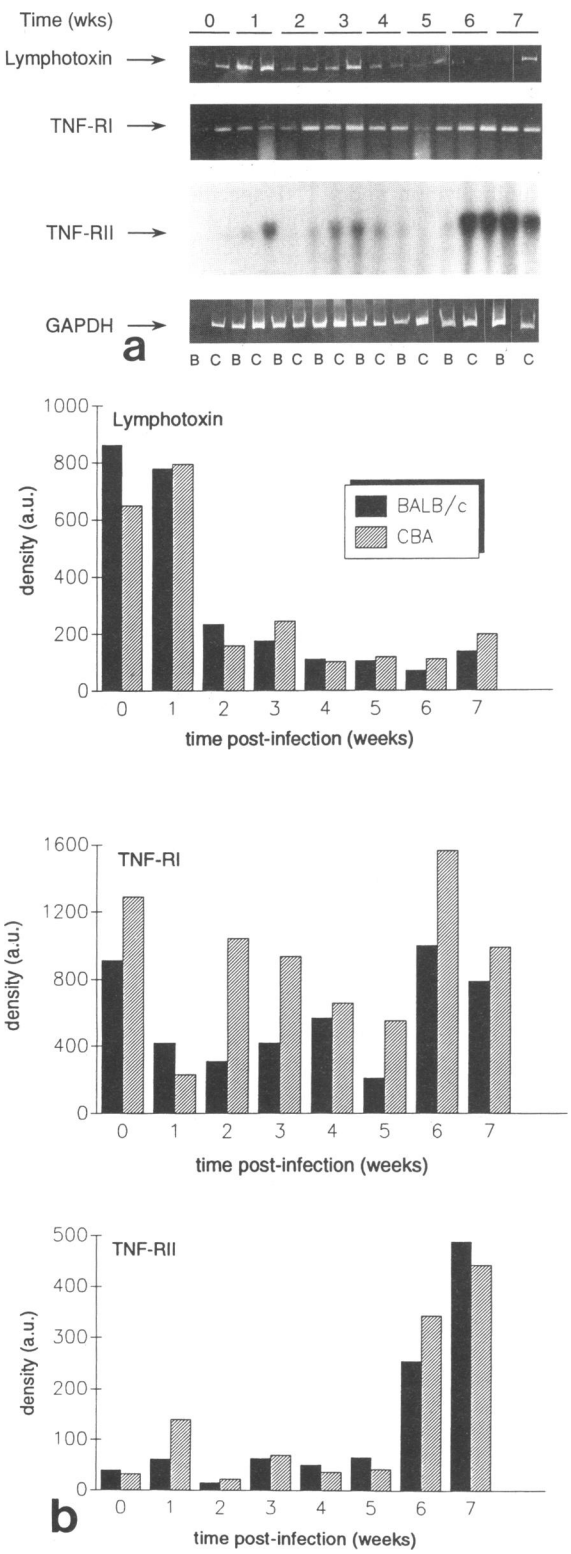

FIG. 4. Detection of cytokine and cytokine receptor mRNA expression by PCR analysis in BALB/c (lanes B) and CBA (lanes C) mice. (a) LT, TNF-RI, and TNF-RII mRNA expression and GAPDH expression were analyzed. The equivalent of 200 ng of total RNA was reverse transcribed and amplified for 35 cycles in the presence of [α-^{32}P]dCTP. One-tenth of the amplification reaction mixture was loaded on an agarose gel. Bands were revealed by ethidium bromide staining of the gel. In the case of TNF-RII, the bands were too faint to be appropriately revealed after 35 cycles, so the gel was directly exposed to XAR-5 film at room temperature for 1 h. (b) The radioactivity of the PCR products was quantitated with a PhosphorImager, and the intensities of the signals (expressed as arbitrary units [a.u.]) were normalized against the GAPDH amplification products (run in parallel) as described in the text.

27% in cell suspensions from BALB/c and CBA mice, respectively. The anti-CD8 treatment led to cell decreases of 28 and 18% in cell suspensions from BALB/c and CBA mice, respectively. The surviving cells were counted and purified by centrifugation through a Ficoll gradient. RNA was extracted and reverse transcribed. PCR analysis was performed to detect the presence of TNF in the different cell populations. Unselected lymph node cell populations showed comparable levels of TNF mRNA in both BALB/c and CBA mice when tested directly ex vivo. The depletion of CD4$^+$ T cells did not change the level of TNF mRNA in lymph node cells from susceptible BALB/c mice. In sharp contrast, the same treatment led to the total disappearance of the TNF messenger in lymph nodes from resistant CBA mice. Elimination of CD8$^+$ T cells resulted in a slight reduction of the signal in both strains of mice (Fig. 5).

DISCUSSION

The results obtained in the present study confirm that neutralization of TNF by administration of anti-TNF antibody to resistant CBA mice infected with *L. major* enhances and prolongs lesions without, however, interfering with the final outcome of disease. Similar treatment of susceptible mice has no significant effect on lesion development. Despite this different effect of TNF neutralization in resistant and susceptible mice, no evidence that mice from these two strains produce different amounts of TNF during infection was obtained. This was assessed by both mRNA expression studies and production of the protein in vitro. Moreover, in CBA but not in BALB/c mice, TNF mRNA expression was dependent on CD4$^+$ T cells. There was no significant strain difference in the expression of LT, TNF-RI, and TNF-RII mRNAs.

Previous results have shown that resistance and susceptibility to infection with *L. major* in mice correlated with the amount of TNF produced by lymphoid cells upon specific stimulation in vitro (24, 35). Combined with the findings that recombinant TNF and anti-TNF antibodies have, respectively, a beneficial and detrimental effect on the course of disease, these observations led to the suggestion that TNF plays an important role in host resistance against cutaneous leishmaniasis. Although the present study confirms the detrimental effect of anti-TNF antibody in resistant mice, no evidence that resistance to *L. major* infection correlates with an increased TNF production capacity or susceptibility with a deficient TNF production was obtained. Our observations are in agreement with those previously reported by Moll et al. (29), who showed that after stimulation of spleen cells with live *L. major* promastigotes in vitro, TNF activity was equally expressed in both infected resistant and susceptible mice. It is shown here in addition that, compared with those in resistant mice, the levels of TNF mRNA were not lower but were even higher in the spleens of infected susceptible mice. Higher levels of TNF mRNA in the spleens of infected BALB/c mice are probably due to the augmented number of infiltrating macrophages. However, elevated RNA levels do not necessarily translate into higher levels of protein. Upon restimulation in vitro, spleen cells from both BALB/c and CBA infected mice produced

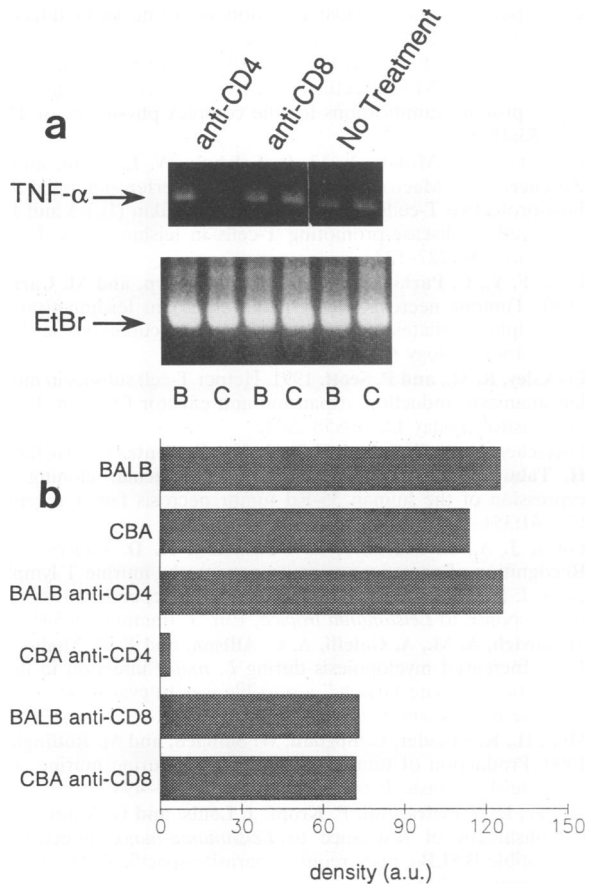

FIG. 5. Effect of depletion of $CD4^+$ and $CD8^+$ T cells in vitro on TNF mRNA expression in lymph node cells from 10 *L. major*-infected BALB/c (lanes B) and 10 CBA (lanes C) mice. (a) Three weeks after s.c. infection, draining lymph nodes were collected. T-cell subsets were selectively depleted with specific antibodies and complement. RNA extracted from surviving cells was reverse transcribed, and TNF mRNA expression was assessed by PCR. EtBr, ethidium bromide. (b) Arbitrary units of incorporated radioactivity in TNF amplification products were obtained with a PhosphorImager and normalized against those of GAPDH gene amplification products run in parallel. The experiment was repeated once with comparable results.

equivalent amounts of TNF. In experimental murine cutaneous leishmaniasis, it has also been shown that the release of biologically active TNF depends upon the presence of T lymphocytes (29), although the phenotype of the T cells involved has not yet been determined. The present paper extends the findings based upon in vitro restimulation assays by studying the cytokine gene expression in vivo. In CBA but not in BALB/c mice, the actual TNF mRNA expression in lymph nodes was shown to require the presence of $CD4^+$ T cells.

Since the protective role of TNF is evidenced by the in vivo neutralization data and since comparable levels of the molecule are made by both strains, it is possible that resistant and susceptible mice differ in terms of membrane-anchored TNF. In the context of experimental leishmaniasis, it has indeed been shown in resistant C57BL/6 mice that 5 to 25% of lymph node $CD4^+$ T lymphocytes express the membrane-associated form of TNF (34). This surface TNF can induce antileishmanial defense through a cell contact-dependent mechanism and provide a means of directing activation signals to macrophages in an antigen-specific manner (34). Therefore, membrane-bound TNF might represent a mechanism by which to restrict the effects of TNF on target cells expressing the specific antigen, thus reducing pathological consequences that can be engendered by systemic release of this cytokine. It is tempting to speculate that membrane-anchored TNF is present on $CD4^+$ T lymphocytes in infected CBA mice and can exert physiopathological effects which differ from those induced by soluble TNF. Along this line, susceptible BALB/c mice may have no or insufficient membrane-bound TNF on the surface of their T cells; this contention is supported by the observation that removal of $CD4^+$ T cells did not alter the amount of TNF mRNA in lymph node cells (Fig. 5). The TNF mRNA found to be expressed in lymphoid organs from infected mice of this strain might result in secreted TNF, produced by macrophages, B cells, or other cell types, rather than in membrane-bound TNF on T cells. Indeed, there is a strong B-cell and macrophage recruitment in the draining lymph nodes of infected susceptible mice (28) which could account for the observed TNF levels.

Differences in the relative quantities of soluble TNF inhibitors, consisting of the shed extracellular domain of TNF-RI and TNF-RII (for reviews see references 17 and 36), between resistant and susceptible mice could account for the apparent lack of effect of TNF on parasite multiplication in susceptible animals. It has been reported that soluble TNF receptors can act either as antagonists of TNF function, by competing with the binding of TNF to its membrane receptor, or as agonists, by prolonging the stability of TNF bioactivity in the serum (1). This might be particularly relevant in the context of a chronic infection, such as leishmaniasis. The relative amounts of soluble, shed receptors during the course of infection would therefore be expected to have complex, indeed unpredictable, effects on the outcome of the disease. Indeed, it has recently been shown that pretreatment of macrophages with lipophosphoglycan purified from *Leishmania donovani* leads to a strongly reduced binding of radiolabeled TNF to its receptors (14). The contribution of the two types of TNF receptors to signal transduction and cellular activation is not fully understood. At first glance our results showing similar levels of TNF-RI and TNF-RII mRNA expression in resistant and susceptible mice could imply that the amounts of TNF receptors present at the cell surface are identical. However, post-transcriptional modulations which could alter the amount of receptors actually expressed on the membrane might occur.

The information resulting from these investigations supports the notion that in vivo, TNF by itself is not the decisive factor responsible for the resistant versus susceptible phenotype in leishmania infection.

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