Impact of Fibronectin Fragments on the Transendothelial Migration of HIV-Infected Leukocytes and the Development of Subendothelial Foci of Infectious Leukocytes

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J Immunol 2004; 173:2746-2754; doi: 10.4049/jimmunol.173.4.2746
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References

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Impact of Fibronectin Fragments on the Transendothelial Migration of HIV-Infected Leukocytes and the Development of Subendothelial Foci of Infectious Leukocytes

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Leukocyte infiltrates that can serve as viral reservoirs, and sites for viral replication are found in many organs of HIV-1-infected patients. Patients whose blood leukocytes migrate across confluent endothelial monolayers ex vivo and transmit infectious virus to mononuclear leukocytes (MNLs) lodged beneath this endothelial barrier have a worse prognosis. We evaluated the ability of 110- to 120-kDa fibronectin fragments (FNf), which are found in the blood of >60% of HIV-1-infected patients, to stimulate transendothelial migration and drive productively infected MNLs into a potential perivascular space. FNf induced MNLs to release TNF-α in a dose-dependent fashion; the resulting increase in lymphocyte and monocyte transendothelial migration could be blocked with soluble TNF receptor I. Rather than penetrate deeply into the subendothelial matrix, as is seen with untreated controls, FNf-treated MNLs clustered just below the endothelial monolayer. Treatment with FNf during migration increased subsequent recovery of HIV-infected cells from the subendothelial compartment. FNf treatment also significantly increased the numbers of HLA-DRbright, dendritic-type cells that reverse-migrated from the subendothelial depot to the apical endothelial surface 48 h after migration. Fibronectin fragments can be produced by viral and host proteases in the course of inflammatory conditions. The ability of FNf to stimulate transendothelial migration of HIV-1-infected MNLs may help to explain the dissemination of this infection into cardiac, renal, and CNS tissues.

Materials and Methods

**MNLS and transendothelial migration under static conditions**

Peripheral blood MNLS, prepared by Ficoll/Hypaque density centrifugation, were added to the apical surfaces of confluent monolayers of HUVECs (gift from Dr. C. W. Smith, Baylor College of Medicine, Houston, TX). HUVECs, isolated weekly (32), were used at the first or second passage after an ~3-fold expansion in serum- and growth factor-containing endothelial cell medium (proprietary recipe; Cell Systems, Kirkland, WA).

Endothelial monolayers were grown on a matrix of hydrated collagen (Vitrogen; Celtrix, Palo Alto, CA) containing 20 pg/ml native FN (Sigma-Aldrich, St. Louis, MO) (19). MNLS, suspended in RPMI 1640 (Invitrogen Life Technologies, Grand Island NY) with 10% FCS (HyClone, Logan UT) were allowed to migrate, typically for 2 h, through the endothelial monolayer into the collagen matrix that supported the endothelial cells. To assess how deeply migrating MNLS penetrated the collagen gel beneath the HUVECs, we used an inverted microscope to count the number of MNLS per high power field in serial planes, spaced at 10-μm intervals through the collagen pad. Three series of planes were counted for each well.

To enumerate the migratory population, nonadherent cells were washed away with Ca²⁺- and Mg²⁺-free HBSS, and cells that were adherent, but had not migrated through the endothelium, were then removed with trypsin (19). Migratory MNLS were subsequently harvested by digesting the subendothelial matrix with collagenase (Sigma-Aldrich). To enumerate the original and migratory populations, cells were incubated with fluorescent-labeled lymphocyte-specific (CD45) and monocyte-specific (CD14) mAbs (LeucoGATE; Coulter, Hialeah, FL) and were counted by flow cytometry using FlowQuanta beads (Coulter) as an internal reference.

To assess the ability of MNLS that had migrated below the endothelial barrier to reaggregate back from the basal to the apical surface, we allowed MNLS to migrate for the customary 2 h. After the nonmigratory cells were washed away with complete medium, we refed the cultures with medium 199 (Invitrogen Life Technologies) containing 20% human type AB serum. Two days later, the reverse-migrating MNLS were collected by gently washing the surface of the HUVECs with warm complete medium so as not to disturb the integrity of the endothelium.

**Treatment of MNLS**

The media, cytokines, and Abs used in these experiments contained <0.03 endotoxin unit/ml endothaxtin as determined by the Limulus amoebocyte assay (Associates of Cape Cod, Woods Hole, MA). Purified human FN enriched for the 110- to 120-kDa chymotrypsin fragment, hereafter called FNf, was obtained from Invitrogen Life Technologies (Gaithersburg, MD) or Upstate Biotechnology (Lake Placid, NY) and TNF-α was obtained from R&D Systems (Minneapolis, MN). Controls for the FNf included intact FN (Sigma-Aldrich, Temecula, CA) and the connecting segment (CS-1) (33) fragment of FN gift from R. Baughn, Baylor College of Medicine, Houston, TX).

To evaluate its effects on transendothelial migration, FNf was mixed with MNLS on the apical surface of the HUVECs. This approach to treatment with FNf was critical, because monocytes become highly adhesive within 30 min of contact with FNf. If pretreated in a polypropylene test tube, many monocytes will adhere to the walls of the tube and be lost from the assay. Because of the adhesiveness of activated monocytes, we used siliconized tubes (SigmaAldrich) for cell culture and flow cytometric analyses. In addition, we added 0.1% gelatin (Sigma-Aldrich) to the Dulbecco’s PBS wash solutions after Ab staining to further reduce the loss of monocytes from nonadhesive attachment.

In some experiments TNF-α was mixed with the MNLS as they were added to the endothelial surface. Monocytes exposed to TNF-α are not as adhesive as those exposed to FNf. This allowed us to test the effects of pulse treatment with TNF-α to discriminate between its direct effects on MNLS and those on HUVECs. In pulse treatment experiments, TNF-α (10 ng/ml) was added with MNLS suspended at 3–5 × 10⁶/ml in RPMI 1640 with 10% FCS at 37°C for the indicated times. Sham-treated MNLS were exposed to medium alone. Pulse-treated MNLS were washed with a 10-fold and then a 100-fold excess of HBSS without Ca²⁺ or Mg²⁺ and adjusted to 1 × 10⁶/ml in RPMI 1640 with 10% FCS for the migration assay.

We blocked the effect of TNF-α by adding 0.64 μg/ml soluble TNF-α receptor I (TNF sR1Fc chimera, TNFRSF1A; R&D Systems) to the MNLS and HUVECs before the addition of FNf or TNF-α. This quantity of TNF sR1 should block ~160 pg/ml TNF-α, which is 10-fold more TNF-α than that released by MNLS as they migrate through HUVECs. To quantify the TNF-α released by migrating MNLS, aliquots of the supernatant were collected at the end of the 2-h migration assay and tested with a chemiluminescent TNF-α kit (R&D Systems).

**Preparation and assay of HIV-1-infected MNLS**

Normal donor MNLS were infected with HIV-1 at a multiplicity of infection of 0.01 in the absence of mitogens or added IL-2 (19). This protocol generates ~0.1–0.01% provirus-positive cells, a fraction comparable to what we found in HIV+ patients’ freshly isolated MNLS (19). Two strains of HIV-1 were used, the M-tropic UC5 and the T-tropic Pblp (a field isolate propagated in MT2 cells). Cells inoculated in this manner do not produce virus until further stimulated, and there is no detectable p24 in their cytoplasm as measured by flow cytometry. After 3 days, the HIV-inoculated MNLS were washed, resuspended in fresh medium, and used in transmigration studies. Cell viability at that time was >95%, determined with trypan blue. Just before the migration assay, the 3-day-old HIV-infected MNLS were treated with medium alone. To the same donor as a source of fresh resting monocytes. This is necessary because monocytes cultured for 3 days begin to differentiate toward macrophages and are no longer representative of circulating monocytes from peripheral blood. We estimated the frequency of infectious cells in the starting and migratory populations with the limiting dilution assay in which serial dilutions of MNLS are added to duplicate wells containing 0.5 × 10⁴ PHA-stimulated allogeneic lymphoblasts in the presence of IL-2 (17). After 7 days we measured the p24 Ag released into the supernatant (ELISA; Coulter Hialeah, FL) to determine which cultures had received MNLS with infectious virus. The frequency of infected cells was calculated by reference to published tables (17).

**Migration under shear stress conditions**

MNLS were added to medium flowing at 2 dynes/cm² across a HUVEC monolayer in a parallel plate flow chamber (34, 35). HUVEC monolayers used to assay migration under shear conditions were routinely prestimulated overnight with 10 ng/ml TNF-α, because leukocytes will not migrate across unstimulated endothelium under these flow conditions. The endothelial monolayers were washed before the assay began and were videotaped for 10 min after the leukocytes began to flow over the surface. Leukocytes that rolled, adhered, or migrated were identified as previously described (34, 35), except that we used more stringent criteria to define adherent cells (full arrest for 1 min rather than 5 s). We did not observe the formation of strings of cells as has been reported previously (36, 37), but did note that several cells would often migrate through the same spot on the endothelial monolayer. Migrating cells typically assumed a polarized shape, extended a cellular process, funneled through a narrow constriction, used the constriction to maintain a phase-dark line below the monolayer. After migration, they localized in a focal plane below the endothelial surface and remained phase-dark as they moved below the endothelial cells.

To verify that MNLS were migrating below the endothelium, we stained MNLS with an FITC-based intracytoplasmic dye (CellTracker Green; Molecular Probes, Eugene, OR) before the migration. After migration, we fixed the endothelial monolayers that provided the base of the flow chamber with paraformaldehyde, then added PE-conjugated anti-CD45 to the apical surface for 5 min. Cells adherent to the apical side of the HUVECs gave both an FITC and a PE signal. Cells that had migrated through the endothelium gave off an FITC fluorescence color resulting from the cytoplasmic staining dye (CellTracker, Brown Deer, WI), at a ratio of 0.1% provirus-positive cells, a fraction comparable to what we found in HIV+ patients’ freshly isolated MNLS (19). Because they were shielded from PE-anti-CD45 Ab by the overlying endothelium, they did not show any PE signal. The subendothelial position of the FITC-positive, PE-negative population was further confirmed by demonstrating that they were located in the focal plane beneath that occupied by the FITC-positive, PE-positive cells that adhered to the apical side of the HUVECs.

**Flow cytometry**

Flow cytometric analyses were performed as previously described (38) on an EPICS XL cytometer calibrated daily with Flow-Check fluorospheres (Beckman Coulter, Miami, FL). Photomultiplier tube voltages were adjusted each day to a target range of fluorescent intensities by means of...
FNf induced adherence of MNLs to HUVECs. MNLs were added to the medium above confluent HUVEC monolayers in the presence (dobutamine) or the absence (control) of 5 μg/ml FNf. Visual inspection confirmed that all MNLs had settled onto the surface of the HUVECs by 15 min. At 20, 40, and 60 min, MNLs that had not adhered to HUVECs were recovered with gentle washing and enumerated by flow cytometry. Error bars are the SD of triplicate determinations. FNf treatment induced a significant decrease in the number of nonadherent leukocytes, indicating a significant increase in the number adherent to HUVECs within 20 min for monocytes and within 40 min for lymphocytes (*, p = 0.05, by Mann-Whitney U test).

Flow-Set fluorospheres (Beckman Coulter) to remove variability attributable to day-to-day fluctuations in instrument performance. mAbs (Beckman Coulter) included anti-CD14 (clone M5E2), anti-CD11b (CR3, clone Bear1), CD49d (clone HP2/1), CD49e (clone Sam1), CD62L (clone DREG56), CD45 (clone J33), CD4 (clone SFC12T4D11), and CD8 (clone B9.11). Isotype control Abs were obtained from BD Biosciences (San Jose, CA) as were mAbs to HLA-DR (clone L243), CD3 (clone UCHT1), CD19 (clone J4.119), CD56 (NCAM16.2), and CD11a (clone G-25.2). We also used anti-CD14 from DakoCytomation (clone TUK4; Carpinteria, CA).

Statistical analysis and graphic representations of data were performed using STATISTICA 6 (StatSoft, Tulsa, OK).

Results
Impact of FNf on MNL transendothelial migration
In the presence of FNf, MNLs adhere in greater numbers to endothelial cells, leaving significantly fewer nonadherent cells in the overlying medium. Monocyte adherence, reflected as a depletion of nonadherent cells, was significantly increased within 20 min, and lymphocyte adherence was significantly increased within 40 min after FNf treatment (p = 0.01, by Mann-Whitney U test; Fig. 1). When allowed to remain with the endothelium for 2 h, stimulation with as little as 1 μg/ml FNf significantly increased the numbers of lymphocytes and monocytes that subsequently migrated through the HUVEC monolayer (p < 0.05, by Mann-Whitney U; Fig. 2). Doses of FNf >20 μg/ml caused a small, but statistically significant, additional increase in the numbers of migratory monocytes, whereas lymphocyte migration reached a plateau with 20 μg/ml FNf. Migration was not affected by intact FN (in doses up to 80 μg/ml) or by the CS-1 fragment of FN (in doses up to 250 μg/ml; Fig. 3). The CS-1 fragment binds VLA-4, but does not include the RGD sequence and does not bind VLA-5 (33). FNf increased the migration of both CD4 and CD8 T cells (Fig. 4; p = 0.05, by Mann-Whitney U test). On the average, two-thirds of the migratory T cells express CD45RO, and one-third express CD45RA, and the migration of both increases with FNf stimulation (not shown).

Exposure to FNf also increased, in a dose-dependent manner, the quantity of TNF-α released by MNLs as they migrate (Fig. 5A). HUVEC monolayers exposed to FNf without addition of MNLs produced <1 ng/ml TNF-α during this 2-h period (data not shown). FNf induced transendothelial migration of monocytes and lymphocytes. MNLs were added to confluent monolayers of HUVECs in the presence of increasing quantities of FNf for 2 h, and the migratory lymphocytes and monocytes were enumerated by flow cytometry. Data from three experiments are included, each normalized to the number of MNLs in that experiment that migrated in the absence of FNf. FNf significantly increased lymphocyte and monocyte migration at all doses tested (p < 0.05, by Mann-Whitney U test).

Effect of intact FN and different FN fragments on MNL migration. MNLs were allowed to migrate across confluent monolayers of HUVECs in the presence of medium alone (no Rx), 5 μg/ml FNf (110-kDa fragment), 20 μg/ml intact FN, or 250 μg/ml CS-1 fragment of FN. After 2 h, the numbers of migratory monocytes (open bars) and lymphocytes (closed bars) were assessed by flow cytometry. Error bars are the SD of triplicate determinations. FNf caused a significant increase in leukocyte migration (*, p = 0.05, by Mann-Whitney U test), but intact FN and the CS-1 fragment did not.

Effect of FNf on the migration of CD4 and CD8 T cells. MNLs were allowed to migrate across confluent HUVEC monolayers in the presence or the absence of FNf (5 μg/ml). After 2 h, the migratory MNLs were harvested, stained with Abs to CD3 and CD4 or CD8, and enumerated by flow cytometry. The results of two experiments under each condition are shown. Error bars are the SD of triplicate determinations. FNf treatment induced a significantly greater number of both CD4 and CD8 T cells to migrate in each case (p = 0.05, by Mann-Whitney U test).
Depleted MNLs were stimulated with FNf (5 ng/ml). The increase in MNL migration, we added TNF-sRI, a highly avid soluble receptor for TNF.

To determine whether TNF-α or lymphocytes vs endothelial cells in terms of migration, we treated them separately or together before or during the migration assay (Fig. 8). When assaying the effect of TNF-α on lymphocyte migration, monocytes were removed because they are a constitutive source of TNF-α (19, 31). Pulse treatment of purified lymphocytes with TNF-α before the migration assay induced a 1.86 ± 0.6-fold increase in migration (mean ± SD of nine donors; p = 0.015, by t test), indicating that TNF-α can directly affect lymphocyte migration (Fig. 8, compare result B to A). Pulse treatment was more effective than continuous treatment of the lymphocytes during the migration (Fig. 8, result B vs C; p = 0.05, three donors, by Mann-Whitney U test). Activation of the endothelium alone with TNF-α also increased migration (Fig. 8, compare result D to A). However, even when the endothelium was maximally stimulated by prior exposure to TNF-α for 18 h, addition expressed diminished quantities of CD11a and CD49e (Fig. 7). The expression of CD49d did not change significantly on the MNLs exposed to FNf or TNF-α.

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To determine whether TNF-α was responsible for the FNf-driven increase in MNL migration, we added TNF sRI, a highly avid soluble receptor for TNF-α that abolishes the biological activity of this cytokine (18, 31). TNF sRI completely blocked the migration-enhancing effects of FNf (Fig. 6). Addition of nonspecific IgG as a control fusion protein for the TNF sRI/Fc chimera had no effect on lymphocyte or monocyte migration (not shown).

TNF-α produced by FNf-stimulated MNLs drives transendothelial migration

To determine whether TNF-α was responsible for the FNf-driven increase in MNL migration, we added TNF sRI, a highly avid soluble receptor for TNF-α that abolishes the biological activity of this cytokine (18, 31). TNF sRI completely blocked the migration-enhancing effects of FNf (Fig. 6). Addition of nonspecific IgG as a control fusion protein for the TNF sRI/Fc chimera had no effect on lymphocyte or monocyte migration (not shown).

TNF-α could increase migration by activating MNLs, activating endothelium, or both; our data indicate that it activates both. FNf and TNF-α modulate the expression of leukocyte adhesion molecules known to be involved in transendothelial migration and translocation through protein matrices. Stimulation of monocytes with either TNF-α or FNf for 1 h increased the quantity of CD11b on their membranes while decreasing the expression of CD62L (Fig. 7). We have previously shown that FNf also induces monocytes to shed CD49e (23). Lymphocytes exposed to TNF-α or FNf expressed diminished quantities of CD11a and CD49e (Fig. 7). The expression of CD49d did not change significantly on the MNLs exposed to FNf or TNF-α.
FIGURE 8. TNF-α stimulates lymphocyte migration directly. Purified lymphocytes were treated with TNF-α for 10 min (Pretreat) or throughout the migration assay (During migration). Lymphocytes were added to unstimulated HUVECs (−) or HUVECs that had been pretreated with TNF-α overnight and washed (Pretreat). Bars indicate the percentage of added lymphocytes that migrated (mean ± SD triplicate wells). TNF-α treatment, we pulse-treated unfractionated MNLs. TNF-α stimulates lymphocyte migration (mean ± SD; triplicate wells). TNF-α induced a significant increase in the number of cells that migrated, but did not change the number that only rolled or adhered without migrating (Fig. 10). Fig. 10 shows results with pulse-treated cells. The same outcome occurred when TNF-α was present throughout the assay. The continuous presence of TNF-α caused a 1.68 ± 0.64-fold increase in the numbers of migratory cells (mean ± SD; seven donors; p = 0.0009, by paired t test) without affecting the number of cells that only rolled or adhered to the HUVECs.

Impact of FNf on localization of MNLs in subendothelial matrices

Considering that FNf modulates the migration of monocytes in FN-rich tissue matrices (23), we investigated the effect of FNf treatment on the behavior of cells after they had passed through the endothelium. Untreated MNLs tended to migrate deep into the collagen pad during the 2-h period, whereas FNf caused MNLs to accumulate just below the endothelial monolayer (Fig. 11). After FNf treatment, there were significantly more leukocytes in the first 100 μm of the collagen pad and significantly fewer in the region 300–400 μm below the endothelial monolayer (p < 0.05, by t test).

Monocytes that encounter proinflammatory stimuli after they migrate through vascular barriers in vitro may differentiate into dendritic cells and reverse-migrate through the endothelial monolayer to the apical surface (17, 40). This reverse migration is thought to model the movement of dendritic cells from tissue into lymphatics en route to lymph nodes (40). To evaluate whether FNf

FIGURE 9. TNF-α stimulates lymphocyte and monocyte migration. MNLs or lymphocytes were treated with TNF-α, washed, and allowed to migrate for 2 h across unstimulated HUVECs. Bars indicate the percentage of added cells that migrated (mean ± SD triplicate wells). TNF-α treatment of unfractionated MNLs caused a significant increase in monocyte and lymphocyte migration (p < 0.05, by Mann-Whitney U test). The results shown are representative of those from nine donors.
Treatment enhances reverse migration, we washed away the non-migratory cells and re-fed the cultures. Two days later we collected the cells that emerged from the apical side of the HUVEC monolayer. We found that FNf increased both the number of HLA-DRdim monocytoid cells that reverse-migrated and the fraction that expressed high levels of HLA-DR, a characteristic of dendritic cells (Fig. 12). The number of lymphocytic cells (CD3-positive, CD19-positive, or CD56-positive cells) that reverse-migrated was not significantly increased by FNf treatment.

Impact of FNf on transendothelial migration of HIV-infected MNLs in vitro

HIV-1-infected MNLs display increased quantities of CD11a, CD18, CD29, and CD49d and become more adherent to endothelial cells, more likely to engage in homotypic adhesive interactions, and more likely to migrate across endothelial barriers (19, 20). Activated normal donor lymphocytes are also more responsive to the migration-enhancing effects of TNF-α (31). Consequently, we postulated that stimulation with FNf might increase migration of HIV-infected cells to a greater degree than uninfected cells. To test this hypothesis, we infected normal donor MNLs with the T-tropic (Phlp) or the M-tropic (UC5) strain of HIV-1 for 2 days without exogenous mitogens or cytokines. Under these conditions, ~0.01–0.1% of the leukocytes became infected. These HIV-infected MNLs were then allowed to migrate across endothelial monolayers in the presence or the absence of FNf. We measured the numbers of HIV-infected MNLs recovered from the collagen pad by means of a limiting dilution assay in which MNLs were incubated with highly susceptible PHA-stimulated lymphoblasts in the presence of IL-2. The limiting dilution assay is designed to detect any cell capable of transmitting HIV, including leukocytes with latent virus that can be reactivated with stimulation. With both T-tropic and M-tropic HIV-1 strains, FNf treatment increased the total number of migratory MNLs and the number of HIV-infected MNLs recovered from the collagen pads 2–24 h later (Fig. 13). Fig. 13 shows the effects of FNf on these infected MNLs, expressed as the fold increase in the number of migratory cells after FNf compared with the increase in the number of infected MNLs treated with medium alone. FNf treatment induced a 2- to 5-fold increase in the number of HIV-infected cells that migrated (p = 0.02 for cells collected after 24 h; p = 0.008 for 2 and 24 h harvests analyzed together; by Wilcoxon test). At the same time, FNf induced only a <2-fold increase in the total number of cells that migrated. If infected and uninfected cells responded in the same way to FNf, then the fold increase in migration caused by FNf should be the same for HIV-infected and uninfected cells. However, as shown in Fig. 13, the increase in recovery of HIV-infected cells among the migratory cells consistently exceeded the increase in the total number of MNLs recovered from the subendothelial compartment. This was equally true for cultures that were...
harvested within 2 h of migration (see inset, Fig. 13). This demonstrates that FNf preferentially promotes the migration of HIV-1-infected cells. The original cell populations in these experiments contained varying numbers of infected cells, ranging from 82–1122/million MNLs (Table I). However, the level of infection did not appear to influence the total number of MNLs, the number of HIV-infected cells that migrated, or the response of these cells to stimulation with FNf.

**Discussion**

These studies were undertaken to investigate why some, but by no means all, HIV-1-infected men whose blood contains productively infected leukocytes have circulating MNLs that can transport infectious virus across endothelial barriers in vitro and disseminate HIV-1 among a large number of uninfected lymphocytes and monocytes drawn to the same sites, presumably by agents that caused the infected cells to migrate in the first place (17). The clinical significance of these observations was initially obscure until, with continued observation of this cohort of patients, it became apparent that complications associated with progression of HIV-1 infection occurred more often in those subjects whose MNLs exhibited this behavior (17). Recognition that FN fragments are present, at least intermittently, in the plasma of many HIV-1-infected patients (38) and that these FNf are among the triggers that cause monocytes to release cytoplasmic stores of TNF-α (26–28) caused us to consider the possibility that FNf may be one of the agents that induces MNLs, particularly HIV-1-infected MNLs, to migrate across endothelial barriers.

The experiments presented in this report suggest that an important effect of FNf is to stimulate MNLs to release TNF-α. The central role of TNF-α in driving MNL migration is confirmed by demonstrating that the increase in MNL migration induced by FNf is blocked by TNF sR1. A focal increase in TNF-α has many effects. It up-regulates endothelial expression of selectins, ICAMs, and VCAM-1, facilitating adhesive interactions that promote leukocyte transendothelial migration (31). The experiments presented in this report suggest that the TNF-α may also directly stimulate MNL migration, even when the endothelium is not activated and even under shear stress conditions. One mechanism may be the modulation of adhesion molecules involved in transendothelial migration and translocation across protein matrices. TNF-α and FNf induce monocytes to shed CD49e and CD62L and to increase the expression of CD11b. They also induce lymphocytes to decrease their expression of CD11a and CD49e, but have little effect on CD49d. One can visualize several ways in which changes in adhesion molecule expression might affect leukocyte motility. Migration involves a dynamic process of attachment and detachment. It is possible that a diminution in cell surface adhesins may prevent a cell from being tethered and allow it to migrate more easily. The phenotypic data shown in Fig. 7 are a single snapshot after 60 min of stimulation. These markers continue to change with time after exposure to FNf. For example, at 24 h, monocyte CD62L and CD11b return to normal, but CD49d and CD49e become elevated on FNf-treated cells. Lymphocyte CD11a and CD49e return to normal, but CD11b decreases to levels below those seen on untreated MNLs (not shown).

Stimulation with FNf increased the recovery of HIV-infected cells among the migratory population. Infection with HIV activates leukocytes. HIV-infected cells express increased quantities of integrins (20, 41–43), adhere in greater numbers to endothelial monolayers (20, 41–43), and have a competitive advantage over uninfected cells when migrating across monolayers of resting endothelium (19). It is not surprising then that HIV-infected cells would also migrate preferentially over endothelium that has been activated by FNf released in response to FNf. A similar effect was seen using endothelium stimulated with LPS to up-regulate vascular adhesion molecules (data not shown). In one experiment, 12-fold more HIV-infected MNLs traversed the LPS-activated endothelium compared with resting endothelium, whereas the total number cells that migrated was only 13% higher for LPS-stimulated HUVECs (325 HIV-infected cells and 1.58 million total MNLs migrated across unstimulated HUVECs compared with 4491 HIV-infected cells and 1.79 million total MNLs migrating across LPS-stimulated HUVECs).

In addition to enhancing the transendothelial migration of HIV-infected cells, FNf may have stabilized cell surface HIV, thereby enhancing transmission of virus to the PHA-stimulated lymphoblasts. Recent reports suggest that diverse forms of matrix FN can stabilize virions of HIV-1 on the surface of infected cells and help facilitate the transmission of infectious virus (24, 25). We have also found that addition of FNf to cultures of in vitro infected MNLs stimulated with IL-2 increases by 2- to 4-fold the quantity of p24 generated over the next 3 days (data not shown).

Leukocyte integrins are involved in interactions with FN. Leukocytes can interact with immobilized FN using both VLA-4 (CD29/CD49d) and VLA-5 (CD29/CD49e). The 110-kDa FN fragment used in these studies includes the RGD sequence and binds to CD49e (27). The other FN fragment we used, CS-1, does not contain the RGD sequence and does not bind to VLA-5. The CS-1 fragment is closer to the C terminus of the FN molecule and binds to VLA-4. Neither the CS-1 fragment nor intact FN induced TNF-α release or affected the migration of MNLs.

After exposure to FNf, monocytes shed CD49e and are less able to migrate through collagenous matrices rich in native FN (23). FNf-induced loss of CD49e may also explain the failure of treated MNLs to penetrate deeply into FN-collagen matrices below the endothelial barrier in the present report. Colocalization of large numbers of infected and uninfected cells in a compact site is likely to promote interactions that facilitate the transfer of HIV-1. Accumulation of these foci of infected and uninfected MNLs just under the endothelial surface may also enhance reverse migration

### Table 1. Effect of FNf on migration total MNLs and HIV-infected MNLs

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<td>18</td>
<td>1,122</td>
<td>6,058,400</td>
</tr>
</tbody>
</table>

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a These same data are shown in Fig. 13, but presented there as the fold increase in FNf-treated cultures compared to sham-treated cultures.

To calculate the numbers of HIV-infected cells that migrated under each condition, we multiplied the total number of migratory cells by the frequency of HIV-infected MNLs in that population, as measured with the limiting dilution assay. For the starting population, data are reported as the number of HIV-infected cells per million MNLs.

In each experiment, 11 HUVEC wells, representing 11 million starting MNLs, were treated with FNf, and an equal number of wells were treated with medium. Migratory cells from the 11 replicate wells were pooled for the limiting dilution assay. The data shown here are the numbers of sham-treated cells that migrated; Fig. 13 shows the fold increase induced by FNf treatment.
of the subset of infected MNLs that develop characteristics of dendritic cells. To the extent that this process is recapitulated in vivo, these experiments suggest that circulating FNI in HIV-1-infected patients could help disseminate HIV-1 in extravascular soft tissue depots within an infected host. Whether the development of these perivascular foci help to protect the infected cells within from the actions of antiretroviral drugs remains to be determined.

Cell surface display of HLA-DR, a phenotypic characteristic of monocytes that have begun to differentiate into dendritic cells (44), is prominently seen on FNI-stimulated monocytes, especially those that reverse-migrated. Release of TNF-α, as a result of FNI stimulation, may induce monocytes to differentiate toward dendritic cells (40, 45). If these developing dendritic cells were to take up HIV-1 from the subendothelial depots, they would be highly effective at infecting T cells that entered the same subendothelial microenvironment (46).

Proteolytic enzymes released by replicating virus and those produced by host inflammatory responses to HIV-1 or to secondary microbial infections may help to break down plasma or tissue FN (41, 47–49). Prospective studies of the prevalence of circulating FN fragments in HIV-1-infected subjects together with additional studies of their effects on the functioning of cells of the immune response system may deepen our understanding of the ability of these byproducts of the inflammatory response to influence the development of extravascular foci of infection that appear to participate in the cardiac, renal, and CNS complications of HIV-1 infections.

Acknowledgments

We thank Dr. C. Wayne Smith (Division of Leukocyte Biology, Baylor College of Medicine) for the generous donations of HUVECs.

References


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