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*J Immunol* 2002; 168:5722-5729; doi: 10.4049/jimmunol.168.11.5722
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Matrix Fibronectin Increases HIV Stability and Infectivity

Giampaolo Greco,* Sampa Pal,† Renata Pasqualini,‡ and Lynn M. Schnapp*§

HIV particles are detected extracellularly in lymphoid tissues, a major reservoir of the virus. We previously reported that a polymerized form of fibronectin (FN), superfibronectin (sFN), as well as a fragment of FN, III1-C, enhanced infection of primary CD4+ T cells by HIV-IIIIB. We now show that sFN enhances infection of primary CD4+ T cells by both R5 and X4 strains of HIV-1. Using HIV pseudotyped with different envelope glycoproteins (gp120) and HOS cells transfected with various chemokine receptors alone or in combination with the CD4 molecule, we show that sFN-mediated enhancement requires the CD4 receptor and does not alter the specificity of gp120 for different chemokine receptors. Because the III1-C fragment also resulted in enhancement, we asked whether proteolysis of FN generated fragments capable of enhancing HIV infection. We found that progressive proteolysis of FN by chymotrypsin correlates with an enhancement of HIV infection in both primary CD4+ T cells and the IG5 reporter cell line. Furthermore, incubation of HIV with sFN significantly prolonged infectivity at 37°C compared with dimeric FN or BSA. In conclusion, these results indicate that polymerized (matrix) or degraded (inflammation-associated), but not dimeric (plasma), FN are capable of enhancing infection by HIV-1, independent of the coreceptor specificity of the strains. Moreover, virions bound to matrix FN maintain infectivity for longer periods of time than do virions in suspension. This study suggests that matrix proteins and their conformational status may play a role in the pathogenesis of HIV.

The extracellular environment provides the context in which HIV interacts with its target cells. In the early stages of infection, HIV establishes a persistent infection within lymphoid tissues, where virus continues to replicate and infect naive cells. With progressive HIV infection, there are significant alterations in the architecture of the lymph node, which change the microenvironment in which viral-cell interactions occur. The extracellular matrix is a network of glycoproteins and proteoglycans that provide structural support to tissues as well as the environmental clues for processes such as cell migration, differentiation, and proliferation. The extracellular matrix of lymph nodes contains abundant fibronectin (FN), particularly in the interfollicular (T cell-rich) compartment (1). FN is secreted as a soluble, dimeric form, which circulates in the blood. However, in the extracellular matrix, FN is incorporated as an insoluble, multimeric complex of fibrils. In vitro, multimerization can be mimicked by the addition of a recombinant fragment derived from the first type III repeat (III1-C) of FN to soluble dimeric FN. This results in spontaneous cross-linking in vitro to form superfibronectin (sFN), a multimeric form of FN that resembles matrix FN (2). sFN is functionally distinct from the soluble dimeric FN in that it enhances cell adhesiveness, reduces cell migration, and possesses antimetastatic properties (3, 4).

We previously reported that sFN, an in vitro model for matrix FN, significantly enhances HIV infection of lymphocytes (5). We showed that gp120 envelope protein of HIV binds to III1-C of FN and results in increased viral adhesion to lymphocytes. In the present study, we expand our initial observations on the role of matrix FN in the infection of lymphocytes to show that sFN enhances infection by different macrophage tropic strains of HIV. sFN does not alter chemokine receptor specificity or substitute for the CD4 molecule on the cell surface. We show that HIV bound to sFN maintains its infectivity significantly longer than unbound virus, suggesting a role for matrix proteins in stabilization of viral particles. Because we observed enhancement of infection with III1-C fragment alone, we speculate that proteolysis of FN releases fragments that enhance infection. We found progressive enhancement of infectivity with progressive proteolysis of FN. These results suggest that FN contains a cryptic functionality, capable of enhancement of HIV infection and maintenance of viral infectivity. We speculate that matrix FN in lymph nodes plays a role in trapping and stabilizing viral particles and facilitates de novo infection of lymphocytes. Matrix remodeling during HIV infection may result in the release of proteolytic fragments of FN that also facilitate infection. Thus, the dynamic interplay of the extracellular environment and HIV may contribute to viral pathogenesis.

Materials and Methods

Cells and reagents

PBMC were isolated from buffy coats by Ficoll-Hypaque (Pharmacia, Peapack, NJ) density gradient centrifugation and depleted of monocytes by several rounds of adherence to tissue culture plastic. CD4+ T lymphocytes were isolated using magnetically labeled anti-CD4 Abs (Miltenyi Biotec, Auburn, CA) and cultured in RPMI 1640 (Cellgro, Herndon, VA) supplemented with 10% FCS (Cellgro), penicillin-streptomycin (Life Technologies, Gaithersburg, MD) and 100 U/ml IL-2 (Proluekin; Chiron, Emeryville, CA). Cells were verified as 95% CD4+ by flow cytometric analysis. In some experiments, CD4+ T lymphocytes were harvested from lymph nodes containing abundant fibronectin, particularly in the interfollicular (T cell-rich) compartment. FN is secreted as a soluble, dimeric form, which circulates in the blood. However, in the extracellular matrix, FN is incorporated as an insoluble, multimeric complex of fibrils. In vitro, multimerization can be mimicked by the addition of a recombinant fragment derived from the first type III repeat (III1-C) of FN to soluble dimeric FN. This results in spontaneous cross-linking in vitro to form superfibronectin (sFN), a multimeric form of FN that resembles matrix FN (2). sFN is functionally distinct from the soluble dimeric FN in that it enhances cell adhesiveness, reduces cell migration, and possesses antimetastatic properties (3, 4).

Received for publication April 26, 2001. Accepted for publication April 1, 2002.

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1 This work was supported by grants from the National Institutes of Health (R01 HL57890) (to L.M.S.).

2 Address correspondence and reprint requests to Dr. Lynn M. Schnapp, Pulmonary and Critical Care Medicine, University of Washington, Seattle, WA 98104; and Genitourinary Oncology, University of Texas, M. D. Anderson Cancer Center, Houston, TX 77030.

3 Address correspondence and reprint requests to Dr. Lynn M. Schnapp, Pulmonary and Critical Care Medicine, University of Washington, Box 359762, 325 Ninth Avenue, Seattle, WA 98104. E-mail address: lschnapp@u.washington.edu

4 Abbreviations used in this paper: FN, fibronectin; III1-C, recombinant first type III repeat; sFN, superfibronectin; LTR, long terminal repeat; GFP, green-fluorescent protein; C/A, chymotrypsin/aprotonin; DC-SIGN, dendritic cell-specific ICAM-3 grabbing nonintegrin; PI, preimmune serum.

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0022-1767/02/$02.00

The Journal of Immunology
were activated for 3 days with PHA (3 μg/ml). HOS-CCR5, HOS-CXCR4, HOS-CD4-CCR5, and HOS-CD4-CXCR4 cell lines were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and contributed by Dr. N. Landau (6, 7). We confirmed receptor expression by flow cytometry and found >95% expression in all cell lines. The HOS cell lines were cultured in DMEM (Cellgro) supplemented with 10% FCS, penicillin-streptomycin, and puromycin (1 μg/ml) (Sigma-Aldrich, St. Louis, MO). The cell line 1G5, a Jurkat derivative, contains a stably integrated HIV-1 long terminal repeat (LTR)-luciferase expression construct and was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and contributed by Drs. E. Aguilar-Cordova and J. Belmont. The 293T cells were obtained from the National Institutes of Health AIDS Research and Reference Reagents Program (Rockville, MD) and were cultured in DMEM supplemented with 10% FCS. BSA was purchased from Amersham (Piscataway, NJ). FN was purchased from Roche (Indianapolis, IN) or isolated from human plasma. A 70-kDa FN fragment was purchased from Sigma-Aldrich. HIV-IIB11B and HIV-IIBa-L were obtained from Advanced Biotechnology (Rockville, MD) and from the AIDS Research and Reference Reagent Program. The recombinant III-I-C peptide and sFN were made as previously described (2). The polyclonal anti-III-C Ab was previously characterized (5). The anti-mouse IgG-HRP conjugate was purchased from DAKO (Carpinteria, CA).

**HIV infection in the presence of matrix proteins**

Viruses (5 × 10^7 TCID₅₀) were incubated for 10 min at room temperature with BSA (50 μg/ml), FN (50 μg/ml), 5 μM III-I-C, or sFN (5 μM III-I-C added to 50 μg/ml FN) and then added to PHA-stimulated CD4⁺ lymphocytes. After 2 h, cells were washed and incubated at 37°C. The amount of virus produced was quantitated in cell-free supernatants by p24 ELISA (Science Applications International, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). One hundred ofilized virus particles were generated as described elsewhere (8). Briefly, 10⁷ 293T cells were plated in DMEM with 10% FCS. The following day, cells were transfected with Lipofectamine 2000 (Life Technologies) in DMEM with three plasmids: 12 μg of pHr CMV/eGFP (gift of L. Gisella, Mount Sinai School of Medicine, New York, NY) (9); 5 μg of a plasmid encoding the gp120 envelope protein of either a macaque tropic strain, Ada, or a T-tropic strain, HXB2 (10) (gift of D. R. Littman, Skirball Institute of Molecular Biology, New York, NY); and 8 μg of the packaging plasmid pCMVAR8.2 (11). Sixteen hours after transfection, the medium was replaced with complete medium supplemented with 10 mM sodium butyrate and cultured for 6 h at 37°C in 5% CO₂. Cells were then washed and incubated in fresh medium without sodium butyrate. Virus supernatants were collected 30 h after transfection, filtered through a 0.45-μm pore size membrane to remove cellular debris, and stored in aliquots at −70°C. Equal titers of pseudotyped viral particles were used to infect HOS cells. From 2 to 3 days after infection, the number of infected cells was obtained by counting the number of green fluorescent protein (GFP)-positive cells per low power field (six fields per condition) or by flow cytometry. Cells were analyzed with a FACSscan flow cytometer with CellQuest software (BD Biosciences, Mountain View, CA).

**Stability assay**

Ninety-six-well plates were coated overnight at 4°C with 100 μl of BSA (10 μg/ml), FN (10 μg/ml), III-I-C (1 μM), or sFN (1 μM III-I-C and 10 μg/ml FN). HIV-IIB (Advanced Biotechnology) was resuspended in PBS at 3 × 10⁶ TCID₅₀/ml, and 100 μl were added to the precoated wells. After incubating virus for various times at 37°C in a humidified chamber, 4–5 × 10⁶ 1G5 cells were added to each well in 100 μl of medium. Cells and virus were incubated for 4 days. During the incubation period, one-half of the supernatant was replaced with medium every other day. At the end of the incubation, cells were washed and lysed using the Luciferase Assay System (Promega, Madison, WI). Luminescence was measured with MiniLumat LB9506 (EG&G Berthold, Berthold, Australia). Data are reported as the percentage of luciferase activity at harvest time/luciferase activity at time 0.

**FN proteolysis**

FN was degraded by incubating 100 μl of FN (1 mg/ml) or sFN with 2.5 μg of chymotrypsin (100 μg/ml; Roche) at room temperature. At set times, proteolysis was stopped by adding 5 μg of aprotinin (Sigma) and placing the tube at 0°C. To verify proteolysis, aliquots of digested FN were re-solved by SDS-PAGE containing a gradient of 6–18% acrylamide and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were probed with anti-III-I-C Ab followed by HRP-conjugated anti-mouse and visualized with ECL chemiluminescent system (Amersham). After cessation of proteolysis, HIV (5 × 10⁵ TCID₅₀/10⁴ CD4⁺ T cells or 3 × 10⁵ TCID₅₀/10⁵ 1G5 cells) was added to digested fragments (equivalent of 30–40 μg of native molecule), for 10 min at room temperature. PHA-stimulated CD4⁺ T lymphocytes, unstimulated lymphocytes, or 1G5 cells were added to the virus mixtures in a total volume of 200 μl and incubated at 37°C for 2 h, then washed. As controls, III-I-C fragment or chymotrypsin/aprotinin (C/A) mixture were added to cells. In some experiments, III-I-C peptide and fragments obtained from the 6-h FN digest were incubated with anti-III-I-C Ab or preimmune serum (PH) for 15 min before addition to cells. Cell-free supernatants were collected from CD4⁺ T lymphocytes 3 and 6 days postinfection, and the amount of virus was quantitated by p24 ELISA. Cell lysates were made from 1G5 cells 3 days postinfection using the Luciferase Assay System with Reporter Lysis Buffer (Promega), and luciferase activity was measured. Data are reported as the mean p24 Ag levels or mean luciferase activity of triplicate wells ± SD (nanograms per milliliter). In parallel experiments, HIV infection was quantitated by detection of HIV viral DNA by PCR from total cellular lysate of unstimulated CD4⁺ T cells 3 days after infection (see below).

**Detection of HIV viral DNA by PCR**

Total cellular DNA was purified using a Qiagen (Chatsworth, CA) DNA isolation kit. DNA concentrations were determined by spectrophotometric analysis of samples at 260 nm and ethidium bromide staining after gel electrophoresis. Approximately 500 ng of DNA were amplified from each sample. The presence of HIV-1 IIB viral DNA was determined using the primers sense, 5’-GTGAACTGCTGGTACTAAT-3’ (nt 477–497); and antisense, 5’-CCACAGATCAAGGATATCTTG-3’ (nt 539–516), which amplify a 120-bp product in the LTR coding region of circularized, extrachromosomal HIV DNA (12). PCR were conducted in 50-μl reaction mixtures containing PCR buffer (PerkinElmer, Palo Alto, CA), 2 mM MgCl₂, 0.2 mM dNTPs, 50 pmol of each primer, and 1.25 U of Taq DNA polymerase (Taq Gold; PerkinElmer). The reaction mixture was incubated for 1 h at room temperature and then subjected to 40 cycles, consisting of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C, followed by a single cycle of 10 min at 72°C. The PCR-amplified products were analyzed by 2% agarose Tris-acetate gel electrophoresis.

**Immunohistochemistry**

Human subject approval was obtained from Institutional Review Board, Mount Sinai School of Medicine, to obtain archival paraffin-embedded, fixed lymph node tissues from normal and AIDS patients and laboratory data. Sections of 5 μm were cut, deparaffinized, blocked with BSA, and incubated with 1:5000 anti-FN Ab (F3648; Sigma-Aldrich) for 2 h at 37°C. After washing in PBS, sections were incubated with peroxidas-conjugated goat anti-rabbit secondary Ab (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The reaction product was visualized after incubation of the sections with diaminobenzidine (DAB Plus kit; Zymed Laboratories, San Francisco, CA). Sections were counterstained with hematoxylin.

**FN detection**

Total RNA was isolated from lymphocytes with TRizol reagent (Amersham Pharmacia Biotech, Piscataway, NJ), and equal amounts were separated on formaldehyde denaturing gels. As a positive control, RNA was isolated from the fibroblast cell line W138. A 1500-kb FN fragment was labeled using the Brightstar Psoralen-Biotin System and detected with the Brightstar BioDetect Kit (Ambion, Austin, TX). To detect secreted FN and III-I-C-containing FN fragments, lymphocytes were placed in AIM V serum-free medium overnight, and conditioned medium was collected and analyzed by Western blotting with anti-III-I-C Ab.

**Zymography**

Cells were placed in AIM V serum-free medium overnight, and conditioned medium was subjected to gel electrophoresis in 7.5% polyacrylamide gels containing 1 mg/ml gelatin or casein under nonreducing conditions. After electrophoresis, the gel was incubated in 2.5% Triton X to remove SDS and then incubated for 24 h at 37°C in substrate buffer (50 mM Tris; 150 mM NaCl; 10 mM CaCl₂, pH 7.4). Enzyme activity was identified as negative staining with Coomassie blue R250.
Results

sFN enhances HIV infection by R5 and X4 strains of HIV-1 and is independent from viral replication

We previously showed that infection of primary CD4+ cells by the HIV strain IIIB was enhanced by sFN (5). Because this effect requires the binding of the gp120 molecule to sFN, we asked whether the enhancement was related to the specificity of the gp120 protein for certain chemokine receptors or was independent of the type of chemokine receptor used. To address this point, we infected PHA-stimulated CD4+ T cells with the Bal (R5) and IIIB (X4) strains of HIV-1, which enter the cells thorough CCR5 and CXCR4, respectively, in the presence of sFN, FN, BSA, or medium only. Viral replication was monitored by testing the levels of p24 Ag in the supernatants every 3 days through 9 days after infection (Fig. 1). The results show that by day 6, sFN resulted in enhanced p24 levels in cultures infected with either the R5 or the X4 strains, compared with FN, BSA, or medium only. In this particular experiment, IIIB did not show infectivity except with sFN. We found that there is donor to donor variation in infection rates. Previous experiments with HIV-IIIB showed consistently lower levels of infection with FN and BSA than with sFN (5). Infection was also performed with a replication-incompetent HIV-1-based vector, encoding for the reporter gene eGFP and pseudotyped with the envelope protein of another macrophage-tropic, R5 strain of HIV-1 (Ada). HOS cells expressing CD4 and CCR5 were infected with this viral vector in medium alone or containing BSA, FN, III1-C, or sFN. The total number of cells was similar in all conditions. Infection was measured by counting the number of GFP-positive cells by fluorescence microscopy or by flow cytometry. A significantly higher number of GFP-positive cells resulted from infection of HOS cells in the presence of sFN than in the presence of FN, BSA, or medium only (Fig. 2). These results indicate that sFN-mediated enhancement is not restricted to a particular HIV strain or to the use of a specific chemokine receptor. Furthermore, because the pseudotyped viral vector was replication defective, the increase in fluorescent cells with sFN is independent from viral replication.

sFN does not alter the specificity for chemokine receptors or substitutes for the CD4 molecule

We previously showed that gp120 envelope protein binds to the III1-C region of sFN (5). Because gp120 also binds to CD4 and chemokine receptors, we asked whether the interaction of sFN with HIV affects the mechanism of entry into the cell. We examined whether sFN alters the specificity of gp120, allowing the virus to use different chemokine receptors, or substitutes for the CD4 molecule. To test these possibilities, we infected HOS cells transfected with CCR5, CXCR4, CD4/CCR5, or CD4/CXCR4 with the GFP coding vector pseudotyped with the gp120 from an R5 strain (Ada) of HIV-1 (which requires CCR5 receptor) or with the gp120 from an X4 strain (HXB2) of HIV-1. Infection was measured as the percentage of GFP-positive cells by flow cytometry (Table I). Using R5-pseudotyped virus, sFN enhanced the infection of HOS cells expressing both CD4 and CCR5 as expected. However, sFN did not allow infection of cells lacking either CD4 or CCR5. Using X4 pseudotyped virus, sFN enhanced the infection of HOS cells expressing both CD4 and CXCR4 as expected. Again, sFN did not allow infection of cells lacking either CD4 or CXCR4. We conclude that sFN does not alter the chemokine receptor specificity of gp120 or substitute for CD4 receptor during HIV infection.

HIV bound to sFN maintains infectivity

We asked whether binding to sFN affects the stability of HIV particles by measuring the infectivity over time of virus incubated at 37°C with various matrix proteins. HIV was incubated up to 4 days in wells previously coated with BSA, FN, III1-C, or sFN. At the end of incubation, 1G5 cells, a reporter cell line that contains the luciferase gene under the control of the LTR promoter, were added to the wells. Four days later, luciferase activity was measured. We previously showed that immobilized sFN enhanced HIV infection, but adhesion of cells to sFN alone did not affect luciferase activity.

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Table I. Effect of sFN on chemokine receptor and CD4 usage

<table>
<thead>
<tr>
<th>HOS Transfected with</th>
<th>Ada (CCR5) Protein</th>
<th>HXB2 (CXCR4) Protein</th>
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<tr>
<td></td>
<td>CSA</td>
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<tr>
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</tr>
<tr>
<td>CXCR4</td>
<td>0.01</td>
<td>7.81</td>
</tr>
</tbody>
</table>

*Viral particles coding for eGFP and pseudotyped with gp120 of the R5 strain Ada or X4 strain HXB2 were preincubated with BSA or sFN for 10 min and then added to HOS cells transfected with CD4 and CCR5, with CD4 and CXCR4, with CCR5 alone (Ada only) or with CXCR4 alone (HXB2 only). Cells were washed and incubated for 3 days at 37°C. Percentage of eGFP-positive cells as determined by flow cytometry is shown.
the activation state of lymphocytes, proliferation, or viability of cells up to 3–5 days after plating on sFN compared with other matrix proteins (Ref. 5 and our unpublished data). When HIV-infected cells were subsequently plated on sFN, there was no difference in luciferase activity or viral replication. Cell spreading was comparable on FN and sFN. The data from two representative experiments are shown (Fig. 3). The levels of luciferase activity obtained with virus incubated for various days at 37°C in the different conditions are reported as percentages of the luciferase activity resulting from infection with the virus incubated for <1 day (12 or 6 h) in the same conditions. The data are presented in this manner to adjust for the enhanced infection with sFN. The data show a significantly slower decline of infectivity when HIV-1 is incubated with sFN, compared with other matrix proteins. Therefore, sFN-bound virus maintains infectivity longer than unbound virus.

Effect of FN proteolysis on HIV infection

The III1-C region that mediates HIV binding is thought to be cryptic in soluble, dimeric FN (13). Because the III1-C peptide used to generate sFN also increases HIV infection (5), we hypothesized that this activity is cryptic in soluble FN and is revealed after proteolysis. To test this, we digested FN with chymotrypsin for increasing periods of time, up to 6 h. Proteolysis was stopped by adding the protease inhibitor aprotinin. To verify proteolysis, FN fragments were resolved by SDS-PAGE on a gradient gel and probed with an anti III1-C Ab (Fig. 4A). A progressive increase in fragments of low molecular mass containing the III1-C epitope, including fragments that are the same size of III1-C fragment, was observed with increasing proteolysis (Fig. 4B). To test the effect of proteolytic FN fragments on HIV infection, we incubated HIV-1 for 10 min at room temperature with the undigested FN, FN fragments, C/A, or the III1-C peptide. After incubation, the virus/protein mix was added to unstimulated CD4 cells, PHA-stimulated CD4 cells, or Iγ5 cells. Levels of infection were detected by PCR analysis of circular extrachromosomal viral DNA, p24 ELISA, and luciferase activity (Fig. 4, C–E).

In unstimulated CD4+ T cells, we detected 120 bp viral extrachromosomal DNA only in cells infected with fragments of FN digested for longer than 1 h (Fig. 4C). The analyses of p24 Ag and luciferase activity also show a progressive increase of HIV infection with increased digestion of FN (Fig. 4, D and E). We observed increased infection with intact sFN and an additional increase after chymotrypsin digest (Fig. 4F). These results show that proteolysis of FN leads to the release of fragments that enhance HIV infection. To determine whether the observed increase was due to the release of III1-C-containing fragments, we treated the 6-h FN digest with an anti-III1-C Ab, or with PI. Treatment of the 6-h FN digest with anti-III1-C Ab eliminated the observed enhancement (Fig. 4G). To determine whether other FN fragments could increase HIV infection, we tested whether purified N-terminal 70-kDa FN fragment affected HIV infection. We previously showed that III-1 peptide (another type III FN repeat) and RGD peptide did not enhance infection (5). Likewise, no enhancement was seen with the 70-kDa fragment. The data suggest that release of III1-C fragments are responsible for the observed enhancement.

Matrix FN is increased in lymph nodes of HIV-infected patients

To verify changes in matrix protein expression, we examined lymph node sections obtained from eight individuals with AIDS and two normal lymph nodes (Fig. 5). In the normal lymph node, FN immunoreactivity is visualized in the interfollicular areas and in a trabecular pattern within the follicular centers (Fig. 5A). With HIV infection, a progressive alteration in the normal lymph node architecture is observed. Five representative patients are shown. Biopsies from all patients revealed increase in FN staining, along with destruction of the normal lymph node architecture. Evidence of follicular involution and atrophic follicles was observed. In several cases, the lymph nodes were largely acellular and had been replaced with fibrotic tissue (i.e., Fig. 5F). Special stains and cultures for infectious organisms were negative (data not shown). CD4 counts were available on some of the patients at the time of biopsy and ranged from 40 (Fig. 5B), 80 (Fig. 5E), 200 (Fig. 5D), and 740 (not shown). Although the aim was not to determine a correlation between FN expression and CD4 counts, the more substantial changes occurred in patients with the lowest CD4 count.

FN synthesis by lymphocytes

To test whether HIV-infected lymphocytes were directly responsible for increased FN deposition, we analyzed samples by Northern analysis for FN mRNA levels after HIV infection. CD4+ lymphocytes were activated by PHA for 3 days, before HIV infection. Uninfected cells were also subjected to PHA activation. RNA was isolated from cells 7 and 12 days after HIV infection. HIV infection was confirmed by measurement of p24 Ag. We found no detectable message in either control lymphocytes or HIV-infected lymphocytes (Fig. 6A). As a positive control, RNA obtained from fibroblasts demonstrated significant FN mRNA levels. We examined levels of FN and FN fragments in conditioned medium from infected lymphocytes. Small amounts of FN were detected in conditioned medium of both infected and uninfected cells (Fig. 6B). However, there was no difference between infected and uninfected cells or evidence of III1-C-containing fragments in the conditioned medium of infected or uninfected cells. Finally, to determine whether infected lymphocytes altered their secretion of matrix-degrading enzymes, we performed zymography on conditioned

FIGURE 3. Effect of adhesion to matrix proteins on HIV-1 IIIB stability. HIV was incubated at 37°C for 1–4 days in 96-well plates previously coated with BSA, FN, III1-C, or sFN. Iγ5 cells were added to the wells and incubated at 37°C. After 4–5 days, cells were washed and lysed, and luciferase activity was measured. Data are presented as the average (three wells/condition) percentage of luciferase activity at harvest time/luciferase activity time 0. Two independent experiments are shown.

![Image](http://www.jimmunol.org/DownloadedFrom/5725/5725.jpg)
Using gelatin zymography, we found no difference in the basal level of expression of matrix metalloproteinase-9 after HIV infection of lymphocytes (Fig. 6C). Casein zymography did not reveal any stromolysin activity in either uninfected or HIV-infected cells (data not shown). Thus, HIV-infected lymphocytes are not directly responsible for increased FN matrix deposition.

**FIGURE 4.** Effect of FN proteolysis on HIV infection. A, FN proteolysis. FN (100 µg) was digested with 0.25 µg of chymotrypsin. After 15 min, 30 min, 1 h, 2 h, or 6 h, proteolysis was stopped by adding 5 µg of aprotinin. Digested FN and the III-C peptide were resolved in a gradient of 6–18% acrylamide and probed after blotted with anti-III-C Ab. B, Increased exposure of the same gel revealing low molecular mass fragments at the size of III-C peptide. C–F, HIV-1 IIIB was preincubated for 10 min at room temperature with FN fragments generated by progressive proteolysis of FN, sFN, or as controls, the III-C peptide or C/A. C, The HIV/FN fragment mixtures were added to unstimulated CD4+ T cells for 2 h. Then, cells were washed, and total cellular DNA was extracted 3 days after infection. DNA was amplified by PCR using HIV-LTR-specific primers. PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining. The expected 120-bp amplification product is detected only in samples with digestion longer than 1 h. PCR amplification with β-globin-specific primers is shown in the bottom panel to verify equal loading of DNA samples. D, The HIV-FN fragment mixtures were added to PHA-stimulated CD4+ T cells. Culture supernatants were tested for virus by p24 ELISA at day 6 postinfection. Data are shown as the mean p24 levels (nanograms per milliliter) ± SD measured in supernatants of triplicate samples. E, The HIV-FN fragment mixtures were added to 1G5 cells. Three days after infection, cell lysates were made, and luciferase activity was measured using the Luciferase Assay System. Data are reported as the average luciferase activity of triplicate samples ± SD. F, Chymotrypsin digest of sFN. The HIV-sFN fragment mixtures were added to 1G5 cells. Three days after infection, cell lysates were made, and luciferase activity was measured using the Luciferase Assay System. Data are reported as the average luciferase activity of triplicate samples ± SD. G, The 6-h FN digest or III-C peptide were incubated with PI sera or anti-III-C Ab and then added to HIV and 1G5 cells as above. As controls, HIV was mixed with the 70-kDa N-terminal fragment of FN or C/A. Three days after infection, cell lysates were made, and luciferase activity was measured using the Luciferase Assay System. Data are reported as the average luciferase activity of triplicate samples ± SD.
Discussion

Studies examining HIV entry have focused on the interaction of the virus envelope protein with its cell surface receptors and subsequent changes in protein conformation that leads to membrane fusion. However, factors that increase virus attachment to the cell surface play a critical step in infection (14, 15). In this study, we provide additional data on the enhancement effect of matrix FN (sFN) on HIV infection. First, we show that sFN, a model of matrix FN, enhances infection by different strains of HIV-1 that use either CCR5 or CXCR4. Second, HIV-1 attached to sFN is infectious for longer periods of time than is HIV in solution or attached to dimeric FN or other proteins. Third, proteolysis of FN generates fragments that increase HIV infection. Finally, among the morphological changes occurring in the lymph nodes of AIDS patients, we detected a remarkable increase in the expression of FN.

We report that sFN (matrix FN) enhances infection of lymphocytes by two different M-tropic gp120 envelope proteins (BaL and Ada) that use CCR5 receptor, in addition to T-tropic strain HIV-IIIb. Thus, sFN enhances infectivity by strains using the two principal coreceptors for HIV. M-tropic strains are responsible for >90% of sexual transmission of HIV, and CCR5 is the main coreceptor usage (16). The ability of matrix FN to enhance infection of M-tropic strains suggests that viral adhesion to matrix at sites of initial transmission such as mucosal surfaces, or areas of ulceration where a FN-rich matrix is deposited, facilitates the transmission of infection. Adhesion of virions to matrix FN at mucosal surfaces may trap and stabilize viral particles and promote uptake by dendritic cells, which then disseminate infection to lymphoid tissues, the major reservoir of virus. Likewise, matrix-bound virus may be presented to receptor-positive target cells for infection.

The enhancement of infection occurred using a replication incompetent virus; therefore, it is unlikely that sFN enhances infection by priming the cells to become more permissive for viral replication. This result, along with our previous studies, suggests that sFN increases HIV infection by increasing attachment of virus to target cells. This mechanism has been previously implicated in the increased efficiency of retroviral gene transfer induced by soluble FN (17, 18). However, these studies did not implicate the III-1 region as playing a role in FN-mediated enhancement, as suggested by our studies.

We asked whether sFN alters the mechanisms of viral entry. Because we previously reported that sFN binds to gp120 envelope protein near a region involved with CD4 binding, we hypothesized that sFN may alter the conformation of the envelope protein and thus modify the chemokine receptor specificity or requirement for CD4 as seen with some strains of HIV-1 and HIV-2 shown to be CD4 independent (19-21). We show that sFN does not alter the specificity for chemokine receptors or substitute for the CD4 molecule (Table I), suggesting that the primary mechanism of enhancement is through increased cell attachment. An additional mechanism of HIV adhesion to matrix FN may be through functional adhesion receptors acquired from host cells (22).

We show that HIV bound to sFN is stable significantly longer than unbound viral particles. Recently, two related lectin molecules, dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) and DC-SIGNR, expressed on dendritic cells and endothelial cells, respectively, were shown to bind HIV and facilitate infection in trans (23, 24). DC-SIGN plays a role in initiating the immune responses from resting T cells (25). When HIV was bound to DC-SIGN, HIV remained infective for up to 2-3 days without being internalized (24). Like sFN, DC-SIGN binds to HIV through gp120 envelope protein. We speculate that because sFN binds viral particles through gp120 envelope protein (5), this prevents the shedding of gp120 and subsequent loss of infectivity (26). Interestingly, although III1-C fragment enhanced infectivity of HIV, it did not increase stability of HIV. Therefore, the three-dimensional structure of multimeric FN appears to be critical for viral stability. The half-life of free HIV particles has been estimated at <6 h (27). Matrix-bound virus may represent a longer lived compartment of viral particles that remain infectious for a prolonged period. Other studies showed that HIV particles immobilized through incorporated adhesion receptors are more infections than free virus (28). Thus, adhesion of viral particles to appropriate ligands (i.e., extracellular matrix or DC-SIGN) may be a method for the virus to escape cytotoxic immunological responses while maintaining infectivity. Lymphoid tissue is the site of continued productive infection throughout the course of infection. Evidence suggests that virus is transmitted locally from one cell to another cell in its vicinity (29). Trapping of virus particles by matrix proteins may increase local concentration of particles and facilitate binding of

FIGURE 5. Expression of FN in the lymph nodes of HIV-infected individuals. Immunohistochemical analysis for FN was performed on lymph node biopsies from eight AIDS patients and two normal individuals. Immunohistochemistry from five representative AIDS patients (B–F) and one normal patient (A) are shown. Sections were counterstained with H&E. Magnification, ×20.
the virus to its receptors, whether on lymphocytes or on follicular dendritic cells. Thus, the local extracellular environment, by trapping and stabilizing particles, may facilitate localized propagation of infection.

Progressive proteolysis of FN, with a corresponding reduction in size of the fragments containing the III-1 C region, correlates with the enhancement of HIV infection. Addition of purified FN fragments (70-kDa N-terminal fragment, RGD-containing fragment, III11) that do not contain III-1 C region did not enhance HIV infection. Furthermore, the enhancement was eliminated when proteolytic fragments were pretreated with an Ab directed against the III1-C epitope. This agrees with our previous finding that III-1 C fragment itself can increase HIV infection. This finding may be important during inflammation or wound healing where proteases are released that generate proteolytic fragments with potential biological activities (30). Proteolysis of extracellular matrix releases cryptic activities that can signal differently than the intact protein (31). Fragments of FN are important modulators of inflammation, for example, by affecting monocyte migration and the expression of integrin receptors (32). During inflammatory arthritides, FN fragments are increased in synovial fluids and can induce chemotaxis and proliferation of CD4+ lymphocytes (33). We propose that during any inflammatory process, there is proteolysis of FN, which releases fragments that enhance HIV infection of lymphocytes, independent from the intact FN. It is documented clinically that intercurrent infections increase the progression of HIV disease. The release of proteolytic fragments during the associated inflammation may be a contributory factor.

We show the HIV-associated lymph node pathology is accompanied by significant increases in FN deposition. We previously reported that the III1-C epitope is exposed in lymph nodes by immunohistochemistry (5). Typically, in untreated patients, there is a progression of lymph node pathology from florid follicular hyperplasia to follicular involution to lymphocyte depletion, which correlates with disease progression (34). Even in patients with high CD4 counts and unenlarged lymph nodes, alterations in normal lymph node architecture are observed (35). With highly active antiretroviral therapy, there is a decrease in lymph node viral RNA levels, but histological abnormalities observed at the onset of therapy remained unchanged (35). After cessation of highly active antiretroviral therapy, lymph nodes rapidly progress and become hyperplastic within 1–2 mo (36). Thus, the lymph node microenvironment is subject to continued alterations throughout the course of HIV infection.

Our studies do not support a direct role of HIV-infected lymphocytes in FN deposition or matrix remodeling. We did not detect a difference in FN protein levels or degradation products in cultured supernatants between infected and infected lymphocytes, nor did we detect FN mRNA. We speculate that HIV-infected lymphocytes are indirectly involved in matrix remodeling, i.e., by secretion of profibrotic cytokines. The cytokines would act on local stromal cells and fibroblasts, which are responsible for matrix remodeling. This is analogous to fibrotic processes in many other organs, such as the lung where activated mononuclear cells secrete TGF-β1, which causes enhanced FN secretion by alveolar interstitial cells.

In summary, we show that components of the matrix may have significant impact on viral infection by trapping and stabilizing viral particles. Lymph nodes are the major reservoir of HIV. HIV bound to matrix FN within lymph nodes provides a longer lasting source of virions that can infect target cells that encounter the extracellular matrix. Furthermore, matrix remodeling, i.e., during inflammation, wound healing, or lymph node remodeling, may release FN fragments that also enhance HIV infection. Therefore, the extracellular matrix is a dynamic environment that impacts on the ability of HIV to infect cells.

Acknowledgments

We thank Dr. Luca Gusella for the pHR CMV/EGFP plasmid and for critical advice and Dr. Wadh Arap for advice. We also thank Arevik Miosoian and Joan Dragavon for technical assistance.

References


