The Journal of Immunology

Norepinephrine Accelerates HIV Replication Via Protein Kinase A-Dependent Effects on Cytokine Production

Steve W. Cole, Yael D. Korin, John L. Fahey and Jerome A. Zack

J Immunol 1998; 161:610-616; ;
http://www.jimmunol.org/content/161/2/610

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Norepinephrine Accelerates HIV Replication Via Protein Kinase A-Dependent Effects on Cytokine Production[1]

Steve W. Cole,[2]*‡§ Yael D. Korin,*¶ John L. Fahey,*†§ and Jerome A. Zack*‡§

To explore the role of sympathetic nervous system activation in HIV pathogenesis, we examined the effect of the neuroeffector molecule norepinephrine (NE) on HIV-1 replication in quiescently infected PBMCs that were subsequently activated with Abs to CD3 and CD28. NE accelerated HIV-1 replication at concentrations ranging from 10^{-8} to 10^{-5} M. This effect could be mimicked by protein kinase A (PKA) activators (forskolin or dibutyryl-cAMP) and abrogated by β-adrenoreceptor antagonists or the PKA inhibitor rp-cAMP, indicating transduction via the adrenoreceptor signaling pathway. NE reduced cellular activation and altered the production of several HIV-modulating cytokines: IL-10 and IFN-γ were markedly suppressed; TNF-α, IL-1β, IL-2, IL-4, and IL-6 were mildly suppressed; and levels of IL-12 were not significantly altered. The addition of either exogenous IFN-γ or IL-10 abrogated the effect of NE on virus production. Thus PKA-dependent suppression of cytokine production appears to mediate the enhancement of HIV-1 replication by NE. ***The Journal of Immunology,*** **1998, 161: 610–616.**

Stress has long been believed to influence viral infections, but the biologic mechanisms underlying these effects remain largely unknown (1, 2). One possible mode of interaction involves sites of direct contact between cells of the peripheral nervous system and cells of the immune system. All primary and secondary lymphoid organs contain the termini of neurons from the sympathetic division of the autonomic nervous system (SNS)[3] (3, 4). When activated by stress or other stimuli, these neurons release micromolar quantities of norepinephrine (NE) into sites that are rich in T lymphocytes and macrophages (3, 5). β_2-adrenoreceptors on the lymphocyte plasma membrane bind NE and signal the cellular interior via the $G_{\alpha S}$ subunit of a heterotrimeric G protein linked to the adenylyl cyclase-cAMP-protein kinase A (PKA) signaling cascade (6–8). Adrenoreceptor ligation alters several aspects of lymphocyte function in vitro, including cellular activation, cytokine production, cell traffic and adhesion, and cytotoxic activity (6, 9–15). However, little is known about the effects of SNS activation on disease pathogenesis.

The innervation of lymphoid tissue by the SNS may be particularly relevant to HIV infection, since lymphoid organs represent the primary site of HIV pathogenesis. Long before the onset of clinical illness, high concentrations of virus and large numbers of infected $CD4^+$ T lymphocytes are present in the lymph node paracortex, splenic periarteriolar lymphoid sheath, and a variety of other secondary lymphoid structures (16–18). These sites are also richly innervated by SNS neurons (3, 4), raising the possibility that SNS activation could conceivably influence HIV pathogenesis. In the present study, we examine the effect of the SNS neuroeffector molecule NE on HIV-1 replication in an in vitro model of $CD4^+$ T lymphocyte infection.

In vivo the vast majority of T lymphocytes are quiescent. These cells are vulnerable to infection by HIV-1 but are incapable of fully reverse-transcribing the viral genome (19, 20). Activation of infected T lymphocytes leads to complete reverse transcription and rapid viral replication (20, 21, 73). To model this process, we infected quiescent PBMCs with a CXCR-4-tropic strain of HIV-1 and activated those cells with Abs to CD3, the signaling component of the T cell receptor, and Abs to CD28, a critical costimulatory molecule (22–25). To examine the effect of SNS activation on HIV-1 replication, NE was added simultaneously with CD3/CD28 costimulation. Previous research indicates that the pharmacologic inducers of cAMP can increase HIV-1 replication in continuously cycling cell lines (26, 27). However, it is not known whether physiologic cAMP inducers such as NE can increase HIV-1 replication or whether such effects occur in normal lymphocytes that are activated in a physiologically relevant manner. In addition, the mechanism by which cAMP affects HIV-1 replication remains unknown. Here, we demonstrate that NE accelerates HIV-1 replication in freshly infected PBMCs. We also show that this effect is transduced via the β-adrenoreceptor-adenylyl cyclase-cAMP-PKA signaling pathway and is mediated by the pronounced suppression of IFN-γ and IL-10.

Materials and Methods

Infection, activation, and cell culture

Healthy donor PBMCs were infected with HIV-1_{NL4-3} (0.05 infectious units/cell) for 2 h in the presence of 10 μg/ml polybrene. Following infection, cells were washed and costimulated with Abs to CD3 (0.1 μg/ml adhered to flask by goat anti-mouse Ab; Southern Biotechnology Associates, Birmingham, AL) and CD28 (0.1 μg/ml soluble; Biodesign, Kennebunkport, ME). Unstimulated control cells received no exogenous Abs. Cells were cultured for 6 to 8 days at 3×10^5/ml in RPMI 1640 supplemented with 10% (v) human AB serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine at 37°C in an atmosphere of 5% CO_2. NE ([-]-Arterenol; Sigma, St. Louis, MO) and other reagents were added once, at activation. Other reagents added at activation included the adenylyl cyclase activator forskolin (Sigma) (28); the membrane-permeable

*University of California at Los Angeles (UCLA) AIDS Institute, †Center for Interdisciplinary Research in Immunology and Disease, and Departments of ‡Medicine, §Microbiology, Immunology, and Molecular Genetics, and ¶Pathology and Laboratory Medicine, UCLA School of Medicine, Los Angeles, CA 90095

Received for publication December 15, 1997. Accepted for publication March 12, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by National Institute of Allergy and Infectious Diseases Grant AI 33259 and by the Norman Cousins Program in Psychoneuroimmunology at UCLA.

[2] Address correspondence and reprint requests to Dr. Steve W. Cole, Division of Hematology-Oncology, Department of Medicine, Factor 11–934, UCLA School of Medicine, Los Angeles, CA 90095-1678. E-mail address: coles @nicco.sscnet.ucla.edu

[3] Abbreviations used in this paper: SNS, sympathetic nervous system; NE, norepinephrine; PKA, protein kinase A; db-cAMP, dibutyryl-cAMP; rp-cAMP.

0022-1767/98/$02.00

cAMP analogue N^6, 2′-*O*-dibutyryl adenosine-3′, 5′-cyclic monophosphate (db-cAMP; Sigma) (29); the membrane permeable PKA antagonist adenosine-3′, 5′-cyclic monophosphothioate, rp isomer (rp-cAMP; Calbiochem, La Jolla, CA) (30); human IFN-γ (R&D Systems, Minneapolis, MN), IL-2 (Genzyme, Cambridge, MA), IL-4 (Genzyme), and IL-10 (R&D Systems). To determine whether the effects of NE were mediated by β-adrenoreceptors, infected PBMCs were incubated in the presence of the receptor antagonist sotalol (ICN Biomedicals, Costa Mesa, CA) or dl-propranolol HCl (ICN Biomedicals) for 1 h before activation in the presence of NE. Cell viability was assessed by trypan blue exclusion and did not differ between NE-treated and untreated cultures at 2, 4, 6, or 8 days postinfection. Cell viability was also not altered by the addition of adrenoreceptor blockers (propranolol, sotalol, phentolamine) or PKA antagonists (rp-cAMP) to NE-treated cultures. Apoptosis rates were assessed by a flow cytometric measurement of annexin V-FITC (Brand Applications) and propidium iodide binding. Apoptosis rates did not differ across cultures of costimulated cells, cultures costimulated in the presence of NE, or NE supplemented by adrenoreceptor blockers or PKA antagonists.

HIV-1 quantification

Virus production was quantified by ELISA for HIV-1 p24 core protein (Coulter, Maastricht, The Netherlands). p24 levels were compared across treatments by ANOVA followed by Duncan's multiple range test for specific comparisons. Dose-response and kinetic profiles were analyzed by linear regression on log p24 values. p24 levels were graphed as the mean plus SE. To confirm the equality of viral inoculation, we also examined the proviral load at 14 h postinfection using PCR (AA55 and M667 primer sequences for the R/U5 region of the viral long terminal repeat, quantified relative to input cell number as indicated by β-globin primers LA1 and LA2) (20). Briefly, one primer of each pair was radio end-labeled, and amplification was conducted for 25 cycles in parallel with control standards consisting of linearized cloned HIV-1 DNA and known quantities of cellular DNA. Radiolabeled, amplified products were resolved on a 6% polyacrylamide gel and quantified by radioanalytic image analysis in comparison with standard curves.

Cytokine quantification and identification of cytokine-producing cells

ELISAs were used to measure the supernatant concentrations of human IL-1β (R&D Systems), IL-2 (Genzyme), IL-4 (Genzyme), IL-6 (Innogenetics, Zwijndrecht, Belgium), IL-10 (Immunotech, Westbrook ME), TNF-α (Genzyme), IFN-γ (Biosource International, Westlake Village, CA), and IL-12 p70; capture Ab 12H4 and detection Ab C8.6 were kindly provided by G. Trinchieri, (Wistar Institute, Philadelphia, PA). Concentrations of each cytokine were determined in parallel at 0, 2, 4, and 6 days postinfection across three independent experiments and graphically expressed as the mean plus SE. Differences in cytokine production were analyzed by the Student *t* test. Total cytokine production was quantified as the area under the curve of the cytokine level over time (as in Fig. 3); the statistical significance of differences in total cytokine production was determined by the Student *t* test.

Cells producing IFN-γ were identified by flow cytometric analysis of intracellular binding of anti-cytokine Abs following a 6-h incubation in the presence of 10 μg/ml brefeldin A (following the protocol of the Ab manufacturer). The $CD4^{+/-}$ and $CD8^{+/-}$ phenotype of cytokine-producing cells was determined by surface staining with anti-CD4 and anti-CD8 Abs (Becton Dickinson, Mountain View, CA) before intracellular cytokine staining. All flow cytometric data were acquired using a FACScan instrument (Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson); dead cells and debris were excluded on the basis of forward scatter vs side scatter profiles.

Assessment of cellular activation

At 3 days postinfection, cellular activation was quantified by 1) the flow cytometric analysis of cell cycle stage via intracellular staining of RNA and DNA, 2) the cell surface expression of CD25 (IL-2R; expressed early in the cell cycle) and CD71 (transferrin receptor; expressed late in the cell cycle) by flow cytometry (Becton Dickinson Immunocytometry protocol), and 3) 6 h of [^{3}H]thymidine uptake. In cell cycle staging, cells were permeabilized with saponin (0.004% w) and stained with 7-amino actinomycin D (Sigma) and pyronin Y (Calbiochem) to quantify intracellular DNA and RNA, respectively (31, 73). Cycling cells move from a 2N DNA/RNA^{low} state (G_0/G_{1A}) through a 2N DNA/RNA^{high} state (G_{1B}) to a 4N DNA/RNA^{high} state (G_2/M). DNA/RNA quadrants were set on the basis of a 2N DNA/RNA^{low} negative control that had been established by activating PBMCs in the presence of 5 mM *N*-butyrate (Sigma), which blocks cell cycle progression in G_{1A} (32). The identification of cycling cells via RNA/DNA staining was highly specific, with <1% of cells falling in the cycling quadrant in the absence of costimulation (vs >65% of costimulated cells). The results of cell cycle staging correlated closely with the expression of the CD25 and CD71 activation Ags and with [^{3}H]thymidine uptake. Dose-response relationships between NE concentration and the fraction of cycling cells were evaluated by linear regression.

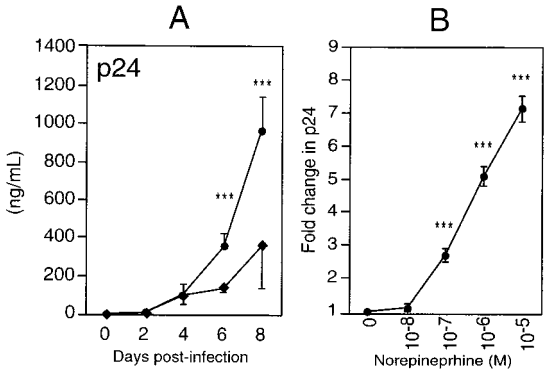

FIGURE 1. Effect of NE on HIV-1 replication. *A*, Kinetics of the effect of NE on HIV-1 replication was assessed by ELISAs of supernatant p24 levels at 0, 2, 4, 6, and 8 days following the infection of quiescent PBMCs and subsequent costimulation by Abs to CD3 and CD28. The simultaneous addition of 10 μM NE (●) at costimulation significantly accelerated viral replication relative to costimulated controls (♦); *d* p24/*dt* = 100.8 ng/ml^{-1} day^{-1} vs 48.9 ng/$ml^{-1}day^{-1}$ in costimulated controls ($p = 0.03$). Data represent the mean plus SE of 3 experiments and are representative of 15 experiments in which day 6 p24 levels in NE-treated cultures averaged 337% of costimulated controls ($p = .0002$). The statistical significance of comparisons at specific time points is indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. *B*, Dose-dependence of the effects of NE on HIV-1 replication was assessed by supernatant p24 levels at 6 days following the infection of quiescent PBMCs and subsequent costimulation in the presence of varying concentrations of NE. Regression analysis indicated a significant dose-response relationship, with p24 levels increasing 2.1-fold per log increase in NE concentration over the range 0.01 to 10 μM ($p = 0.002$).

Results

NE accelerates HIV-1 replication

To examine the effects of NE on HIV-1 replication, p24 levels were assessed at 2, 4, 6, and 8 days postinfection (Fig. 1*A*). As expected, costimulating HIV-1_{NL4-3}-infected PBMCs with Abs to CD3 and CD28 markedly increased viral replication relative to unstimulated cultures. Identical costimulation in the presence of 10 μM NE led to a significant additional increase in HIV replication. This effect emerged reliably across 15 independent experiments, with NE increasing day 6 p24 levels to an average of 337% of costimulated control levels ($p < 0.0001$). In some donors, NE increased day 6 p24 levels by >11-fold. The effect of NE on HIV-1 replication was dose-dependent, with day 6 p24 levels increasing approximately twofold with each log increase in NE concentration over the range 10^{-8}–10^{-5} M (Fig. 1*B*) ($p = 0.002$). These differences emerged despite the fact that a PCR analysis of the HIV-1 proviral load confirmed equivalent initial viral inoculation (no difference in proviral penetrance at 14 h postinfection; data not shown). PCR analysis corroborated p24 ELISA results in documenting increased proviral spread in NE-treated cultures at 6 to 8 days postinfection (data not shown).

Table I. *Effects of PKA-inducing and-blocking agents on HIV replication*[a]

Agent	p24 at 6 Days Postinfection	Difference from Costimulated Control
NE (10^{-5} M)	337.3% (62.5)	$p < .05$
NE + β-adrenergic blocker		
Sotalol (10^{-5} M)	100.4% (16.7)	NS
Propranolol (10^{-7} M)	87.9% (8.7)	NS
NE + α-adrenergic blocker		
Phentolamine (10^{-5} M)	327.6% (70.7)	$p < .05$
PKA-activating agents		
Forskolin (10^{-5} M)	283.7% (184.7)	$p < .05$
db-cAMP (10^{-4} M)	358.4% (291.8)	$p < .05$
NE + PKA inhibitor		
rp-cAMP (10^{-4} M)	109.7% (6.8)	NS

[a] PKA inducers or inhibitors were added postinfection and prior to CD3/CD28 costimulation. Data represent the mean (SE) percent change in p24 levels relative to costimulated controls (equaling 100%) at 6 days postinfection. NS, not significant.

Effect of NE on HIV-1 replication is mediated by the β-adrenoreceptor-adenylyl cyclase-cAMP-PKA signaling pathway

In the classical NE signaling pathway, ligation of the cell surface β-adrenoreceptor activates adenylyl cyclase, which triggers increased synthesis of cAMP and thereby activates the cAMP-dependent kinase PKA. To determine whether the effect of NE on HIV-1 replication is transduced by ligation of the β-adrenoreceptor, infected PBMCs were incubated in the presence of the β antagonists sotalol (10 μM) or propranolol (0.1 μM) for 1 h before activation in the presence of 10 μM NE. Both adrenoreceptor antagonists abrogated the effects of NE on HIV-1 replication, reducing p24 concentrations to levels that were statistically indistinguishable from costimulated control cultures (Table I). In contrast, the α-adrenoreceptor antagonist phentolamine (10 μM) failed to block the enhancement of HIV replication by NE (Table I).

To determine whether activation of the adenylyl cyclase-cAMP-PKA signaling pathway is capable of accelerating HIV-1 replication, infected PBMCs were costimulated in the presence of the adenylyl cyclase activator forskolin (10 μM) or the membrane-permeable cAMP analogue db-cAMP (100 μM). Both of these PKA activators increased p24 concentrations to levels similar to those observed in NE-treated cultures (Table I).

To determine whether the effects of NE on HIV-1 replication were mediated by increased activation of PKA, infected PBMCs were incubated in the presence of the PKA inhibitor rp-cAMP (100 μM) for 1 h before costimulation in the presence of NE. PKA blockade strongly inhibited the effect of NE on HIV-1 replication (Table I), reducing p24 concentrations to levels that were statistically indistinguishable from costimulated control cultures. Thus, the activation of the PKA signaling cascade via the cell surface β-adrenoreceptor appears to mediate the effects of NE on HIV-1 replication.

Effect of NE on HIV-1 replication is not mediated by increased cellular activation

Cellular activation is critical to efficient HIV-1 replication (20, 21). NE and other cAMP-inducing agents partially suppress cellular activation in uninfected PBMCs (9), but their effect on cellular activation in HIV-infected lymphocytes is unknown. A flow cytometric assessment of cell cycling by analysis of intracellular DNA and RNA indicated that NE decreased cellular activation in HIV-1-infected PBMCs (Fig. 2). These effects were dose-dependent, with the fraction of cycling cells declining by an average of 15.3% per log increase in NE concentration over the range 10^{-7} to 10^{-5} M ($p < 0.0001$ across four experiments). Similar effects emerged in flow cytometric analyses of the early CD25 and the late CD71 activation Ags and in measurements of 6-h [^{3}H]thymidine uptake (data not shown). Thus the effect of NE on HIV-1 replication does not appear to be mediated by increased cellular activation in HIV-infected cultures.

NE alters cytokine production profiles

Previous research indicates that NE can suppress the production of IL-2 and IFN-γ by activated (HIV-uninfected) PBMCs and mouse Th1 clones while sparing or enhancing IL-4 production by activated PBMCs and Th2 clones (13, 33). The effects of NE on cytokine production in HIV-infected PBMCs are unknown. However, effects on cytokine production could play a role in altered viral replication, since HIV-1 production can be suppressed by several immunoregulatory cytokines (e.g., IL-10, and IL-12) and enhanced by several other proinflammatory cytokines (e.g., TNF-α, IL-6, and IL-1β) (34–36).

Consistent with the partial suppression of cellular activation by NE, ELISAs of supernatant cytokine concentrations showed a moderate suppressive effect of NE on the production of IL-2 and IL-4 (Fig. 3). The total production of each cytokine over time (area under curve) was reduced by 49% and 64% for IL-2 and IL-4, respectively, with neither suppression reaching statistical significance ($p = 0.32$ and 0.10, respectively). NE similarly suppressed the proinflammatory cytokines IL-1β (by 48% over time, $p = 0.017$), IL-6 (48%, $p = 0.008$), and TNF-α (41%, $p = 0.052$) (Fig. 3). However, the most pronounced

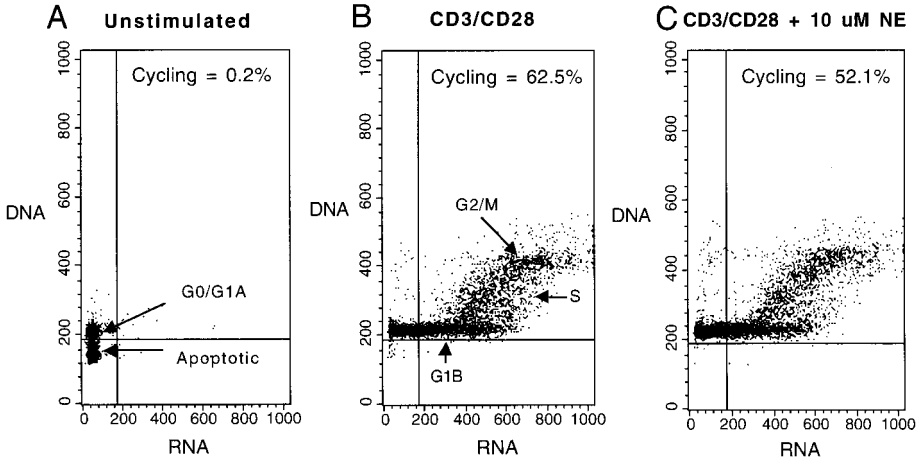

FIGURE 2. Effect of NE on cellular activation in HIV-1-infected PBMCs. Cell cycle stage was determined by flow cytometric assessment of intracellular DNA (vertical axis) and RNA (horizontal axis). Costimulation increased the fraction of cycling lymphocytes (i.e., those outside the G_0/G_{1A} quadrant that are identified by arresting costimulated cells at G_{1A} with 5 mM *N*-butyrate) from a mean of 0.3% (*A*) to 65.2% (*B*). Identical costimulation in the presence of 10 μM NE reduced the cycling fraction to 44.5% (*C*) across four experiments ($p < 0.01$).

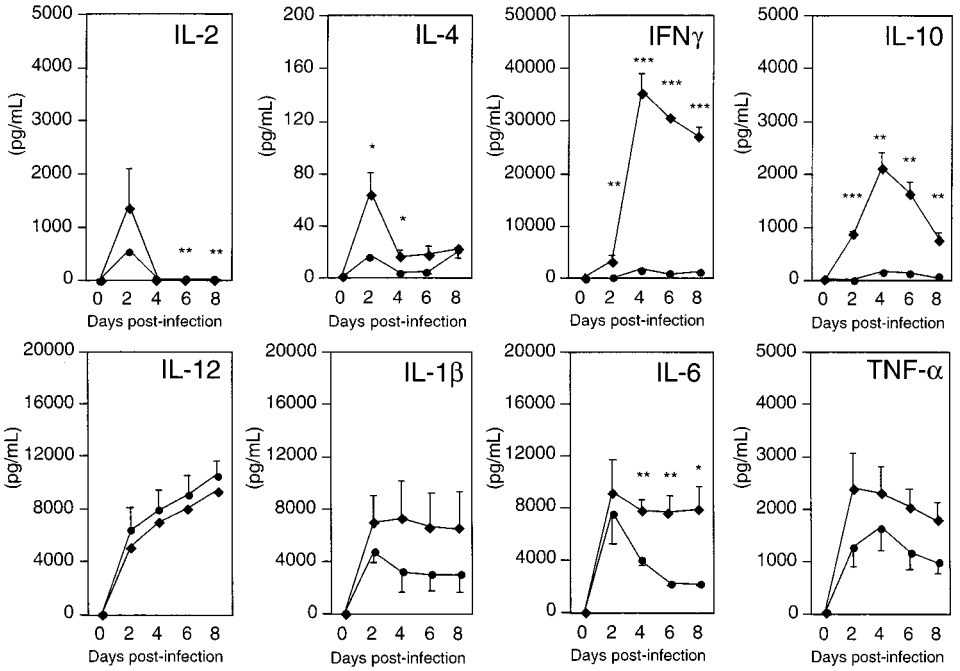

FIGURE 3. Effect of NE on cytokine production. Cytokine levels were determined by ELISA at 0, 2, 4, 6, and 8 days following the infection of quiescent PBMCs and subsequent costimulation in the presence (10 μM) (●) or absence (♦) of NE. NE suppressed the total production of IFN-γ and IL-10 by 96% ($p = 0.006$) and 94% ($p = 0.014$), respectively. NE moderately suppressed the total production of TNF-α, IL-6, and IL-1β ($p \lesssim .05$) and did not significantly alter the total production of IL-2, IL-4, or IL-12 (all $p > 0.10$). The statistical significance of comparisons at specific time points is indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

suppressive effects of NE were on IFN-γ and IL-10 (Fig. 3), with the total production of IFN-γ suppressed by 96% ($p = 0.006$) and the total production of IL-10 suppressed by 94% ($p = 0.014$). In contrast to its suppressive effects on other cytokines, NE did not significantly alter the level of IL-12 produced by HIV-infected PBMCs (Fig. 3) ($p = 0.610$).

To determine whether the pronounced effects of NE on IFN-γ and IL-10 production were mediated by activation of the PKA signaling pathway, we examined the ability of the PKA antagonist rp-cAMP to abrogate NE-induced cytokine suppression. Preincubation with rp-cAMP blocked the NE-induced suppression of IFN-γ by 79% and the NE-induced suppression of IL-10 by 92% across three independent experiments (both $p = 0.008$; data not shown). Concentrations of both cytokines were restored to levels that were statistically indistinguishable from those of costimulated control cultures (both $p > 0.41$). Thus, the suppression of IFN-γ and IL-10 by NE appears to be mediated by the cAMP-PKA signaling pathway.

To determine which cellular sources of IFN-γ were affected by NE, cytokine-producing cells were identified by flow cytometric analysis of intracellular cytokine staining. As shown in Figure 4, NE suppressed IFN-γ production by both $CD4^+$ and $CD8^+$ lymphocytes (*A* and *B*), with 10 μM NE almost totally abrogating the induction of IFN-γ-producing cells (*C–E*).

Effects of NE on HIV-1 replication are mediated by IFN-γ and IL-10

To determine whether altered cytokine production mediates the effects of NE on HIV-1 replication, NE-treated cultures were supplemented with either 500 pg/ml of exogenous IFN-γ or 500 pg/ml of exogenous IL-10 (concentrations similar to those found in costimulated control cultures). The restoration of either cytokine alone was sufficient to completely abrogate the effect of NE on p24 levels (Fig. 5), indicating that the acceleration of HIV-1 replication by NE is mediated by the simultaneous suppression of both IFN-γ and IL-10. This effect is specific to IFN-γ and IL-10, in that the addition of either 500 pg/ml of IL-2 or 50 pg/ml of IL-4 to NE-treated cultures did not suppress viral replication (Fig. 5).

Discussion

In the present study, we find that the SNS neuroeffector molecule NE can accelerate HIV-1 replication by up to 11-fold in acutely infected PBMCs. The effect of NE on viral replication is transduced via the β-adrenoreceptor-adenylyl cyclase-cAMP-PKA signaling cascade and is mediated by a pronounced suppression of IFN-γ and IL-10. The present results confirm previous observations that cAMP can increase HIV-1 replication (26, 27) and identify a basis for those effects in the suppression of HIV-limiting cytokines. Consistent with its partial suppression of cellular activation, NE also moderately suppressed the production of several proinflammatory cytokines that are known to increase HIV-1 replication (IL-1β, IL-6, and TNF-α). However, any replication-retarding effects of reduced cellular activation or proinflammatory cytokine production appear to be offset by the replication-enhancing effects of suppressing IFN-γ and IL-10. The effects of NE on viral replication were dose-dependent over a physiologically relevant range (3, 5), raising the possibility that the activation of SNS neurons innervating lymphoid tissue could conceivably influence HIV disease pathogenesis.

The simultaneous suppression of both IFN-γ and IL-10 was necessary for accelerated HIV-1 replication. Replacement of either cytokine alone abrogated the effects of NE. In humans, both IFN-γ and IL-10 regulate cellular immune responses (37–40). However, altered cellular immunity is not likely to explain the present data, because viral replication differences emerged over periods of time that were too short to support the development of an acquired immune response in vitro (41–43). Both IFN-γ and IL-10 are produced at high rates throughout HIV infection (44–48). Both of these cytokines also regulate HIV-1 replication (35, 49–53), although the mechanisms by which they do so remain incompletely understood (54–57). In the present studies, virus production kinetics lagged behind IFN-γ and IL-10 expression kinetics, even though these cytokines were shown to mediate the effects of NE on viral replication. Because the HIV life cycle takes ~2 days under optimal conditions, effects on virus production can lag up to two

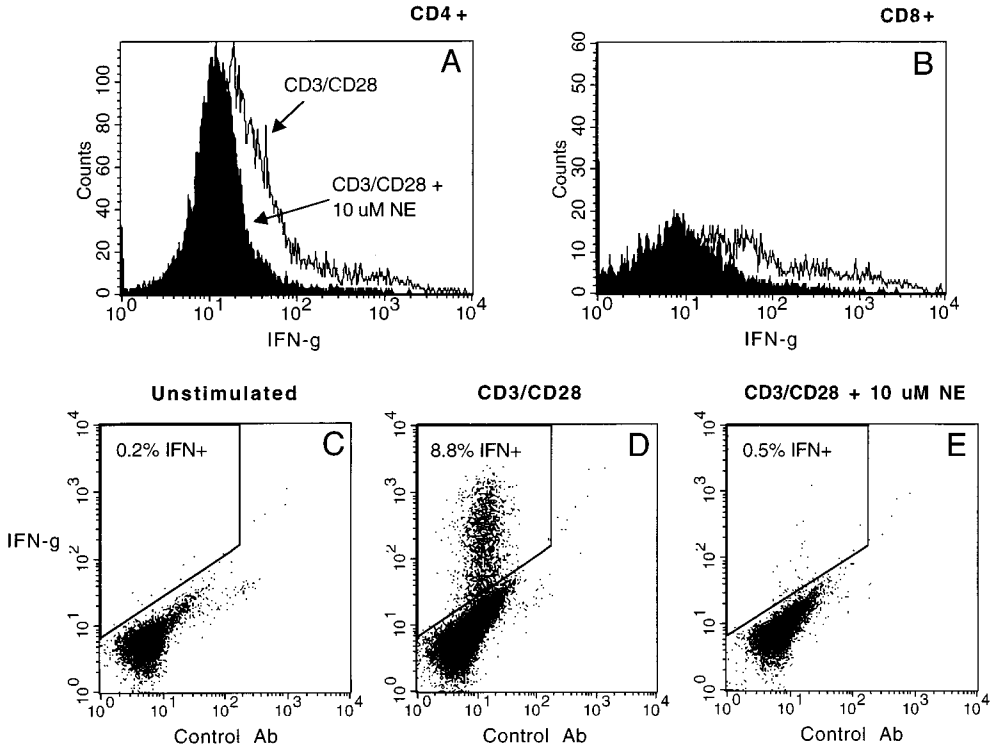

FIGURE 4. Effect of NE on IFN-γ production by $CD4^+$ and $CD8^+$ cells. Cytokine-producing lymphocytes were identified by intracellular staining with anti-cytokine Abs at 3 days following the infection of quiescent PBMCs and subsequent costimulation. *A*, Relative to costimulated controls (open histogram) 10 μM NE (solid histogram) significantly reduced the fraction of $CD4^+$ lymphocytes expressing IFN-γ. *B*, NE similarly reduced IFN-γ expression in $CD8^+$ lymphocytes. Data are representative of three experiments in which NE reduced the fraction of $CD4^+$ cells staining positive for IFN-γ by an average of 68% ($p = 0.016$) and reduced the fraction of $CD8^+$ cells staining positive for IFN-γ by an average of 66% ($p = 0.023$). Because histogram comparisons can confound alterations in cytokine production with alterations in cell size, we used anti-IFN-γ (FL-2) and an irrelevant, isotype-matched control Ab (FL-1) to spatially separate the effects on cell size from effects on cytokine induction. Relative to unstimulated cells (*C*), costimulation markedly increased IFN-γ expression (*D*). Identical costimulation in the presence 10 μM NE (*E*) suppressed the fraction of lymphocytes staining positive for IFN-γ by an average of 82% across three experiments ($p = 0.006$). The higher suppression rate in the later analyses stems from the greater number of costimulated cells identified as IFN-γ^+; this increased identification is the result of the capacity of two-color analyses to distinguish between small IFN-γ^+ cells and nonspecific staining of large cells.

days behind any potential cytokine influences. In addition, cytokine alterations may influence the viral life cycle via downstream events that require further time to develop. The identification of such downstream mediators represents an important focus for further research.

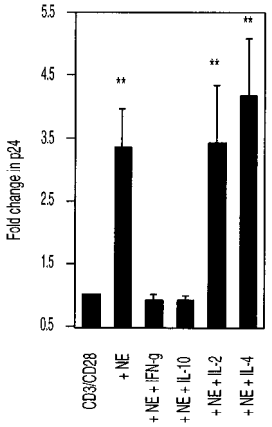

FIGURE 5. Effect of exogenous IL-10 or IFN-γ on NE-induced acceleration in HIV-1 replication. Quiescently infected PBMCs were costimulated in the presence of 10 μM NE or NE plus the indicated cytokines (500 pg/ml human IFN-γ, IL-10, or IL-2; 50 pg/ml human rIL-4). Data represent the mean fold increase in supernatant p24 levels at 6 days postinfection relative to costimulated controls; the statistical significance of comparisons at specific time points is indicated as $^{**}p < 0.01$.

The suppression of IFN-γ by NE appears to occur at the level of the cytokine-producing cell itself (primarily $CD8^+$ and $CD4^+$ T lymphocytes), because the production of IL-12, the major exogenous trigger for IFN-γ production (58–60), was not altered. A direct effect on the cytokine-producing cell is consistent with the presence of a cAMP response element in the IFN-γ promoter (61–63) as well as with the NE-induced suppression of IFN-γ production in isolated murine T cell clones (13). Direct suppressive effects of NE on human IL-10 production have not been demonstrated previously, but the IL-10 promoter does contain a cAMP response element (64). The presence of cAMP-linked β_2-adrenoreceptors on both $CD4^+$ and $CD8^+$ T lymphocytes provides a structural basis for direct effects of NE on cytokine-producing cells (8, 13).

The simultaneous suppression of both IFN-γ and IL-10 by NE is consistent with an emerging body of evidence suggesting that the innervation of lymphoid tissue by SNS may function as a natural mechanism for selectively suppressing cellular immune responses while sparing or enhancing other aspects of immune system function (e.g., humoral immune responses) (33, 65–69). As such, analyzing the mechanisms by which the SNS alters lymphocyte function may lead to novel strategies for selectively manipulating immune responses in HIV infection and other disease settings.

Our interest in the effects of SNS activation on the basic HIV life cycle led us to examine a model of $CD4^+$ T cell infection in the absence of acquired immune responses (e.g., CD8 antiviral factors or HIV-specific CTLs) (41–43). Basic viral replication rates play a critical role in driving HIV pathogenesis and long-term disease progression (70). The present data indicate that the products of SNS activation can alter HIV-1 replication in activated PBMCs. Such effects underscore the critical role of immunomodulatory factors in altering HIV replication (35) and raise the possibility that the course of HIV could conceivably be affected by factors associated with SNS activation (e.g., physical or psychologic stress) (71, 72). Such results also suggest that it may be productive to address immunoregulatory influences from outside the Ag-immune system axis in attempts to suppress HIV replication over the extended periods of time that are necessary for sustained clinical benefit.

Acknowledgments

We thank Najib Aziz and Pari Nishanian for their assistance and G. Trinchieri for providing anti-IL-12 Abs.

References

1. Cohen, S., D. A. J. Tyrrell, and A. P. Smith. 1991. Psychological stress and susceptibility to the common cold. *N. Engl. J. Med. 325:606.*
2. Cohen, S., and G. M. Williamson. 1991. Stress and infectious disease in humans. *Psychol. Bull. 109:5.*
3. Felten, D. L., S. Y. Felten, D. L. Bellinger, S. L. Carlson, K. D. Ackerman, K. S. Madden, J. A. Olschowka, and S. Livnat. 1987. Noradrenergic sympathetic neural interactions with the immune system: structure and function. *Immunol. Rev. 100:225.*
4. Felten, D. L., S. Y. Felten, L. S. Carlson, J. A. Olschowka, and S. Livnat. 1985. Noradrenergic and peptidergic innervation of lymphoid tissue. *J. Immunol. 134: 755s.*
5. Shimizu, N., T. Hori, and H. Nakane. 1994. An interleukin-1β-induced noradrenaline release in the spleen is mediated by brain corticotropin-releasing factor: an in vivo microdialysis study in conscious rats. *Brain Behav. Immun. 7:14.*
6. Kammer, G. M. 1988. The adenylate cyclase-cAMP-protein kinase A pathway and regulation of the immune response. *Immunol. Today 9:222.*
7. Kobilka, B. 1992. Adrenergic receptors as models for G protein-coupled receptors. *Annu. Rev. Neurosci. 15:87.*
8. Landmann, R. 1992. Beta-adrenergic receptors in human leukocyte subpopulations. *Eur. J. Clin. Invest. 22:S1:30.*
9. Bourne, H. R., L. M. Lichtenstein, K. L. Melmon, C. S. Henney, Y. Weinstein, and G. M. Shearer. 1974. Modulation of inflammation and immunity by cyclic AMP. *Science 184:19.*
10. Crary, B., S. L. Hauser, M. Borysenko, I. Kutz, C. Hoban, K. A. Ault, H. L. Weiner, and H. Benson. 1984. Epinephrine-induced changes in the distribution of lymphocyte subsets in peripheral blood of humans. *J. Immunol. 131: 1178.*
11. Ottaway, C. A., and A. J. Husband. 1992. Central nervous system influences on lymphocyte migration. *Brain Behav. Immun. 6:97.*
12. Ottaway, C. A., and A. J. Husband. 1994. The influence of neuroendocrine pathways on lymphocyte migration. *Immunol. Today 15:511.*
13. Sanders, V. M., R. A. Baker, D. S. Ramer-Quinn, D. J. Kasprowicz, B. A. Fuchs, and N. E. Street. 1997. Differential expression of the β_2-adrenergic receptor by Th1 and Th2 clones: implications for cytokine production and B cell help. *J. Immunol. 158:4200.*
14. Van Tits, L. J., M. C. Michel, H. Grosse-Wilde, M. Happel, F. W. Eigler, A. Soliman, and O. E. Brodde. 1990. Catecholamines increase lymphocyte β_2-adrenergic receptors via a β_2-adrenergic, spleen-dependent process. *Am. J. Physiol. 258:E191.*
15. Katz, P., A. M. Zaytoun, and A. S. Fauci. 1982. Mechanisms of human cell-mediated cytotoxicity: modulation of natural killer cell activity by cyclic nucleotides. *J. Immunol. 129:287.*
16. Embertson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz, and A. T. Haase. 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature 362:359.*
17. Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci. 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature 362:355.*
18. Haase, A. T., K. Henry, M. Zupancic, G. Sedgewick, R. A. Faust, H. Melroe, W. Cavert, K. Gebhard, K. Staskus, Z. Zhang, P. J. Dailey, H. H. Balfour, A. Erice, and A. S. Perelson. 1996. Quantitative image analysis of HIV-1 infection in lymphoid tissue. *Science 274:985.*
19. Sun, Y., L. M. Pinchuk, M. B. Agy, and E. A. Clark. 1997. Nuclear import of HIV-1 DNA in resting $CD4^+$ T cells requires a cyclosporin A-sensitive pathway. *J. Immunol. 158:512.*
20. Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Y. Chen. 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell 61:213.*
21. McDougal, J. S., A. Mawle, S. P. Cort, J. K. A. Nicholson, G. D. Cross, J. A. Scheppler-Campbell, D. Hicks, and J. Sligh. 1985. Cellular tropism of the human retrovirus HTLV-III/LAV: role of T cell activation and expression of the T4 antigen. *J. Immunol. 135:3151.*
22. Asjo, B., D. Cefai, P. Debre, Y. Dudoit, and B. Autran. 1993. A novel mode of human immunodeficiency virus type 1 (HIV-1) activation: ligation of CD28 alone induces HIV-1 replication in naturally infected lymphocytes. *J. Virol. 67:4395.*
23. Pinchuk, L. M., P. S. Polacine, M. B. Agy, S. J. Lkaus, and E. A. Clark. 1994. The role of CD40 and CD80 accessory cell molecules in dendritic cell-dependent HIV infection. *Immunity 1:317.*
24. Roederer, M., P. A. Raju, D. K. Mitra, L. A. Herzenberg, and L. A. Herzenberg. 1997. HIV does not replicate in naive CD4 T cells stimulated with CD3/CD28. *J. Clin. Invest. 99:1555.*
25. Smithgall, M. D., J. G. P. Wong, P. S. Linsley, and O. K. Haffar. 1995. Costimulation of $CD4^+$ T cells via CD28 modulates human immunodeficiency virus type 1 infection and replication in vitro. *AIDS Res. Hum. Retrovir. 11:885.*
26. Nokta, M. A., and R. B. Pollard. 1992. Human immunodeficiency virus replication: modulation by cellular levels of cAMP. *AIDS Res. Hum. Retrovir. 8:1255.*
27. Chowdhury, M. I., Y. Koyanagi, S. Horiuchi, O. Hazeki, M. Ui, K. Kitano, D. W. Golde, K. Takada, and N. Yamamoto. 1993. cAMP-stimulated human immunodeficiency virus (HIV-1) from latently infected cells of monocyte-macrophage lineage: synergism with TNF-α. *Virology 194:345.*
28. Seamon, K. B., W. Padgett, and J. W. Daly. 1981. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. Natl. Acad. Sci. USA 78:3363.*
29. Strom, T. B., A. P. Lundlin, and C. B. Carpenter. 1977. The role of cyclic nucleotides in lymphocyte activation and function. In *Progress in Immunology*. Vol. 3. R. S. Schwartz, ed. Grune and Stratton, New York, p. 115.
30. Parker-Bothelo, L. H., J. D. Rothermel, R. V. Coombs, and B. Jastorff. 1988. cAMP analog antagonists of cAMP action. *Methods Enzymol. 159:159.*
31. Toba, K., E. F. Winton, T. Koike, and A. Shibata. 1995. Simultaneous three-color analysis of surface phenotype and DNA-RNA quantitation using 7-amino-actinomycin D and pyronin Y. *J. Immunol. Methods 382:193.*
32. Gilbert, K. M., and W. O. Weigle. 1993. Th1 cell cycle anergy and blockade in G_{1A} phase of the cell cycle. *J. Immunol. 151:1245.*
33. Elenkov, I. J., D. A. Papanicolaou, D. L. Wilder, and G. P. Chrousos. 1996. Modulatory effects of glucocorticoids and catecholamines on human interleukin-12 and interleukin-10 production: clinical implications. *Proc. Assoc. Am. Physicians 108:374.*
34. Fahey, J. L., and R. Mitsuyasu. 1996. Immunomodulatory approaches to the therapy of AIDS. *Clin. Immunother. 6:39.*
35. Fauci, A. S. 1996. Host factors and the pathogenesis of HIV-induced disease. *Nature 384:529.*
36. Guenounou, M. 1997. *HIV and Cytokines.* Les Editions Institut National de la Santé et de la Recherche Médicale, Paris.
37. Del Prete, G., M. De Carli, F. Almerigogna, M. G. Guidici, R. Biagiotti, and S. Romagnani. 1993. Human IL-10 is produced by both type 1 helper T clones and type 2 helper T clones and inhibits their antigen-specific proliferation and cytokine production. *J. Immunol. 150:353.*
38. Moore, K. W., A. O'Garra, R. W. Malefyt, P. Vieira, and T. R. Mossman. 1993. Interleukin-10. *Annu. Rev. Immunol. 11:165.*
39. Romagnani, S. 1992. Human Th1 and Th2 subsets: regulation of differentiation and role in protection and immunopathology. *Int. Arch. Allergy Appl. Immunol. 98:279.*
40. Sornasse, T., P. V. Larenas, K. A. Davis, J. E. de Vries, and H. Yssel. 1996. Differential stability of T helper 1 and 2 cells derived from naive human neonatal $CD4^+$ T cells, analyzed at the single-cell level. *J. Exp. Med. 184:473.*
41. D'Souza, M. P., and B. J. Mathieson. 1996. Early phases of HIV type 1 infection. *AIDS Res. Hum. Retrovir. 12:1.*
42. Jassoy, C., and B. D. Walker. 1997. HIV-1-specific cytotoxic T lymphocytes and the control of HIV-1 replication. *Springer Semin. Immunopathol. 18:341.*
43. Levy, J. A., C. E. Mackewicz, and E. Barker. 1996. Controlling HIV pathogenesis: the role of the noncytotoxic anti-HIV response of $CD8^+$ T cells. *Immunol. Today 17:217.*
44. Breen, E. C., J. F. Salazar-Gonzalez, L. P. Shen, J. A. Kolberg, M. S. Urdea, O. Martinez-Maza, and J. L. Fahey. 1997. Circulating CD8 T cells show increased interferon-gamma mRNA expression in HIV infection. *Cell. Immunol. 178:91.*
45. Fan, J., H. Z. Bass, and J. L. Fahey. 1993. Elevated IFN-γ and decreased IL-2 gene expression are associated with HIV infection. *J. Immunol. 151:5031.*
46. Emilie, D., M. Peuchmaur, M. C. Maillot, M. C. Crevon, N. Brousse, J. F. Delfraissy, J. Dormont, and P Galanaud. Production of interleukins in human immunodeficiency virus-1-replicating lymph nodes. *J. Clin. Invest. 86:148.*
47. Graziosi, C., G. Pantaleo, K. R. Gantt, J. Fortin, J. F. Demarest, O. J. Cohen, R. P. Sekaly, and A. S. Fauci. 1994. Lack of evidence for the dichotomy of TH1 and TH2 predominance in HIV-infected individuals. *Science 265:248.*
48. Maggi, E., M. Mazzetti, A. Ravina, F. Annunziato, M. De Carli, M. P. Piccinni, R. Manetti, M. Carbonari, A. M. Pesce, G. Del Prete, and S. Romagnani. 1994. Ability of HIV to promote a TH1 to TH0 shift and to replicate preferentially in TH2 and TH0 cells. *Science 265:244.*
49. Emilie, D., M. C. Maillot, J. F. Nicolas, R. Fior, and P. Galanaud. 1992. Antagonistic effect of interferon-γ on tat-induced transactivation of HIV long terminal repeat. *J. Biol. Chem. 267:20565.*

50. Koyanagi, Y., W. A. O'Brien, J. Q. Zhao, D. W. Golde, J. C. Gasson, and I. S. Y. Chen. 1988. Cytokines alter production of HIV-1 from primary mononuclear phagocytes. *Science 241:1673.*
51. Saville, M. W., K. Taga, A. Foli, S. Broder, G. Tosato, and R. Yarchoan. 1994. Interleukin-10 suppresses human immunodeficiency virus-1 replication in vitro in cells of the monocyte/macrophage lineage. *Blood 83:3591.*
52. Uittenbogaart, C. H., D. J. Anisman, J. A. Zack, A. Economides, I. Schmid, and E. F. Hays. 1995. Effects of cytokines on HIV-1 production by thymocytes. *Thymus 23:155.*
53. Weissman, D., G. Poli, and A. S. Fauci. 1994. Interleukin 10 blocks HIV replication in macrophages by inhibiting the autocrine loop of tumor necrosis factor alpha and interleukin 6 induction of virus. *AIDS Res. Hum. Retrovir. 10:1199.*
54. Constantoulakis, P., M. Campbell, B. K. Felber, G. Nasioulas, E. Afonina, and G. N. Pavlakis. 1993. Inhibition of Rev-mediated HIV-1 expression by an RNA binding protein encoded by the interferon-inducible 9–27 gene. *Science 259: 1314.*
55. Francis, M. L., M. S. Meltzer, and H. E. Gendelman. 1992. Interferons in the persistence, pathogenesis, and treatment of HIV infection. *AIDS Res. Hum. Retrovir. 8:199.*
56. Pitha, P. M. 1991. Multiple effects of interferon on HIV-1 replication. *J. Interferon Res. 11:313.*
57. Hovanessian, A. G., B. Krust, and E. M. Coccia. 1997. On the mechanism of anti-HIV action of interferon. In *HIV and Cytokines.* M. Guenounou, ed. Les Editions Institut National de la Santé et de la Recherche Médicale, Paris, pp. 465–470.
58. O'Garra, A., and K. Murphy. 1995. Role of cytokines in determining T lymphocyte function. *Curr. Opin. Immunol. 6:458.*
59. Seder, R. A., and W. E. Paul. 1994. Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. *Annu. Rev. Immunol. 12:635.*
60. Trinchieri, G. 1995. Interleukin 12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and adaptive immunity. *Annu. Rev. Immunol. 13:251.*
61. Aune, T. M., L. A. Penix, M. R. Rincon, and R. A. Flavell. 1997. Differential transcription directed by discrete gamma interferon promoter elements in naive and memory (effector) CD4 T cells and CD8 T cells. *Mol. Cell. Biol. 17:199.*
62. Cippitelli, M., A. Sica, V. Viggiano, J. Ye, P. Ghosh, M. J. Birrer, and H. A. Young. 1995. Negative transcriptional regulation of the interferon-gamma promoter by glucocorticoids and dominant negative mutants of c-Jun. *J. Biol. Chem. 270:12548.*
63. Penix, L. A., M. T. Sweetser, W. M. Weaver, J. P. Hoeffler, T. K. Kerppola, and C. B. Wilson. 1996. The proximal regulatory element of the interferon-γ promoter mediates selective expression in T cells. *J. Biol. Chem. 271:31964.*
64. Platzer, C., C. Meisel, K. Vogt, M. Platzer, and H. D. Volk. 1995. Up-regulation of monocytic IL-10 by tumor necrosis factor-alpha and cAMP-elevating drugs. *Int. Immunol. 7:517.*
65. Ader, R., N. Cohen, and D. Felten. 1995. Psychoneuroimmunology: interactions between the nervous system and the immune system. *Lancet 345:99.*
66. Besedovsky, H. O., and A. del Rey. 1992. Immune-neuroendocrine circuits: integrative role of cytokines. *Front. Neuroendocrinol. 13:61.*
67. Esquifino, A. I., and D. P. Cardinali. 1994. Local regulation of the immune response by the autonomic nervous system. *Neuroimmunomodulation 1:265.*
68. Fuchs, B. A., K. S. Campbell, and A. E. Munson. 1988. Norepinephrine and serotonin content of the murine spleen: its relationship to lymphocyte beta-adrenergic receptor density and the humoral immune response in vivo and in vitro. *Cell. Immunol. 117:339.*
69. Sanders, V. M., and F. E. Powell-Oliver. 1992. β_2-adrenoreceptor stimulation increases the number of antigen-specific precursor B lymphocytes that differentiate into IgM-secreting cells without affecting burst size. *J. Immunol. 148:1822.*
70. Mellors, J. W., C. R. Rinaldo, Jr., P. Gupta, R. M. White, J. A. Todd, and L. A. Kingsley. 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science 272:1167.*
71. Cole, S. W., and M. E. Kemeny. 1997. Psychobiology of HIV infection. *Crit. Rev. Neurobiol. 11:289.*
72. Cole, S. W., M. E. Kemeny, S. E. Taylor, B. R. Visscher, and J. L. Fahey. 1996. Accelerated course of human immunodeficiency virus infection in gay men who conceal their homosexual identity. *Psychosom. Med. 58:219.*
73. Korin, Y. D., and J. A. Zack. 1998. Progression to the G_{1B} stage of the cell cycle is required for completion of HIV-1 reverse transcription in T cells. *J. Virol. 72:3161.*