Seminal Plasma Anti-HIV Antibodies Trigger Antibody-dependent Cellular Cytotoxicity: Implications for HIV Transmission

Matthew S. Parsons, PhD,* Vijaya Madhavi, MSc,* Fernanda Ana-Sosa-Batiz, BSc,* Rob J. Center, PhD,† Kim M. Wilson, PhD,‡ Torsak Bunupuradah, MD,§ Kiat Ruxrungtham, MD,¶ and Stephen J. Kent, MD*.

Abstract: Recent evidence from HIV vaccine trials in humans and non-human primates suggests that nonneutralizing antibody functions, such as antibody-dependent cellular cytotoxicity (ADCC), are an important component of vaccine-mediated protection. Whether anti-HIV ADCC antibodies are present in seminal fluid, however, is not known. We assessed whether anti-HIV antibodies within seminal plasma mediate ADCC and activate natural killer (NK) cells. Using matched blood and seminal plasma samples, we detected anti-HIV IgG within samples from all 11 HIV-infected donors. Furthermore, anti-HIV antibodies within the seminal plasma triggered detectable ADCC in 9 of 11 donors and activated NK cells in 6 of 11 donors. The ability of seminal plasma-derived IgG to activate NK cells in an anti-HIV antibody-dependent manner was enhanced when IgG were enriched and other seminal plasma components were removed. These observations have relevance for understanding natural immunity to HIV infection and provide assistance with HIV vaccine design.

Key Words: semen, ADCC, NK cells, antibody

(J Acquir Immune Defic Syndr 2016;71:17-23)

INTRODUCTION

The moderate protection conferred by the RV144 HIV vaccine regimen was associated with the presence of antiviral IgG directed to the V1–V2 regions of the HIV envelope, and low levels of antiviral IgA.1 Furthermore, the ability of antiviral IgG to mediate antibody-dependent cellular cytotoxicity (ADCC) was associated with protection in vaccinees that also had low levels of IgA antibodies. The potential involvement of ADCC in protecting from HIV infection is not surprising given that this immune response has been linked to slower progression towards AIDS in HIV-infected elite controllers and simian immunodeficiency virus (SIV)-infected macaques.3,4 Furthermore, ADCC was demonstrated in macaques infected with a live-attenuated SIV variant and correlated with protection from subsequent challenge with pathogenic virus.4

Although the analyses of immune responses in RV144 vaccinees focused exclusively on antibodies within peripheral blood, a highly successful HIV vaccine may require establishment of antiviral immunity within mucosal sites of exposure. Indeed, passive administration of broadly neutralizing antibodies to the vagina provides protection from infection upon challenge with simian-HIV.5 A potentially analogous requirement for anti HIV antibodies at mucosal sites of exposure was recently observed in human babies who remain HIV-uninfected after oral exposure to HIV-infected breast milk. Breast milk from HIV-infected mothers not transmitting HIV to their infants carried antibodies that mediated more robust ADCC than that from HIV-infected mothers transmitting HIV to their infants.6 As women in both transmitting and non-transmitting groups had high viral loads, it is unlikely that these antibodies were providing protection through utilization of ADCC effector cells within the mother. A more likely hypothesis is that ADCC effector cells within infants were able to use breast milk antibodies to eliminate infected cells in the early phase of transmission.

Similar to breast milk, semen is both a potent vehicle of HIV transmission and contains anti-HIV antibodies.7 Seminal plasma can contain neutralizing antibodies,8 but whether seminal plasma contains ADCC antibodies is unknown. We studied seminal plasma samples from HIV-infected donors and found that most seminal plasma samples
had anti-HIV antibodies capable of activating NK cells and/or killing gp120-coated target cells.

MATERIALS AND METHODS

Participants
Study participants included 11 HIV-infected individuals recruited for the HIV STAR trial in Thailand, as well as one donor with a known HIV-negative status. The HIV-infected subjects included 6 individuals on failing non-nucleoside reverse transcriptase inhibitor–based highly active antiretroviral therapy and 5 individuals who had been switched from failing non-nucleoside reverse transcriptase inhibitor–based highly active antiretroviral therapy to effective (LPV/r) regimens. Clinical data for these individuals were collected within a 45-day window period of the sampling date. Table S1, Supplemental Digital Content, http://links.lww.com/QAI/A731, shows the clinical characteristics of the HIV-infected subjects. Blood and seminal plasma samples were collected as previously described and used in the Western blot, enzyme-linked immunosorbent assay (ELISA), rapid fluorometric ADCC (RFADCC) and NK cell activation assays. Whole blood from 2 additional HIV-uninfected donors was used to derive peripheral blood mononuclear cells (PBMC) that served as effector cells in the RFADCC and NK cell activation assays. The National Serology Reference Laboratory (Fitzroy, Victoria, Australia) provided 2 additional blood plasma samples (1 from an HIV-infected and 1 from an HIV-uninfected donor), which served as quality controls for the HIV Western blot. Informed consent was obtained from all participants before collection of biological samples. The participating institutions approved all experiments.

HIV Western Blot
HIV Western blots were performed using a modification of a previously described method. HIV-1 IIB propagated in Hut 78 cells was used as a viral lysate (ZeptoMetrix Corporation, New York, NY). The lysate was sonicated and an optimized amount of 250 µg was used per gel for Western blot analysis of samples. The HIV-1 viral lysate was run on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a Protean II large format electrophoresis apparatus (Bio-Rad Laboratories Inc.) run at 100 V at 200 V for 6–8 hours. The proteins were transferred to nitrocellulose (0.45 µm pore size) overnight at 30 V in a Trans-Blot Cell (Bio-Rad Laboratories Inc., Hercules, CA).

The nitrocellulose was blocked on a shaker for 2 hours at room temperature in 0.3% skim milk powder in TBS (Tris-HCl-buffered saline pH 7.4) and cut into 3-mm vertical strips. Plasma samples derived from whole blood were diluted 1:100 in 0.1% skim milk powder in TBS, and each sample was incubated with a strip at room temperature for 4.5 hours. Seminal plasma samples were diluted 1:10 in blotto (50 mM Tris-HCl pH 8.0 containing 5% skim milk powders, 2 mM calcium chloride, 80 mM sodium chloride, and 0.2% NP40) and incubated overnight at room temperature on a shaking platform. Strips were washed 3 times in TBS-T (Tris-HCl-buffered saline containing 0.05% Tween 20), incubated for 1 hour at room temperature in a 1:20,000 dilution of biotinylated goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), and diluted in 0.1% skim milk powder in TBS. Strips were again washed before the addition of a 1:20,000 dilution of streptavidin–alkaline phosphatase (Molecular probes by Life Technologies, Carlsbad, CA) in 0.1% skim milk powder in TBS and incubation for 30 minutes at room temperature. Strips were washed extensively in TBS-T and the BCIP/NBT phosphatase substrate system (Kirkegaard & Perry laboratories, Gaithersburg, MD) was used to visualize bands. Extensive washing in water stopped the reaction, and the strips were air-dried overnight at room temperature.

ELISA for HIV Envelope Specific Antibodies
Modifying a previously published method, ELISA plates were coated with 100 ng per well of HIV-1 gp120 in carbonate: bicarbonate coating buffer (Sigma, St. Louis, MO) overnight at 4°C. The next day, plates were washed 3 times with wash buffer (PBS + 0.1% Tween 20) and blocked in blocking buffer (PBS + 5% bovine serum albumin [BSA] + 0.1% Tween 20) for 1 hour at 37°C. After 3 washes, seminal and blood plasma samples were added as log_{10} dilutions (1:100–1:1,000,000) in dilution buffer (PBS + 0.1% BSA + 0.2% Tween 20 + 0.5% NP40) and incubated for 90 minutes at 37°C. Incubation was followed by 6 washes, which was followed by a 1-hour incubation with horseradish peroxidase-conjugated antibody against human IgG. Plates were washed 6 times and developed with TMB substrate (Sigma). Samples were considered positive for anti-HIV IgG when OD was at least 2 times the background OD obtained from the HIV-uninfected control sample.

IgG Enrichment
As previously described for blood plasma, IgG was purified from seminal plasma samples with the Protein G HP Multitrap and antibody buffer kit (both from GE Healthcare, Buckinghamshire, UK), as per the manufacturer’s protocol. Eluates were washed with PBS in 30k Amicon Ultra-4 Centrifugal Filter Units (Millipore, Bedford, MA), and reconstituted within a volume of PBS identical to the volume of the original sample. A Nanodrop 2000 was used to determine IgG concentrations.

NK Cell Activation Assay
The NK cell antibody-dependent activation assay was performed as previously described. Briefly, the CEM.NK-CCR5 cell line was coated with HIV-1 Tat gp120 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) (3 µg/mL) for 90 minutes at 4°C. The gp120 formulation of envelope was used for this assay because we have previously observed gp120 antigens to elicit higher anti-HIV antibody-dependent NK cell activation than gp140 antigens. Next, freshly isolated HIV-negative donor PBMC and coated or uncoated CEM.NK-CCR5 cells were incubated together at 10:1 ratio in the presence of a source of human antibodies, 5 µg/mL Brefeldin A (Sigma), 6 µg/mL.
monensin (BD, Biosciences, San Jose, CA), and APC-conjugated anti-CD107a antibody (BD) for 5 hours at 37°C. Antibody sources included seminal or blood plasma from infected or uninfected donors, which were at final dilutions of 1:10 and 1:1000, respectively. For some renditions of the assay, IgG enriched from seminal plasma of HIV-infected donors was used as an antibody source at a 1:10 dilution. Control conditions included all reagents excluding the human antibody source or PBMC alone. After incubation, fluorochrome-conjugated antibodies against cell surface antigens [Per-CP-conjugated anti-CD3 (BD) and PE-Cy7-conjugated anti-CD56 (BD)] were added to the cells. Next, cells were fixed in formaldehyde, washed and incubated in permeabilization buffer (BD). Cells were then stained with Alexa Fluor 700-conjugated anti-IFNγ antibody (BD), fixed in formaldehyde and acquired using a BD FACSCanto II flow cytometer. Analysis was performed with FlowJo software (Tree Star Inc., Ashland, OR). Plasma samples were considered capable of triggering anti-HIV antibody-dependent NK cell activation when the percent NK cells activated in the presence of plasma and gp120-coated CEM.Nkr-C5R5 cells was at least 3 times the percent NK cells activated in the presence of plasma and uncoated CEM.Nkr-C5R5 cells.

**RESULTS**

**Anti-HIV Antibodies Within Seminal Plasma**

Previous research has demonstrated anti-HIV antibodies within the semen of infected donors. These antibodies occurred at lower titers than those observed in blood plasma, and the antibodies were mostly of the IgG isotype. To assess anti-HIV antibodies within seminal and blood plasmas, we performed Western blots to detect anti-Env (gp160, gp120, and gp41), anti-Gag (p24 and p18), anti-RT (p66 and p51), and anti-IN (p32) antibodies. As depicted in Figure 1A, seminal and blood plasma samples from all 11 HIV-infected HIV STAR trial donors exhibited anti-Env antibodies (ie, positive bands for gp160, gp120, and/or gp41), and all donors exhibited positive responses against at least one other HIV gene product (ie, Gag, RT, and/or IN). No responses were detected in either the seminal or blood plasma of the HIV-uninfected donor (Fig. 1A). Next, we used an ELISA to screen the seminal plasma of the 11 HIV-infected HIV STAR trial donors for anti-HIV gp120 IgG. Antibodies against HIV gp120 were observed within both the blood and seminal plasmas of 11 of 11 donors (Fig. 1B). The anti-HIV gp120 antibody titers were higher in the blood plasma than the paired seminal plasma within 9 of 11 samples. In one donor anti-gp120 IgG titers were higher in seminal than blood plasma, and an additional donor demonstrated an equal titer in both plasmas.

**Anti-HIV Antibodies Within Seminal Plasma Mediate ADCC**

Given that IgG can mediate ADCC, we next assessed if seminal plasma could mediate ADCC using the RFADCC assay. Both blood and seminal plasma at dilutions ranging from 1:10 to 1:100,000 were used as sources of antibodies in assays containing PBMC effectors and CEM.Nkr-C5R5 targets cocultured at a 10:1 ratio. The implemented gating strategy is depicted in Figure 2A. Figure 2B depicts the RFADCC readout of an HIV-infected donor, who exhibits the highest level of blood plasma-mediated ADCC at 1:1000 dilution and the highest level of seminal plasma ADCC at a 1:10 dilution, and an HIV-uninfected donor. Similar to the HIV-infected donor represented in Figure 2B, the seminal plasma samples eliciting ADCC responses in the RFADCC assay mostly peaked at the 1:1000 dilution, whereas the majority of seminal plasma samples that triggered ADCC responses peaked at the 1:10 dilution. Indeed, we detected ADCC-mediated killing (ie, at least 3 times the background signal observed on combination of uncoated CEM.Nkr-C5R5, a 1:10 dilution of HIV-positive blood plasma and PBMC) in 11 of 11 subject’s blood plasma at 1:1000 dilution in the presence of gp120 [66.1% (41.9%–79.3%), P < 0.0001; Figure 2C left plot]. ADCC-mediated killing (ie, at least 3 times the background signal observed on combination of uncoated CEM.Nkr-C5R5, a 1:10 dilution of HIV-positive seminal plasma and PBMC) was also detected in 9 of 11 subject’s seminal plasma samples at 1:10 dilution in the presence of gp120 [33.0% (6.7–48.7%), P < 0.0001; Figure 2C right plot]. Collectively, these ADCC

**Statistical Analyses**

All data analyses were conducted using GraphPad Prism version 4.0. Paired data sets were compared with Wilcoxon matched pairs tests. Differences between groups were considered significant when P < 0.05. Data throughout the article are presented in the [median (range)] format.

Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.
Anti-HIV Antibodies Within Seminal Plasma Activate NK Cells for Degranulation and Cytokine Production

Antiviral antibodies bound to target cells bearing HIV envelope can trigger ADCC mediated by NK cells and/or monocytes. Additionally, stimulation of NK cells leads to expression of the CD107a degranulation marker and production of cytokines and/or chemokines. Activation of NK cells to produce chemokines inhibits viral replication. Given the potential importance of NK cell activation for inhibiting HIV replication, we assessed whether stimulation of NK cells with gp120-pulsed CEM.NK-CCR5 cells, in the presence of anti-HIV antibodies, activated NK cells to express the CD107a degranulation marker and produce IFNγ. Since the RFADCC assay demonstrated robust blood plasma-mediated ADCC at the 1:1000 dilution and robust seminal plasma-mediated ADCC was observed at the 1:10 dilution, we used these respective antibody dilutions to screen for NK cell activation elicited by each bodily fluid. The gating strategy used to identify activated NK cells is depicted in Figure 2D. Similar to the RFADCC assay, blood plasma from 11 of 11 individuals activated NK cells to express CD107a and/or produce IFNγ against CEM.NK-CCR5 cells coated with gp120 [19.4% (9.7%-28.2%)], but not against uncoated CEM.NK-CCR5 [0.4% (0.3%-0.5%)]. Also, similar to the RFADCC assay, seminal plasma was able to trigger antibody-dependent NK cell activation. Indeed, we
FIGURE 2. Assessment of the ability of anti-HIV antibodies within seminal and blood plasma to trigger ADCC and activate NK cells. A-C, Both blood and seminal plasma samples were assessed for their ability to trigger anti-HIV ADCC using the RFADCC assay. A, FACS plots depict the gating strategy used. Gating was on CFSE+ cells followed by gating on CD3+ CD14+ monocytes and analysis of PKH26 expression for these monocytes. The graph on the bottom right depicts an example of the comparison of PKH26 expression by CD14+ monocytes in the presence of HIV-positive seminal plasma (black histogram) or HIV-negative seminal plasma (gray histogram). B, Assessment of monocytes for PKH26 expression after RFADCC conditions containing a range of dilutions of blood or seminal plasma samples for HIV-positive subject 10 (left graph) and for HIV-negative subject (right graph). C, Percentage of CD14+ monocytes that have gained PKH26 expression after RFADCC assays performed using blood plasma at 1:100 dilution (left graph) and seminal plasma at 1:10 dilution (right graph) from 11 HIV-positive individuals against CEM.NK-CCR5 targets coated with HIV-1sa gp120, as compared with blood plasma and seminal plasma at 1:10 dilutions against CEM.NK-CCR5 targets not coated with HIV-1sa gp120. Dotted lines represent the percentage of PKH26+ monocytes observed in conditions containing an HIV-negative blood or seminal plasma sample. Comparisons were made using Wilcoxon matched pairs tests. Solid lines in the graphs represent the medians. D, The ability of anti-HIV antibodies within blood and seminal plasma to activate NK cells to express CD107a and/or produce IFN-γ was assessed with the NK cell activation assay. As shown in the FACS plots, gating was initially on the CD3+ CD56+ NK cell population. Next, NK cells were assessed for their CD107a expression and/or IFN-γ production upon stimulation with gp120-coated or uncoated CEM.NK-CCR5 target cells in the presence of seminal (top) or blood (bottom) plasma. Graphs on the right depict the percentages of NK cells expressing CD107a and/or IFN-γ upon stimulation with CEM.NK-CCR5 cells coated or uncoated with gp120 in the presence of blood (bottom) or seminal (top) plasma. E, To determine whether factors within seminal plasma vs. blood plasma were key to the observed expression of CD107a and/or IFN-γ production, at least 3 times above the background activation seen against uncoated CEM.NK-CCR5 target cells in the presence of the same seminal plasma source, in 6 of 11 individuals [1.6% (1.2%-7.8%) against coated cells vs. 0.3% (0.2%-0.4%) against uncoated cells (ie, for the 6 responsive donors)] (Fig. 2D).

As antibody-dependent NK cell activation induced by seminal plasma was only observed in small fractions of NK
cells, we next enriched IgG from the seminal plasmas of 8 donors. Given that previous research has demonstrated that factors within seminal plasma can inhibit ADCC to non-HIV antigens, we predicted that enriched IgG from semen would induce better activation of NK cells than whole seminal plasma. Indeed, in all 8 donors tested seminal plasma-derived IgG induced more profound NK cell activation than whole seminal plasma [14.3% (3.0%–25.4%) vs. 1.8% (0.1%–3.4%), P = 0.0078] (Fig. 2E). Indeed, seminal plasma-derived IgG from 3 donors, whose seminal plasma did not induce anti-HIV antibody-dependent NK cell activation, induced anti-HIV antibody-dependent NK cell activation. These results demonstrate that anti-HIV antibodies within seminal plasma can trigger NK cell activation but that inhibitory factors within semen can dampen these responses.

**DISCUSSION**

To our knowledge, this is the first demonstration that anti-HIV antibodies within seminal plasma can activate NK cells and trigger ADCC. We found that seminal plasma contained variable amounts of anti-HIV Env-specific antibodies, and these antibodies trigger anti-HIV ADCC. This suggests ADCC antibodies enter seminal plasma as a proportion of total antibodies. Our data have ramifications for exploring mechanisms of protective immunity to HIV.

What is the potential significance of detecting ADCC within seminal plasma? It is plausible ADCC antibodies present within seminal plasma in HIV-infected donors may potentially be “used” by HIV-negative sexual partners to limit HIV transmission. We speculate that ADCC antibodies within seminal plasma may be one mechanism underlying the generally low rate of HIV transmission per coital act. Indeed, if the semen recipient has appropriate effector cells, such as NK cells or monocytes with a particularly favorable ability to mediate ADCC, seminal plasma antibodies capable of triggering ADCC could potentially underlie the relative resistance to HIV infection seen in some HIV-exposed seronegative subjects. Indeed, previous studies have demonstrated higher NK cell functional potential in HIV-exposed seronegative subjects. Although protection in HIV-exposed uninfected subjects is probably multifactorial, involving several immune responses, studying such donor–recipient pairs for ADCC antibodies in donor semen and NK cell functionality in the recipient is an avenue of future research. In addition to NK cells, monocytes are potent mediators of anti-HIV ADCC. As such, future assessments of ADCC effector cells in HIV-exposed uninfected donors should also incorporate assessments of monocyte-mediated ADCC. In addition to seminal plasma, studying the potential of antibodies in cervicovaginal fluids to trigger ADCC will also be of interest in this context. Indeed, a recent study demonstrated antibodies within cervicovaginal lavages of HIV-infected women trigger ADCC. Furthermore, assessing seminal plasma antibodies for their ability to initiate other Fc-receptor-dependent effector functions may prove important. It is interesting to note that recent work on the protective mechanisms involved in live-attenuated SIV infections of macaques suggests a possible role for Fc-mediated functions of antibodies at the mucosal level.

Although our results provide evidence that anti-HIV antibodies within seminal plasma can trigger ADCC and activate NK cells, the observation that IgG enriched and used in the absence of the other components of seminal plasma triggers higher NK cell activation suggests seminal plasma carries factors that inhibit antibody-dependent activation. Indeed, these data fit with previous work demonstrating seminal plasma to inhibit non-HIV ADCC. That some seminal plasma samples are able to overcome these inhibitory factors to produce detectable responses implies that seminal plasma antibodies could exhibit in vivo utility, as discussed above. The ability of seminal plasma to inhibit antibody-dependent NK cell activation, however, raises intriguing questions about the ability of semen to inhibit the protective potential of vaccine-induced or passively transferred antibodies within HIV-uninfected recipients of HIV-infected semen. This possibility requires evaluation in future investigations. In conclusion, our work illustrates HIV-specific antibodies that initiate ADCC are present in seminal plasma, but that additional factors within seminal plasma may dampen the ability of these antibodies to activate NK cells in an anti-HIV antibody-dependent manner. Defining the role of such antibodies in protective immunity is an important priority for future research.

**REFERENCES**


