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Journal
The Journal of Infectious Diseases, 210(7)

ISSN
0022-1899

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Publication Date
2014-10-01

DOI
10.1093/infdis/jiu220

Peer reviewed

BRIEF REPORT

Interaction of Fibronectin With Semen Amyloids Synergistically Enhances HIV Infection

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Semen harbors amyloids that enhance human immunodeficiency virus type 1 (HIV-1) infection. We set out to identify factors that bind these amyloids and to determine whether these factors modulate amyloid-mediated HIV-enhancing activity. Using biochemical and mass spectrometric approaches, we identified fibronectin as a consistent interaction partner. Although monomeric fibronectin did not enhance HIV infection, it synergistically increased the infectivity enhancement activity of the amyloids. Depletion of fibronectin decreased the enhancing activity of semen, suggesting that interfering with the binding interface between fibronectin and the amyloids could be an approach to developing a novel class of microbicides targeting the viral-enhancing activity of semen.

Keywords. semen; HIV; SEVI; semenogelin; fibronectin; amyloid.

Although much progress has been made in combating the AIDS epidemic, approximately 2.5 million people continue to be newly infected with human immunodeficiency virus (HIV) each year [1]. The majority of infections result from sexual contact between an infected individual and their uninfected partner. A considerable body of recent literature suggests that semen, the vector for sexual transmission of HIV, markedly enhances infectivity of the virus in vitro [2–11]. Screening of a peptide library generated from human semen identified multiple fragments from prostatic acid phosphatase that formed amyloid fibrils that enhance HIV infection [2, 12]. These amyloids, termed semen-derived enhancer of viral infection (SEVI), are cationic and enhance HIV infection by electrostatically promoting attachment of the virions to target cells [2, 4]. More recently, we identified a second set of semen amyloids comprising fragments from semenogelin 1 (SEM1) and SEM2, the major components of the semen coagulum [6]. Like SEVI, SEM amyloids are cationic and enhance HIV attachment. The relative levels of amyloidogenic SEVI and SEM peptides in semen samples from different individuals correlate with the relative viral-enhancing activity of the samples [5, 6], suggesting that both amyloids contribute to the ability of semen to enhance HIV infection.

METHODS

Detailed protocols are provided in the Supplementary Materials.

RESULTS

To determine whether the viral-enhancing activity of semen amyloids can be modulated by naturally occurring factors in semen, we set out to identify interacting proteins of the SEM1 amyloids in pull-down experiments. Biotinylated SEM1 amyloids, whose fibrillar structure and HIV-enhancing activity were confirmed by thioflavin T binding, electron microscopy, and infectivity assays (data not shown), were conjugated to streptavidin-conjugated beads and incubated with seminal plasma (SP). Following extensive washing, bound proteins were identified by mass spectrometry. Fibronectin (FN) was identified as the top high-confidence interacting factor (defined as the hit exhibiting the highest level of enrichment in 3 of 3 independent experiments; Supplementary Materials). Of note, FN is also a prominent component of the SEM1-containing semen coagulum. The levels of seminal FN bound to SEM1 fibrils was significantly more than that bound to control samples containing only beads or the corresponding monomeric SEM1 peptide (Figure 1*A*). In the reciprocal experiment, pull-down of FN from SP resulted in coisolation of peptides from the amyloidogenic region of SEM1. Immunoprecipitation of FN resulted in enrichment of peptides from the amyloidogenic region of SEM1 [6] by 3.1-fold relative to control samples, in which albumin was immunoprecipitated. Dot and western blotting analyses confirmed that purified FN physically interacted with SEM1 amyloids (Figure 1*B*). The ability of FN to bind SEM1 amyloids is consistent with the previous reported ability of this protein to

Received 24 January 2014; accepted 1 April 2014; electronically published 8 April 2014.
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The Journal of Infectious Diseases 2014;210:1062–6

DOI: 10.1093/infdis/jiu220

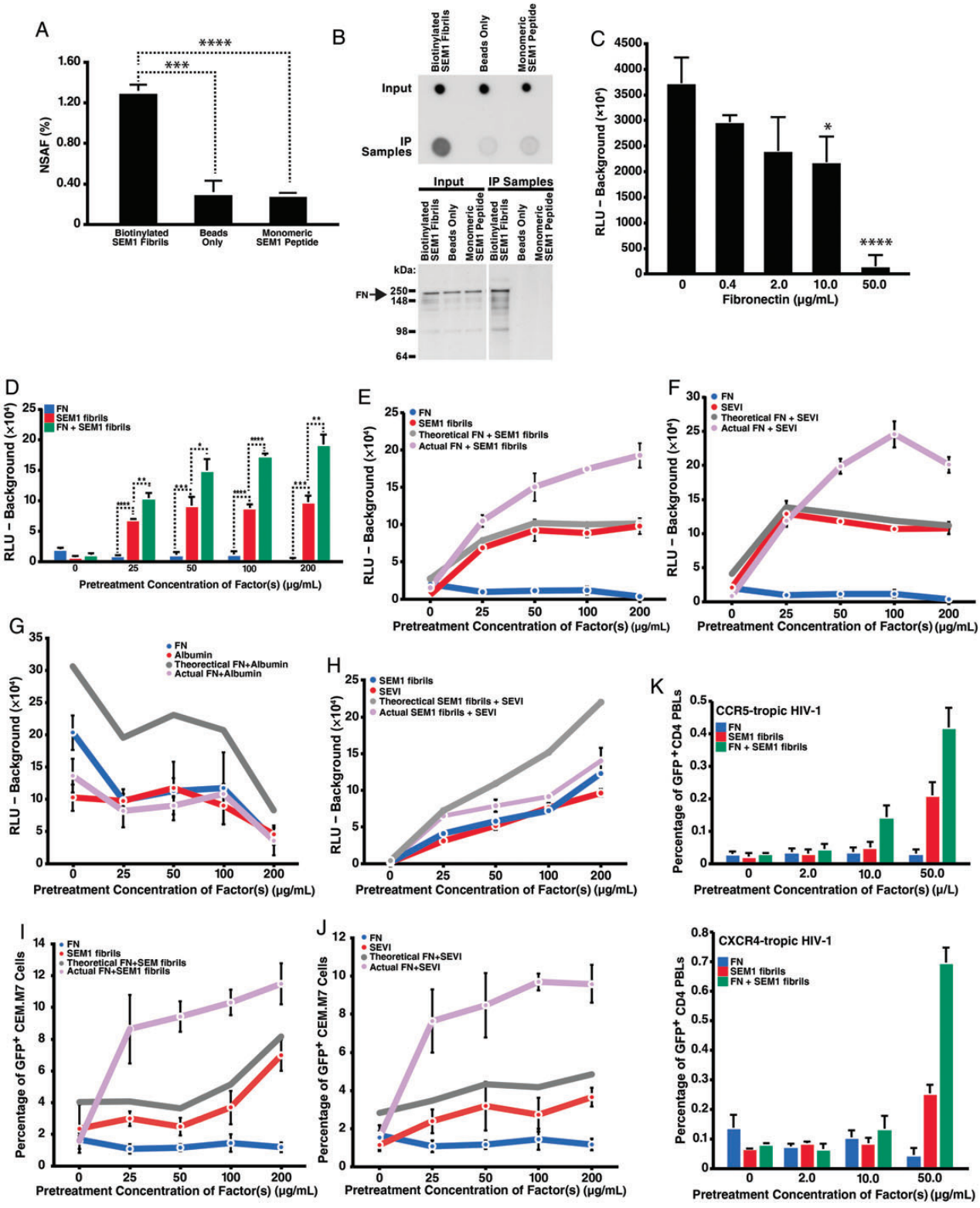

Figure 1. Fibronectin (FN) binds semen amyloids and augments their ability to enhance human immunodeficiency virus type 1 (HIV-1) infection. *A*, Label-free mass spectrometry quantification of endogenous seminal FN bound to biotinylated semenogelin 1 (SEM1) fibrils, beads, or the monomeric SEM1 peptide. NSAF, normalized spectral abundance factor. ***$P < .001$, ****$P < .0001$ (by 2-tailed *t* tests). *B*, SEM1 fibrils but not monomeric SEM1 peptide bind purified FN. Beads conjugated to SEM1 fibrils or monomeric peptide were incubated with FN, washed, and then assessed by dot blot (top) or western blot (bottom). IP, immunoprecipitated materials. *C–H*, HIV-1 infection of TZM-bl cells. CCR5-tropic 81A HIV-1 virions (100 ng/mL $p24^{Gag}$) were incubated for 5 minutes with the indicated concentrations of the corresponding factors, diluted 15-fold, and then added to TZM-bl cells. Infection was assessed 3 days later by quantitating β-galactosidase activity. Background signal corresponding to uninfected cells was subtracted from shown values. *C*, FN inhibits HIV-1

bind the general amyloid fold of fibrils, including bacterial curli [13].

We next assessed whether binding of FN to SEM1 amyloids affects the ability of the fibrils to enhance HIV infection. Infection of TZM-bl cells with HIV-1 was performed as previously described [6]. When added alone, FN inhibited HIV infection (Figure 1*C*), a finding consistent with FN-mediated interference of gp120 binding to the CD4 receptor [14]. Conversely, addition of FN to SEM1 amyloids resulted in viral enhancement activity that was significantly higher than the effect of the SEM1 amyloids alone (Figure 1*D*). Synergy between the 2 factors was confirmed in a complete dose-response curve, wherein the theoretical sum of infection levels in the presence of either factor alone was significantly less than that observed experimentally (Figure 1*E*). The synergistic enhancing effects between FN and the amyloids was not due to increased proliferation or viability of the cells, as assessed by cellular adenosine triphosphate levels (Supplementary Figure 1*A*). Synergistic enhancement of HIV infection was also observed between FN and SEVI amyloids (Figure 1*F*), suggesting that FN may generally boost the viral-enhancing activity of semen amyloids. In contrast, FN together with purified human albumin, a highly prevalent protein in semen, did not enhance HIV infection (Figure 1*G*), demonstrating specificity in the response. Furthermore, SEM1 and SEVI amyloids did not synergize with each other (Figure 1*H*), suggesting that synergistic viral enhancement is a rather unique response elicited by a combination of FN and the amyloids. FN similarly boosted the ability of semen amyloids to enhance HIV-1 infection of CEM.M7 cells and primary activated $CD14^{-}CD4^{+}$ peripheral blood leukocytes, demonstrating that the synergy also occurred in nonadherent and primary cells (Figure 1*I*–*K*). Furthermore, enhancement occurred with both R5- and X4-tropic viruses (Figure 1*K*).

To test whether specific domains of FN mediated the synergistic enhancing activity, proteolytic fragments of FN were tested for their ability to increase SEM1 fibril-mediated enhancement of HIV infection. The N-terminal 29-kDa FN fragment, generated from cleavage of FN by cathepsin D and partial tryptic digestion, proved sufficient to synergize with SEM1 amyloids in HIV infection assays (Supplementary Figure 1*B*). In contrast, a 40-kDa chymotryptic fragment of FN, beginning at approximately residue 300 and located downstream of the N-terminal 29-kDa fragment, failed to synergize with the amyloids (Supplementary Figure 1*B*). These findings indicate that synergistic stimulation of HIV infection with semen amyloids is mediated through the N-terminus of FN.

The HIV-enhancing activity of semen amyloids is highly dependent on their cationic nature, since anionic polymers abrogate their ability to enhance HIV infection [4, 6]. To determine whether FN overcomes this inhibitory activity of anionic polymers, we assessed whether pretreatment of the 29-kDa N-terminal FN fragment with the polyanion heparin affected its ability to synergize with SEM1 amyloids. As shown in Supplementary Figure 1*C*, heparin abrogated the synergistic enhancement of HIV-1 infection mediated by the FN fragment. Therefore, the complex consisting of the cationic amyloids with the active FN fragment is still dependent on electrostatic interactions for enhancement activity.

Next, studies were performed to determine whether FN altered the binding of the fibrils to either HIV-1 virions or target cells. To assess whether FN increased the ability of SEM1 fibrils to bind HIV-1, we conducted a virion pull-down assay as described elsewhere [8]. SEM1 amyloids were incubated with HIV-1 81A in the absence or presence of FN, followed by low-speed centrifugation and assessment of $p24^{Gag}$ in the pellet measured by ELISA. As shown in Supplementary Figure 2*A*, FN did not significantly increase the ability of SEM1 amyloids to bind and pellet HIV-1 virions. These data argue against the notion that FN increases HIV infection by altering the fibrils in a way that facilitates their binding to viral particles. FN also did not increase binding of SEM1 fibrils to TZM-bl cells (Supplementary Figure 2*B*). Furthermore, electron microscopy did not reveal marked alterations in SEM1 fibril length or clustering following addition of FN (Supplementary Figure 2*C*). Together, these findings indicate that the action of FN likely does not involve altering amyloid structure or increasing amyloid attachment to either virions or target cells.

Finally, we set out to examine the activity of endogenous seminal FN by assessing the effect of FN blockade or depletion

Figure 1 continued. infection of TZM-bl cells. **P* < .05, *****P* < .0001 (by the Bonferroni multiple comparisons test, compared with infection in the absence of FN). *D*, FN increases the ability of SEM1 amyloids to enhance HIV-1infection. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001 (by 2-tailed *t* tests). *E*, Data are similar as those in panel *D* but also show the theoretical additive value of infection levels in the presence of FN and SEM1 amyloids (grey line). The experimental infectivity levels in the presence of both FN and SEM1 amyloids (purple line) is higher than the theoretical value (grey line), demonstrating a synergistic effect. *F*, FN increases the ability of semen-derived enhancer of viral infection (SEVI) to enhance HIV-1 infection. *G*, FN and albumin do not enhance HIV infection. *H*, SEM1 and SEVI amyloids do not synergistically enhance HIV-1 infection. *I* and *J*, HIV-1 infection of CEM.M7 cells. CCR5-tropic 81A HIV-1 virions (100 ng/mL $p24^{Gag}$) were incubated for 5 minutes with the indicated concentrations of the corresponding factors, diluted 15-fold, and then added to suspension CEM.M7 cells. Infection was assessed 3 days later by flow cytometry. FN increases the ability of SEM1 amyloids (*I*) and SEVI (*J*) to enhance HIV-1 infection of CEM.M7 cells. *K*, FN increases the ability of SEM1 amyloids to enhance both CCR5-tropic (top) and CXCR4-tropic (bottom) HIV-1 infection of activated $CD4^{+}$ peripheral blood leukocytes (PBLs). $CD14^{-}CD4^{+}$ cells were purified from buffy coats and activated with PHA/IL-2. Two days later, nef-IRES-GFP HIV-1 virions (20 ng/mL $p24^{Gag}$) were pretreated for 5 minutes with the indicated concentration of FN or SEM1 amyloids, diluted 15-fold, and added to the cells. Infection was assessed 3 days later by flow cytometry. Abbreviation: RLU, relative light unit.

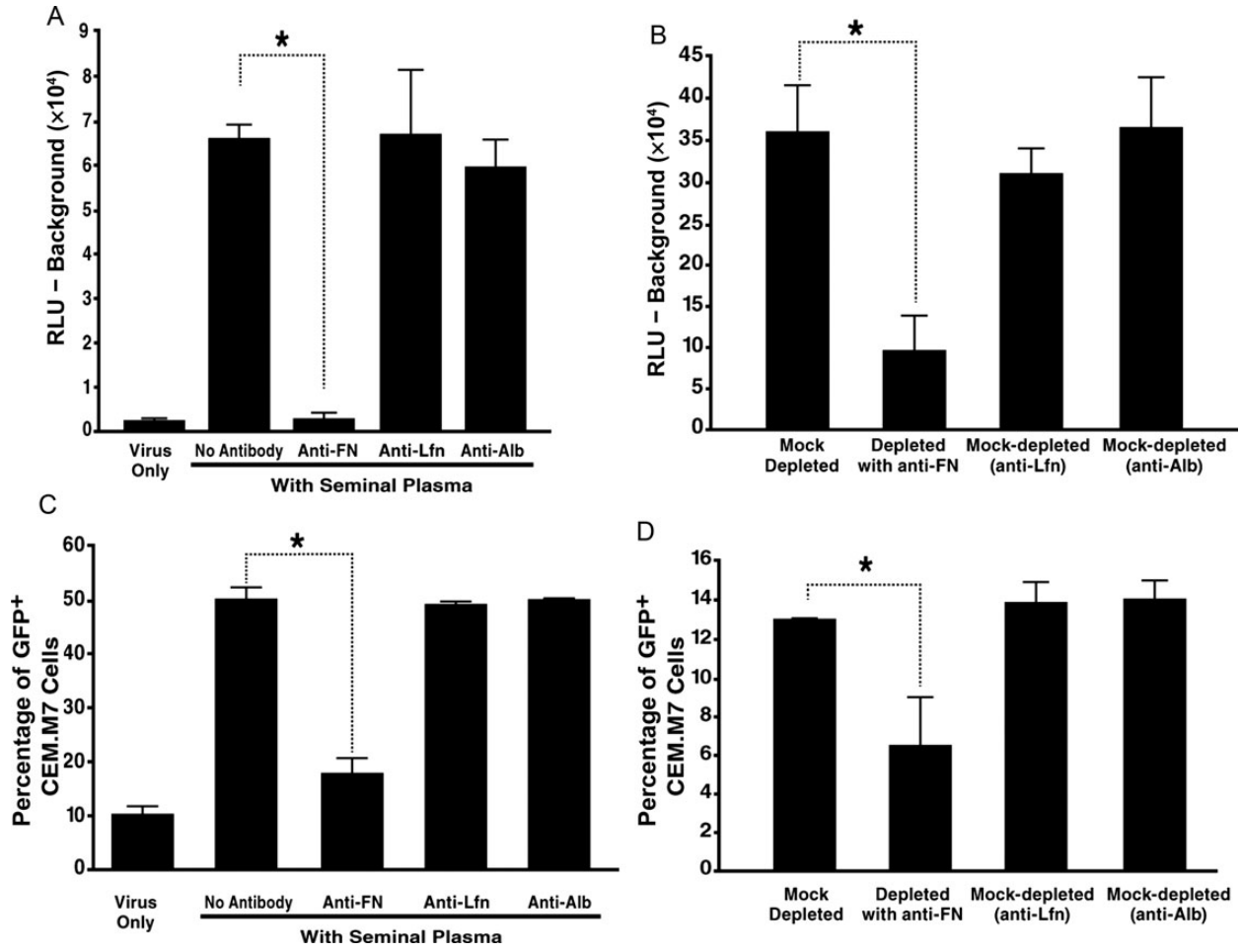

Figure 2. Blockade or immunodepletion of fibronectin (FN) decreases the ability of seminal plasma (SP) to enhance human immunodeficiency virus type 1 (HIV-1) infection. *A*, Incubation of SP with antibodies against FN but not against lactoferrin (Lfn) or albumin (Alb) decreases SP-mediated enhancement of HIV infection in TZM-bl cells. SP was diluted with phosphate-buffered saline to 2% and incubated for 12 hours at 4°C with anti-FN, anti-Lfn, or anti-Alb (all antibody concentrations, 60 μg/mL). HIV was then added, followed by TZM-bl cells, and infection was assessed 3 days later by quantitating β-galactosidase activity. *B*, Mock-treated SP or SP immunodepleted of FN (Supplementary Figure 3) was diluted to 2% and assessed for the ability to enhance HIV-1 infection of TZM-b cells, using methods described in panel *A*. *C* and *D*, Data are similar to those in panels *A* and *B*, except that CEM.M7 target cells were used. Infection levels were determined by assessing GFP expression on a LSRII flow cytometer.

on the ability of SP to enhance HIV infection. SP was mixed with HIV-1 in the absence or presence of anti-FN and then added to TZM-bl cells, and infection levels were assayed 3 days later by luminescence. Anti-FN concentrations of up to 200 μg/mL did not diminish the viral-enhancing activity of SP relative to treatment with control antibodies (data not shown). However, because under these experimental conditions the virions may have competed with anti-FN for binding to the FN/amyloid complex, we tested whether an overnight treatment of SP with anti-FN in assay plates, followed by addition of HIV-1 and target cells, would lead to different results. Remarkably, under these conditions, SP markedly enhanced HIV infection in a manner inhibited by anti-FN (Figure 2*A*). In contrast, antibodies against lactoferrin or albumin, 2 other abundant proteins in human semen, did not inhibit the SP response (Figure 2*A*). Immunodepletion of FN from semen through the use of anti-FN (Supplementary Figure 3) also decreased the ability of SP to enhance viral infection (Figure 2*B*). Blockade or immunodepletion of FN under similar conditions likewise diminished the ability of SP to enhance HIV infection of the nonadherent CEM.M7 cells (Figure 2*C* and 2*D*). Together, these data suggest that blockade or depletion of FN can diminish SP's viral-enhancing activity.

DISCUSSION

Although semen amyloids have been well characterized for their ability to enhance HIV-1 infection [2–9], their ability to bind to

and be modulated by other proteins in semen has not been studied. Here, we identify FN as a binding partner of HIV-enhancing SEM1 amyloids. FN increases the ability of SEM1 and SEVI amyloids to enhance HIV-1 infection, which may explain the marked infection-enhancing activity of semen (which contains all 3 factors) [2, 5]. The mechanism by which FN augments the activity of the amyloids is unclear but does not appear to be mediated by increased virion attachment to the fibrils and is recapitulated by the N-terminal 29-kDa region of FN. Because heparin abrogates the viral-enhancing activity of the FN/fibril complex, we speculate that FN may facilitate the binding of the fibrils to heparan sulfate proteoglycans on the surface of target cells in a manner that facilitates subsequent viral entry. In fact, FN contains a heparin-binding domain located within the 29-kDa N-terminal region [15]. The studies described herein report an unprecedented ability of FN to further augment the HIV-enhancing activity of semen amyloids. Design of site-specific disruptors targeting the interface between FN and seminal amyloids may be useful for developing microbicides that impair the ability of semen to enhance HIV infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank J. Wong, for generating electron micrographs; C. Miller, for assistance in confocal microscopy; T. Roberts, for assistance in preparing the figures; S. Cammack and R. Givens, for administrative assistance; and Dr Susan J. Fisher, for her support and helpful discussions.

Financial support. This work was supported by the National Institutes of Health (NIH; grants 5K12 DK083021-04 KURe and K99/R00 1K99AI104262 to N. R. R., grant 1PO1 AI083050-01 to W. C. G., and grant R01HD074511 to N.R.R. and W.C.G.), the UCSF Hellman Family Award (grant to N. R. R.); the Department of Defense (grant W81XWH-11-1-0562 to W. C. G.); and the UCSF–Gladstone Center for AIDS Research (grant P30-AI027763 to H. L.). Mass spectrometry analysis was performed by the UCSF Sandler-Moore Mass Spectrometry Core Facility, which acknowledges support from the Sandler Family Foundation, the Gordon and Betty Moore Foundation, and the National Cancer Institute, NIH (grant P30 CA082103).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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