Norepinephrine Enhances Adhesion of HIV-1-Infected Leukocytes to Cardiac Microvascular Endothelial Cells

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Recent reports have indicated that norepinephrine (NE) enhances HIV replication in infected monocytes and promotes increased expression of select matrix metalloproteinases associated with dilated cardiomyopathy (DCM) *in vitro* in co-cultures of HIV-infected leukocytes and human cardiac microvascular endothelial cells (HMVEC-C). The influence of NE on HIV infection and leukocyte-endothelial interactions suggests a pathogenic role in AIDS-related cardiovascular disease. This study examined the effects of norepinephrine (NE) and HIV-1 infection on leukocyte adhesion to HMVEC-C. Both flow and static conditions were examined and the expression of selected adhesion molecules and cytokines were monitored in parallel. NE pretreatment resulted in a detectable, dose-dependent increase of leukocyte-endothelial adhesion (LEA) with both HIV-1-infected and -uninfected peripheral blood mononuclear cells (PBMCs) relative to media controls after 48 hr in co-culture with HMVEC-C *in vitro*. However, the combination of NE plus HIV infection resulted in a significant ($P < 0.0001$) 18-fold increase in LEA over uninfected media controls. Increased levels in both cell-associated and -soluble ICAM-1 and E-Selectin but not VCAM-1 correlated with increased LEA and with HIV-1 infection or NE pretreatment. Blocking antibodies specific for ICAM-1 or E-Selectin inhibited HIV-NE-induced LEA. These data suggest a model in which NE primes HIV-1-infected leukocytes for enhanced adhesion and localization in HMVEC-C where they can initiate and participate in vascular injury associated with AIDS-related cardiomyopathy. Exp Biol Med 228:730–740, 2003

Key words: HIV; norepinephrine; leukocytes; endothelial cells; heart disease

Since early reports during the AIDS epidemic in North America (1), there has been an increase in the reported number of cases of AIDS-related cardiovascular disease (CVD). It has been proposed that the introduction of more effective antiretroviral therapies and the increasing number of newly diagnosed cases of AIDS have contributed to the overall increase in the number and variety of AIDS-related chronic diseases, including CVD (2). It is known that HIV-1 infection promotes leukocyte interactions with the vascular endothelium, primarily by causing enhanced leukocyte-endothelial adhesion (LEA) (3–6). Increased LEA has been associated with and implicated in the pathogenesis of a number of the AIDS-related cardiovascular complications, including myocarditis, perivascular disease, dilated cardiomyopathy (DCM), CVD, pulmonary hypertension, and Kaposi's sarcoma (2, 7–10).

The pathogenesis arising from leukocyte-endothelial interactions, which drive viral replication (11–13) and may contribute to AIDS-related heart disease, can evolve along several separate and overlapping pathways. These pathways may involve cytokines expressed by virally infected T cells that mediate endothelial cell (EC) activation, increased production of nitric oxide (NO) (14–17), and the expression of natriuretic peptides (18, 14), resulting in cardiotoxicity (19–22). AIDS-related heart disease can also be mediated by the actions of HIV-related viral proteins. HIV-1 TAT protein expressed by virally infected leukocytes induces angiogenesis and has been implicated in the development of Kaposi's sarcoma (23, 24). In addition, the HIV-gp-120/160 env glycoprotein has been shown to induce apoptosis of ECs, thus contributing to HIV-1-induced vasculopathies (25, 26). HIV-1 infection can also result in the increased expression of matrix metalloproteinases (MMPs), which participate in cardiac tissue remodeling involved in DCM (27,28). In addition, leukocyte-EC interactions have been shown to induce monocyte activation, resulting in an increase in HIV-1 replication within monocytes (29).

AIDS-related CVD, as well as HIV disease progression, may also be aggravated by stress-associated auto-

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nomic nervous system (ANS) neurotransmitters, e.g., norepinephrine (NE) (30). NE has been shown to enhance HIV replication by stimulation through β-adrenoceptors expressed on infected monocytes(31). Furthermore, certain coexisting conditions, e.g., cocaine abuse, which is often associated with HIV infection (32, 33), have also been shown to contribute to ischemic heart disease by mediating increased levels of NE (34–36). NE mediates direct cardiotoxic effects by regulating vasoconstriction through its binding to α-adrenoreceptors on cardiovascular smooth muscle cells and by regulating chronotropic and ionotropic contractility responses through its binding to β-adrenoreceptors on cardiac myocytes. Chronic stimulation of adrenoreceptors by NE leads to cardiac dysfunction by causing ischemia and by causing elevated levels of cAMP in cardiac myocytes, resulting in hypertrophy or apoptosis (37). Furthermore, NE, HIV infection, and leukocyte interactions with cardiac microvascular ECs demonstrate overlapping cooperative effects on the enhanced expression of MMPs associated with AIDS-related cardiomyopathies (38, 28). However, the potentiating effects of NE on interactions of HIV-1-infected leukocytes and the cardiac vascular endothelium in models of AIDS-related CVD have not been studied.

In a preliminary effort to address this issue, we carried out studies to test the hypothesis that NE enhances HIV-1-induced leukocyte adhesion to ECs. The results of our studies reported herein indicate that the combination of HIV infection and NE pretreatment significantly enhanced leukocyte adhesion to primary cultures of autologous cardiac microvascular ECs and correlated with increased expression of select adhesion molecules by microvascular ECs in the co-culture system. Taken together, these data provide further support for a pathogenic role for NE in AIDS-related CVD.

Materials and Methods

Reagents and Antibodies. The following polyclonal and monoclonal antibodies to human cytokines and cell surface antigens that were used in this investigation for immunophenotyping and functional studies are described below: mouse (Mo)-anti-CD11a (clone 38), Mo-anti CD11b (clone ICRF44), Mo-antiCD15s (clone AHN1.1), Mo-anti-CD31 (clone 1582B3), Mo-anti-CD40 (clone EA5), Mo-antiCD49d (Clone BU49), Mo anti-CD54 (clone 15.2), Mo-anti-CD62-E (clone 1.2B6), Mo-anti-CD62P (clone G1/G1-4), Mo anti-CD106 (clone 1.G11B1), Mo anti-CD154 (clone 24-31; Ancell, Bayport, MN), sheep anti-von Willebrand Factor (vWF; Biodesign, Kennebunk, ME), rabbit anti-ZO-1 (Zymed, San Francisco, CA), and ac-LDL-DiI (Molecular Probes, Eugene, OR).

HIV-1 Infection of Peripheral Blood Mononuclear Cells (PBMCs). Ficoll-Hypaque-purified PBMCs collected from healthy individuals were adjusted to 10^6/ml in RPMI 1640 media containing 10% fetal bovine serum (FBS), 2 m*M* L-glutamine, and 50 μg/ml gentamicin, and were then incubated with 2 μg/ml phytohemagglutinin (Sigma Chemicals, St. Louis, MO) for 48 hr at 37°C in a 7% CO_2 humidified atmosphere. The resulting activated PBMCs were then washed, resuspended at 10^6/ml, and incubated overnight in complete media or complete media containing HIV-1_{LAV} at a multiplicity of infection (MOI) of approximately 0.005. After virus absorption, the infected PBMCs were washed to remove cell-free virus and were resuspended in fresh complete media and incubated under the same culture conditions in co-culture experiments described below.

Isolation of Microvascular Endothelial Cells from the Heart. Cardiac human microvascular endothelial cells (HMVEC-Cs) were isolated and purified from the explanted hearts of transplant recipients at Emory University Hospital using a variation of a previously described method (39). Briefly, a two-square-inch transmural myocardial section from the left ventricle was diced and covered with sterile Dispase I, 0.6 U/ml in PBS, and was then incubated for 60 min at 37°C. The HMVEC-Cs were then sterilely expressed from the dispase-digested heart muscle by mechanical depression with the blunt end of a sterile plastic disposable syringe. The disaggregated microvascular cells were then washed twice with Hank's balanced salt solution (HBSS) containing 5% FBS and antibiotics. The cell suspension was then adjusted to 10^7 cells/ml and the HMVEC-Cs were positively selected using immunomagnetic beads coated with anti-CD31 antibody (Dynal, Lake Success, NY). The enriched HMVEC-Cs were then seeded into vented tissue culture flasks precoated with 0.2% sterile gelatin (Sigma) and expanded in EGM-2MV media (Clonetics, Walkersville, MD). In some cases, positive selection was repeated to enhance purity of the HMVEC-C population. Experiments performed to characterize and compare the levels of expression of select EC-specific antigens (CD62E, vascular-endothelial cadherin, Zonula Occludens-1, CD31, and vWF) or endothelial-specific functional activities (e.g., uptake of acetylated forms of LDL, cobblestone morphology of confluent monolayers, and spontaneous capillary-like tubule formation on three-dimensional gels of type I and type III bovine collagen; Vitrogen, Cohesion Technologies, Palo Alto, CA) supported the observation that the primary HMVEC-Cs remained functionally and phenotypically stable at passages 3 through 10 (see "Results").

Static Adhesion Assays. Ficoll-Hypaque gradient-purified PBMCs collected from heart transplant recipients and experimentally infected with HIV-1 as described above or left uninfected were adjusted to 5×10^6/ml in RPMI with 10% FBS, 2 m*M* L-glutamine, and 50 μg/ml gentamicin, and were incubated for 30 min at 37°C with 5 μ*M* calcein AM (Molecular Probes). Afterward, the cells were washed twice in prewarmed (37°C) RPMI media and were then adjusted to 10^6 cells/ml. A total of 10^5 labeled PBMCs was then added to confluent HMVEC-Cs in quadruplicate wells in 96-well tissue culture plates and cell binding was allowed to proceed for 48 hr at 37°C. Unbound cells were removed

by carefully washing the wells four times with Dulbecco's PBS with Ca^{++} and Mg^{++}. The bound labeled PBMCs as well as HMVEC-Cs were then solubilized in the tissue culture plate with 0.1% SDS in distilled water. The plates were then read with a fluorescence plate reader with filter settings of 485 nm for excitation and 530 nm for emission. For certain experiments, a titration of fluorescently labeled PBMCs in replicate wells was set up for each donor. Regression analysis was then performed to correlate the observed fluorescence with the number of labeled PBMCs. For purposes of control, certain adhesion experiments (including LEA inhibition with blocking antibodies described in "Results") were repeated using PBMCs labeled with 5-chloromethylfluorescein diacetate (or Cell Tracker Green; Molecular Probes) to confirm the results obtained with calcein AM-labeled PBMCs.

***In Vitro* Flow Adhesion Assays.** HMVEC-C were grown to confluence on parallel plates in a sealed culture chamber (*NUNC Cell Culture Systems*, Naperville, IL) containing inflow and outflow ports connected by sterile presized tubing to a regulated flow pump (Harvard Apparatus, Holliston, MA) as described elsewhere (40–42). Briefly, premeasured numbers of HIV-1-infected autologous PBMCs along with measured amounts of NE were sterilely introduced through an injection port. The co-culture system mounted onto a stage of an inverted microscope was maintained in a controlled culture environment of 37°C and 5% CO_2. Adhesion experiments were conducted under regulated flow rates of 1 ml/sec and 0.1 ml/sec corresponding to sheer stress pressures of approximately 3.0 and 0.3 dynes/cm^2. Phase microscopic imaging was optically interfaced with a video camera system capable of monitoring in real time leukocyte-endothelial interactions on both the upper and lower plates of the culture chamber. Digital video images taken at 1-sec intervals were interpreted by Optima Image Analysis Software capable of measuring both the distance and direction of leukocyte dislocation on the EC surface. Leukocytes that appeared stationary for at least 10 sec by producing a single-cell image were counted as adherent. Adhesion data (mean number of adherent cells per field ± SEM) was collected from three sets of autologous PBMC and HMVEC sets.

Detection of Cell Adhesion Molecules (CAMs) by ELISA. The induction of CAMs in co-cultures with HMVEC-Cs, NE, and HIV-1-infected or -uninfected PBMCs was detected by QuantiKine quantitative sandwich enzyme immunoassays (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Measurement of cell-associated CAMs on HMVEC-Cs was performed by capture ELISA using preparation of cellular lysates. Cell lysates were prepared by subjecting confluent monolayers of HMVEC-Cs grown in 25-cm^2 tissue culture flasks to gentle rotation for 30 min in a volume of 0.5 ml of ice-cold Tris-HCl extraction buffer containing Tris-buffered saline, pH 7.5, containing 1 m*M* phenylmethylsulfonyl fluoride, 40 U/ml aprotinin, 15 μg/ml leupeptin, and 1% (v/v) Triton X. The cellular lysates were spun down to remove undissolved cellular debris and the lysate mixture was aliquoted and stored at −80°C until testing.

Analysis of Inflammatory Cytokines by Flow Cytometry. Quantitative measurements of the levels of human IL-1β, IL-6, IL-8, IL-10, IL-12 p70, and TNF-α in culture supernatants were made using a human inflammation cytometric bead array (CBA) according to the manufacturer's instructions (BD Biosciences, San Diego CA). Briefly, supernatant fluids collected from different experimental co-culture groups were added to a mixture of CBA beads that possessed unique fluorescein fluorescence intensities and that had been precoated with capture antibodies specific for the panel of inflammatory cytokines being measured, and with PE-conjugated detection antibodies specific for each cytokine. After incubation at room temperature for 3 hr, the beads were washed and then resolved in the FL3 channel of a BD FACScan flow cytometer with CellQuest software (BD BioSciences). A regression analysis of the mean channel fluorescence versus picograms per milliliter of a dilution series for human inflammation cytokine standards was used to calculate the quantitative levels of cytokines in supernatant fluids from experimental and control groups.

PBMC Subset Analysis of Cytokine-Producing Cells by Flow Cytometry. Detection of IL-6/8 expression by PBMC subpopulations was done with BD FastImmune three-color analysis as described previously (43). Briefly, cells from different experimental coculture groups were treated for ~12 hr with Brefeldin A (2.5 μg/ml), and then harvested, washed, and immunostained with fluorescently labeled antibodies specific for human $CD4^+$ (T cells), $CD8^+$ (T cells), $CD14^+$ (macrophage/monocytes), or $CD56^+$ (natural killer cells). The cells were then treated with Perm-Fix solution (BD BioSciences), immunostained with fluorescently labeled antibodies specific for either IL-6 or IL-8, and resolved by three-color analysis using a BD FACScan flow cytometer and CellQuest Software (BD BioSciences).

Statistical Analysis. Statistical analysis of replicate measurements of independent samples taken at single or multiple time points was performed after descriptive tests (e.g., the Shapiro-Wilk W test) were conducted to verify that the observed measurements were normally distributed. Differences between means of multiple independent normally distributed samples were assessed by one-way analysis of variance (ANOVA) with contrasts, and *P* values were assigned using Tukey's 95% confidence intervals. For measurements of CAM expression where absolute values were not reported, statistically significant differences were determined by the nonparametric method of Kruskal-Wallis one-way ANOVA by ranks, where all *P* values were computed by chi-square approximation with correction for ties. All statistical analyses was performed using Analyze-It software version 1.62 for Microsoft Excel (Leeds, UK).

Results

Characterization of HMVEC-Cs. The purity of isolated HMVEC-Cs used in this investigation was determined based on morphological and functional characteristics. Furthermore, HMVEC-Cs, which were routinely used between passages 5 and 7 for the described experiments, remained phenotypically stable. A rigorous series of studies was performed to ensure that the procedures used by our laboratory yielded purified HMVEC-Cs. The phenotypic profile resulting from these studies showed that the primary HMVEC-Cs formed contact-inhibited confluent monolayers with typical cobblestone morphology (Fig. 1A), demonstrated 100% positive immunostaining for vWF and Factor VIII (Fig. 1D) as determined by IFA (isolated human coronary artery smooth muscle cells subjected to IFA staining under the same conditions were negative for vWF), demonstrated positive immunostaining for platelet EC adhesion molecule (PECAM) and Zonula Occludens–1 (ZO-1), both of which are associated with EC intercellular junctions, spontaneously organized into capillary-like tubules when cultured on collagen gels within 5 days after seeding HMVEC-Cs on three-dimensional gels of Type I and Type III bovine collagen (Fig. 1B), and demonstrated an ability to actively internalize modified (acetylated) LDL (ac-LDL) by essentially 100% of the cells (Fig. 1C). Thus, based on these observed phenotypic and functional characteristics, the primary HMVEC-Cs used in the studies reported herein were considered as highly pure and homogeneous.

Effects of NE and HIV-1 Infection on Leukocyte Adhesion to HMVEC-Cs under Static Culture Conditions. Initial attempts to assess the effects of NE on leukocyte adhesion were performed in static adhesion assays as described in "Materials and Methods." Previous reports from our laboratory indicated that the maximal effects of NE on enhanced expression of MMPs in co-cultures of HIV-infected leukocytes and HMVEC-Cs could be observed after 48 hr (28). Therefore, in these series of experiments, measurements of the combined effects of NE, HIV-1 infection, and leukocyte-endothelial interactions on LEA were conducted using HMVEC-Cs and HIV-1-infected PBMCs in co-culture with increasing concentrations of NE for the same 48-hr time period. The results depicted in

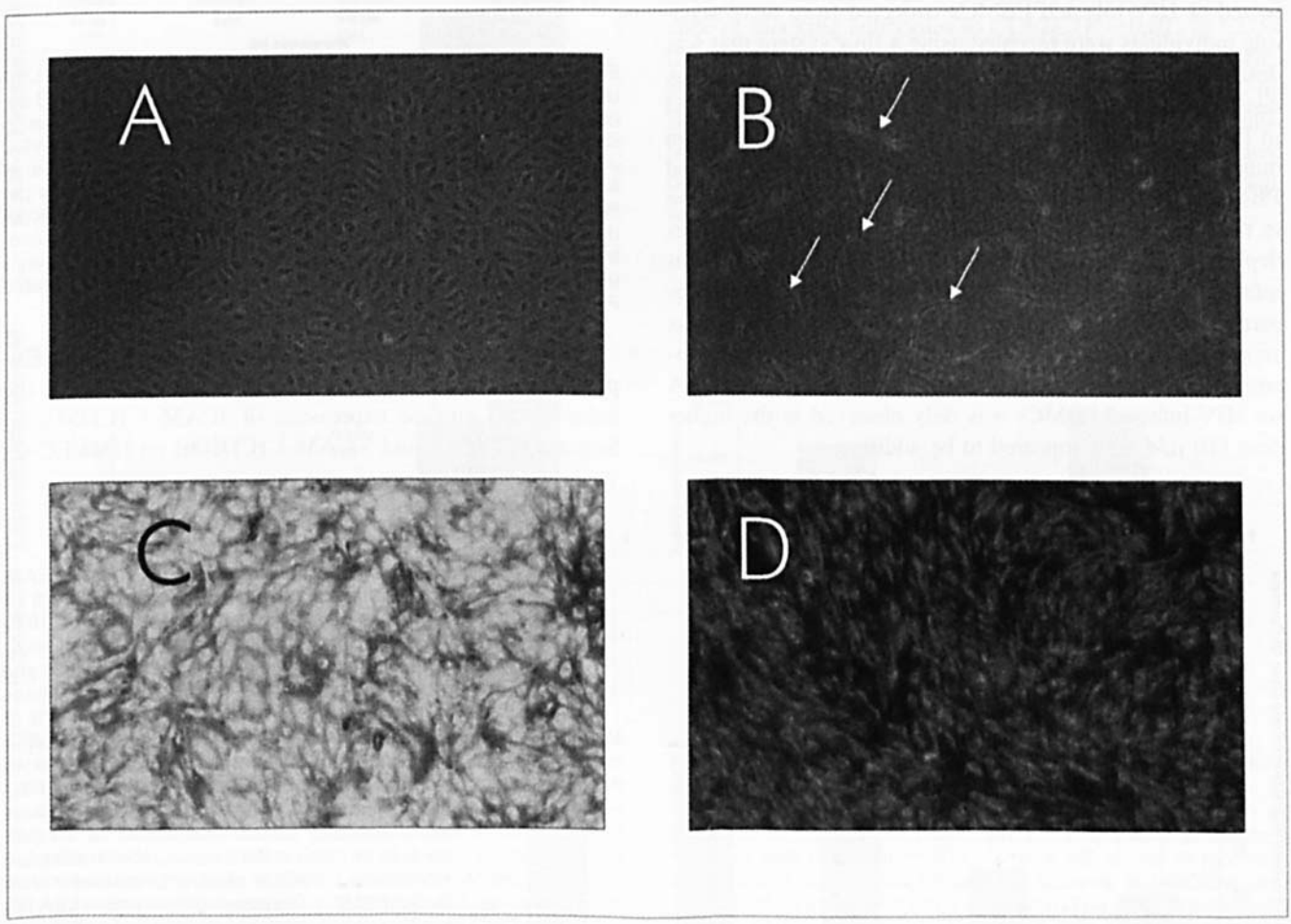


Figure 1. Phenotypic characterization of HMVEC-C. (A) Confluent monolayers of HMVEC-Cs grown on gelatin-coated tissue culture-treated polystyrene form contact-inhibited confluent monolayers with typical cobblestone morphology. (B) HMVEC-Cs grown on three-dimensional collagen gels form capillary-like tubules. White arrows indicate microvascular tubule formations. (C) HMVEC-Cs take up DiI-ac-LDL via cell surfaced expressed ac-LDL scavenger receptors. (D) Immunofluorescent staining of cytoplasmic vWF expressed in HMVEC-Cs using specific FITC-conjugated anti-human vWF antibodies.

Figure 2 are presented as the percentage increase in the mean number of adherent HIV-1-infected PBMCs (from quadruplicate co-cultures) relative to uninfected leukocytes using autologous HMVEC-Cs isolated from 14 individual heart transplant recipients. Under these experimental conditions, NE demonstrated a clear and significant ($P < 0.0001$) dose-dependent effect on the augmentation of adherence of HIV-infected PBMCs to HMVEC-Cs. Control experiments conducted with shorter incubation periods supported the requirement of a 48-hr co-culture period for the development of detectable responses (data not shown).

Dose-Dependent Effects of NE and HIV-1 Under Flow Culture Conditions. NE-mediated LEA observed under static conditions was unable to discriminate between strong and weak leukocyte endothelial interactions. Also, the results from static adhesion assays did not control for the potential effects of intracellular fluorescent dyes on leukocyte adhesion. Furthermore, the direct effects of NE on LEA in the absence of HIV-1 infection under 48-hr co-culture conditions remain to be elucidated. Therefore, to more carefully assess the significance of NE-mediated LEA, adhesion studies using HMVEC-Cs and unlabeled autologous uninfected or HIV-infected PBMCs collected from three separate individuals were repeated using a flow system that was designed to model physiologic sheer stress conditions as described in "Materials and Methods." The results depicted in Figure 3 are presented as the fold increase in the mean number of adherent HIV-1-infected (Fig. 3B) or -uninfected PBMCs (Fig. 3A) relative to adherent PBMCs in co-culture in the absence of NE (media control). Although NE dose-dependent enhancement of LEA was observed for both uninfected and HIV-infected PBMCs, the NE effect was apparent at lower doses (5 μM) for uninfected PBMCs. As shown in Figure 2, HIV infection alone contributes to increased LEA, therefore, the ability of NE to enhance LEA for HIV-infected PBMCs was only observed at the higher dose (10 μM) and appeared to be additive.

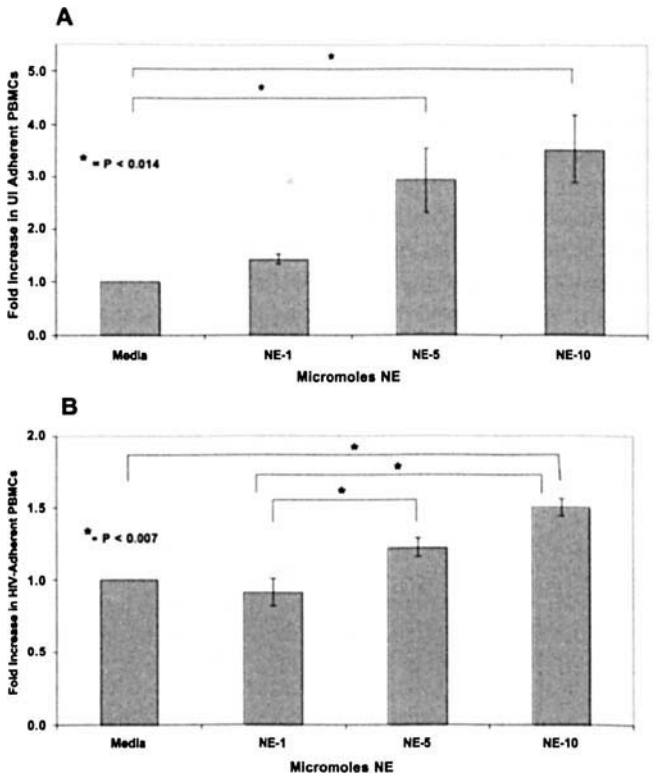


Figure 3. Effect of NE enhances on LEA- and HIV-1-mediated LEA under flow conditions. Flow studies were conducted in a sealed co-culture system as described in "Materials and Methods." Adhesion data were collected from three sets of autologous PBMC-HMVEC pairs. LEA is represented as the fold increase in the mean number of NE-treated adherent (A) uninfected or (B) HIV-infected PBMCs per field relative to adherent untreated (media only) PBMCs. Significant differences in LEA between experimental groups and untreated controls as calculated by one-way ANOVA with contrasts using Tukey's 95% confidence intervals are indicated by brackets with associated P values.

Effects of NE and HIV-1 Infection on the Expression of Adhesion Molecules. An analysis of the induced cell surface expression of ICAM-1 (CD54), E-Selectin (CD62E), and VCAM-1 (CD106) on HMVEC-Cs

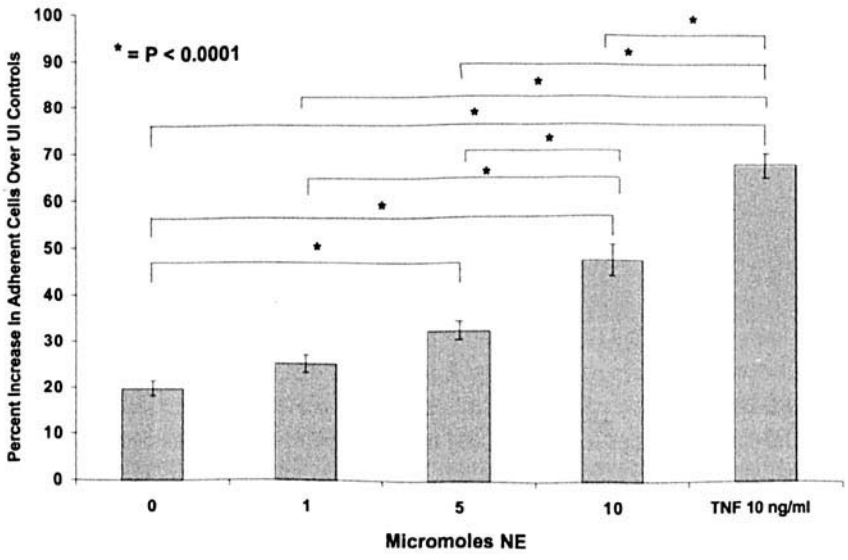


Figure 2. Effect of NE on HIV-1-mediated LEA in static adhesion assays. PBMCs isolated from 14 individual transplant recipients were HIV-1infected or left uninfected and then placed in co-culture with confluent autologous HMVEC-Cs and increasing doses of NE in quadruplicate cultures in microtiter tissue culture plates. Coculture of PBMCs with HMVEC-Cs pretreated with TNF-α (10 ng/ml) served as a positive control. After 48 hr, nonadherent PBMCs were removed and the mean number of adherent leukocytes per well was measured. LEA is represented as the percentage increase in the mean number of adherent HIV-infected PBMCs relative to adherent uninfected PBMCs. Significant differences in LEA between experimental groups and untreated controls as calculated by one-way ANOVA with contrasts using Tukey's 95% confidence intervals ($P < 0.0001$) are indicated by brackets.

was performed as described in "Materials and Methods" in attempts to reveal possible underlying mechanisms involved in the observed NE-HIV-mediated LEA. Culture supernatants and cell lysates, prepared from 48-hr co-cultures of autologous HMVEC-Cs and adherent HIV-infected or -uninfected PBMCs with or without NE (n = 14), were examined for total protein expression of both soluble (in culture supernatant fluids) and membrane-bound forms (in cell lysates) of CAMs. Expression of VCAM-1 was not detected under any of these experimental conditions. The addition of NE (5 μM) to co-cultures of HMVEC-Cs and uninfected PBMCs resulted in elevated levels of expression of ICAM-1 approaching statistical significance ($P < 0.06$; Fig. 4A), but not in the expression of E-Selectin (Fig. 4B). However, expression of both ICAM-1 (Fig. 4A) and E-Selectin (Fig. 4B) was strongly increased ($P < 0.0003$) relative to media controls in co-cultures of HIV-1-infected PBMCs and HMVEC-Cs (P values computed from Kruskal-Wallis statistics as described in "Materials and Methods"). Furthermore, CAM levels in these co-cultures were comparable with those measured in TNF-α-pretreated HMVEC-C-positive controls. Although the addition of NE (5 μM) to appeared to slightly augment expression of both ICAM-1 and E-Selectin, these CAM levels did not significantly differ from the (maximal) levels measured for co-cultures of HIV-1-infected PBMCs and HMVEC-C alone. LEA is mediated by the interaction of matched sets of adhesion molecules on both leukocytes and vascular ECs. However, NE had no detectable effect levels of expression of CD11a, CD11b, CD15s, CD49d, CD154, and CD62P on HIV-infected or -uninfected PBMCs after 48 hr in culture without HMVEC-Cs (data not shown).

Static adhesion experiments were performed in the presence and absence of blocking antibodies to CD62E or CD54 (10 μg/ml) to address the functional relationship between the increased expression of these CAMs and the observed increases in LEA. As shown in Figure 5, adhesion of HIV-infected PBMCs to HMVEC-Cs after 48 hr in the presence of NE (5 μM) was significantly reduced in the presence of blocking antibodies to CD62E ($P < 0.02$) or CD54 ($P < 0.04$; P values computed from Kruskal-Wallis statistics as described in "Materials and Methods"). However, no significant differences in antibody-mediated adhesion inhibition could be detected between NE-treated and -untreated co-cultures of HIV-infected PBMCs and HMVEC-Cs (data not shown).

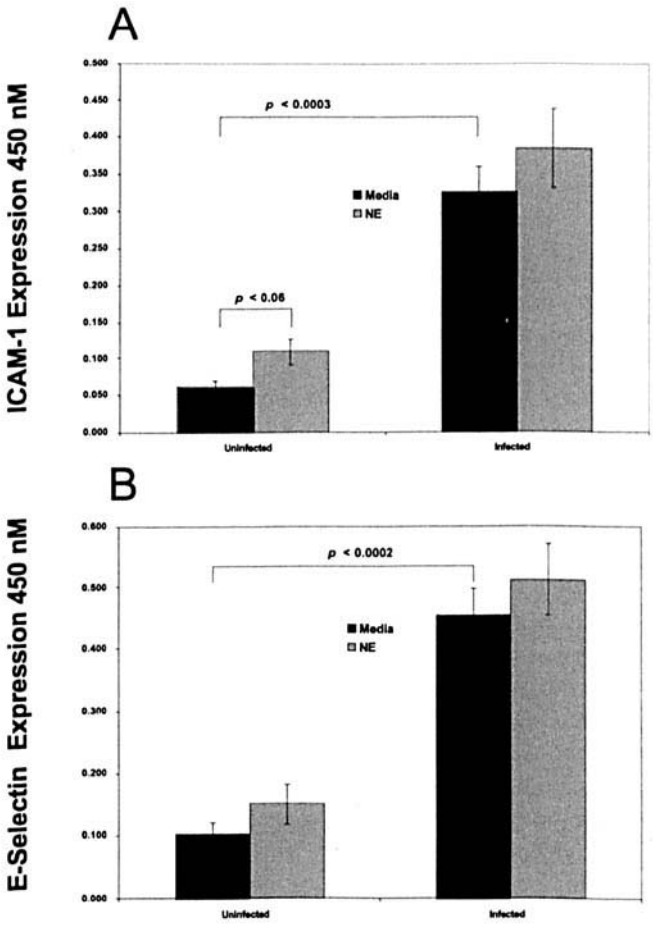


Figure 4. Effect of HIV-1 infection and NE on the induction of CAM expression by HMVEC-Cs in coculture with PBMCs. PBMCs isolated from 14 individual transplant recipients were HIV-1 infected or left uninfected and then placed in coculture with confluent autologous HMVEC-Cs with and without NE (5 μM) in quadruplicate cultures in microtiter tissue culture plates. After 48 hr, total (i.e., both soluble and cell-associated) levels of ICAM-1 expression (A) and E-Selectin (B) expression were measured by capture ELISA as described in "Materials and Methods." Significant differences in CAM expression between NE-treated and untreated groups as calculated by Kruskal-Wallis one-way ANOVA by ranks as described in "Materials and Methods."

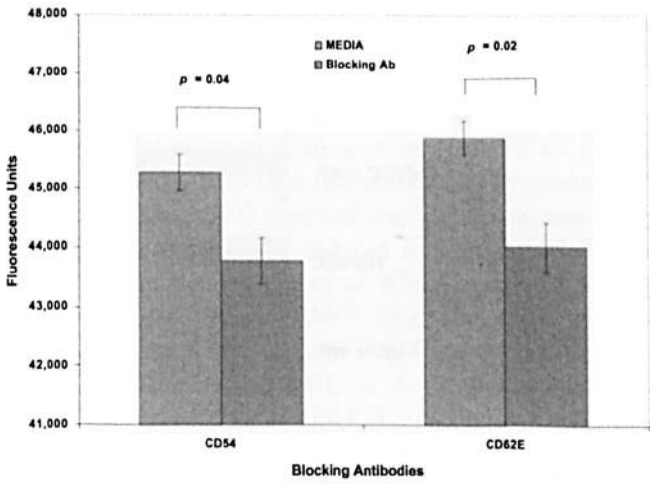


Figure 5. Inhibition of LEA by anti-CD62E- or anti-CD54-blocking antibodies. PBMCs isolated from four individuals were HIV-1 infected and then fluorescently labeled with Cell-Tracker-Green as described in "Materials and Methods," and then placed in coculture for 48 hr with confluent HMVEC-Cs plus NE (5 μM) in multi-well tissue culture plates in the presence or absence of anti-CD54- and anti-CD62E-blocking antibodies (10 μg/ml). The mean fluorescence values and standard errors of adherent cells are represented for indicated experimental groups. Statistical analysis was conducted using Kruskal-Wallis one-way ANOVA by ranks as described in "Materials and Methods."

Effect of NE- and HIV-1-Infected PBMCs in Co-Culture with HMVEC-Cs on the Expression of Inflammatory Cytokines. Certain pro-inflammatory cytokines are known to induce EC activation and CAM expression. Therefore, experiments were conducted to determine the effects of NE and HIV infection on expression of a select group of these cytokines in co-cultures of HMVEC-Cs and HIV-infected PBMCs with and without NE. Single cultures of HIV-infected or -uninfected PBMCs and co-cultures of HMVEC-Cs and HIV-1-infected or -uninfected phytohemagglutinin (PHA)-activated PBMCs from four individuals were set up in triplicate in the presence or absence of NE (5 μM). After 48 hr, supernatant fluids were assayed for IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12 p70 by a quantitative flow cytometric cytokine binding assay as described in "Materials and Methods." A typical result is depicted in Figure 6A. No significant differences in the levels of IL-1β, IL-10, IL-12, or TNF-α were detected in any of the experimental culture groups (Fig. 6B). However, co-culture of PHA-activated or HIV-infected PBMCs with

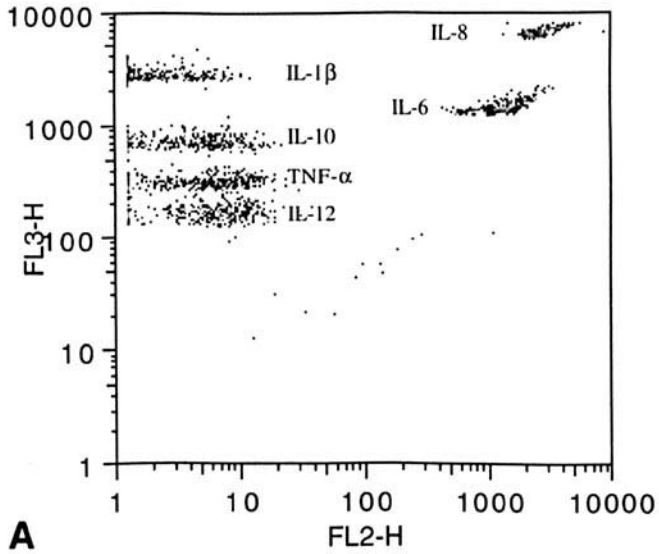
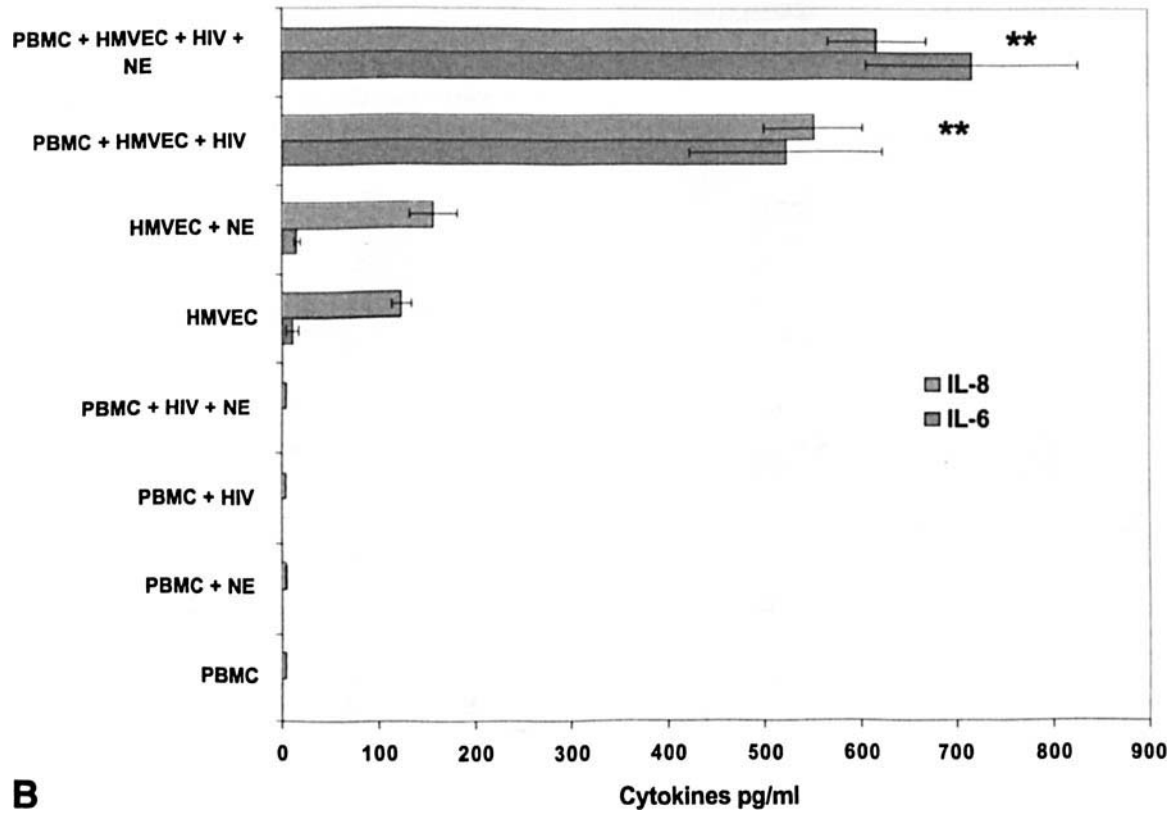


Figure 6. Effect of HIV-1 infection and NE on the induction of proinflammatory cytokines by PBMCs in coculture with HMVEC-Cs. PBMCs isolated from four individuals were HIV-1 infected or left uninfected and then cultured individually or placed in coculture with confluent HMVEC-Cs in the presence or absence of NE (5 μ*M*) in multi-well tissue culture plates. After 48 hr, the levels of human IL-1β, IL-6, IL-8, IL-10, IL-12 p70, and TNF-α were measured in culture supernatant fluids by CBA and flow cytometry as described in "Materials and Methods." No detectable levels of IL-1β, IL-10, IL-12 p70, and TNF-α were measured. (A) A representative profile obtained from CBA flow cytometry analysis is shown in. (B) Significantly elevated levels of IL-6/8 detected in culture supernatant fluids (picograms per milliliter) from the indicated experimental groups are depicted. **Indicates $P < 0.0001$ as determined by ANOVA.



HMVEC-Cs resulted in a significant increase ($P < 0.0001$) in expression of both IL-8 and IL-6 relative to PBMCs or HMVEC-Cs cultured separately. Some constitutive levels of IL-6/8 were detected in HMVEC-C cultured separately. Although levels of IL-6/8 appeared slightly elevated with the addition of NE (5 μM), differences in the levels of expression between NE-treated and -untreated co-culture groups or HMVEC-Cs were not significant.

Because PBMCs represented a heterogeneous population of mononuclear leukocytes, intercellular cytokine analysis (ICA) for IL-6/8 expression was performed on $CD4^+$ (T cells), $CD8^+$ (T cells), $CD14^+$ (macrophage/monocyte), and $CD56^+$ (natural killer cells) to ascertain the predominant subpopulation of leukocytes involved in the observed increases in cytokine expression. After 48 hr in co-culture with HMVEC-C, both PHA-activated and HIV-infected $CD4^+$ T cells predominantly expressed IL-8, although some expression was also detected in $CD8^+$ T cells. IL-6 expression was also detected in $CD4^+$ T cells, although to a lesser degree than IL-8. IL-6 and IL-8 have been shown to be involved in increased viral replication in monocytes and in mediating strong adhesion of monocytes under flow conditions, respectively (13, 42, 44).

Discussion

HIV-1 infection leading to AIDS and expression of the stress associated-catecholamine NE both contribute to the development of CVD (45–50) through separate and overlapping pathogenic pathways involving leukocyte-cardiovascular interactions (9, 38, 51, 52). The results of this study suggest that NE can contribute to the pathogenesis of AIDS-related CVD by enhancing leukocyte adhesion to HMVEC-Cs (Figs. 2 and 3). Enhanced adhesion of HIV-infected monocytes to ECs has been previously reported. Both HIV-1 infection or the effects of HIV-1 proteins, e.g., HIV-1 tat, have been shown to promote LEA by inducing the expression of relevant CAMs and cytokines on both monocytes and ECs (3, 53–55). However, these reports were based on studies involving allogeneic cells or promonocytic cell lines and human umbilical vein-derived ECs (HUVECs), not HMVEC-Cs. The knowledge that there are marked differences between HUVECs and microvascular ECs questioned the relevance of these findings to our understanding of the potential pathogenic mechanisms within the heart. The results presented in this report are based on leukocyte-endothelial interactions between matched pairs of autologous PBMCs and highly characterized primary cultures of HMVEC-Cs derived from heart transplant recipients. This experimental approach was chosen to minimize potential biases due to allointeractions or to endothelial lineage-specific phenotypic differences. An example of the relevance of endothelial lineage to LEA responses has recently been described. In a report by Gan *et al.* (56), cocaine was shown to enhance LEA and CAM expression in human brain microvascular ECs, but not in HUVECs. Clinical situations in which both AIDS and chronic cocaine abuse are involved present an increased risk for DCM (57), therefore, similar findings of cocaine-mediated increased LEA using HMVEC-Cs would have been of significance for this investigation. However, we were unable to detect increased LEA or induced expression of CAMs on HMVEC-Cs after pretreatment with cocaine (10 nM-1 μM) over a 48-hr period (data not shown), thus suggesting differential responses to cocaine by specific tissue lineages of microvascular ECs *in vitro*. Nevertheless, our findings do suggest that cocaine could indirectly influence LEA between leukocytes and HMVEC-Cs *in vivo* by its ability to induce increased physiologic levels of NE. Cardiovascular manifestations of intranasal or intravenous administration of cocaine, including tachycardia, hypertension, and vasospasms, have been attributed to excessive adrenergic stimulation due to increased levels of catecholamines (35). Cocaine enables the accumulation of NE in the heart by inhibiting the NE reuptake transporter and by increasing the sympathetic nerve activity-mediated expression of NE in peripheral nerve terminals (35). Furthermore, infection with HIV alone causes increased expression of NE. Pattarini *et al.* (58) have shown that binding of the HIV envelope protein gp120 to *N*-methyl-D-aspartate receptors mediates the release of NE from central nervous system nerve endings.

In this investigation, a significant and NE dose-dependent enhancement of LEA was observed in co-cultures of HIV-infected PBMCs and HMVEC-Cs (Fig. 2). The fact that NE increased LEA in a manner that appeared additive rather than synergistic suggested a mechanism(s) independent of HIV-1-mediated LEA. Furthermore, because the NE effect required a prolonged (48 hr) co-culture period, coordinated mechanisms between NE treatment, HIV infection, and leukocyte interactions with HMVEC-Cs appeared to be involved.

The effects of such complex interactions were reflected in the patterns of CAM expression by HMVEC-Cs in this co-culture model. LEA is mediated primarily by a system of interacting CAMs with matched specificities expressed by leukocytes and ECs. NE induced a significant increase ($P < 0.03$) in the expression of ICAM-1 on ECs in co-culture with uninfected PBMCs (Fig. 4A). Co-culture of HMVEC-C with HIV-infected PBMCs lead to an even greater increase ($P < 0.0003$) in the expression of both ICAM-1 and E-Selectin by HMVEC-Cs (Fig. 4, A and B) to levels comparable with TNF-α-pretreated positive controls. Furthermore, the addition of NE in co-culture appeared to further augment CAM expression, although the increase did not achieve statistical significance, possibly due to the fact that CAMs were already at their maximal levels of expression. Inhibition of increased LEA by specific blocking antibodies suggested that CD62E and CD54 may be involved in mediating the observed increases in both NE-mediated and HIV-mediated LEA. Therefore, although the effects of NE appeared to work cooperatively with HIV infection and leukocyte endothelial cellular interactions to increase expres-

sion ICAM-1 and perhaps E-Selectin on HMVEC-Cs, they failed to convincingly explain the observed significant NE dose-dependent increases in LEA or the contributions of other induced nonclassical adhesion molecules.

It is known that the expression of ICAM-1 and E-Selectin, which are involved in the tethering of leukocytes and neutrophils to the vascular endothelium, may be induced on ECs by various stimuli, including flow shear stress (59) and pro-inflammatory cytokines (60). Leukocyte interactions with the vascular endothelium *in vivo* occur in the dynamic environment of the circulatory system. Under these conditions, LEA occurs in what has been described as a multi-step adhesion cascade. The prototypic model defines a specific sequence of events: tethering, leukocyte rolling, arrest, strong adhesion, and finally, leukocyte transendothelial migration (61). When the adhesion studies were repeated under flow conditions with physiologically defined flow shear stress pressures, the same patterns of NE-mediated enhancement of LEA were observed for both HIV-1-infected and -uninfected PBMCs (Fig. 3). E-Selectin-CD15s and ICAM-1-LFA-1 interactions that were involved in the observed LEA in this investigation (Fig. 4) are associated with tethering, leukocyte rolling, and arrest along the vascular endothelium. However, strong adhesion associated with NE-induced LEA observed under flow conditions suggested that other adhesive responses may be involved. Co-culture of PHA-activated or HIV-infected PBMCs and HMVEC-Ls resulted in significantly elevated levels of IL-6/8, but not in detectable levels of IL-1β, IL-10, IL-12, or TNF-α (data not shown). IL-8 is a CXC chemokine that triggers firm adhesion of monocytes to the vascular endothelium under flow shear stress conditions (42, 44). Furthermore, HIV-1 viral replication has been show to increase (as measured by p24 levels) in monocytes interacting with vascular ECs and endothelial-derived IL-6 (13). Although levels of IL-6/8 in supernatants of HIV-infected PBMCs and HMVEC-Cs appeared slightly higher in the presence of NE, no statistically significant differences between NE-treated and control groups were detected. Therefore, in the dynamic flow system and most certainly *in vivo*, other (NE-dependent) events influencing LEA are likely to be involved.

The results of this investigation suggest a model in which NE-mediated leukocyte interactions with the vascular endothelium as a potentially central triggering event in the progression toward AIDS-related DCM. In this model, NE enhances LEA of PBMCs and of HIV-1-infected leukocytes by increasing CAM expression. These events ultimately lead to extravasation of HIV-1-infected leukocytes into the myocardium. NE enhances MMP expression in HIV-1-infected PBMCs, which may also facilitate their diapedesis (28). MMPs expressed by HIV-1-infected PBMCs could also contribute to degradation of the cardiac extracellular matrix, triggering reparative cardiac tissue remodeling and ultimately ventricular dilatation (38).

The conclusions of this investigation are focused on the effects of HIV-1 infection and NE on LEA measured in a defined co-culture system. Although further *in vivo* studies should be conducted to more carefully define the precise physiologic significance, there is increasing evidence supporting a significant role for stress-related ANS neurotransmitters, e.g., NE, in HIV disease progression (30). It has been reported that HIV infection alone increases monocyte adhesion to ECs (3) and that NE increases HIV viral replication in monocytes (31). Our data suggest that the NE effect is additive as opposed to synergistic. However, because all adhesion studies described in this report were conducted within 72 hr postinfection, the contribution of NE-mediated increases in HIV replication in adherent leukocytes toward the enhancement of LEA could not be thoroughly addressed. Therefore, further studies are required to more precisely define the mechanisms involved. Nevertheless, the findings presented in this report suggest that NE can promote increased leukocyte interactions with the cardiac microvascular endothelium and suggest a mechanism by which NE may influence the progression of AIDS-related heart disease.

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