

TUMOR NECROSIS FACTOR PLAYS A PROTECTIVE ROLE IN EXPERIMENTAL MURINE CUTANEOUS LEISHMANIASIS

BY RICHARD G. TITUS,* BARBARA SHERRY,† AND ANTHONY CERAMI‡

From the *Department of Tropical Public Health, Harvard School of Public Health, Boston, Massachusetts 02115; and the †Laboratory of Medical Biochemistry, The Rockefeller University, New York, New York 10021

The cytokine, cachectin/tumor necrosis factor (TNF), was identified originally on the basis of its ability to induce hemorrhagic necrosis of tumors and to contribute to the severe wasting (cachexia) that accompanies chronic parasitic and viral infections and neoplastic disease. Recently, it has become clear that TNF is an inducer of shock and a potent pro-inflammatory mediator capable of stimulating in vitro functions of monocytes, eosinophils, neutrophils, endothelial cells, and fibroblasts (1).

Depending upon the disease model, the effect of TNF may be either beneficial or harmful. For example, TNF has been shown (a) to be involved in resistance to *Listeria monocytogenes* (2) and Bacillus Calmette Guerin (BCG)¹ (3), (b) to cause killing of virus-infected cells (4–6), (c) to be capable of enhancing the cytotoxicity of both eosinophils (7) and platelets (8) for schistosomes, and (d) to be able to activate neutrophils to kill *Candida albicans* (9) and macrophages to kill *Trypanosoma cruzi* (10). In contrast to these beneficial effects, in the case of malarial infection, TNF seems to be an important mediator of the host-destructive pathogenesis that accompanies murine cerebral malaria (11).

We observed initially that lymph node cells (LNC) from mice infected with, or sensitized to, the protozoan parasite *Leishmania major*, could produce, upon stimulation with either parasite antigens or mitogens in vitro, substantial amounts of a substance that was cytotoxic for L929 cells. We therefore investigated the role of TNF in the course of murine cutaneous leishmaniasis induced by *L. major*. This article shows that C3H mice (resistant to *L. major*) produced significant amounts of TNF during the course of infection with *L. major*. In contrast, TNF production by BALB/c mice (susceptible to *L. major*) was never detected. In addition, repeated injection of recombinant human TNF (rHuTNF) into both strains of mice infected with *L. major* had a therapeutic effect on the course of infection. In contrast, injection of

This work was supported by National Institutes of Health grants 1 R29 AI-24511, 5 PO1 AI-22794, AI-21359 (5RO1), and by the Rockefeller Foundation (RF85072 No. 125) and the Chiron Corporation.

Address correspondence to Dr. Richard Titus, Department of Tropical Public Health, Harvard School of Public Health, 665 Huntington Ave., Boston, MA 02115.

¹ Abbreviations used in this paper: anti-TNF, neutralizing rabbit anti-murine TNF; BCG, Bacillus Calmette Guerin; LNC, lymph node cells; MTT, 3(4,5-dimethyl-thiazoyl-2-yl)2,5 diphenyltetrazolium bromide; mTNF, native murine TNF; nrIg, normal rabbit Ig; rHuTNF, recombinant human TNF.

a neutralizing anti-murine TNF antibody was shown to markedly exacerbate the course of infection with the parasite.

Materials and Methods

Animals. C3H/HeN and BALB/c mice were obtained from Taconic Farms (Germantown, NY) and were used as sex-matched young adults at 8–10 wk old.

Leishmania major and Infection with *L. major*. *L. major* promastigotes (LV 39) were maintained as described (12), and when used, were taken from stationary phase cultures (13). The numbers of promastigotes stated in the text were injected subcutaneously in the hind footpad and lesion progression was followed by determining the increase in footpad thickness with a vernier caliper relative to the contralateral uninfected control footpad.

Determination of the Number of *L. major* in Infected Mouse Tissues. Enumeration of *L. major* in the infected footpad and draining lymph nodes was performed using a limiting dilution assay described in detail elsewhere (14).

Production of TNF by Mouse LNC. Mice were injected with 5×10^6 *L. major* promastigotes subcutaneously in the hind footpads and at intervals during the course of infection the popliteal and inguinal lymph nodes were removed. Single cell suspensions were produced and 4×10^6 LNC/well were cultured in 48-well plates (Costar, Cambridge, MA) containing 0.5 ml of DME (15) supplemented with 0.5% normal mouse serum. The cultures were stimulated with either 3×10^4 , 10^5 , or 3×10^5 *L. major* promastigotes/milliliter. At varying times thereafter, the supernatants of the cultures were harvested and stored at -70°C until they were analyzed for their content of TNF by the L929 cell cytotoxicity assay.

Recombinant Human TNF (rHuTNF). rHuTNF was kindly provided by Chiron Corp. (Emeryville, CA) and exhibited a specific activity of 2×10^8 U/mg.

Purification of Native Murine TNF (mTNF). The mouse macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD) was maintained in culture according to established procedures (16). Using conditioned medium from LPS-stimulated RAW 264.7 cells as the starting material, mTNF for use as a standard and for immunization purposes was purified to homogeneity in a manner identical to that described previously (17).

Preparation of Neutralizing Rabbit Anti-Murine TNF (anti-TNF) and Normal Rabbit Ig (nrIg). Female New Zealand white rabbits were immunized with mTNF according to a regimen described previously (17). An immune Ig fraction pooled from five rabbits (anti-TNF) was obtained by ammonium sulfate precipitation according to established procedures (18). The precipitate was extensively dialyzed, and the protein content was determined by Bradford assay (19). This anti-TNF preparation is not known to react with any cytokines other than TNF (our unpublished observations). nrIg was obtained by pooling sera collected from normal New Zealand white rabbits and preparing the Ig fraction in a manner identical to that described above.

Assay for Quantifying TNF. A clone of the fibroblast line L929 (kindly provided by Dr. A. Glasebrook, Lilly Research Laboratories, La Jolla, CA), selected for its sensitivity to the cytotoxic effects of TNF, was used. L929 cells were cultured in 96-well plates (Costar, Cambridge, MA) at 2×10^5 /well in DME with 5% FCS (Gibco Laboratories, Grand Island, NY). The cells were cultured for 36 h at which time, the culture medium was replaced with medium containing a final concentration of $1 \mu\text{g/ml}$ actinomycin D (Sigma Chemical Co., St. Louis, MO) and various dilutions of test supernatants obtained from the LNC of infected mice as described above. The plates were then cultured overnight, at which time the degree of cytotoxicity of the supernatants for the L929 cells was determined by the 3(4,5-dimethyl-thiazoyl-2-yl)2,5 diphenyltetrazolium bromide (MTT) colorimetric assay for cell viability (20).

The OD obtained with L929 cells cultured with medium alone (negative control) or with the supernatant of LNC not restimulated with *L. major* in vitro did not differ significantly. Therefore, the percent cytotoxicity effected by supernatants containing TNF activity was calculated using the following formula: percent cytotoxicity = $[1 - (\text{OD TNF-treated}/\text{OD negative control})] \times 100$.

1 U of TNF was defined as the amount of material resulting in 50% cytotoxicity for the

L929 monolayers. Thus, by comparison to a standard curve obtained with dilutions of purified mTNF, the number of units of TNF in each test supernatant could be calculated.

Results

To assess whether there exists a correlation between resistance to experimental murine cutaneous leishmaniasis and the ability of infected animals to produce TNF in response to parasite antigens, C3H (resistant) and BALB/c (susceptible) mice were infected with *L. major*, and at intervals thereafter assessed for the capacity of their LNC to produce TNF in response to parasite challenge. Results in Fig. 1 *A* demonstrate that LNC isolated from C3H mice at various time intervals after infection with *L. major* were able to produce measurable amounts of TNF when restimulated with the parasite in vitro. The highest TNF levels were observed in cultures of LNC obtained from C3H mice infected with *L. major* for 63 d (Fig. 1 *A*), a time when cutaneous lesions were essentially healed in the animals (Fig. 1 *B*). In contrast, BALB/c LNC were not able to produce significant levels of TNF at any time during the course of infection.

The experiments above indicated that the ability of LNC from mice infected with *L. major* to produce TNF in vitro in response to challenge with the parasite correlated with the resistance of the mice to infection with the parasite in vivo. This suggested that host production of TNF might have a protective effect on the course of cutaneous leishmaniasis. We therefore examined the effects of injecting rHuTNF

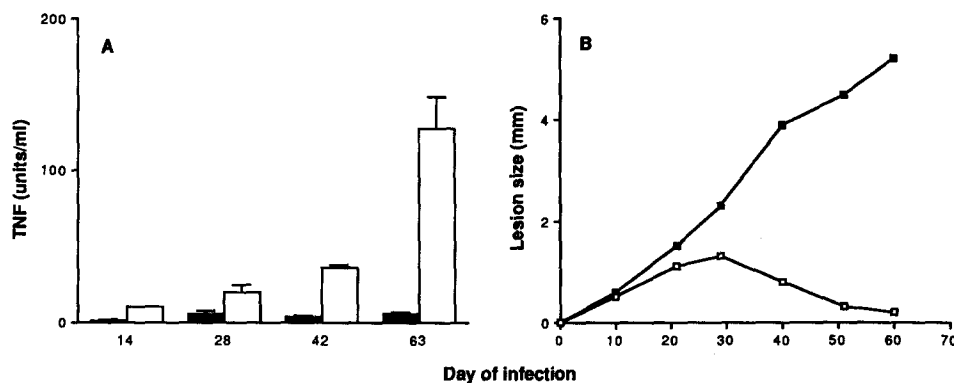


FIGURE 1. TNF production by LNC isolated from C3H and BALB/c mice infected with *L. major*. (A) At the indicated day of infection, LNC were harvested from duplicate C3H (open bars) and BALB/c (shaded bars) mice and stimulated in vitro for 24 h with 10^5 parasites/ml. The concentration of TNF present in the supernatants of the LNC cultures was determined in the L929 cytotoxicity assay (see Materials and Methods). The data presented were obtained in triplicate L929 cultures \pm SD. Similar data were obtained with other doses of parasites and after 48 h of stimulation in vitro. When a culture supernatant displayed cytotoxic activity for L929 cells, the fact that this was due strictly to the presence of TNF in the supernatant was confirmed by including anti-TNF in the culture. In each case, the L929 cytotoxic capacity of a positive supernatant was completely blocked by a final concentration of either 200 or 400 μ g/ml of anti-TNF. (B) Groups of five C3H (\square) and five BALB/c (\blacksquare) mice each were injected subcutaneously in the hind footpad with 5×10^6 *L. major* promastigotes. The figure depicts the mean increase in the thickness of the infected footpad compared with the thickness of the control uninfected footpad (see Materials and Methods for techniques).

or anti-TNF antibodies in both C3H (resistant) and BALB/c (susceptible) mice experimentally infected with *L. major*. Representative results of three experiments with C3H mice are presented in Fig. 2. As can be seen, administering rHuTNF impeded lesion development (Fig. 2 A) while treatment with anti-TNF significantly exacerbated lesion development (Fig. 2 B). Similar results were obtained using BALB/c mice (Fig. 3 A and B). In experiments using anti-TNF, groups of mice were also injected with nrIg, which controlled for any nonspecific effects that might occur due to the injection of mice with rabbit Ig. Results depicted in Fig. 2 B reveal there was no difference in lesion development in nrIg-treated and untreated control mice.

The experiments presented above demonstrated that TNF could impede lesion development in cutaneous leishmaniasis. It therefore became important to determine whether this effect of TNF on lesion size was accompanied by a parallel change in the numbers of *L. major* parasites in the lesion. If parasite multiplication were also inhibited by TNF, this would indicate that TNF was protective for the host not only through control of pathological damage but also by inhibition of parasite replication. Representative results of three experiments are given in Table I. At day 21 of infection, mice treated with rHuTNF had 12-fold fewer parasites in their lesions than did control mice. In contrast, treatment with anti-TNF resulted in a 20-fold increase in the number of parasites in the lesions. In addition, at day 39 of infection, whereas substantial numbers of parasites were still detectable in mice treated with anti-TNF, no parasites were detected in control lesions or in lesions of mice treated with nrIg (data not shown). Similar results were obtained when lymph nodes draining the lesions were examined for their content of *L. major*. For example, the number

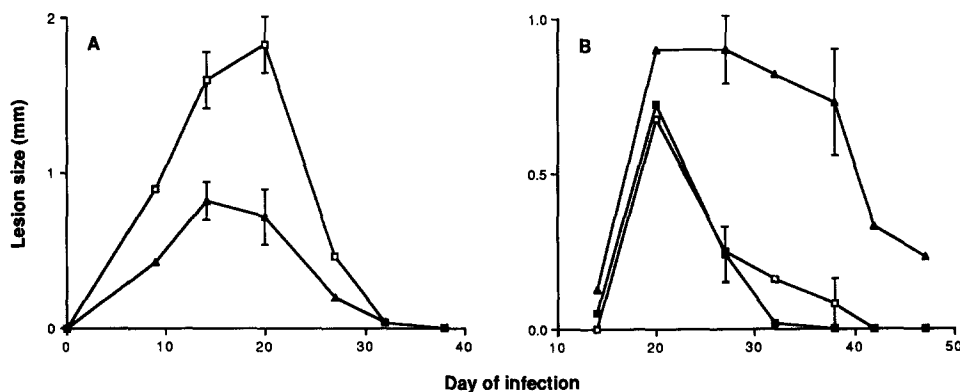


FIGURE 2. Effect of rHuTNF and α TNF on the course of murine cutaneous leishmaniasis in C3H mice. (A) Five C3H mice were injected subcutaneously in one hind footpad with 20×10^6 *L. major* and were treated with rHuTNF (Δ) by intravenous injection of 2 μ g rHuTNF on days 7, 10, and 13 of infection; 3 μ g rHuTNF on days 16, 19, and 22 of infection; and 4 μ g rHuTNF on days 24, 27, and 30 of infection. Control mice (\square) were not treated with rHuTNF. (B) Groups of five C3H mice each were injected subcutaneously in one hind footpad with 0.5×10^6 *L. major* and were treated with anti-TNF (\blacktriangle) or nrIg (\blacksquare) by intraperitoneal injection of 200 μ g of either material every 2 d beginning at day 7 of infection. Control mice (\square) were challenged with *L. major* but were otherwise untreated. Injection of 200 μ g α TNF every 2nd day yielded detectable circulating anti-TNF in the treated mice when assayed after five injections of anti-TNF. The figure depicts the mean increase in the thickness of the infected footpad (\pm SE) compared with the thickness of the control uninfected footpad (see Materials and Methods for techniques).

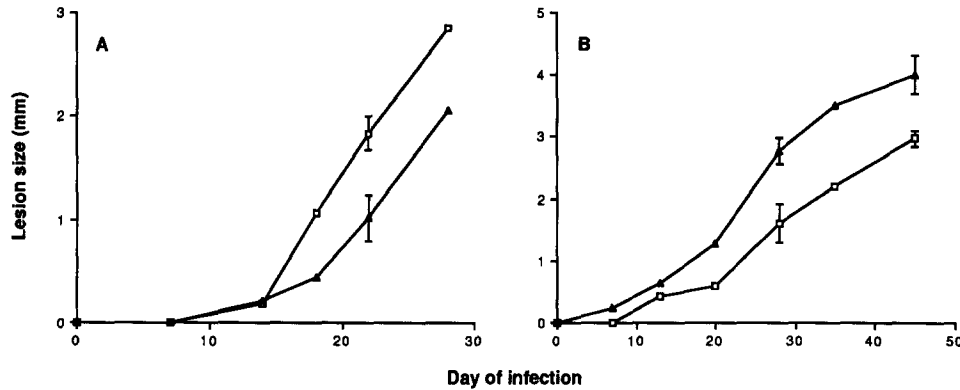


FIGURE 3. Effect of rHuTNF and α TNF on the course of murine cutaneous leishmaniasis in BALB/c mice. (A) BALB/c mice challenged with 2×10^6 *L. major* and treated with rHuTNF (Δ); control mice not treated with rHuTNF (\square). (B) BALB/c mice challenged with 0.5×10^6 *L. major* and treated with anti-TNF (\blacktriangle); control mice not treated with anti-TNF (\square). Techniques were as described in the legend of Fig. 2.

TABLE I
Number of *Leishmania major* in the Lesions and Lymph Nodes
of Mice Treated with Anti-TNF Antibody or With TNF

Challenge	Treatment	Number of <i>L. major</i> ($\times 10^{-5}$) in lesion \pm SE	Number of <i>L. major</i> ($\times 10^{-2}$) in LNC \pm SE
0.5×10^6 <i>L. major</i>	None	10.2 ± 3.6	9.2 ± 2.4
0.5×10^6 <i>L. major</i>	nrIg	11.8 ± 5.0	2.3 ± 0.6
0.5×10^6 <i>L. major</i>	Anti-TNF	190.9 ± 43.0	76.5 ± 25.8
20×10^6 <i>L. major</i>	None	1212.0 ± 273.7	10.5 ± 3.3
20×10^6 <i>L. major</i>	rHuTNF	102.6 ± 28.7	9.2 ± 2.4

Groups of C3H mice were treated with anti-TNF, nrIg, or rHuTNF as described in the legend of Fig. 2. At day 21 of infection, duplicate mice were assessed for the number of *L. major* present in the lesions and draining lymph nodes as described in Materials and Methods.

of parasites in the lymph nodes of mice treated with anti-TNF was 35-fold higher than the number present in mice treated with nrIg (Table I).

Discussion

The role of TNF in immunity in experimental murine cutaneous leishmaniasis was investigated. We found that whereas LNC from mice genetically susceptible to infection with *L. major* (BALB/c) produced little TNF in response to the parasite, LNC from infected C3H mice (genetically resistant) produced ever increasing quantities of TNF through the course of the disease (Fig. 1 A). It is not known why resistant mice produced the greatest amounts of TNF late in the course of infection (i.e., 63 d of infection; Fig. 1 A); however, several explanations are possible. Early in the course of infection of resistant mice with *L. major*, LNC draining the lesion contain large numbers of parasites, whereas at later timepoints few parasites can be found

(14). It is possible therefore that at earlier timepoints TNF is consumed by the cultures, whereas as the parasites are cleared from the LNC, excess amounts of TNF are produced. Alternatively, since it is not known what cell is producing the TNF in the LNC cultures (i.e., macrophages or T cells), it is possible that parasite-specific T cells are the major source of the TNF. The frequency of parasite-specific T cells in lymph nodes draining the lesion increases with the duration of infection (21). Therefore, it is possible that the ability of the LNC to produce TNF increases as the frequency of parasite-specific T cells increases.

Since the ability of the host to produce TNF in response to infection with *L. major* correlated with resistance of the host to infection, we also investigated the effect of injecting TNF or a neutralizing anti-TNF antibody into mice infected with *L. major*. Treatment with TNF was beneficial for the host and anti-TNF treatment was detrimental for the host in both C3H (resistant) and BALB/c (susceptible) mice infected with the parasite, although the effect was more pronounced in resistant mice (compare Figs. 2 and 3). In addition, the beneficial/detrimental effect of TNF or anti-TNF on cutaneous lesions of *L. major* was accompanied by a decrease or increase, respectively, in the number of parasites present in the lesions. Taken together, these results suggest that in the *L. major*-infected host, TNF can be protective for the host through its capacity to inhibit parasite multiplication, and thus reduce damage to the host resulting from the pathological changes associated with the disease.

The mechanism by which TNF exerts its protective effect on the course of cutaneous leishmaniasis is currently under investigation. To determine whether TNF has a direct cytotoxic effect on *L. major*, we have cultured *L. major* promastigotes with as much as 1,000 U/ml of TNF for as long as 10 d. TNF did not alter the multiplication rate of the parasites under any condition. Since the macrophage is the mammalian host cell for *L. major* (22) and since TNF can exist as a transmembrane protein in macrophages (23), we have also investigated whether the detrimental effect of injecting anti-TNF into mice infected with *L. major* might be due to lysis of infected macrophages via complement-mediated cytotoxicity. Lysis of infected macrophages could have resulted in rapid dissemination of *L. major* to uninfected macrophages and might also have inhibited presentation of parasite antigens by macrophages thus inhibiting the development of specific T cell immunity. To determine whether anti-TNF could affect macrophages, we injected mice intraperitoneally every second day for a total of 10 d with either anti-TNF or nrIg and the resident peritoneal cells or spleens were harvested from both groups of mice. The cell yields from both groups of mice were identical and there were >95% esterase-positive cells (24) in both peritoneal cell populations, suggesting that injection of anti-TNF had not depleted macrophages in the animals. In addition, there was no difference in the ability of spleen cells from either group of mice to act as antigen-presenting cells in vitro for an *L. major*-specific T cell line. Taken together, these results suggest that the beneficial effect of TNF on cutaneous leishmaniasis is due to its ability to activate infected macrophages to destroy *L. major* or its ability to interact with other cells and lymphokines of the immune system resulting in enhanced resistance to *Leishmania*. In fact, preliminary experiments are revealing that TNF is able to inhibit parasite replication in macrophages in vitro, which suggests that the beneficial effect of TNF on cutaneous leishmaniasis may be mediated through its ability to activate macrophages. These results are similar to those recently reported by Wirth and Kierszenbaum (10) in

which TNF was found to activate macrophages to destroy *Trypanosoma cruzi*. Thus, in addition to IFN- γ , TNF may activate macrophages to destroy many intracellular pathogens.

Summary

The ability of mice to resist infection with *L. major* correlated directly with the capacity of their LNC to produce TNF in response to in vitro parasite challenge. Blocking TNF in vivo by passively administering anti-TNF antibodies exacerbated the course of *L. major* infection, resulting in substantially larger cutaneous lesions and elevated numbers of parasites within those lesions. In addition, treatment of infected mice with exogenous rHuTNF afforded host protection as evidenced by smaller lesion size and decreased parasite counts. Taken together, these results suggest a central role for TNF in resistance to *L. major*.

The excellent technical assistance of Ms. Mary McGurn is gratefully acknowledged.

Received for publication 21 June 1989 and in revised form 16 August 1989.

References

1. Beutler, B., and A. Cerami. 1988. Tumor necrosis, cachexia, shock and inflammation: a common mediator. *Annu. Rev. Biochem.* 57:505.
2. Havell, E. A. 1987. Production of tumor necrosis factor during murine listeriosis. *J. Immunol.* 139:4225.
3. Kindler, V., A.-P. Sappino, G. Grau, P.-F. Piguet, and P. Vassalli. 1989. The inducing role of tumor necrosis factor in the development of bacterial granulomas during BCG infection. *Cell.* 56:731.
4. Mestan, J., W. Digel, S. Mittnacht, H. Hillen, D. Blohm, A. Moller, H. Jacobsen, and H. Kirchner. 1986. Antiviral effect of recombinant tumour necrosis factor in vitro. *Nature (Lond.)* 323:816.
5. Wong, G. H. W., and D. V. Goeddel. 1986. Tumour necrosis factors α and β inhibit virus replication and synergize with interferons. *Nature (Lond.)* 323:819.
6. Koff, W. C., and A. V. Fann. 1986. Human tumor necrosis factor-alpha kills herpesvirus-infected but not normal cells. *Lymphokine Res.* 5:215.
7. Silberstein, D. S., and J. R. David. 1986. Tumor necrosis factor enhances eosinophil toxicity to *Schistosoma mansoni* larvae. *Proc. Natl. Acad. Sci. USA.* 83:1055.
8. Damonville, M., J. Wietzerbin, V. Pancre, M. Joseph, A. Delanoye, A. Capron, and C. Auriault. 1988. Recombinant tumor necrosis factors mediate platelet cytotoxicity to *Schistosoma mansoni* larvae. *J. Immunol.* 140:3962.
9. Djeu, J. Y., D. K. Blanchard, D. Halkias, and H. Friedman. 1986. Growth inhibition of *Candida albicans* by human polymorphonuclear neutrophils: Activation by interferon- γ and tumor necrosis factor. *J. Immunol.* 137:2980.
10. Wirth, J. J., and F. Kierszenbaum. 1988. Recombinant tumor necrosis factor enhances macrophage destruction of *Trypanosoma cruzi* in the presence of bacterial endotoxin. *J. Immunol.* 141:286.
11. Grau, G., L. F. Fajardo, P.-F. Piguet, B. Allet, P.-H. Lambert, and P. Vassalli. 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. *Science (Wash. DC).* 237:1210.
12. Titus, R. G., R. Ceredig, J.-C. Cerottini, and J. A. Louis. 1985. Therapeutic effect of anti-L3T4 monoclonal antibody GK1.5 on cutaneous leishmaniasis in genetically-

- susceptible BALB/c mice. *J. Immunol.* 135:2108.
13. Sacks, D. L., and P. V. Perkins. 1984. Identification of an infective stage of *Leishmania* promastigotes. *Science (Wash. DC)*. 223:1417.
 14. Titus, R. G., M. Marchand, T. Boon, and J. A. Louis. 1985. A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. *Parasite Immunol.* 7:545.
 15. Maryanski, J. L., J. van Snick, J.-C. Cerottini, and T. Boon. 1982. Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815 III. Clonal analysis of the syngeneic cytolytic lymphocyte response. *Eur. J. Immunol.* 12:401.
 16. Wolpe, S. D., G. Davatelis, B. Sherry, B. Beutler, D. G. Hesse, G. T. Nguyen, L. L. Moldawer, C. F. Nathan, S. F. Lowry, and A. Cerami. 1987. Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. *J. Exp. Med.* 167:570.
 17. Sherry, B., J. Gelin, Y. Fong, M. Marano, H. Wei, A. Cerami, S. F. Lowry, K. G. Lundholm, and L. L. Moldawer. 1989. Anticachectin/tumor necrosis factor- α antibodies attenuate development of cachexia in tumor models. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:1956.
 18. Beutler, B. A., I. W. Milsark, and A. Cerami. 1985. Passive immunization against cachectin/tumor necrosis factor (TNF) protects mice from the lethal effects of endotoxin. *Science (Wash. DC)*. 229:869.
 19. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248.
 20. Green, L. M., J. L. Reade, and C. F. Ware. 1984. Rapid colorimetric assay for cell viability: Application to the quantitation of cytotoxic and growth inhibitory lymphokines. *J. Immunol. Methods.* 70:257.
 21. Milon, G., R. G. Titus, J.-C. Cerottini, G. Marchal, and J. A. Louis. 1986. Higher frequency of *Leishmania major*-specific L3T4⁺ T cells in susceptible BALB/c as compared with resistant CBA mice. *J. Immunol.* 136:1467.
 22. Howard, J. G. 1986. Immunological regulation and control of experimental leishmaniasis. *Int. Rev. Exp. Pathol.* 28:79.
 23. Kriegler, M., C. Perez, K. DeFay, I. Albert, and S. D. Lu. 1988. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. *Cell.* 53:45.
 24. Kaplow, L. S. 1981. Cytochemical identification of mononuclear macrophages. In *The Manual of Macrophage Methodology*. H. B. Herscovitz, H. T. Holden, J. A. Bellanti, and A. Ghaffar, editors. Marcel Dekker, Inc., New York. 199.