rin, and selenium (ITS, 1 ml/liter Collaborative Research, Cambridge, MA). Conditioned medium was collected in a sterile manner on wet ice, clarified by centrifugation, and stored at −70°C. Upon thawing, 0.2- to 0.5-liter aliquots were adjusted to *p*H 3.0 with trifluoracetic acid (TFA) and, at 4°C, sequentially ultrafiltered through 30-kD and 5-kD YM membranes (Amicon Inc., Danvers, MA). The concentrated (50×) YM-5 retentate was buffer-exchanged with TFA in Milli-Q water (*p*H 3.0), lyophilized, dissolved in anion-exchange column equilibration buffer (10 m*M* tris-HCl, 6*M* urea, and 0.01% Tween 80, *p*H 8.0), and separated on a Mono Q HR 5/5 column. The LAI activity recovered in the unbound material was concentrated by Centricon 10 ultrafiltration (Amicon), diluted with cation-exchange buffer (25 m*M* sodium acetate, 6*M* urea, and 0.01% Tween 80, *p*H 5.0), and separated on a Mono S HR 5/5 column. Bound proteins were eluted with a three-stage linear gradient of NaCl in equilibration buffer (0.15*M* NaCl in 5 min, 0.5*M* NaCl in 40 min, 1*M* NaCl in 50 min; flow rate, 0.5 ml/min). Column fractions (2 ml) were prepared for bioassay by spin dialysis against RPMI with bovine albumin (0.4 mg/ml, Cohn Fraction V). Twenty-four–hour conditioned medium was treated similarly, except that YM-30 filtration and lyophilization were omitted and 0.15*M* NaCl was added to the Mono S equilibration buffer.

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7. The LAI activity was quantified by a modified endothelial-leukocyte adhesion assay (*2, 4*). Confluent HEC monolayers in 96-well microtiter plates were preincubated (37°C, 4 hours) with or without rh IL-1β (5 or 10 U/ml) (*6*) and washed, and LAI or control preparations were added. Human polymorphonuclear leukocytes (>97% neutrophils) labeled with 2′,7′-bis-(2-carboxyethyl)-5 (and -6) carboxyfluorescein, acetoxymethyl ester (BCECF) (Molecular Probes, Eugene, OR) were then added (final concentration, 2×10^5 neutrophils per well, final volume, 0.1 ml). After 10 min at 37°C, plates were sealed, inverted, and centrifuged (250*g*, 5 min), and supernatants were removed. Microscopic monitoring indicated that, at this time interval, the majority of adherent leukocytes were attached to the apical surface of the monolayer (*2, 4*). The number of adherent neutrophils was calculated from monolayer-bound fluorescence read in an automated microtiter plate fluorimeter.
8. Electrophoresis of nonreduced samples in 12% acrylamide gels was performed as described by H. Schagger and G. von Jagow [*Anal. Biochem.* **166**, 368 (1987)].
9. Protein samples were sequenced by a modification of the method of P. Edman and G. Begg [*Eur. J. Biochem.* **1**, 80 (1967)] with 0.1*M* Quadrol (*p*H 10.0), phenylisothiocyanate (Beckman Instruments) and TFA (Applied Biosystems) as reagents. Samples were applied in solution to a reversed-phase sequencing column and washed with water before sequencing. The reversed-phase cartridge was then loaded onto a prototype gas-liquid phase sequencer (EP-257735). The 2-anilino-5-thiazoiinone from each cycle was converted to the phenylthiohydantoin derivatives for identification on a Hewlett-Packard 1090 L liquid chromatograph.
10. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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18. IL-8 cDNA, identical in coding sequence to that described by J. Schmid and C. Weissmann [*J. Immunol.* **139**, 250 (1987)], was isolated from a phorbol ester–induced human peripheral blood lymphocyte cDNA library [P. W. Gray *et al.*, *Nature* **312**, 721 (1984)] by screening with a synthetic DNA oligonucleotide probe based on the NH_2-terminal amino acid sequence of IL-8. An 800-bp Hpa II–Nhe I fragment spanning the entire coding region of IL-8 was inserted into the mammalian expression vector pRK5 between the Cla I and the Xba I sites in the multiple cloning region downstream from the cytomegalovirus promotor. The resulting plasmid, pRK.hg.8k, was used to transfect human 293 cells by the $CaPO_4$/DNA precipitation method (10 μg of plasmid DNA/100 mm culture dish). Conditioned medium was harvested after 72 hours and centrifuged to remove cell debris before chromatography on S-Sepharose.
19. Neutrophil-mediated damage was assessed by a modification of the monolayer adhesion assay (*7*). Confluent HEC monolayers (control or after 4-hour treatment with rh IL-1β at 10 U/ml) were washed and incubated with unstimulated neutrophils (100:1, 50:1, 20:1, 10:1 neutrophils to endothelial cells) in RPMI with 1% fetal bovine serum (FBS) at 37°C for 10, 30, 60, 90 or 120 min. Purified endothelial-derived LAI or recombinant IL-8 was added with the neutrophil suspension. The assay was terminated by centrifugation, and the contents of each well were fixed and stained for microscopic evaluation.
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Conserved Repetitive Epitope Recognized by CD4$^+$ Clones from a Malaria-Immunized Volunteer

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T cell clones obtained from a human volunteer immunized with *Plasmodium falciparum* sporozoites specifically recognized the native circumsporozoite (CS) antigen expressed on *P. falciparum* sporozoites, as well as bacteria- and yeast-derived recombinant falciparum CS proteins. The response of these CD4$^+$CD8$^-$ cells was species-specific, since the clones did not proliferate or secrete gamma interferon when challenged with sporozoites or recombinant CS proteins of other human, simian, or rodent malarias. The epitope recognized by the sporozoite-specific human T cell clones mapped to the 5′ repeat region of the CS protein and was contained in the NANPNVDPNANP sequence.

IMMUNIZATION BY EXPOSURE TO THE bites of irradiated malaria-infected mosquitoes induces protective anti-sporozoite immunity in rodents, monkeys, and human volunteers that involves both humoral and cell-mediated effector mechanisms (*1*). Initial efforts to develop a sporozoite malaria vaccine focused on the induction of antibodies directed against an immunodominant B cell epitope, $(NANP)_3$, located within the repeat region of the CS surface protein of *P. falciparum* (*2*). Immunization of human volunteers with an $(NANP)_3$–tetanus toxoid synthetic peptide vaccine induced a modest level of antibodies to sporozoites in 70% of the recipients and some protection against *P. falciparum* sporozoite challenge (*3*). Studies in rodents (*4*) and humans (*5, 6*), however, have since indicated that T cells of most individuals do not recognize the NANP repeats of the CS

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protein. The inclusion of parasite-specific T cell epitopes is required for an effective malaria vaccine, since protection requires both helper (*7*) and cytotoxic (*8*) T cells, as well as gamma interferon (IFN-γ), a potent inhibitor of parasite development within hepatocytes (*9*).

In an effort to characterize the cell-mediated immune (CMI) responses to sporozoites, four volunteers at the Center for Vaccine Development, University of Maryland, were immunized by multiple exposures to the bites of irradiated *Anopheles* mosquitoes infected with the NF54 strain of *P. falciparum* (*10*). Peripheral blood lymphocytes (PBLs) of the three seropositive volunteers proliferated in vitro (stimulation index > 10) when challenged with a yeast-derived recombinant protein (rPfCS) containing 70% of the total *P. falciparum* CS sequence (*11*). The fourth volunteer, who did not develop antibodies to sporozoites or CMI after exposure to a small number of infected mosquitoes, developed an allergy to insects that precluded booster immunizations.

The highest CMI response occurred in a volunteer whose PBLs gave a stimulation index of 4.8 after his first exposure to the bites of 49 *P. falciparum*–infected mosquitoes and reached a peak of 110 after four exposures to a total of 244 infected mosquitoes. A T cell line was derived from this individual by in vitro expansion of his PBLs with rPfCS and interleukin-2 (IL-2). Forty $CD4^+CD8^-$ clones were obtained by limiting dilution (*12, 13*), and five clones were chosen for further characterization.

All clones had a similar pattern of response after challenge with malarial antigens (Table 1). T cell proliferation was obtained after challenge with rPfCS protein but not after challenge with recombinant vivax CS (rPvCS) protein (*14*). The clones also did not respond when challenged with a recombinant CS protein of rodent malaria (*15*) or a control antigen extracted from sham-transformed *Saccharomyces cerevisiae* (*16*).

The clones recognized the native CS protein expressed by the malaria parasite and proliferated when challenged with extracts of as few as 5×10^2 sporozoites per milliliter, corresponding to approximately 100 *P. falciparum* sporozoites per well (Table 1). No proliferation was detected when the T cell clones were challenged with sporozoite extracts of rodent (*P. berghei*) or simian (*P. cynomolgi*) malaria (*16*).

Lymphokine production correlated with antigen-induced proliferation. All of the clones secreted IFN-γ when challenged with the rPfCS or the native CS protein from *P. falciparum* sporozoites. No IFN-γ was detected in supernatants of cultures challenged with CS proteins derived from heterologous species of malaria (Table 1). All of the clones also secreted IL-2 after antigen-specific stimulation with recombinant or native *P. falciparum* CS protein (*16*).

Mapping of the T cell epitope recognized by the sporozoite-specific $CD4^+$ clones was carried out with a series of recombinant CS proteins produced in *Escherichia coli* (*17*). All of the clones proliferated when challenged with a recombinant protein (CSFEC) containing amino acids 103–151 plus 300–408 of the *P. falciparum* CS protein sequence (Fig. 1). In contrast, when the clones were challenged with a recombinant CS protein containing amino acids 103–127 plus 300–408, which lacked the repeat region except for a single NANP sequence, proliferation and lymphokine secretion were reduced to 10% to 13% of the levels obtained with CSFEC (*16*).

The failure to stimulate the clones with the truncated recombinant protein suggested that the T cell epitope was contained

Table 1. Proliferation and IFN-γ production by T cell clones derived from a sporozoite-immunized volunteer. Clone 1C1 or 1C2 (2×10^4 T cells per well) were incubated with 5×10^4 irradiated autologous PBLs as antigen presenting cells (iAPC). After 48 hours, the cultures were treated overnight with 1 μCi of [^{3}H]thymidine. The amount of ^{3}H incorporated (in counts per minute) was determined with a scintillation counter. IFN-γ (U/ml) was measured in 48-hour supernatants with a commercial radioimmunoassay (Centocor, Malvern, PA). The antigens were diluted in complete RPMI 1640 containing 10% AB^+ serum. Recombinant *P. falciparum* CS protein (rPfCS) and recombinant *P. vivax* CS protein (rPvCS) were expressed in transformed yeast (*11, 12*). *P. falciparum* sporozoite (Spz) extracts, obtained by freeze-thawing, were sterilized by irradiation. The Spz extract concentration is expressed as equivalent number of sporozoites per milliliter. The level of [^{3}H]thymidine incorporation and IFN-γ in antigen wells containing only T cells or iAPC alone was not above the background values obtained with medium controls.

Antigen	Amount per milliliter	1C1 + iAPC		1C2 + iAPC	
		^{3}H	IFN-γ	^{3}H	IFN-γ
rPfCS	10.0 μg	6,501	43.0	26,295	74.2
	1.0 μg	1,593	8.4	20,065	62.8
	0.1 μg	521	<0.1	10,341	43.6
rPvCS	10.0 μg	175	<0.1	234	<0.1
P. falciparum Spz	10^4	3,138	21.4	20,271	74.6
	10^3	1,678	9.6	15,365	65.8
	10^2	465	1.2	6,688	26.2
Medium		207	<0.1	166	<0.1

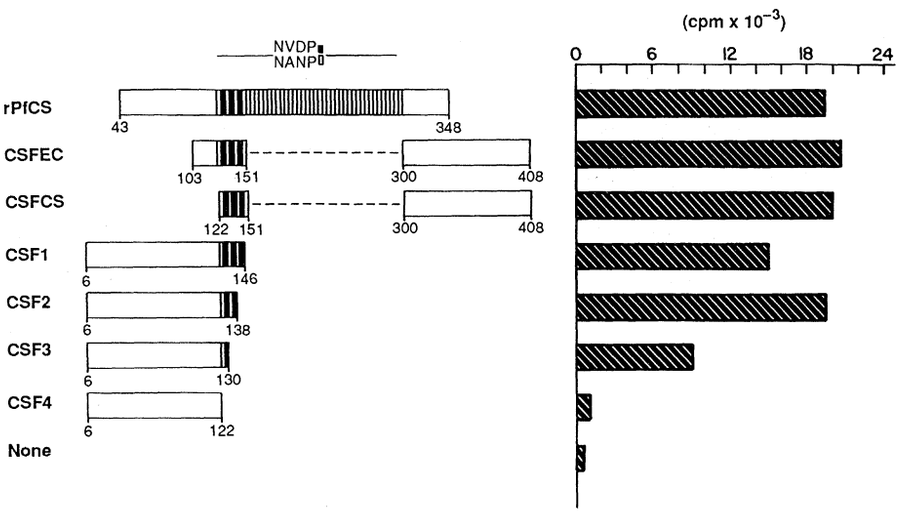

Fig. 1. The proliferative responses to a series of CS recombinant proteins of varying size were assayed as described in Table 1. Incorporation of [^{3}H]thymidine by a representative T cell clone challenged with 2 μg/ml of the different recombinant proteins is shown in the bar graph. The regions of the *P. falciparum* CS protein contained in the different recombinants is illustrated. All of the recombinant CS proteins were derived from the amino acid sequence of the *P. falciparum* T4 isolate (*24*). The recombinant rPfCS was derived from plasmid-transformed yeast (*11*) and contains amino acids 43 to 348 of the *P. falciparum* CS protein, including the entire repeat region $(NANPNVDP)_3$ plus $NANP_{38}$. Bacteria-derived recombinant CS proteins (CSFEC, CSFK, CSFCS, and CSF1 to CSF4) were expressed in *E. coli* as fusion proteins with a polyhistidine peptide $(His)_6$ at the COOH-terminus (*17*). Five of the fusion proteins, CSFCS and CSF1 to CSF4, also contain at their NH_2-terminus 196 amino acids of the mouse dihydrofolate reductase sequence.

within the 5′ repeat region of the CS protein. In order to define the epitope, we challenged the clones with a series of recombinant CS proteins that contained various multiples of the 5′ tandem repeat sequence NANPNVDP (Fig. 1). Challenge with recombinant protein CSFCS (amino acids 122–151 + 300–408) gave proliferative responses comparable to those obtained with CSFEC, an indication that the NH_2-terminal amino acids that precede the repeats were not required for antigen recognition by the T cell clones. Significant proliferation was obtained with CSF1, which lacks amino acids 147 to 151 of the repeat sequence contained in CSFEC and CSFCS. A similar level of proliferation was obtained with CSF2, which contains an incomplete duplicate tandem repeat sequence (NANPNVDPNANPNVD).

In contrast, a significant loss of stimulation occurred if the repeat region was reduced to NANPNVD, as shown by the response to the recombinant protein CSF3. When the repeat region was totally deleted (CSF4), proliferation was reduced to less than 10% of that observed with proteins containing the 5′ repeats (CSFEC and CSFCS).

Identification of the T cell epitope within the 5′ repeat region was confirmed with a synthetic 12–amino acid peptide that contained only the NANPNVDPNANP sequence. All of the clones showed a similar pattern of fine specificity. The response of a representative clone is shown in Table 2. The 12–amino acid synthetic peptide stimulated the T cell clone to the same extent as the rPfCS protein did. In contrast, the $(NANP)_3$ peptide, which contains the immunodominant B cell epitope of the *P. falciparum* CS protein (*2*), did not induce proliferation or interferon production. Furthermore, peptides containing larger multiples of the NANP repeats, $(NANP)_{19}$ and $(NANP)_{50}$, did not stimulate any of the T cell clones (*16*).

Recent studies had shown that the $(NANP)_n$ repeats of the *P. falciparum* CS protein are recognized by PBLs of a small percentage of individuals living in *P. falciparum* malaria endemic areas (*5, 6*), and little or no proliferative response to the $(NANP)_3$ sequence was detected in volunteers immunized with a synthetic peptide vaccine, $(NANP)_3$-TT (*3, 18*). The PBLs obtained from the sporozoite-immunized volunteers in our study recognized neither the $(NANP)_3$ nor the $(NANP)_{50}$ peptide (*19*). Furthermore, the murine immune response to the $(NANP)_n$ sequence is genetically restricted, and only one haplotype ($H\text{-}2^b$) of nine congenic mouse strains developed NANP-specific T cells (*4*).

In view of these findings, previous attempts to define the T cell epitope (or epitopes) on the falciparum CS protein had focused on regions outside of the repeats. Synthetic peptides representing amino acid sequences 5′ and 3′ of the central repeat region, exclusive of the NANPNVDP repeats, were used to examine the CMI responses of individuals living in endemic malaria areas (*5, 6, 20*) and to establish T cell clones from individuals who had not experienced a malaria infection (*6, 21*). Since several of the T cell epitopes that were identified were located in polymorphic regions of the *P. falciparum* CS protein, the possible role of strain-related amino acid variations in the generation of the immune response to *P. falciparum* sporozoites was emphasized (*5, 22*).

No amino acid substitutions have been found in the NANP and NVDP repeats in the *P. falciparum* isolates cloned thus far (*23–26*). Two or more tandem repeats of the NANPNVDP sequence are present in the 5′ repeat region of all isolates, although there are strain variations in the total number of repeats and in the localization of the NVDP sequences. The presence of this epitope in different *P. falciparum* strains is consistent with the ability of the sporozoite-specific T cell clones to recognize a recombinant CS protein based on the CS sequence of the T4 (Thailand) strain, as well as a sporozoite extract of the NF54 (African) strain. Recognition of a nonvariant epitope is also consistent with the strain cross-reactivity of protection noted in earlier reports of sporozoite-immunized volunteers who successfully resisted challenge with *P. falciparum* sporozoites of diverse geographical isolates (*26*).

Earlier studies examined the murine and human T cell response to a bacteria-derived recombinant protein, R32tet32, which contains one copy of the NANPNVDPNANP sequence in addition to multiple NANP repeats (*4, 27–29*). However, R32tet32 is a fusion protein and contains, in addition to the CS sequence, 32 amino acids (tet32) derived from the *E. coli* expression plasmid. The recent identification of both helper (*4*) and suppressor (*29*) epitopes in the bacteria-derived sequence complicates the interpretation of the response to the R32tet32 recombinant protein. In a limited number of studies with a related protein, R32LR, which does not contain the bacterial tet32 sequence, positive CMI responses were obtained with cells from individuals living in an area of endemic malaria (*20*), as well as volunteers immunized with R32tet32 (*28*). Since most human T cells do not recognize an epitope within the NANP repeats (*3, 5, 6, 19*), it is plausible that these proliferating cells may have recognized the NANPNVDPNANP sequence within R32LR.

We recently obtained evidence that the presence of a T cell epitope within the repeat region is not unique to the *P. falciparum* CS protein. Using T cell lines derived from PBLs of a chimpanzee immunized with *P. vivax* sporozoites, we identified a T cell epitope within one of the repeat sequences in the *P. vivax* CS protein (*30*).

In conclusion, T cell clones derived from a *P. falciparum* sporozoite–immunized volunteer were found to be directed against an epitope contained in the NANPNVDPNANP sequence of the 5′ repeat region of the CS protein. This epitope, which is contiguous but not cross-reactive with the immunodominant B cell epitope $(NANP)_3$, has not been previously defined as a T cell epitope. The role of NANPNVDPNANP-specific $CD4^+$ cells in sporozoite immunity, and the frequency with which this conserved epitope is recognized by immune individuals, can now be investigated with T cells from other sporozoite-immunized volunteers and individuals living in areas of endemic *P. falciparum* malaria.

Table 2. Clone specificity determined with synthetic peptides representing the repeat region of *P. falciparum* CS protein. T cells (2×10^4) of clone 1C2 were cocultured with 1×10^4 irradiated (7500 rad) Epstein-Barr virus–transformed autologous B cells (*31*) as antigen presenting cells (iAPC). Proliferation and IFN–assays were carried out as described for Table 1. Recombinant *P. falciparum* CS protein (rPfCS) is described in the legend to Fig. 1. Synthetic peptides were prepared by standard techniques of solid-phase peptide synthesis (*32*). The 12–amino acid synthetic peptides are shown as tetramer subunits of the repeat region of the *P. falciparum* CS protein. ND, not determined.

Antigen	Amount (μg/ml)	1C2 + iAPC		1C2		iAPC	
		^3H	IFN-γ	^3H	IFN-γ	^3H	IFN-γ
rPfCS	20.0	12,799	43.4	1,120	<0.1	684	<0.1
	2.0	9,266	13.8	211	ND	972	ND
NANPNVDPNANP	20.0	17,938	63.8	244	<0.1	742	<0.1
	2.0	19,406	18.2	360	ND	929	ND
	0.2	2,578	0.4	275	ND	820	ND
$(NANP)_3$	20.0	1,003	<0.1	286	<0.1	986	<0.1
	2.0	929	<0.1	209	ND	877	ND
None		942	<0.1	217	<0.1	866	<0.1

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

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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.

THE TAT PROTEIN FROM HUMAN IMmunodeficiency 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 μg/ml in distilled water containing 10 m*M* β-mercaptoethanol. A reducing agent was included to help prevent oxidation, which can lead to formation of disulfide-linked 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 [^{3}H]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, statistically significant (range; $P < 0.0055$ to $P < 0.00001$; 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 μg/ml and 50% with ~0.5 μg/ml. Only a single dose of Tat was needed during the 6-day 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

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