Leonardo and D. Baltimore, *Cell* **58**, 227 (1989). The (−453/+80) HIV-LUC reporter was derived from the HIV–chloramphenicol acetyltransferase (CAT) reporter used by Nabel and Baltimore, whereas the ΔNF-κB (−453/+80)HIV-LUC reporter is derived from their mutant HIV-CAT reporter containing three–base pair substitutions in each NF-κB binding site.
8. P. A. Baeuerle and D. Baltimore, *Science* **242**, 540 (1988); *Cell* **53**, 211 (1988); *Genes Dev.* **3**, 1689 (1989).
9. Y. Devary, R. A. Gottlieb, T. Smeal, M. Karin, *Cell* **71**, 1081 (1992).
10. P. Angel and M. Karin, *Biochim. Biophys. Acta.* **1072**, 129 (1991).
11. L. Cantley *et al.*, *Cell* **64**, 281 (1991).
12. L. Feig and G. Cooper, *Mol. Cell. Biol.* **8**, 3235 (1988); W. Kolch, G. Heidecker, P. Lloyd, U. R. Rapp, *Nature* **349**, 426 (1991); M. Kamps and B. M. Sefton, *Mol. Cell. Biol.* **6**, 751 (1986). Transfection experiments were done as previously described (*9*).
13. S. M. Ruben *et al.*, *Science* **251**, 1490 (1991); G. P. Nolan, S. Ghosh, H. C. Liou, P. Tempst, D. Baltimore, *Cell* **64**, 961 (1991).
14. S. Ghosh *et al.*, *Cell* **62**, 1019 (1990); M. Kieran *et al.*, *ibid.*, p. 1007.
15. F. Mercurio, J. A. DiDonato, C. Rosette, M. Karin, *Genes Dev.* **7**, 705 (1993); *DNA Cell Biol.* **11**, 523 (1992). Note that the antiserum to p50 has only a small effect on DNA binding by the p50:p65 heterodimer.
16. J. A. DiDonato and C. Rosette, unpublished results.
17. S.-C. Sun, P. A. Ganchi, D. W. Ballard, W. C. Greene, *Science* **259**, 1912 (1993); K. Brown, S. Park, T. Kanno, G. Franzoso, U. Siebenlist, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2532 (1993).
18. C. Rosette and Y. Devary, unpublished results.
19. A. Levitzki, A. Gazit, N. Osherou, I. Posner, C. Gilon, *Methods Enzymol.* **201**, 347 (1991).
20. J. Szeberenyi, H. Cai, G. M. Cooper, *Mol. Cell. Biol.* **10**, 5324 (1990).
21. M. Karin and T. Smeal, *Trends Biochem. Sci.* **17**, 418 (1992); W. J. Boyle *et al.*, *Cell* **64**, 573 (1991); B. Binétruy, T. Smeal, M. Karin, *Nature* **351**, 122 (1991).
22. H. Cai *et al.*, *Mol. Cell. Biol.* **12**, 5329 (1992).
23. Phosphatidylcholine turnover was measured as described [F. Forrum, *Biochem. J.* **115**, 465 (1969)]. HeLa cells were labeled with [^{3}H]choline (2 μCi/ml) for 16 hours. One hour after UV exposure, the phospho-[^{3}H]choline released into the medium was collected and purified by organic extraction and measured by scintillation counting. In wt PC12 cells, UV irradiation gave rise to a 65 ± 5% increase in phosphocholine release, whereas in Ha-*rasN17*–transfected PC12 cells it had an insignificant effect on phosphocholine release.
24. T. David-Pfeuty and Y. J. Nouviar-Dooghe, *J. Cell Biol.* **111**, 3097 (1990); M. C. Willingham, I. Pastan, T. Y. Shih, E. M. Scolnick, *Cell* **19**, 1005 (1980).
25. A. Radler-Pohl *et al.*, *EMBO J.* **12**, 1005 (1993).
26. G. Poste, *Exp. Cell Res.* **73**, 273 (1972). Cells were incubated in cytochalasin B (20 μg/ml; Sigma) for 2 hours, washed, and detached from culture dishes with Versene, then resuspended in Percoll (1.05 g/ml; Pharmacia) in phosphate-buffered saline (PBS) containing cytochalasin B (5 μg/ml). After centrifugation for 1 hour at 33°C, 24,000 rpm (77,000*g*) in a Beckman SW 28 rotor, enucleated cells were recovered from the midpoint of the gradient, whereas whole cells were recovered from the bottom. Cell fractions were washed twice with PBS, resuspended in culture medium containing 10% fetal bovine serum, then replated onto 60-mm plates and allowed to recover for 1 hour at 37°C before UV treatment. We monitored the degree of enucleation by staining fixed cells and cytoplasts with 4,6-diamino-2-phenylindole (Boehringer Mannheim) and viewing under phase contrast with a UV filter in a Nikon epifluorescent microscope.
27. M. Hibi, A. Lin, T. Smeal, M. Karin, *Genes Dev.*, in press; 30 μg of total cell extract were incubated for 3 hours at 4°C with a glutathione-S-transferase (GST)–c-Jun(1–223) fusion protein coupled to glutathione-agarose beads, followed by five washes with HBB [20 mM Hepes (pH 7.7), 50 mM NaCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% Triton X-100]. The resuspended beads were then used to phosphorylate recombinant c-Jun (10 ng) by incubation for 30 min at 30°C in 20 mM Hepes (pH 7.5), 20 mM β-glycerophosphate, 20 mM *p*-nitrylphenyl phosphate, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM Na_3VO_4, 2 μCi of γ-^{32}P–labeled adenosine triphosphate (ATP), and 20 μM unlabeled ATP. The reaction was stopped by the addition of SDS gel loading buffer, boiled for 5 min, and was run on a 10% SDS polyacrylamide gel.
28. R. Schreck, P. Rieber, P. A. Baeuerle, *EMBO J.* **10**, 2247 (1991); R. Schreck and P. A. Baeuerle, *Trends Cell Biol.* 1, 39 (1991); F. J. T. Staal, M. Roederer, L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9943 (1990).
29. D. W. Ballard *et al.*, *Cell* **63**, 803 (1990).
30. H. Land, A. C. Chen, J. P. Morganstern, L. F. Parada, R. A. Weinberg, *Mol. Cell. Biol.* **6**, 1917 (1986).
31. We thank P. Baeuerle for suggesting the enucleation experiment, L. Herzenberg and M. T. Anderson for discussion and unpublished results, G. Cooper for the Ha-*rasN17*–transfected PC12 cell line, W. Greene for the antibodies to p65, and J. H. Brown for help with measurements of PC turnover. Supported by a Minority Supplement for Research from the National Institutes of Health (DK38527-06S2) (to C.R.) and by an NIH postdoctoral fellowship (5F32 DK08584-03) (to J.D.). Research was supported by grants from the Department of Energy (DE-FG03-86ER60429) and the NIH (ES04151, CA50528).

20 April 1993; accepted 13 July 1993

Inhibition of Viral Replication by Interferon-γ–Induced Nitric Oxide Synthase

Gunasegaran Karupiah,* Qiao-wen Xie, R. Mark L. Buller, Carl Nathan, Cornelio Duarte, John D. MacMicking*

Interferons (IFNs) induce antiviral activity in many cell types. The ability of IFN-γ to inhibit replication of ectromelia, vaccinia, and herpes simplex-1 viruses in mouse macrophages correlated with the cells' production of nitric oxide (NO). Viral replication was restored in IFN-γ–treated macrophages exposed to inhibitors of NO synthase. Conversely, epithelial cells with no detectable NO synthesis restricted viral replication when transfected with a complementary DNA encoding inducible NO synthase or treated with organic compounds that generate NO. In mice, an inhibitor of NO synthase converted resolving ectromelia virus infection into fulminant mousepox. Thus, induction of NO synthase can be necessary and sufficient for a substantial antiviral effect of IFN-γ.

Several cytokines, including interferons (IFN-α, -β, -γ, and -ω) and tumor necrosis factors (TNF-α and -β) display antiviral activity (*1*). The antiviral effects of the IFNs have been attributed in part to their induction of (i) P1 kinase, which phosphorylates and inactivates eukaryotic protein synthesis initiation factor eIF-2α; (ii) 2′,5′-oligoadenylate synthetase, whose products activate a latent endoribonuclease; (iii) indoleamine 2,3-dioxygenase, which depletes cell cultures of tryptophan; (iv) Mx proteins, which inhibit influenza and vesicular stomatitis viruses; and (v) the 9-27 protein, which binds to the Rev-responsive element of human immunodeficiency virus (HIV) (*1*). Nevertheless, the mechanisms of action of IFNs remain incompletely understood.

The antimicrobial and antiproliferative actions of cytokines such as IFN-γ are undergoing reassessment in light of their ability to induce the expression of *iNOS*, a gene encoding an isoform of nitric oxide synthase (NOS) that produces large amounts of the radical gas, NO, from a guanidino nitrogen of L-arginine (*2*, *3*). Induction of *iNOS* contributes to IFN-γ's antiproliferative actions (*4*) and to its enhancement of macrophage cytotoxicity toward tumor cells, bacteria, fungi, protozoa, and helminths (*2*, *3*). The inducibility of high-output NO production among many other cell types, such as hepatocytes, smooth and cardiac myocytes, keratinocytes, endothelium, mesangial cells, tumor cells, and some fibroblasts (*2*, *5*), prompted the hypothesis that the induction of *iNOS* might also defend the host against the one class of pathogens that can infect all nucleated cells—viruses (*2*).

To test this hypothesis, we first focused on ectromelia virus (EV). The replication of this orthopoxvirus in C57BL/6 mice is restricted by IFN-γ (*6*), yet EV may inhibit both the P1 kinase and the 2′,5′-oligoadenylate synthetase pathways (*7*). In vitro, IFN-γ inhibits EV replication in mouse RAW 264.7 macrophage-like cells and in primary mouse macrophages but not in L929 mouse fibroblasts or 293 human renal epithelial cells (Fig. 1A). Thus, an additional, IFN-γ–inducible antiviral pathway is likely to exist and be selectively ex-

G. Karupiah, R. M. L. Buller, C. Duarte, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.
Q.-w. Xie, C. Nathan, J. D. MacMicking, Beatrice and Samuel A. Seaver Laboratory, Division of Hematology-Oncology, Department of Medicine, Cornell University Medical College, New York, NY 10021.
*To whom correspondence should be addressed.

pressed. The differential antiviral effect of IFN-γ in these four cell types correlated with their ability to produce NO, measured as the stable oxidation product, nitrite (NO_2^-) (*8*) (Fig. 1B).

In macrophages, the antiviral effect of IFN-γ was substantially reversed by inclusion in the cultures of N^ω-methyl-L-arginine (L-NMA), a guanidino-*N*-substituted L-arginine analog that acts as a competitive inhibitor of iNOS (9) (Fig. 2, A to D). In control macrophages, EV, the closely related vaccinia virus (VV), and herpes simplex virus type–1 (HSV-1) produced between 2.0 and 2.5 $\log_{10}$ progeny virus over a 24-hour period. Treatment of the macrophages with IFN-γ severely restricted replication of all three viruses. In the presence of L-NMA, IFN-γ–induced inhibition of viral replication was reversed by 73 to 100%. The ability of L-NMA to nullify the protective effect of IFN-γ was itself substantially antagonized by the addition of excess L- but not D-arginine. Stereospecificity was further evidenced by the inability of N^ω-methyl-D-arginine to reverse IFN-γ–induced inhibition of viral replication. In the absence of IFN-γ, L-NMA, D-NMA, L-arginine, and D-arginine did not affect viral yields (*10*). Production of NO_2^- by EV-infected RAW 264.7 cells or peritoneal macrophages was similarly inhibited by L-NMA and restored by excess L-arginine (Fig. 2, E and F). Two additional NOS inhibitors, N^ω-nitro-L-arginine (NNA) and *N*-iminoethyl-L-ornithine (NIO), also reversed IFN-γ–induced inhibition of EV replication in RAW 264.7 cells (*10*). The effects of all three NOS inhibitors were concentration-dependent (with EV, the concentration giving 50% of the maximum effect was 0.24 mM for L-NMA, 0.90 mM for NNA, and 0.17 mM for NIO in the presence of 0.82 mM L-arginine).

Because inhibition of iNOS blocked antiviral activity, we next tested whether NO, a product of iNOS, could confer antiviral activity. The NO was furnished either genetically or pharmacologically with similar results (Fig. 3). First, 293 cells were transfected with a plasmid (*11*) encoding the functional "long-form" of *iNOS* cDNA cloned from RAW 264.7 cells (*12*). As controls, other 293 cells were transfected with the vector only or with catalytically inactive "short-form" *iNOS* cDNA (*11*, *12*). The short form is 22 amino acids shorter than the long form, but otherwise the two differ in only 11 of 1122 amino acids (*12*). Both isoforms of recombinant iNOS were expressed in 20 to 25% of 293 cells, as judged by indirect immunofluorescence with an antibody to iNOS (*12*). Even though most 293 cells did not express iNOS, the presence of enzymatically active iNOS within the cultures (the supernatants of which accumulated 15 nmol of NO_2^- per 10^6 cells within 6 hours) resulted in 45 to 100% inhibition of replication of EV, VV, and HSV-1 relative to untransfected control cells. In contrast, transfection with enzymatically inactive iNOS or vector alone (no NO_2^- accumulated) reduced viral replication by 0 to 18% (Fig. 3, A to C). Thus, iNOS-expressing cells appear to confer an antiviral state on neighboring cells. Similarly, the NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) (*13*) reduced viral titers almost to the level of the inoculum, whereas the control compound *N*-acetyl-penicillamine had no effect (Fig. 3, D to F). The antiviral effect of SNAP did not reflect toxicity to the cells, in that viral titers returned to the levels seen in control cultures within 20 to 24 hours after SNAP was removed (*10*).

These results support two conclusions. First, activity of iNOS is both necessary and sufficient for a substantial antiviral effect of IFN-γ in vitro. Second, although iNOS has many potential enzymatic actions—depletion of L-arginine and NADPH (reduced form of nicotinamide adenine dinucleotide phosphate), oxidation of tetrahydrobiopterin, production of hydrogen peroxide and citrulline, and reduction of cytochrome P-450 (*2*)—provision of NO alone is sufficient to inhibit viral replication.

Depletion of L-arginine by macrophage-derived arginase has been proposed as an antiviral mechanism (*14*), but in the present experiments the concentration of L-arginine fell by <15% in the medium of IFN-γ–stimulated, virus-infected RAW 264.7 cells

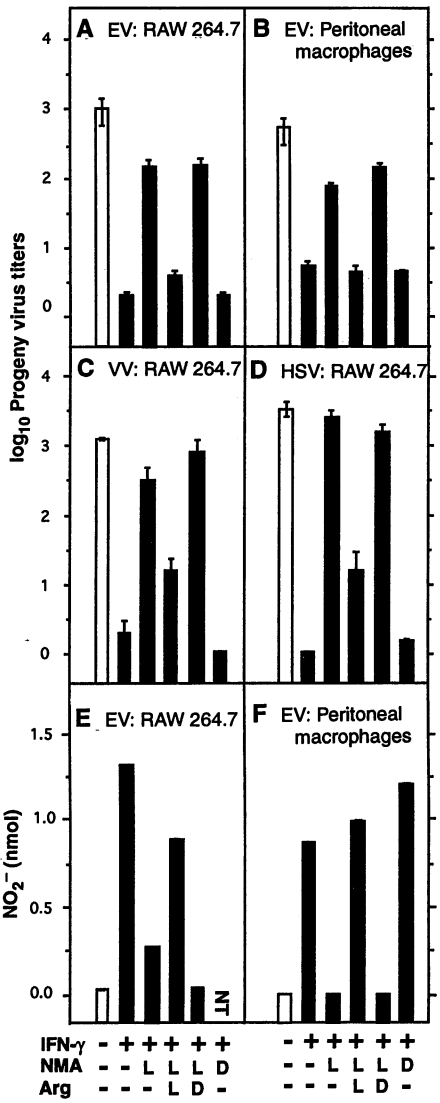

Fig. 2. Dependence of IFN-γ–induced antiviral activity in macrophages on their production of NO. The RAW 264.7 cells (**A**, **C**, **D**, and **E**) or peritoneal macrophages (**B** and **F**) were cultured for 18 hours with (solid bars) or without (open bars) IFN-γ alone [25 U/ml (EV and VV) or 5 U/ml (HSV-1)]; IFN-γ together with 300 μM L-NMA; IFN-γ, L-NMA, and 2.5 mM (EV and VV) or 1 mM (HSV-1) L-arginine; IFN-γ, L-NMA, and 2.5 mM D-arginine; or IFN-γ and 300 μM D-NMA. The cells were then infected with the indicated viruses. Panels (A to D) show the titers of viral progeny (*23*) at 24 hours. Panels (E) and (F) show the NO_2^- accumulation in the medium at 24 hours. The L-NMA, D-NMA, L-arginine, and D-arginine were from Sigma. Results are presented as the mean ± SEM of triplicate cultures. Results were similar in each of two or three additional experiments with each cell type. NT, not tested.

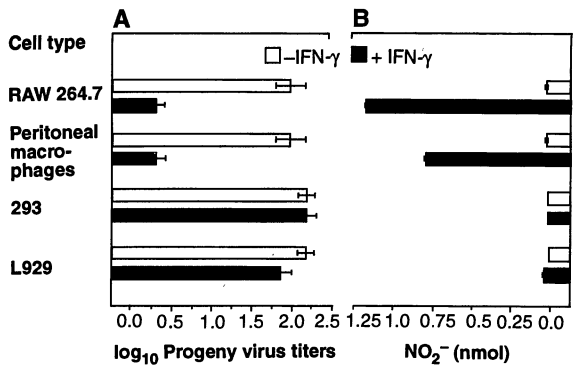

Fig. 1. Differential sensitivity of EV to IFN-γ in various cell types and correlation with NO production. (**A**) Cells were cultured for 18 hours with or without recombinant IFN-γ, infected with EV, and assayed for infectious progeny virus 24 hours later (*23*). Progeny virus is expressed as the mean ± SEM of $\log_{10}$ viral titer in triplicate cultures minus $\log_{10}$ of infectious virus recovered from parallel cultures 1 hour after the addition of virus to the culture. (**B**) Accumulation of NO_2^- in the medium of the same cell cultures, measured by the Griess assay (*8*). Results, presented as the mean ± SEM of triplicate cultures, were similar in each of two or three additional experiments with each cell type.

and inhibition of viral replication was not reversed by addition of excess L-arginine (*10*).

Finally, we tested the effect of an NOS inhibitor on viral replication in vivo. Inoculation of the virulent Moscow strain of EV into the footpad of C57BL/6 mice reproduces a natural, self-limited infection with a well-characterized immune response and pathology (*15*). Treatment of uninfected mice with L-NMA or D-NMA (four mice per group) had no effect on organ histology or survival through day 14. Of EV-infected control mice treated with vehicle (n = 19) or D-NMA (n = 15), 95% survived through day 14, by which time virus had been cleared from all organs examined (Table 1). In contrast, treatment of 20 EV-infected mice with L-NMA led to 30% mortality (mean time of death was 8.3 days after infection), increased titers of virus in spleen and liver in mice killed at 5 days after infection, and persistence of virus in mice killed at 14 days after infection (Table 1), all hallmarks of fulminant mousepox. However, L-NMA did not inhibit the development of major histocompatibility complex class I–restricted cytolytic T cells with specificity for EV-infected host cells (*16*).

Table 1. Impact of NOS inhibitor on the course of EV infection in mice. The treatment was as follows: Female, 6-week-old C57BL/6NCR mice were infected subcutaneously in the right hind footpad on day zero with 4 × 10^3 PFU of EV. The same footpad was injected with 20 μl of phosphate-buffered saline (PBS) alone or PBS containing D-NMA or L-NMA (5 mg per day) from day 1 until day 5 after infection. Thereafter, the same daily dosage was administered in a 100-μl volume intravenously until the mouse succumbed to disease or was killed (day 14).

Treatment	Day of analysis	Log_{10} virus per gram*	
		Liver	Spleen
PBS	5	6.6 ± 0.4	5.8 ± 0.5
PBS + D-NMA	5	6.6 ± 0.1	6.0 ± 1.1
PBS + L-NMA	5	7.2 ± 0.3	7.4 ± 0.4
PBS	14	<2.0	<2.0
PBS + D-NMA	14	<2.0	<2.0
PBS + L-NMA	14	4.2 ± 0.8	3.4 ± 0.4

*Viral titers (mean log_{10} ± SEM) were determined in homogenates of organs from five mice per group (*23*). Titers were significantly higher in L-NMA–treated mice on day 5 ($P < 0.01$, Student's t test) and day 14 ($P < 0.00001$) compared to either set of controls. The limit of sensitivity of the assay is 2.0 log_{10} PFU per gram.

These findings establish another mechanism through which IFN-γ can exert an antiviral effect. Induction of iNOS is likely to play a role in the antiviral action of other cytokines besides IFN-γ, as IFN-α and -β and TNF-α and -β can synergize with other stimuli to induce iNOS expression (*8*). These results do not discount the participation of previously defined antiviral mechanisms of IFN-γ, nor can all unexplained antiviral effects of cytokines be ascribed to the action of iNOS. For example, IFN-α and -β inhibited EV replication in primary mouse macrophages nearly as well as IFN-γ, and this inhibition was not accompanied by NO_2^- production nor was it reversed by L-NMA (*10*).

The antiviral action described here may explain the paradox of iNOS being widely inducible although autotoxic (*2*). Advantages of NO as an antiviral agent may include (i) its ability to pass readily into neighboring cells, like some viruses but unlike antibody and complement; (ii) its action independent of immune recognition of the infected cell, in contrast to that of antiviral lymphocytes; and (iii) the likely multiplicity of its viral and virally exploited cellular targets, which may limit the capacity of viruses to develop resistance (*2*).

The reactivity of NO and of its higher oxides and nitrosothiol products (*2*, *17*) makes it probable that a variety of molecular targets are involved in its antiviral action. Inhibition of ribonucleotide reductase (*18*) and deamination of viral DNA (*19*) may be important mechanisms. Interference of NO with viral infectivity seems less likely, as treatment of EV, VV, and HSV-1 virions with 1 mM SNAP had no discernible effect (*16*). The ability of NO to activate the transcription factor NF-κB (*20*) opens up two further possibilities: first, that NO could exert antiviral effects by inducing proteins, not just by inactivating them; and second, that NO could enhance the replication of certain viruses, like HIV, that thrive when NF-κB is activated (*21*). Finally, the mutagenic potential of NO (*19*) suggests that, even while inhibiting viruses, NO may further their evolution.

Note added in proof: After this paper was submitted, Croen reported that NO inhibits the replication of HSV-1 virus in cells treated with IFN-γ and bacterial lipopolysaccharide (*22*).

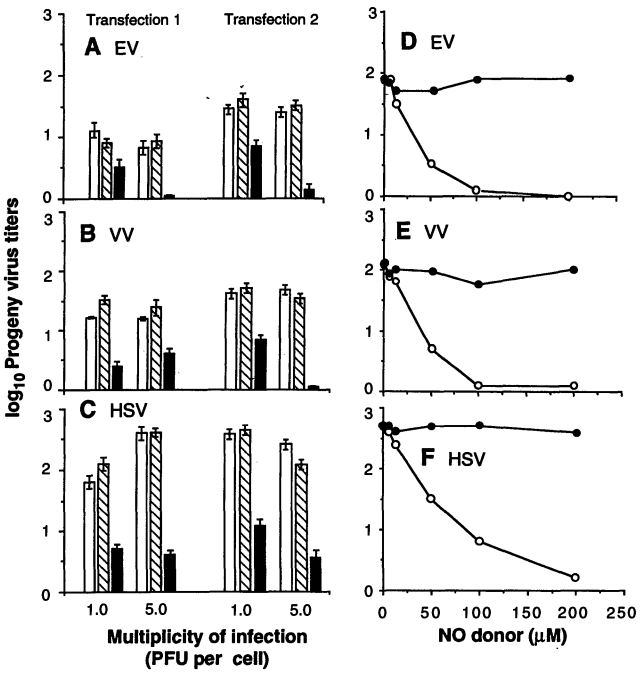

Fig. 3. Inhibition of viral replication in 293 cells provided with NO by transfection with *iNOS* cDNA or by addition of an organic NO donor. (**A** through **C**) The *iNOS* cDNA was introduced by calcium phosphate–assisted transfection of cells in culture dishes 100 mm in diameter with 20 μg of piNOSL8 (*11*) encoding the enzymatically active "long form" of iNOS (solid bars), carried in the pcDNA I vector (Invitrogen, San Diego, California) (*12*). As controls, other cultures were transfected with the pcDNA I vector alone (empty bars) or with vector containing the enzymatically inactive "short form" piNOSS2 (*11*, *12*) (striped bars). After 24 hours, cells were distributed in 24-well plates at a density of 3 × 10^5 (transfection 1) or 5 × 10^5 (transfection 2) cells per well, allowed to adhere for 6.5 hours, and inoculated at the indicated multiplicities of infection. Viral replication was measured 24 hours later (*23*). Results are means ± SEM of triplicate cultures from each of two independent transfections. (**D** through **F**) The 293 cells were infected with virus and incubated with 2.5 mM L-cysteine, together with the indicated concentrations of SNAP (*13*) (open circles) or *N*-acetyl-penicillamine (Sigma) (filled circles). These compounds were replenished every 4 hours until viral titers were determined (at 24 hours). Results (given as the mean ± SEM of triplicate cultures) were similar in two additional experiments.

REFERENCES AND NOTES

1. J. Vilček, in *Peptide Growth Factors and Their Receptors*, M. B. Sporn and A. B. Roberts, Eds. (Springer-Verlag, Berlin, 1990), vol. 2, pp. 3–38; C. E. Samuel, *Virology* **183**, 1 (1991); G. C. Sen and P. Lengyel, *J. Biol. Chem.* **267**, 5017 (1992); C. Nathan, in *Inflammation: Basic Principles and Clinical Correlates*, J. Gallin, I. Goldstein, R. Snyderman, Eds. (Raven, New York, ed. 2, 1992), pp. 265–290; P. Constantoulakis *et al.*, *Science* **259**, 1314 (1993); H. H. Hassanain, S. Y. Chon, S. L. Gupta, *J. Biol. Chem.* **268**, 5077 (1993).
2. C. Nathan, *FASEB J.* **6**, 3051 (1992).
3. S. Moncada, R. M. J. Palmer, E. A. Higgs, *Pharmacol. Rev.* **43**, 109 (1991); C. Nathan and J. B. Hibbs, Jr., *Curr. Opin. Immunol.* **3**, 65 (1991); J. B. Hibbs, Jr., *et al.*, in *Nitric Oxide from L-Arginine: A Bioregulatory System*, S. Moncada and E. A. Higgs, Eds. (Elsevier, Amsterdam, 1990), pp. 189–223.
4. G. Werner-Felmayer *et al.*, *J. Exp. Med.* **172**, 1599 (1990).
5. A. B. Roberts, Y. Vodovotz, N. S. Roche, M. B. Sporn, C. F. Nathan, *Mol. Endocrinol.* **6**, 1921 (1992); D. E. Heck, D. L. Laskin, C. R. Gardner, J.

D. Laskin, *J. Biol. Chem.* **267**, 21277 (1992).
6. G. Karupiah, T. N. Fredrickson, K. L. Holmes, L. H. Khairallah, R. M. L. Buller, *J. Virol.* **67**, 4214 (1993); G. Karupiah, R. V. Blanden, I. A. Ramshaw, *J. Exp. Med.* **172**, 1495 (1990); J. Ruby and I. Ramshaw, *Lymphokine Cytokine Res.* **10**, 353 (1991).
7. E. Paez and M. Esteban, *Virology* **134**, 12 (1984); E. Beattie, J. Taraglia, E. Paoletti, *ibid.* **183**, 419 (1991); H.-W. Chang, J. C. Watson, B. L. Jacobs, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4825 (1992); M. V. Davies, O. Elroy-Stein, R. Jagus, B. Moss, R. J. Kaufman, *J. Virol.* **66**, 1943 (1992); M. V. Davies, H.-W. Chang, B. L. Jacobs, R. J. Kaufman, *ibid.* **67**, 1688 (1993).
8. A. H. Ding, C. F. Nathan, D. J. Stuehr, *J. Immunol.* **141**, 2407 (1988); J.-C. Drapier, J. Wietzerbin, J. B. Hibbs, Jr., *Eur. J. Immunol.* **18**, 1587 (1988).
9. J. B. Hibbs, Jr., R. Taintor, Z. Vavrin, *Science* **235**, 473 (1987).
10. G. Karupiah and J. MacMicking, unpublished results.
11. Q.-w. Xie and C. Nathan, in preparation.
12. Q.-w. Xie *et al.*, *Science* **256**, 225 (1992).
13. L. J. Ignarro *et al.*, *J. Pharmacol. Exp. Ther.* **218**, 739 (1981).
14. P. Wildy, P. G. H. Gell, J. Rhodes, A. Newton, *Infect. Immun.* **37**, 40 (1982); K. K. Sethi, *Immunobiology* **165**, 459 (1983).
15. R. M. L. Buller and G. J. Palumbo, *Microbiol. Rev.* **55**, 80 (1991).
16. G. Karupiah, unpublished results.
17. J. S. Stamler, D. J. Singel, J. Loscalzo, *Science* **258**, 1898 (1992).
18. M. Lepoivre *et al.*, *J. Biol. Chem.* **265**, 14143 (1990); N. S. Kwon, D. J. Stuehr, C. F. Nathan, *J. Exp. Med.* **174**, 761 (1991).
19. D. A. Wink *et al.*, *Science* **254**, 1001 (1991); T. Nguyen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3030 (1992).
20. H. Lander, P. Sehajpal, D. M. Levine, A. Novogrodsky, *J. Immunol.* **150**, 1509 (1993).
21. R. J. Pomerantz, M. B. Feinberg, D. Trono, D. Baltimore, *J. Exp. Med.* **172**, 253 (1990); N. Israël, M.-A. Gougerot-Pocidalo, F. Aillet, J.-L. Virelizier, *J. Immunol.* **149**, 3386 (1992).
22. K. D. Croen, *J. Clin. Invest.* **91**, 2446 (1993).
23. Primary macrophages were lavaged from the peritoneal cavities of C57BL/6NCR mice 6 days after the intraperitoneal injection of 0.1 ml of sterile mineral oil, washed three times in Hanks' balanced salt solution (BioWhittaker Inc., Walkersville, MD), plated at 1×10^5 cells per well in complete medium [RPMI 1640 (Gibco BRL) supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), nonessential amino acids, 500 μM β-mercaptoethanol, and 10% fetal bovine serum (Hyclone Labs)], and allowed to adhere. Complete medium contained endotoxin (20 pg/ml) as determined by a chromogenic limulus amebocyte lysate assay (BioWhittaker). Nonadherent cells (~50% of the total) were washed off before infection. RAW 264.7 cells, L929 cells, and 293 cells (all from American Type Culture Collection) were plated at 2.5×10^5 cells per well in complete medium 24 hours before use. Some cultures were treated with pure recombinant mouse IFN-γ (25 U/ml) (for macrophages, RAW 264.7, and L929 cells) or human IFN-γ (for 293 cells) (Genentech) for 18 hours before and throughout infection. Virus was added at one plaque-forming unit (PFU) per cell and allowed to adsorb for 1 hour at 37°C before monolayers were washed three times with phosphate-buffered saline and repleted with 1 ml of complete medium. After 24 hours at 37°C, cells were scraped into culture supernatant, lysed by three cycles of freeze-thaw and sonication, and serially diluted 10-fold. Aliquots (0.5 ml) were adsorbed to BS-C-1 cell monolayers for 1 hour at 37°C and overlaid with Eagle's minimum essential medium with 10% fetal bovine serum and 0.9% (w/v) methylcellulose (Sigma Chemical). Viral plaques were counted on stained monolayers as described (*6*).
24. We thank J. Manning and M. A. Pospischil for amino acid analysis of culture media, L. Ignarro for SNAP, S. Shah and M. McCoss for NIO, G. Chaudhri for RAW 264.7 cells, M. Challberg for HSV-1, and Genentech, Inc., for IFN-γ. Supported by NIH grant CA43610 (to C.N.) and by the National Institute of Allergy and Infectious Diseases (to R.M.L.B., C.D., and G.K.).

23 March 1993; accepted 1 July 1993

Helper T Cells Without CD4: Control of Leishmaniasis in CD4-Deficient Mice

Richard M. Locksley,* Steven L. Reiner, Farah Hatam, Dan R. Littman, Nigel Killeen

Expression of either the CD4 or CD8 glycoproteins discriminates two functionally distinct lineages of T lymphocytes. A null mutation in the gene encoding CD4 impairs the development of the helper cell lineage that is normally defined by CD4 expression. Infection of CD4-null mice with *Leishmania* has revealed a population of functional helper T cells that develops despite the absence of CD4. These $CD8^-$ αβT cell receptor$^+$ T cells are major histocompatibility complex class II–restricted and produce interferon-γ when challenged with parasite antigens. These results indicate that T lymphocyte lineage commitment and peripheral function need not depend on the function of CD4.

The critical role of T cells in the control of murine *Leishmania major* infection is underscored by the failure of the immune system of T cell–deficient nude or severe combined immunodeficient (SCID) mice to control fatal dissemination of the parasite (*1*). Sustained depletion of $CD4^+$ cells by monoclonal antibodies (mAbs) results in an inability to control disease (*2*), although transient depletion at the time of infection allows the outgrowth of protective type 1 helper T cells (T_H1 cells) in otherwise susceptible BALB/c mice (*3*). This requirement for $CD4^+$ cells may be related to the localization of the organism in a late endosomal compartment in macrophages into which major histocompatibility complex (MHC) class II molecules co-localize (*4*). Mice with a targeted disruption of the CD4 gene have impaired development of the CD4 lineage and decreased helper T cell activity (*5*, *6*). We examined the capacity of such mice to control an *L. major* infection in order to investigate the requirements for $CD4^+$ T cells in the control of this intramacrophage parasite.

Mice homozygous (−/−) and heterozygous (+/−) for the CD4 gene disruption on the (C57BL/6 × 129) F_1 ($H\text{-}2^b$) background were generated and screened (*6*). The parental and hybrid wild-type mice on this genetic background are resistant to infection with *L. major* and develop a small lesion at the site of inoculation that heals in 6 weeks. After receiving an inoculation of stationary-phase promastigotes in the hind footpads, both heterozygote and homozygote mice developed local swelling that peaked during the third to fourth week of infection and resolved thereafter (Fig. 1). Only 1 of 29 $CD4^{-/-}$ mice showed any delay in this pattern of resolution; that mouse had a footpad lesion of 5.1 mm after 9 weeks but was otherwise well. We cultured serial dilutions of footpad and splenic tissues to confirm that parasite replication had been controlled. Eleven infected $CD4^{-/-}$ mice had no recoverable promastigotes; only the animal with a persistent footpad lesion had recoverable parasites (from the footpad cultures only).

Resolution of *L. major* infection can be abrogated if the neutralizing antibody to interferon-γ (IFN-γ) is administered at the time of infection (*7*). When we administered intraperitoneally a single dose of neutralizing mAb to IFN-γ at the same time we inoculated mice with the parasite, healing was blocked in both control and $CD4^{-/-}$ mice. The lesions progressed (Fig. 1) and organisms were recovered from both footpads and spleen in all treated animals.

We assayed mice for delayed type hypersensitivity (DTH), a reaction attributed to $CD4^+$ T_H1 cells in normal mice (*8*), by injecting *L. major* antigens into one footpad 8 weeks after infection, by which time the local lesion had completely resolved. Saline was injected into the other footpad. At 48 hours, infected but not uninfected mice developed swelling typical for DTH only in the antigen-injected footpad (footpad thickness measured with a metric caliper was 2.18 ± 0.22 and 2.06 ± 0.18 mm for saline and 3.48 ± 0.24 and 3.28 ± 0.21 for 50 μg of *L. major* antigen in $CD4^{-/-}$ and

R. M. Locksley, Departments of Medicine and Microbiology and Immunology, University of California, San Francisco, CA 94143–0654.
S. L. Reiner and F. Hatam, Department of Medicine, University of California, San Francisco, CA 94143–0654.
D. R. Littman and N. Killeen, Department of Microbiology and Immunology and Howard Hughes Medical Institute, University of California, San Francisco, CA 94143–0414.
*To whom correspondence should be addressed.