REFERENCES AND NOTES

- 1. V. Nussenzweig and R. S. Nussenzweig, Adv. Immunol. 45, 283 (1989).
- 2. F. Zavala et al., Science 228, 1436 (1985). NANP is Asn-Ala-Asn-Pro.
- D. A. Herrington et al., Nature 328, 257 (1987)
- M. F. Good et al., J. Exp. Med. 164, 655 (1986); G. del Giudice et al., J. Immunol. 137, 2952 (1986).
 M. F. Good et al., Proc. Natl. Acad. Sci. U.S. A. 85, 1199 (1988).
- F. Sinigaglia et al., Eur. J. Immunol. 18, 633 (1988).
 G. Spitalny, J. P. Verhave, J. H. E. Meuwissen, R. S.
- Nussenzweig, Exp. Parasitol. **42**, 73 (1977). 8. L. Schofield et al., Nature **330**, 664 (1987); W. R. Weiss, M. Sedgah, R. L. Beaudoin, L. Miller, M. F. Good, Proc. Natl. Acad. Sci. U.S.A. 85, 573
- (1988); S. Kumar et al., Nature 334, 258 (1988); P. Romero et al., Nature 341, 323 (1989). 9. A. Ferreira et al., Science 232, 881 (1986); L. Schofield, A. Ferreira, R. Altszuler, V. Nussenz-
- weig, R. S. Nussenzweig, J. Immunol. 139, 2020 (1987)10. D. A. Herrington et al., in preparation. 11. The recombinant P. falciparum CS protein was de-
- rived from plasmid-transformed yeast with methods similar to those described for a recombinant P. vivax CS protein (14) and a recombinant P. berghei CS protein (15). Briefly, Saccharomyces cerevisiae (strain 2168) was transformed with an expression plasmid containing DNA encoding amino acids 43 to 348 of the CS protein of *P. falciparum* T4 isolate (24). F. Sinigaglia, H. Matile, J. R. Pink, *Eur. J. Immunol.*
- 12. 17, 187 (1987).
- 13. The T cell phenotype was determined by reacting cells with a monoclonal antibody specific for human CD4 and CD8 determinants [W. Biddison et al., J. Immunol. 131, 152 (1983); E. Reinherz, P. Kung, G. Goldstein, R. Levey, S. Schlossman, Proc. Natl. Acad. Sci. U.S.A. 77, 1588 (1980)] and then staining with fluorescein isothiocyanate-conjugated antibody to mouse IgG.
- P. Barr et al., J. Exp. Med. 165, 1160 (1987).
 P. J. Romero et al., Eur. J. Immunol. 18, 1951 (1988)
- 16. È. H. Nardin et al., unpublished data.
- The expression and purification of the E. coliderived CS proteins are as described by J. Kilgus et al. [Proc. Natl. Acad. Sci. U.S.A. 86, 1629 (1989)] and E. Hochuli, W. Banwarth, H. Dobeli, R. Gentz, D. Stuber [Biotechnology 6, 1321 (1988)]. The bacteria-derived recombinant CS proteins were expressed in E. coli as fusion proteins with a polyhistidine peptide [(His)6] at the COOH-terminus to facilitate protein purification. Five of the fusion proteins, CSFCS and CSF1 to CSF4, in addition to the polyhistidine affinity peptide, also contain at their NH_2 -terminus 196 amino acids of the mouse dihydrofolate reductase sequence. Purification of the expressed CS fusion proteins was carried out with immobilized metal ion affinity and cation exchange chromatography. 18. H. Etlinger et al., J. Immunol. 140, 626 (1988).
- E. Nardin, in preparation.
- S. Hoffman et al., J. Immunol. 142, 1299 (1989). 20.
- M. Guttinger et al., EMBO J. 7, 2555 (1988).
 V. F. de la Cruz, A. Lal, T. McCutchan, J. Biol. Chem. 262, 11935 (1987); M. F. Good et al., J. Immunol. 140, 1645 (1988).
- J. B. Dame et al., Science 225, 593 (1984); M. 23. Lockyer and R. Schwarz, Mol. Biochem. Parasitol. 22, 101 (1987).
- H. del Portillo, R. S. Nussenzweig, V. Enea, Mol. Biochem. Parasitol. 24, 289 (1987).
 P. Caspers et al., ibid. 35, 185 (1989).
 D. R. Clyde, V. McCarthy, R. M. Miller, R. Hornick, Am. J. Med. Sci. 266, 398 (1973).
 J. E. Varnez et al., Scime 229, 952 (1985).

- 27. J. F. Young et al., Science 228, 958 (1985); Webster et al., Am. J. Trop. Med. Hyg. 38, 37 (1988); I. Campbell et al., ibid. 39, 232 (1988).
- W. R. Ballou et al., Lancet 87-i, 1277 (1987).
- D. M. Russo, J. Sundy, J. Young, H. Maguire, W. P. Weidanz, J. Immunol. 143, 655 (1989).
- 30. E. H. Nardin et al., in preparation. 31. G. Miller and M. Lipman, Proc. Natl. Acad. Sci. U.S.A. 70, 109 (1973); B. Sudgen, Rev. Infect. Dis. 4, 1048 (1982)
- 32. B. Merrifield, Science 232, 341 (1986).

33. We thank V. Nussenzweig for helpful comments and suggestions and G. Y. Xu for excellent technical assistance. This work was supported by NIH grants AI25085 and AI62533, by grant DPE-0453-A-

00501200 from the Agency for International Development, and by the McArthur Foundation.

18 October 1989; accepted 21 November 1989

Inhibition of Antigen-Induced Lymphocyte Proliferation by Tat Protein from HIV-1

RAPHAEL P. VISCIDI, KUMUDINI MAYUR, HOWARD M. LEDERMAN, Alan D. Frankel

The purified human immunodeficiency virus type-1 (HIV-1) Tat protein inhibited lymphocyte proliferation induced by tetanus toxoid or Candida antigens by 66 to 97% at nanomolar concentrations of Tat. In contrast, Tat did not cause a significant reduction of lymphocyte proliferation in response to mitogens such as phytohemagglutinin or pokeweed mitogen. Inhibition was blocked by oxidation of the cysteine-rich region of Tat or by incubation with an antibody to Tat before the assay. A synthetic Tat peptide (residues 1 to 58) also inhibited antigen-stimulated proliferation. Experiments with H9 and U937 cell lines showed that Tat can easily enter both lymphocytes and monocytes. The specific inhibition of antigen-induced lymphocyte proliferation by Tat mimics the effect seen with lymphocytes from HIV-infected individuals and suggests that Tat might directly contribute to the immunosuppression associated with HIV infection.

HE TAT PROTEIN FROM HUMAN IM-

munodeficiency virus activates HIV-1 gene expression and is essential for viral replication in vitro (1, 2). Tat can be taken up by cells growing in tissue culture, enter the nucleus, and transactivate genes expressed from the HIV-1 promoter (3). This property of Tat raises questions about the biological importance of extracellular Tat during the course of HIV infection in vivo, but it also provides a simple way to study the effects of Tat in the absence of viral replication. To test whether Tat might play a direct role in immune dysfunction in addition to its role in viral replication, we measured the effect of extracellular Tat in lymphocyte proliferation assays. We now show that Tat inhibits antigen-induced, but not mitogen-induced, lymphocyte proliferation

The Tat protein (amino acids 1 to 72) was expressed in Escherichia coli and purified to >95% homogeneity, as described (3, 4). Lyophilized Tat protein was resuspended at 200 μ g/ml in distilled water containing 10 mM β -mercaptoethanol. A reducing agent was included to help prevent oxidation, which can lead to formation of disulfidelinked multimers and loss of activity (4). We assayed lymphocyte proliferation in the presence or absence of Tat protein. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy HIV-1 seronegative donors and incubated with or without Tat for 1 hour at 37°C. Soluble tetanus toxoid or Candida antigen was then added to the cultures. On day 6, cultures were pulsed with [³H]thymidine and harvested. For mitogen stimulation, 5×10^4 cells were incubated with phytohemagglutinin-P (PHA), concanavalin A (Con A), or Staphylococcus aureus protein A (Sp A) for 3 days or with pokeweed mitogen (PWM) for 7 days.

Tat inhibited the proliferative response to tetanus toxoid by 66 to 97% and the response to Candida antigens by 75 to 91%. Representative data from three individuals are shown in Table 1. Differences in antigen-induced proliferation in the presence or absence of Tat were, in each case, statisticalsignificant (range; P < 0.0055 to lv P < 0.00001; two-tailed t test). Four additional subjects have been tested, and all showed similar responses. The inhibitory activity of Tat was concentration dependent (Fig. 1). Tetanus toxoid-induced proliferation was inhibited 81% with Tat at 10 $\mu g/ml$ and 50% with ${\sim}0.5~\mu g/ml.$ Only a single dose of Tat was needed during the 6day incubation even though the half-life of the protein in tissue culture medium is less than 24 hours (3). The inhibition is unlikely to be due to a cytotoxic effect of the Tat

R. P. Viscidi and K. Mayur, Eudowood Division of Infectious Diseases, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD 21205

H. M. Lederman, Eudowood Division of Immunology, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD 21205. A. D. Frankel, Whitehead Institute for Biomedical Re-

search, Cambridge, MA 02142.

preparation since PHA-induced proliferative responses were not inhibited (Table 1). Other mitogens (Con A, Sp A, and PWM) were also tested and showed no significant inhibition (5). Since PWM requires a 7-day treatment, inhibition cannot simply be due to long-term incubation with Tat. To determine at what time Tat was required for antigen-specific inhibition, we treated PBMC cultures with Tat for 1 hour and washed the cells before stimulation with tetanus. Lymphocyte proliferation was inhibited by 70%; however, when Tat was added to PBMC cultures 1 day after stimulation with tetanus, negligible inhibition was seen (3% inhibition).

Several experiments provided evidence that the immunosuppressive activity was due specifically to Tat. The Tat protein contains a cysteine-rich region that mediates metal-linked dimerization in vitro (4, 6) and is essential for its biological activity as a transactivator (7). Oxidation of the cysteines results in disulfide-linked multimers with decreased biological activity. To test the effect of oxidation, we bubbled 100% O₂ through a Tat protein sample at neutral pH, resulting in almost complete loss of inhibitory activity (Table 2). Incubation of Tat with a polyclonal rabbit antibody to Tat (anti-Tat), but not with preimmune serum, before addition to PBMC cultures resulted in the loss of inhibitory activity for tetanus toxoid-induced proliferative responses (Table 2). A different source of Tat was also tested for inhibition. A synthetic peptide containing Tat residues 1 to 58, which is active for transactivation (8), also inhibited tetanus-stimulated proliferation (Table 2). Concentrations of the peptide ten times as great as for Tat 1-72 were required to inhibit proliferation, correlating with its reduced activity as a transactivator (8). We also tested another Tat protein, which is encoded by the two exons of Tat (residues 1 to 86), to see if there was any functional difference between the two forms of the protein. Both proteins have similar transactivation activity (8). Bacterially expressed Tat 1–86 (0.4 μg per culture) (3) also inhibited tetanus-induced proliferation (55% inhibition) and had no effect on PHAinduced proliferation.

Because Tat is taken up by HeLa cells growing in tissue culture and transactivates the HIV-1 promoter (3), Tat might also directly enter lymphocytes or monocytes, and this might help explain the effect of Tat on lymphocyte proliferation. To test this, we treated H9 lymphoid and U937 promonocytic cells that have an integrated HIV-1 long terminal repeat (LTR)-chloramphenicol acetyl transferase (CAT) reporter with Tat in the presence or absence of chloroquine. Chloroquine has been shown to prevent proteolysis of Tat in HeLa cells (3). Tat concentrations similar to those that inhibited lymphocyte proliferation caused transactivation in the H9 and U937 cells, but in contrast to the effect of chloroquine in HeLa cells, chloroquine had little effect (Fig. 2). The chloroquine-independent entry into lymphocytes and monocytes may reflect differences in cellular metabolism or mechanism of uptake.

Inhibition of antigen-induced proliferation of lymphocytes by Tat represents a novel activity of the protein. However, the mechanism of inhibition is unclear. Proliferation of blood mononuclear cells in response to soluble antigens involves multiple steps that include antigen processing and presentation, T cell activation, and lymphokine secretion (9). The inhibitory effect of

Table 1. Effect of HIV-1 Tat protein on antigen- and mitogen-induced lymphocyte proliferation. PBMCs were isolated by centrifugation over Ficoll-Hypaque. Cells (1×10^5) were incubated in microtiter plate wells with or without Tat $(1.25 \ \mu g/ml)$ for 1 hour at 37°C in an atmosphere of 5% humidified CO₂ in 0.2 ml of RPMI 1640 medium containing 10% autologous serum, $5 \times 10^{-5}M \beta$ -mercaptoethanol, glutamine, penicillin, and streptomycin. Soluble tetanus toxoid $(0.1 - 0.5 \ \mu g/ml)$; Lederle Laboratories) or Candida antigen (1:30 dilution of *Candida albicans* allergenic extract; Greer Laboratories) was then added to the cultures. On day 6, cultures were pulsed for 4 hours with 1 μ Ci of [³H]thymidine. Cells were harvested, and [³H]thymidine incorporation into DNA was measured. For mitogen stimulation, 5×10^4 cells in 0.2 ml of RPMI containing 10% fetal bovine serum were incubated with PHA (Difco Laboratories) for 3 days. Data are mean counts per minute (cpm) ± 1 SD of six replicates. Numbers in parentheses indicate percent inhibition.

Stimulus	[³ H]Thymidine incorporation		
	Subject 1	Subject 2	Subject 3
Tetanus Tetanus + Tat	$\begin{array}{c} 19,874 \pm 4,079 \\ 626 \pm 106 \; (97\%) \end{array}$	$10,916 \pm 1,678 \\ 3,260 \pm 1,125 \ (70\%)$	$\begin{array}{c} 61,789 \pm 3,085 \\ 21,309 \pm 1,767 \; (66\%) \end{array}$
Candida Candida + Tat	$\begin{array}{c} 19,791 \pm 6,304 \\ 1,695 \pm 166 \; (91\%) \end{array}$	$\begin{array}{c} 2,856 \pm 1,355 \\ 702 \pm 146 \; (75\%) \end{array}$	
PHA PHA + Tat	9,793 ± 516 13,125 ± 728 (0%)	$\begin{array}{c} 7,022 \pm 242 \\ 6,454 \pm 432 \; (8\%) \end{array}$	$\begin{array}{c} 20,056 \pm 2,312 \\ 17,835 \pm 2,020 \; (11\%) \end{array}$
Cells alone 3-day culture 6-day culture	224 ± 67 765 ± 232	$436 \pm 86 \\ 581 \pm 190$	$698 \pm 125 \\ 429 \pm 82$
Cells + Tat 3-day culture 6-day culture	221 ± 54 960 ± 163	443 ± 164 1,188 ± 150	392 ± 145 1,171 ± 357

Table 2. Specificity of immunosuppressive effect of Tat. Tat $(1.25 \ \mu g/ml)$, oxidized Tat $(1.25 \ \mu g/ml)$, or Tat $(1.25 \ \mu g/ml)$ treated with rabbit polyclonal anti-Tat or preimmune rabbit serum, was incubated with 1×10^5 PBMCs for 1 hour before addition of tetanus toxoid (TT). Control cultures received tetanus but no Tat. On day 6, [³H]thymidine incorporation was measured. To oxidize Tat, we bubbled 100% O₂ through a protein sample in 10 mM sodium phosphate buffer, *p*H 7.2, and 150 mM NaCl (PBS) for 1 hour at room temperature. Polyclonal anti-Tat was prepared by injecting rabbits with purified Tat (residues 1 to 72; 100 μ g) three times, 2 weeks apart. To measure the effect of rabbit sera, we incubated 0.25 μ g of Tat with 50% serum in 20 μ l of PBS for 2 hours at 37°C before addition to PBMCs. Synthetic Tat 1–58 peptide was synthesized and purified as described (8). Data for oxidation are from one subject and are mean cpm ± 1 SD of quadruplicate replicates. Data for incubation with anti-Tat are from a different subject and are mean cpm ± 1 SD of quintuplicate replicates. Data for incubation with anti-Tat are from a different subject agave similar results. Numbers in parentheses indicate perient inhibition.

Procedure	[³ H]Thymidine incorporation
Experiment 1	
TT	91.849 ± 17.035
$TT + Tat (1.25 \ \mu g/ml)$	$57,503 \pm 14,855$ (37%)
$TT + Tat oxidized (1.25 \mu g/ml)$	$82,940 \pm 18,639$ (10%)
Experiment 2	
TT	$19,920 \pm 6,004$
$TT + Tat (1.25 \ \mu g/ml)$	$1,934 \pm 1,223 (90\%)$
$TT + Tat (1.25 \mu g/ml) + anti-Tat serum$	$10,766 \pm 3,299$ (46%)
$TT + Tat (1.25 \mu g/ml) + preimmune serum$	$3,020 \pm 1,667$ (85%)
Experiment 3	
TT	$18,447 \pm 2,577$
$TT + Tat (2.0 \ \mu g/ml)$	$8,819 \pm 528 (52\%)$
TT + synthetic Tat 1–58 (20 μ g/ml)	$9,729 \pm 1,984 (47\%)$

REPORTS 1607



Fig. 1. Effect of HIV-1 Tat protein on tetanusinduced lymphocyte proliferation. Several concentrations of Tat were incubated with 1×10^5 PBMCs for 1 hour. Tetanus toxoid was then added to the cultures, and cells were grown as described in Table 1. Incorporation of [³H]thymidine was measured on day 6. Results are means ± 1 SD of six replicates.

Tat could occur at any of these steps. Since Tat does not inhibit PHA-induced proliferative responses, it seems reasonable that Tat may interfere with signal transduction via the T cell antigen receptor or with antigen processing or presentation. Alternatively, Tat might induce production of cellular factors or cytokines that mediate the inhibition.

One of the hallmarks of acquired immunodeficiency syndrome (AIDS) is depletion of T4 cells, with the subsequent development of immunodeficiency. However, it is



Fig. 2. Transactivation of the HIV-1 promoter in lymphocytes, promonocytes, and HeLa cells by purified Tat protein. H9 lymphocytes and U937 promonocytes (10⁶ cells) that have an integrated HIV-1 LTR-CAT plasmid [H938 and U38 cells, respectively (12)] were incubated in RPMI 1640 medium containing 10% fetal bovine serum (1 ml in 25-mm wells) at 37°C (no Tat), treated with 5 µg of purified Tat protein (Tat), or treated with 5 $\mu \bar{g}$ of Tat protein and 100 μM chloroquine (Tat + CQ). Cells were harvested 24 hours after Tat treatment and assayed for CAT activity (13). HeLa cells $(10^6$ cells) that have an integrated HIV-1 LTR-CAT plasmid (HL3T1) (12) were incubated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (1 ml in 25-mm wells) and similarly treated with Tat protein, or with Tat and chloroquine, and assayed for CAT activity. Unacetylated (cm) and acetylated (ac) forms of [14C]chloramphenicol were separated by thin-layer chromatography.

does not adequately explain all of the immunopathogenic effects of HIV infection (10). For example, even early in infection, patients' lymphocytes have a defect in their ability to recognize and respond to soluble antigen in vitro, although there are still normal numbers of CD4⁺ T lymphocytes (11). In contrast, lymphocytes from HIV-1infected patients retain the ability to proliferate in response to mitogens. The effect of Tat on in vitro lymphocyte proliferation mimicked this difference between antigenand mitogen-induced proliferation. Under our in vitro conditions, 50 nM Tat was sufficient for 50% inhibition, suggesting that Tat may be a potent immunosuppressive agent. We do not yet know whether Tat must be provided extracellularly or whether Tat produced internally can elicit these effects. If inhibition of lymphocyte function occurs in vivo, Tat produced inside HIVinfected cells may act directly on those cells or Tat may be released extracellularly, perhaps by cell lysis. More studies are needed to determine the biological significance of an immunosuppressive activity of Tat.

recognized that destruction of CD4⁺ cells

REFERENCES AND NOTES

- 1. J. G. Sodroski et al., Science 227, 171 (1985). 2. A. G. Fisher et al., Nature 320, 367 (1986); A. I.
- Dayton et al., Cell 44, 941 (1986). 3. A. D. Frankel and C. O. Pabo, Cell 55, 1189
- (1988)4. A. D. Frankel et al., Science 240, 70 (1988).
- 5. R. P. Viscidi; K. Mayur, H. M. Lederman, A. D.
- Frankel, unpublished observations. 6. A. D. Frankel, L. Chen, R. J. Cotter, C. O. Pabo,
- Proc. Natl. Acad. Sci. U.S.A. 85, 6297 (1988).
- 7. J. A. Garcia et al., EMBO J. 7, 3143 (1988); M. R. Sadaic et al., Proc. Natl. Acad. Sci. U.S.A. 85, 9224 (1988); S. Ruben et al., J. Virol. 63, 1 (1989).
 8. A. D. Frankel, S. Biancalana, D. Hudson, Proc. Natl.
- Acad. Sci. U.S.A. 86, 7397 (1989). 9. A. Weiss et al., Annu. Rev. Immunol. 4, 593 (1986);
- P. A. Allen, Immunol. Today 8, 270 (1987). 10. A. S. Fauci, Science 239, 617 (1988).
- H. C. Lane et al., N. Engl. J. Med. 313, 79 (1985);
 G. M. Shearer et al., J. Immunol. 137, 2514 (1986); R. J. Gurley et al., Proc. Natl. Acad. Sci. U.S.A. 86, 1993 (1989)
- 12. B. K. Felber and G. N. Pavlakis, Science 239, 184 (1988).
- 13. C. M. Gorman, L. F. Moffat, B. H. Howard, Mol. Cell. Biol. 2, 1044 (1982)
- 14. We thank B. Felber and G. Pavlakis for the H938, U38, and HL3T1 cell lines, B. Liu for help with rabbit immunizations, and R. Young and A. Aldovini for helpful comments. This work was supported in part by the Lucille P. Markey Charitable Trust and NIH National Institute of Allergy and Infectious Diseases grant AI29135 (A.D.F.)

7 September 1989; accepted 10 November 1989

Mechanisms for Regulating Expression of Membrane Isoforms of FcyRIII (CD16)

MARGARET L. HIBBS, PERIASAMY SELVARAJ, OLLI CARPÉN, TIMOTHY A. SPRINGER, HELMUT KUSTER, MARIE-HÉLÈNE E. JOUVIN, JEAN-PIERRE KINET

Granulocyte and natural killer (NK) cell Fc receptors for immunoglobulin G (CD16) differ in only a few amino acids, yet have phosphatidylinositol glycan (PIG) or polypeptide membrane anchors, respectively. Mutagenesis shows that anchoring is regulated by a serine residue near the PIG anchor attachment site in the extracellular domain. The NK cell isoform was not expressed on the surface of COS cells unless cotransfected with a subunit that was expressed in NK cells and that was identical to the γ subunit of the high affinity IgE Fc receptor (FceRI). However, the CD16 sequence and not expression of the γ subunit is dominant in regulating PIG reanchoring.

T C RECEPTORS (FCRS) SPECIFIC FOR the Fc domain of immunoglobulin (Ig) play a vital role in the function of the immune system. These receptors belong to the Ig gene superfamily (1). The IgG Fc receptor type III (FcyRIII or CD16) is predominantly found on NK cells, granulocytes, and tissue macrophages and mediates low affinity binding to human IgG1 and IgG3. On granulocytes two alleles, NA1 and NA2, have been described for this receptor. CD16 is anchored to the membrane of granulocytes through a PIG moiety, but on NK cells and tissue macrophages it has a peptide transmembrane anchor (2-10). The type of membrane anchor appears to determine the functional capacity of this receptor, because NK cell CD16 is able to trigger killing and other functions like lymphokine secretion and IL2 receptor expression, whereas granulocyte CD16 is unable to do so (6, 11). There are two similar genes for

M. L. Hibbs, P. Selvaraj, O. Carpén, T. A. Springer, the Center for Blood Research and Department of Patholo-

gy, Harvard Medical School, Boston, MA 02115. H. Kuster, M.-H. E. Jouvin, J.-P. Kinet, Section on Clinical Immunology, National Institutes of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD