and pharyngeal endoderm (on other side of dashed line) were removed from the inner surface of the chordamesoderm. Eggs were fertilized, dejellied, cultured as in (*34*), and staged according to (*35*). Adult frogs were treated in accordance with University of California guidelines.
12. R. E. Keller, *Dev. Biol.* **42**, 222 (1975); *ibid.* **51**, 118 (1976).
13. P. Wilson and R. Keller, *Development* **112**, 289 (1991).
14. M. Jacobson and U. Rutishauser, *Dev. Biol.* **116**, 524 (1986); K. Balak, M. Jacobson, J. Sunshine, U. Rutishauser, *ibid.* **119**, 540 (1987).
15. C. R. Kintner and J. P. Brockes, *Nature* **308**, 67 (1984).
16. Embryos and explants were immunostained with the use of horseradish peroxidase–tagged secondary antibodies (Bio-Rad) as in (*17*) but with shortened washes (30 min). $NiCl_2$ (0.05%) was added to enhance diaminobenzidine (DAB) staining (Polysciences), except when detecting N-CAM and 12-101. Whole mount in situ hybridization was performed as in (*36*).
17. A. Hemmati-Brivanlou and R. M. Harland, *Development* **106**, 611 (1989).
18. G. Oliver, C. V. E. Wright, J. Hardwicke, E. M. DeRobertis, *EMBO J.* **7**, 3199 (1988).
19. C. V. E. Wright, E. A. Morita, D. J. Wilkin, E. M. DeRobertis, *Development* **109**, 225 (1990).
20. D. G. Wilkinson, S. Bhatt, P. Chavrier, R. Bravo, P. Charnay, *Nature* **337**, 461 (1989); N. Papalopulu *et al.*, *Development* **113**, 1145 (1991); M. E. Bolce, A. Hemmati-Brivanlou, P. D. Kushner, R. M. Harland, *ibid.*, in press.
21. Stage 10+ head mesoderm (Fig. 1A) was wrapped with stage 10+ animal cap ectoderm, cultured in MDM until stage 26, and immunostained with *en-2* (4D9) antibodies as in (*16*). 0 of 22 recombinates expressed *en-2*.
22. A. Hemmati-Brivanlou, J. R. de la Torre, C. Holt, R. M. Harland, *Development* **111**, 715 (1991).
23. Histological sections (not shown) show that *en-2*–positive cells of the mesodermal and endodermal portion of explants are internal, and therefore probably mesodermal, because endoderm forms superficial endothelium (*12*). The *en-2*–positive cells of the mandibular arch of intact embryos (*22*) may therefore originate from mesodermal tissue, as in zebrafish (*Brachydanio rerio*) [K. Hatta, T. F. Schilling, R. A. BreMiller, C. B. Kimmel, *Science* **250**, 802 (1990)], rather than from neural crest (*22*).
24. E. M. DeRobertis *et al.*, *Cell* **57**, 189 (1989); M. A. Frohman *et al.*, *Development* **110**, 589 (1990).
25. Rectangles of stage 10+ dorsal ectoderm, about 90° wide, were cut at approximately the border of ectoderm and mesoderm (*12*) and the animal pole. They were cultured in MDM until stage 26 and immunostained (*16*). Because *XlHbox 6* is expressed in posterior mesoderm, it was not possible to use this marker for this test.
26. R. L. Gimlich and J. Braun, *Dev. Biol.* **109**, 509 (1985).
27. We labeled mesoderm donors by injecting 8 nl of FDA (25 mg/ml) into stage 1 embryos. At stage 10+, the presumptive posterior edge of a rectangle of FDA-labeled dorsal mesoderm was grafted onto the presumptive posterior edge of a rectangle of ventral ectoderm (about 60 to 90° wide) from unlabeled stage 10 embryos. Mesoderm was eliminated from the ventral ectoderm by cutting above the thickened region of the marginal zone. After healing for 5 min, recombinates were covered with a piece of cover slip suspended on vacuum grease, cultured in MDM until controls reached stage 22 to 26, and immunostained as in (*16*).
28. Results not shown for *XlHbox 6* recombinates.
29. P. D. Nieuwkoop, *J. Exp. Zool.* **120**, 1 (1952).
30. L. Wolpert, *Dev. Suppl.* **107**, 3 (1989).
31. M. Servetnick and R. M. Grainger, *Dev. Biol.* **147**, 73 (1991).
32. C. London, R. Akers, C. R. Phillips, *ibid.* **129**, 380 (1988); S. Sokol and D. A. Melton, *Nature* **351**, 409 (1991); C. R. Sharpe, A. Fritz, E. M. DeRobertis, J. B. Gurdon, *Cell* **50**, 749 (1987).
33. J. Holtfreter and V. Hamburger, in *Analysis of Development*, B. H. Willier, P. A. Weiss, V. Hamburger, Eds. (Hafner, New York, 1955), pp. 230–297.
34. J.-P. Vincent, G. F. Oster, J. C. Gerhart, *Dev. Biol.* **113**, 484 (1986).
35. P. D. Nieuwkoop and J. Faber, *Normal Table of Xenopus laevis (Daudin)* (North-Holland, Amsterdam, 1967).
36. R. M. Harland, in *Methods in Cell Biology*, B. K. Kay and H. B. Peng, Eds. (Academic Press, San Diego, 1991), vol. 36, pp. 685–695.
37. We thank R. Keller, A. Sater, and J. Shih for discussions and explant lessons; S. Roberts, J. Yost, C. Kenyon, and R. Durbin for comments on the manuscript; A. Hemmati-Brivanlou for teaching immunostaining; R. Harland for in situ hybridization; N. Patel, C. Goodman, C. Wright, E. DeRobertis, and U. Rutishauser for antibodies; D. Wilkinson for the *Krox-20* clone; and R. Gimlich for FDA. Supported by a Jane Coffin Childs fellowship and an NIH (National Institutes of Health) postdoctoral training grant to T.D., National Aeronautics and Space Administration grant NAGW-1719 to C.R.P., and NIH grant GM19363 to J.C.G.

28 January 1992; accepted 7 May 1992

Transforming Growth Factor–β in Leishmanial Infection: A Parasite Escape Mechanism

Manoel Barral-Netto, Aldina Barral, Cari E. Brownell, Yasir A. W. Skeiky, Larry R. Ellingsworth, Daniel R. Twardzik, Steven G. Reed*

The course of infection with the protozoan parasite *Leishmania* is determined in part by their early replication in macrophages, the exclusive host cells for these organisms. Although factors contributing to the survival of *Leishmania* are not well understood, cytokines influence the course of infection. Transforming growth factor–β (TGF-β) is a multipotential cytokine with diverse effects on cells of the immune system, including down-regulation of certain macrophage functions. Leishmanial infection induced the production of active TGF-β, both in vitro and in vivo. TGF-β was important for determining in vivo susceptibility to experimental leishmanial infection.

Parasites of the genus *Leishmania* are responsible for millions of infections in humans, with cutaneous, mucosal, and visceral manifestations. The severity of disease produced by the diverse species that infect humans varies widely, ranging from cutaneous or mucosal involvement that can be self-healing to destructive, to visceral infections that can be mild to fatal. The host or parasite properties responsible for these clinical variations are not known. In experimental models, inbred mice display distinct patterns of susceptibility and resistance to leishmanial infection. For example, *L. major* and *L. amazonensis* can cause self-healing cutaneous infections in C57BL/6 mice but disseminating, fatal infections in BALB/c mice. Another species, *L. braziliensis*, causes cutaneous and mucosal infections in humans but does not cause disease in mice. Host responses that determine whether infections will be healing or nonhealing are not understood, but selective patterns of cytokine production have been associated with murine infections. Thus, infection with *L. major* in susceptible, nonhealing BALB/c mice has a predominantly helper T cell type 2 (T_H2) response, with the production of interleukin-4 (IL-4) and IL-10, whereas infection in resistant C57BL/6 mice results in a predominantly T_H1-type response, with the production of interferon-γ (IFN-γ) and IL-2 (*1–3*). IFN-γ is the cytokine most associated with resistance to leishmanial infection, whereas IL-4 has been linked to a susceptibility to the disease (*1–4*).

Transforming growth factor–β (TGF-β) is a 24-kD protein produced by many cells, including B and T lymphocytes and activated macrophages (*5–7*). It is usually secreted as a latent precursor that requires enzymatic cleavage of carbohydrate groups or transient acidification to release the active cytokine. Among the inhibitory effects of TGF-β on the immune system are decreases in IL-2 receptor induction (*7*) and IFN-γ–induced class II expression (*8*), as well as decreases in IL-1–induced thymocyte proliferation (*9*), B cell differentiation and proliferation (*5*), and cytotoxic and lymphokine-activated killer cell production (*10*). TGF-β also blocks IFN-γ–induced macrophage activation, which diminishes their oxidative responses (*11, 12*). Treatment of macrophages with TGF-β reduces their ability to

M. Barral-Netto and A. Barral, Federal University of Bahia School of Medicine, 40.140 Salvador, Bahia, Brazil, and Seattle Biomedical Research Institute, Seattle, WA 98109.
C. E. Brownell and Y. A. W. Skeiky, Seattle Biomedical Research Institute, Seattle, WA 98109.
L. R. Ellingsworth, Celtrix Laboratories Inc., Palo Alto, CA 94303.
D. R. Twardzik, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA 98121.
S. G. Reed, Seattle Biomedical Research Institute, Seattle, WA 98109, and Cornell University Medical College, New York, NY 10021.

*To whom correspondence should be addressed.

become activated to inhibit the intracellular replication of the protozoan parasite *Trypanosoma cruzi* (*13*). Because of the powerful effects of TGF-β on macrophages, we investigated whether this cytokine was important in the replication of *Leishmania* within these host cells.

We examined TGF-β production during leishmanial infection as a potential way to allow the parasites to survive and proliferate in macrophages. With the use of a sensitive bioassay, it was shown that in vitro infection of normal mouse peritoneal macrophages with *L. amazonensis* (*14*) led to the production of biologically active TGF-β 72 hours after infection (Fig. 1). Although latent TGF-β may be produced by uninfected macrophages in culture, the finding of increased active TGF-β in these supernatants may be relevant for establishing leishmanial infection. We then examined lesions that had developed after leishmanial infection by immunohistochemistry with a monoclonal antibody (MAb) specific for TGF-β (*15*) (Fig. 2). There was local TGF-β production in mouse footpads after leishmanial infection, but no TGF-β was detected in uninfected footpads. Thus, increased TGF-β production was associated with leishmanial infection both in vitro and in vivo.

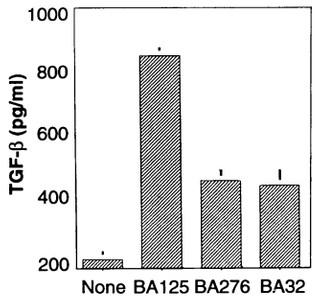

Fig. 1. In vitro *Leishmania*-induced TGF-β production. Active TGF-β was produced by murine macrophages after infection with *L. amazonensis* (*14*). Data represent mean ± SEM (five mice per group) of peak response (obtained 72 hours after infection) of three different isolates of *L. amazonensis*, compared to uninfected cells (labeled "None"). The specificity of the bioassay was confirmed by inhibition with monoclonal anti–TGF-β.

To determine whether TGF-β could alter the course of leishmanial infection, resistant C57BL/6 mice were infected with *L. amazonensis* (*16*) and treated with recombinant TGF-β (Fig. 3A). Whereas in saline-treated mice the infections healed as expected, TGF-β–treated mice developed large, nonhealing lesions. Thus, TGF-β reversed the genetic resistance to leishmaniasis of C57BL/6 mice. Another parasite, *L. braziliensis*, did not produce lesions in BALB/c mice, which are susceptible to infection with other species of *Leishmania*, but TGF-β treatment allowed the replication of *L. braziliensis* and the production of lesions (Fig. 3B). Whereas lesions from saline-treated animals had a predominantly lymphocytic infiltrate (Fig. 4, A and C), lesions from TGF-β–treated animals had many heavily vacuolated and parasitized macrophages among the lymphocytes (Fig. 4, B and D). Thus, local administration of TGF-β led to the production of acute disease in two different models of resistance.

To determine whether endogenously produced TGF-β influenced leishmanial infection in vivo, we treated BALB/c mice with a neutralizing anti–TGF-β antibody during the course of infection with *L. amazonensis*. Similarly, control mice were injected with control antibody (Fig. 5A). In vivo treatment with anti–TGF-β arrested the development of lesions within 5 weeks after infection, which was maintained thereafter. When draining lymph nodes were examined for cytokine production by the polymerase chain reaction (PCR), it was found that control-treated, infected mice had no detectable IFN-γ mRNA but had increased levels of IL-4 mRNA (Fig. 5B). In contrast, healing, anti–TGF-β–treated mice had no increase in IL-4 mRNA but had increased IFN-γ mRNA. Thus, endogenous TGF-β production was directly associated with susceptibility to leishmanial infection and with the development of a nonhealing T_H2 cytokine response that was reversed to a healing T_H1 response after anti–TGF-β treatment.

In the present study, the induction of TGF-β by host macrophages after leishmanial infection was demonstrated both in vitro and in vivo. The in vitro–induced TGF-β was biologically active without further modification. In addition, exogenously administered TGF-β altered the course of parasite infection: genetically resistant mice became susceptible to infection after TGF-β treatment, and avirulent *Leishmania* produced disease. The in vivo dependence of disease on TGF-β was confirmed with the use of neutralizing MAb, which halted the progression of disease in genetically susceptible mice. Inhibition of TGF-β in vivo led to a healing T_H1 cytokine response pattern (*1*, *2*). Thus, inducing TGF-β production may be an important mechanism by which *Leishmania* avoid destruction by host macrophages. No other known cytokine directly influences leishmanial infection in vivo to the extent that TGF-β does. For

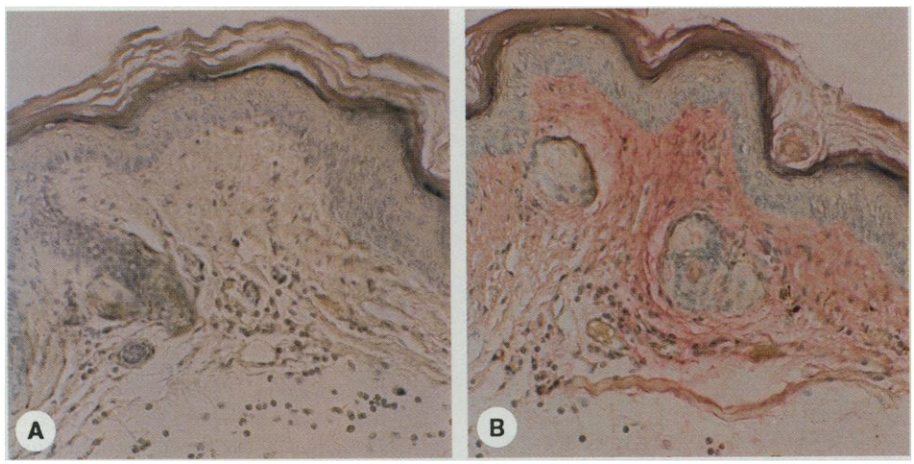

Fig. 2. Local production of TGF-β in the footpads of BALB/c mice after infection with *L. amazonensis* (MHOM/BR/88/125). Footpads from BALB/c mice were obtained (**A**) before infection or (**B**) 24 hours after infection with *L. amazonensis* and processed for immunohistochemical localization of TGF-β (*15*). Infected footpads show a positive (red) reaction diffusely in the subcutaneous tissue, whereas no demonstrable reaction is observed in the uninfected footpad. Similarly, no reactivity was observed in sections from infected footpads reacted with immunoglobulin G1 myeloma protein instead of 1D11.16 MAb (not shown). Original magnification, ×100.

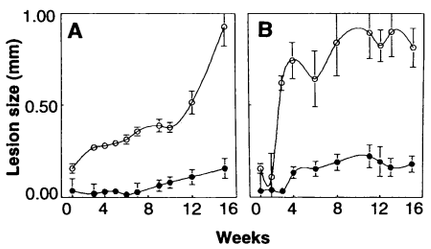

Fig. 3. In vivo effect of TGF-β on the course of cutaneous leishmaniasis. (**A**) C57BL/6 mice (five per group) were infected with *L. amazonensis* (*16*). Recombinant TGF-β (200 ng per mouse) was injected subcutaneously 24 hours and at 6, 7, 8, and 9 weeks after infection (open circles). Similarly, control animals were injected with saline (closed circles). (**B**) BALB/c mice were infected with *L. braziliensis* (*16*), and recombinant TGF-β was injected into the infection site at 24 hours and at 1, 2, 3, 5, 12, and 13 weeks after infection (open circles). Saline-treated controls are shown with closed circles.

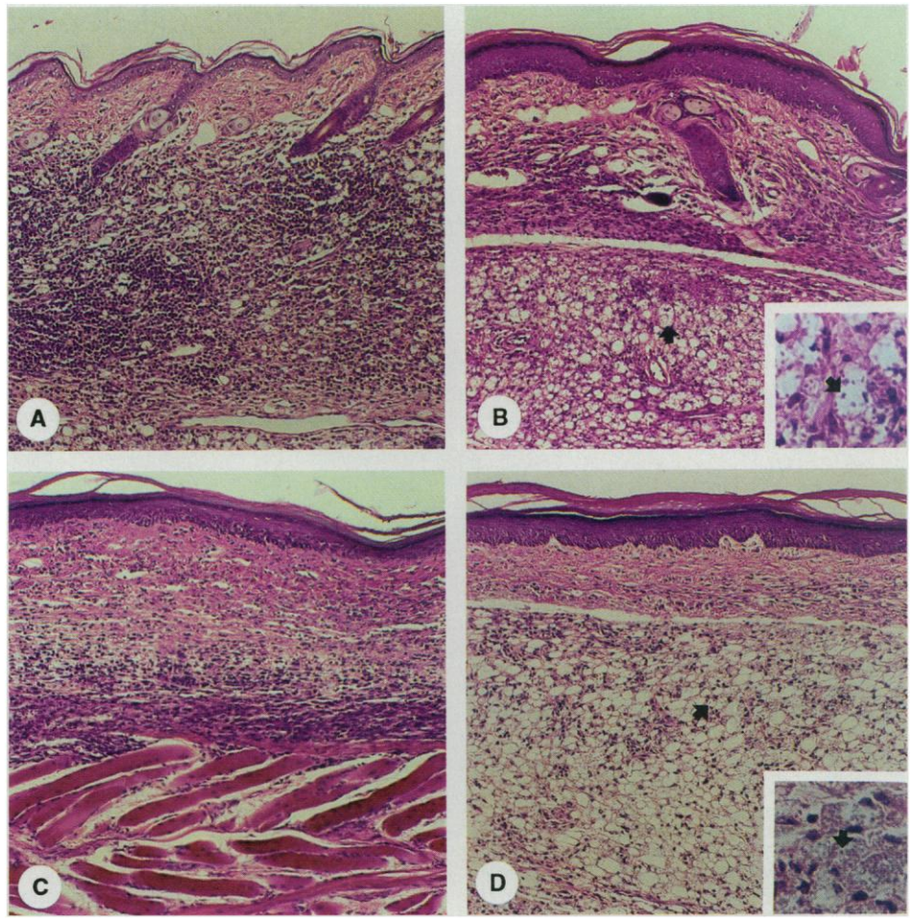

Fig. 4. Effect of TGF-β on the histological picture of *Leishmania*-infected mice. (**A**) Lesions of saline-treated C57BL/6 mice infected with *L. amazonensis* were discrete, with a predominant lymphocytic pattern of few macrophages. (**C**) Lesions of BALB/c animals infected with *L. braziliensis* were even smaller and were composed almost exclusively of lymphocytes. (**B** and **D**) Treatment of infected animals with TGF-β led to lesions exhibiting a large number of vacuolated and parasitized macrophages intermingled with lymphocytic infiltrate [(B), *L. amazonensis*, and (D), *L. braziliensis*]. Original magnification, ×100. Inserts in (B) and (D) are the corresponding sections at original magnifications of ×400. Arrows indicate leishmanial amastigotes.

example, anti–IL-4 treatment increased resistance to *L. major* in mice, but recombinant IL-4 did not alter infection (*17*). Similarly, anti–IFN-γ treatment increased susceptibility in mice, but IFN-γ alone was unable to cure infection. Thus, TGF-β may act to induce exacerbating or macrophage-inactivating cytokines such as IL-4 or IL-10; alternatively, production of these cytokines may result from increased intracellular infection, promoted by TGF-β.

These findings suggest that TGF-β may be involved in early control (or lack thereof) of infection with an obligate intracellular parasite. In related studies, undefined components from sandfly saliva have also been reported to promote infection by *L. major* and *L. braziliensis* (*18*). Such products may have an action similar to that of TGF-β or may act by inducing TGF-β production. Early events may be crucial for determining which response pattern—T_H1 or T_H2—will develop and the ultimate outcome of infection. IFN-γ, associated with T_H1, has been considered to be the cytokine most important in control of leishmanial infection (*4*). It has been used clinically, in conjunction with chemotherapy, to treat both visceral (*19*) and cutaneous (*20*) leishmaniases. However, studies with both experimental *T. cruzi* and leishmanial infections indicate that TGF-β may block the inhibitory effects of IFN-γ (*13*, *21*). The appearance of IFN-γ mRNA in the draining lymph node of anti–TGF-β–treated mice suggests that the healing response could be due to a combination of TGF-β inhibition and increased IFN-γ production. The present study raises the possibility that, in addition to designing therapeutic strategies aimed at macrophage activation, complementary approaches directed toward neutralization of "inactivating" cytokines may be useful in and applicable to a variety of infectious diseases.

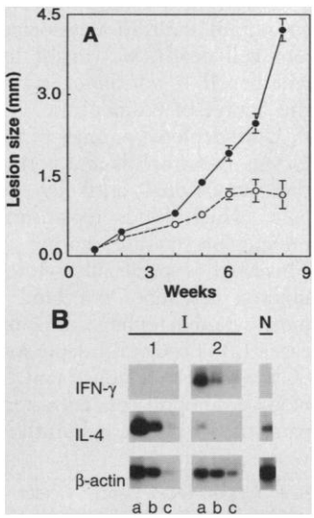

Fig. 5. Protective effect of MAb to TGF-β on the course of cutaneous leishmaniasis. (**A**) BALB/c mice (five per group) were infected with *L. amazonensis* BA-125. During the first 3 weeks after infection mice were treated three times a week with anti–TGF-β MAb (80 μg per dose; open circles) or control antibody (closed circles). Both antibodies were injected subcutaneously at the infection site in a volume of 30 μl. (**B**) Cytokine mRNA production in draining popliteal lymph nodes of infected mice (I) treated with control (lane 1) or anti–TGF-β (lane 2) MAb. Mice were infected and treated as in (A), and lymph nodes were removed 7 weeks after infection. Messenger RNA was isolated from lymph node cells pooled from three mice per group (10^7 cells per mouse) as described (*23*). Complementary DNA was synthesized from 1 μg of total RNA using poly(dT) (Pharmacia) and AMV reverse transcriptase (Bethesda Research Laboratories) in a reaction volume of 20 μl. Complementary DNA samples were brought to 200 μl with water. Five microliters of diluted cDNA were amplified by PCR with Taq polymerase (Perkin-Elmer Cetus), with the use of 0.2 μM of the respective 5′ and 3′ external primers (*22*) in a reaction volume of 50 μl. Samples were amplified for 25 cycles by denaturation at 96°C for 1 min, annealing was at 55°C for 30 s, and extension was at 72°C for 1 min. Semi-quantitative PCR was illustrated with the use of serial dilutions of the cDNA: 1:10 (lanes a), 1:50 (lanes b), and 1:500 (lanes c). Lane N, normal mouse lymph node cDNA (1:10 dilution). PCR products were analyzed by electrophoresis on 1.5% agarose gels, transferred to a nylon membrane, and probed with ^{32}P-labeled internal oligonucleotides (*22*).

REFERENCES AND NOTES

1. P. Scott *et al.*, *J. Exp. Med.* **168**, 1675 (1988).
2. F. P. Heinzel *et al.*, *ibid.* **169**, 59 (1989).
3. F. P. Heinzel *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7011 (1991).
4. C. F. Nathan *et al.*, *J. Exp. Med.* **158**, 670 (1983); M. Belosevic *et al.*, *J. Immunol.* **143**, 266 (1989).
5. J. H. Kehrl *et al.*, *J. Immunol.* **137**, 3855 (1986).
6. R. K. Assoian *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6020 (1987).
7. J. H. Kehrl *et al.*, *J. Exp. Med.* **163**, 1037 (1986).

8. C. W. Czarniecki *et al.*, *J. Immunol.* **140**, 4217 (1988). 9. S. M. Wahl *et al.*, *ibid.*, p. 3026.
10. J. J. Mule, S. L. Schwartz, A. B. Roberts, M. B. Sporn, S. A. Rosenberg, *Cancer Immunol. Immunother.* **26**, 95 (1988).
11. S. Tsunawaki, M. Sporn, A. Ding, C. Nathan, *Nature* **334**, 260 (1988).
12. A. Ding *et al.*, *J. Immunol.* **145**, 940 (1990).
13. J. S. Silva, D. R. Twardzik, S. G. Reed, *J. Exp. Med.* **174**, 539 (1991).
14. Peritoneal macrophages from normal BALB/c mice were obtained after thioglycolate stimulation and plated at a concentration of 1×10^6 per well in 24-well plates. After nonadherent cells were washed, they were infected with 3×10^6 stationary-phase *L. amazonensis* (isolates MHOM/BR/85/BA-32, MHOM/BR/87/BA-125, or MHOM/BR/89/BA-276) promastigotes per well for 3 hours. Extracellular parasites were removed and the cells were cultured for another 72 hours in a humid atmosphere of 5% CO_2 at 35°C. TGF-β was assayed with the use of the CCL64 cell line (*13*).
15. Footpads were fixed in buffered formaldehyde and embedded in paraffin. Sections were treated with hyaluronidase (1 mg/ml; Sigma) for 30 min at room temperature, blocked with 10% normal goat serum in phosphate-buffered saline (PBS) for 30 min, and incubated with biotinylated MAb 1D11.16 (150 μg/ml) or mouse immunoglobulin G overnight at 4°C. After incubation with avidin–alkaline phosphatase (Biogenex, San Ramon, CA; 1:10; 20 min), the substrate [Fast-Red (1 mg/ml; Sigma) in tris-HCl buffer (pH 8.2) with 0.02% naphtol AS-MX phosphate (Sigma)] was applied for 20 min. Slides were counterstained with Mayer's hematoxylin.
16. BALB/c or C57BL/6 mice (five per group) were infected subcutaneously in the left hind footpad with 5×10^6 stationary-phase promastigotes of *L. amazonensis* (BA-125) or *L. braziliensis* (MHOM/BR/87/BA-92). The course of infection was followed by serial measurements of both footpads with a dial gauge caliper (Starret, Athol, MA) and was expressed in millimeters (mean ± SEM) as the difference between the infected and the contralateral footpad. For histological studies, footpads were harvested 5 weeks after infection, fixed in buffered formalin, and processed for hematoxylin-eosin stain.
17. M. D. Sadick, N. Street, T. R. Mosmann, R. M. Locksley, *Infect. Immun.* **59**, 4710 (1991).
18. R. G. Titus and J. M. C. Ribeiro, *Science* **239**, 1306 (1988); J. Samuelson, E. Lerner, R. Tesh, R. Titus, *J. Exp. Med.* **173**, 49 (1991).
19. R. Badaro *et al.*, *N. Engl. J. Med.* **322**, 16 (1990).
20. G. Harms *et al.*, *Lancet* i, 1287 (1989).
21. B. J. Nelson, P. Ralph, S. J. Green, C. A. Nacy, *J. Immunol.* **146**, 1849 (1991).
22. The external primers used were: IFN-γ 5′, CTG-GCT-GTT-ACT-GCC-ACG-GCA-CAG-TC; IFN-γ 3′, TCG-GAT-GAG-CTC-ATT-GAA-TGC-TTG-GCG-CT; IL-4 5′, TCT-CTC-GTC-ACT-GAC-GGC-ACA-GAG-CT; IL-4 3′, TCC-ATT-TGC-ATG-ATG-CTC-TTT-AGG-CT; actin 5′, GAC-TTC-GAG-CAG-GAG-ATG-GCC-AC; actin 3′, CTC-CTG-CTT-GCT-GAT-CCA-CAT-C. Labeled probes were prepared by ^{32}P-labeled PCR with the use of the following internal primers: IFN-γ 5′, TAG-ATG-TGG-AAG-AAA-AGA-GTC-TCT-TCT; IFN-γ 3′, TCT-GAG-GTA-GAA-AGA-GAT-AAT-CTG-GCT; IL-4 5′, TCA-TGG-AGC-TGC-AGA-GAC-TCT-TTC-GGG-CT; IL-4 3′, TCC-AGG-AAG-TCT-TTC-AGT-GAT-GTG-GAC-T; actin: 5′, GAG-CTG-CCT-GAC-GGC-CAA-GTC-ATC-A; actin 3′, GTC-AAC-GTC-ACA-CTT-CAT-GAT-GG.
23. J. S. Silva *et al.*, *J. Exp. Med.* **175**, 169 (1992).
24. Supported by grants AI-25038 (S.G.R.) and AI-16282 (W. Johnson) as well as a training grant (TW-00018) from the National Institutes of Health and a fellowship from the Medical Research Council of Canada (Y.A.W.S.). We thank E. Yong for TGF-β assays and K. Kinch for manuscript preparation.

18 December 1991; accepted 26 May 1992

An Eosinophil-Dependent Mechanism for the Antitumor Effect of Interleukin-4

Robert I. Tepper,* Robert L. Coffman, Philip Leder

Murine interleukin-4 (IL-4) exhibits potent antitumor activity when present at the site of tumor cell challenge. Associated with tumor cell death is the appearance of an inflammatory infiltrate comprised predominantly of eosinophils and macrophages, but with few lymphocytes. Antibodies that specifically block the accumulation of granulocytes at the site of inflammation were injected in vivo to define the cell type responsible for the antitumor action of IL-4. These studies implicate eosinophils in IL-4–mediated tumor cytotoxicity. The lymphoid-independent nature of IL-4 action is supported by the analysis of mutant mouse strains with defined lymphocyte immunodeficiencies. The observed regression of established tumor masses by localized IL-4 action provides a rationale for exploring IL-4–mediated tumor killing as a potential therapy for human malignant disorders.

In a previous report (*1*), tumor cells engineered to produce IL-4 by transfection had reduced or absent tumorigenicity when reintroduced into animals. The antitumor activity of IL-4 was also observed on untransfected tumor cells when they were mixed with IL-4–producing cells before host inoculation. Using these transplantation assays, we showed that the antitumor activity of IL-4 acted in a dose-dependent manner on a wide variety of tumor cell types in vivo but not in vitro. Histologic analyses of five distinct tumor types inhibited by IL-4 (plasmacytomas, melanomas, B cell lymphomas, mammary adenocarcinomas, and glioblastomas) have revealed a characteristic host inflammatory reaction associated with tumor cell killing, which consists of the early influx (within 18 hours of tumor cell inoculation) of large numbers of eosinophils, and then macrophage infiltration and, subsequently, fibroblastic scarring.

Given the invariant histologic finding of eosinophil infiltration associated with tumor cell death, we sought to investigate whether IL-4 antitumor activity required the action of eosinophils. To accomplish this, we depleted animals of mature granulocytes (neutrophils and eosinophils) with the monoclonal antibody (MAb) RB6-8C5, which binds to a surface antigen present on mature murine granulocytes. This MAb is specifically cytotoxic for granulocytes and does not bind cells of the monocyte-macrophage or lymphocyte lineages (*2*). Eosinophil depletion with RB6-8C5 was first demonstrated by measurng blood eosinophil concentrations from mice parasitized with the helminth *Nippostrongylus brasiliensis* in the presence or absence of antibody (Table 1). The marked eosinophilia associated with this infection could be reversed within 48 hours by the administration of a single intraperitoneal dose (0.25 mg or more) of the MAb. Antibody treatment also depleted neutrophils and could be demonstrated by the reduction of blood neutrophils after administering comparable doses of RB6-8C5 to normal mice (>95% reduction within 48 hours).

To study the effect of host granulocyte depletion on the antitumor activity of IL-4, we studied two distinct tumor types, the murine BALB/c-derived plasmacytoma J558L and the murine melanoma B16, a spontaneously arising tumor from a C57BL/6 mouse. Both of these cell lines reproducibly form subcutaneous tumors in syngeneic and immunodeficient hosts (*1*). The localized expression of IL-4 at the site of tumor cell transplantation, either by direct tumor cell transfection with an activated IL-4 gene (*3*) or by mixing tumor cells with IL-4–producing cells, eradicates

Table 1. Effect of RB6-8C5 antibody administration on eosinophilia in *Nippostronglyus* (*Nb*)–infected BALB/c mice. A single intraperitoneal dosage of the antibody was administered on day 0, 12 days after the subcutaneous injection of 750 third-stage *Nb* larvae. Blood was obtained by tail bleeds on the days indicated and eosinophils were counted in Discombe's fluid with a hemacytomer (*12*). Data represent the mean eosinophil counts per milliliter in groups of five mice.

Dosage (mg)	Blood eosinophils per milliliter ($\times 10^{-4}$)		
	Day 0	Day 2	Day 4
2	440	92	1
1	548	26	0
0.5	383	13	1
0.25	271	4	3
0	440	249	123

R. I. Tepper, MGH Cancer Center, Massachusetts General Hospital, Charlestown, MA 02129.
R. L. Coffman, DNAX Research Institute, Palo Alto, CA 94304.
P. Leder, Department of Genetics, Harvard Medical School and Howard Hughes Medical Institute, Boston, MA 02115.

*To whom correspondence should be addressed.