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Short communication

HIV infection of mononuclear cells is calcium-dependent

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Abstract

Strategies that prevent initial HIV infection of cells are greatly needed. In this study, we determined the requirement of divalent cations for HIV infection of and attachment to peripheral blood mononuclear cells (PBMC), which contain several types of HIV-infectable cells—$CD4^+$ T cells, monocytes and dendritic cells. EDTA, added only during PBMC exposure to HIV, reduced infection by an average of 92%. The reduction of infection by EDTA was accompanied by a reduction in HIV binding to PBMC; R5, X4 and dual-tropic HIV binding to PBMC were inhibited by >85%. EGTA similarly reduced HIV binding to PBMC, while addition of Ca^{2+} or Mn^{2+}, but not Mg^{2+}, fully restored binding. Virus attachment was inhibited in a dose-dependent manner by trypsin treatment of PBMC, indicating protein involvement in HIV binding. In contrast, mannan or soluble ICAM-1 did not inhibit HIV binding to PBMC. These data indicate that a Ca^{2+}-dependent cell-surface protein(s) is responsible for the majority of HIV attachment to and infection of PBMC. Further studies of this are likely to reveal novel strategies to prevent infection of PBMC.

Keywords: HIV binding; Calcium; EDTA; EGTA; Peripheral blood mononuclear cells

1. Introduction

Transmission of HIV to susceptible targets is enhanced by its attachment to molecules other than CD4. In particular, Dendritic Cell Specific ICAM Grabbing Non-integrin (DC-SIGN) expressed on dendritic cells binds to glycans on gp120 of HIV (Geijtenbeek et al., 2000). Attachment of HIV to cells via DC-SIGN directly increases both *cis* infection, virus infection of $DC\text{-}SIGN^+/CD4^+$cells (Lee et al., 2001) and *trans* infection, infection by transfer of HIV from $DC\text{-}SIGN^+$ cells to $CD4^+$ lymphocytes (Geijtenbeek et al., 2000). Similarly, macrophages bind HIV via the mannose receptor and transfer infectious virus to lymphocytes (Nguyen and Hildreth, 2003).

DC-SIGN and mannose receptor are C-type lectins and therefore require Ca^{2+} for their function. HIV binding to macrophages and endothelial cells can be mediated by heparan sulfates, resulting in increased *cis* and *trans* infection (Bobardt et al., 2003; Saphire et al., 2001). Both macrophages and T cells express the integrin Leukocyte Function Antigen-1 (LFA-1), and antibodies that activate LFA-1 into a high affinity conformation increase infection by HIV that contains ICAM-1 (Fortin et al., 1998). LFA-1 can also be incorporated into the virus membrane and increases HIV attachment to ICAM-expressing cell lines (Liao et al., 2000).

We previously established that primary isolates of HIV bind to peripheral blood mononuclear cells (PBMC) and this binding facilitates HIV infection to T cells (Olinger et al., 2000). Additionally, this attachment is CD4-independent (Olinger et al., 2002). With the long-term goal of elucidating the identity of this HIV attachment molecule and given that several families of adhesion molecules, including C-type lectins and integrins, are divalent cation-dependent, we examined the requirement for divalent cations in HIV attachment to and infection of PBMC.

2. Materials and methods

PBMC were obtained from healthy donors by Ficoll-Hypaque (Whittaker M.A. Bioproducts, Walkersville, MD) gradient centrifugation of freshly obtained heparanized blood. PBMC were activated by culture with 3 μg/ml phytohemagglutinin (PHA-L; Sigma/Aldrich, St. Louis, MO) in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum and 50 μg/ml gentamicin (complete medium). After 2 days of culture with PHA, PBMC were washed and then cultured in complete medium containing 20 U/ml interleukin-2 (obtained

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through the AIDS Research and Reference Reagent Program [ARRRP], National Institute of Allergy and Infectious Diseases, National Institutes of Health, from Maurice Gately, Hoffman-La Roche, Inc.). THP-1/DC-SIGN cells were obtained from the ARRRP.

The viruses HIV_{TH} (R5), $HIV_{89.6}$ (R5X4), HIV_{NL4-3} (X4) and HIV_{BaL} (R5) were produced in PHA-stimulated PBMC of healthy donors as described (Takefman et al., 1998). HIV_{NL4-3}, $HIV_{89.6}$ and HIV_{BaL} were obtained from the ARRRP. HIV_{TH} was isolated as described (Olinger et al., 2000).

HIV binding to cells was performed by incubating 100 μl of virus-culture supernatant, diluted in serum-free RPMI to contain 2000 pg of p24, with 3×10^6 PBMC, PHA-stimulated PBMC or THP-1/DC-SIGN cells for 45 min on ice. The cells were then washed with 3 ml of phosphate buffered saline (PBS) with a transfer to a new tube between washes to prevent measurement of HIV binding to tubes. Pelleted cells were lysed with 0.5% Triton X-100, and the amount of virus bound to cells was measured by p24 antigen ELISA (National Institutes of Health AIDS Vaccine Program, Frederick, MD). In some experiments, cellular protein content was measured in tubes after washing to determine cell loss by assaying 25 μl of the lysate using the BCA Pierce Micro protein assay, as described by the manufacturer (Pierce, Rockford, IL). The protein assays showed that cell loss was minimal and similar between experimental groups (data not shown).

For experiments where divalent cations were depleted from medium, PBMC and HIV were pre-incubated with 10 mM EDTA or varying doses of EGTA for 10 min. PBMC were washed by centrifugation at $300 \times g$ at 4 °C for 10 min, and binding was performed as described above, except in the presence of the chelating agent. For experiments where divalent cations were added in excess of EGTA, 3 mM of divalent cation was combined with 1 mM EGTA. Three millimolar $MnCl_2$ was also combined with either 10 mM EDTA or 10 mM EGTA for some groups. In some experiments, cells were washed with 10 mM EDTA after HIV binding. Mannan 250 (μg/ml) or ICAM-1 (60, 600 nM) was added during HIV binding in some experiments. Soluble ICAM-1 was from R&D Systems (Minneapolis, MN), while all other inhibitors were from Sigma/Aldrich (St. Louis, MO).

For experiments where trypsin (1:250; Cellgro, Herndon, VA) was used to remove cell-surface protein, 2×10^6 PBMC were pre-treated with 2.5–25 mg/ml trypsin at 37 °C for 10 min. PBMC were washed twice by centrifugation at $300 \times g$ at 4 °C for 10 min, and binding was performed as described above.

For experiments where HIV RNA was measured, HIV binding to PBMC was performed as above. RNA was isolated from PBMC using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from 0.2 μg RNA using the RT-PCR Kit from Clontech (Palo Alto, CA). Amplification of cDNA was performed using SYBR® Green PCR Core Reagents (Applied Biosystems, Foster City, CA) at the following concentrations: 0.05 μM primers, 1× SYBR® Green PCR Buffer, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 0.01 U/μl AmpErase® UNG, 0.02 U/μl of AmpliTaq Gold® DNA Polymerase and 20 ng cDNA in 50 μl total volume. Primers that amplify the HIV LTR, as described by Chun et al. (2003), were used in real-time PCR. 8E5 cells, that contain one HIV provirus copy per cell, were used to construct a standard curve (Folks et al., 1986). An ABI prism 5700 Thermocycler (Applied Biosystems) was used to amplify cDNA with the following thermocycle profile: Stage 1, 50 °C (2 min); Stage 2, 95 °C (10 min); Stage 3 consisting of 50 cycles of 95 °C (45 s), 62 °C (45 s) and 72 °C (45 s).

For infection of PBMC, freshly isolated PBMC were cultured in complete medium with 3 μg/ml PHA for 48 h and then washed prior to HIV binding. Cells were incubated with HIV and washed as described above. PBMC were then transferred to 24-well plates (Corning Inc., Corning, NY) and cultured in medium with 20 U/ml IL-2 in a total volume of 1 ml. HIV p24 in culture supernatants was measured by ELISA on days 3–12. Cultures were fed on days 5, 7 and 10 by removal of 500 μl and replacement with fresh medium containing IL-2. Infection of PBMC by $HIV_{NL4-3.HSA.R^+}$ (ARRRP) was performed as above, except cells were collected on days 4 and 7. Murine CD24 on PBMC was measured by flow cytometry after staining cells with 0.5 μg anti-CD24 FITC (eBioscience, San Diego, CA) on ice for 30 min. $HIV_{NL4-3.HSA.R^+}$ encodes the mouse CD24 gene fused in frame to the *nef* initiator methionine codon (He et al., 1995).

3. Results and discussion

Given that some cell-surface molecules, such as C-type lectins and integrins require divalent cation for their respective functions, and that these molecules also bind HIV efficiently, we evaluated the requirement of divalent ions for efficient HIV infection of PBMC. PBMC were stimulated with PHA for 48 h, and then incubated with virus for 45 min at 4 °C either in the presence or absence of EDTA. Cells were washed thoroughly to remove EDTA and unbound HIV, and then cultured for up to 12 days. The presence of EDTA during exposure of PBMC to virus inhibited infection by >92% measured at day 7 for HIV_{TH}, $HIV_{89.6}$, HIV_{BaL} and $HIV_{NL4-3.HSA.R^+}$ (Fig. 1A–D). To determine if the EDTA treatment was toxic to cells, PHA-stimulated PBMC were pretreated with EDTA for 45 min and washed to remove EDTA prior to incubation with virus. Pretreatment of cells with EDTA did not significantly affect infection (Fig. 1A–C). Similar experiments were performed with an $HIV_{NL4-3.HSA.R^+}$ construct encoding a murine CD24 reporter (He et al., 1995) where infection was detected by flow cytometry. CD24 expression was 8.5% of PBMC on day 7, whereas cells infected in the presence of 10 mM EDTA expressed only 0.8% CD24 (Fig. 1D). To elucidate which divalent cation was required for HIV infection, we chelated Ca^{2+} with EGTA (data not shown). Treatment of PBMC with 1 mM EGTA inhibited HIV_{TH} infection by 95% as measured on day 5 (average of two experiments). Together, these results show that chelation of Ca^{2+} during the initial exposure of PBMC to HIV substantially reduces HIV infection.

Since EDTA and EGTA were present only during exposure of HIV to cells in the above infection experiments, we hypothesized that chelation of Ca^{2+} inhibited HIV binding to PBMC. To measure the effect of Ca^{2+} on HIV attachment to PBMC, HIV was added to freshly isolated PBMC or PHA-stimulated PBMC

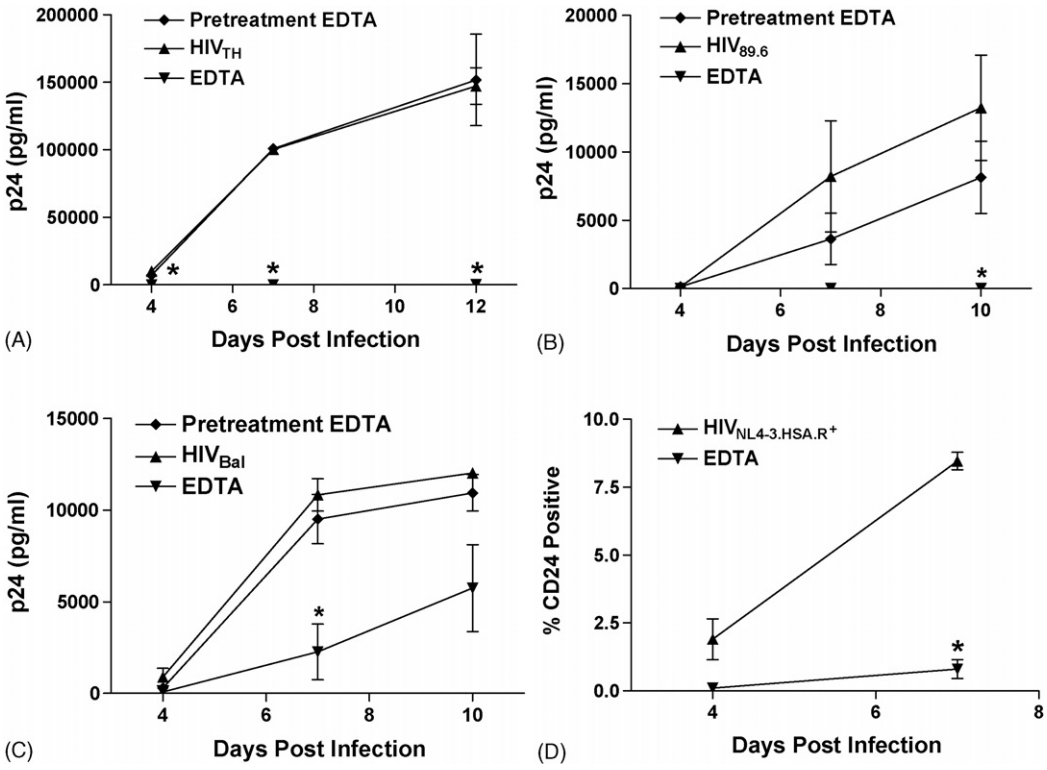

Fig. 1. HIV infection of PBMC is inhibited by chelation divalent-cations. (A–C) PHA-stimulated PBMC were incubated with 2000 pg of p24 (HIV_{TH}, $HIV_{89.6}$ or HIV_{BaL}) in serum-free medium in the absence or presence of 10 mM EDTA for 45 min on ice. After incubation with HIV, cells were washed once in the presence of 10 mM EDTA, and then washed twice with PBS to remove EDTA. Cells were cultured with IL-2 and supernatants were collected on days 4–12. p24 was measured by ELISA. (D) PHA-stimulated PBMC were incubated with $HIV_{NL4-3.HSA.R^+}$ as in (A). CD24 was measured on days 4 and 7 by flow cytometry using anti-CD24. Asterisk represents a p-value <0.05. Results for HIV_{TH} experiments represent the means ± S.E. of triplicate determinations of one experiment representative of three independent experiments. Results for all other experiments are the means ± S.E. of triplicate determinations of one experiment. Asterisk represents a p-value < 0.05.

for 45 min at 4 °C in the presence or absence of EDTA or EGTA. PBMC were washed extensively to remove unbound virus, lysed with 0.5% Triton X-100 and levels of bound HIV were measured by p24 ELISA. Binding of an R5 virus strain (HIV_{TH}) to unstimulated or PHA-stimulated PBMC was reduced >91% by 10 mM EDTA (Fig. 2A). The ability of EDTA to detach bound virus was also tested by washing in 10 mM EDTA after virus was bound. Washing PBMC in 10 mM EDTA reduced the amount of HIV bound to cells by 76% (Fig. 2A). Binding of HIV_{TH} to PBMC was also inhibited by EDTA when measured by real-time RT-PCR for HIV RNA (Fig. 2B), confirming that EDTA inhibits binding of intact virus particles to cells. The ability of EDTA to inhibit binding of R5, X4 and dual-tropic viruses to PBMC was also tested. Treatment with 10 mM EDTA inhibited virus attachment by 99% for HIV_{TH} (R5), 94% for HIV_{NL4-3} (X4), 99% for $HIV_{89.6}$ (X4R5) and 89% for HIV_{BaL} (R5) (Fig. 2C).

To determine the specific cation necessary for HIV binding to freshly isolated PBMC, we bound virus to cells in the presence of EGTA. A dose response showed that incubation with 1 mM EGTA prevented HIV binding to PBMC by 99%, while as little as 0.1 mM EGTA significantly inhibited HIV attachment to PBMC (data not shown), suggesting that virus attachment to PBMC was calcium-dependent. Addition of 3 mM Ca^{2+} to 1 mM EGTA fully restored binding of HIV to freshly isolated PBMC (Fig. 2D) and PHA-stimulated PBMC (data not shown). EGTA chelates Ca^{2+} without substantially chelating Mg^{2+}; however, EGTA can also chelate other divalent cations, including Mn^{2+}. To determine if other divalent-cations affect HIV binding to PBMC, we compared the ability of Ca^{2+}, Mg^{2+} and Mn^{2+} to restore HIV binding to PBMC by adding excess divalent cation to EGTA (Fig. 2D). Addition of 3 mM Mg^{2+} to 1 mM EGTA did not restore HIV binding to PBMC, whereas addition of 3 mM Mn^{2+} or 3 mM Ca^{2+} to 1 mM EGTA increased HIV binding to levels greater than the control. The ability of 3 mM Mn^{2+} to increase HIV binding to PBMC was abrogated by 10 mM EDTA or 10 mM EGTA. These results show that HIV binding to PBMC is specific for Ca^{2+} but not Mg^{2+}. Mn^{2+} is known to increase integrin binding affinity (Leitinger et al., 2000). Thus, the increased binding of HIV to PBMC after addition of Mn^{2+} may be due to an integrin. However, since Mn^{2+} is not present in the culture medium used in our binding experiments, it is not clear if the Ca^{2+}-mediated binding and the Mn^{2+}-mediated binding are due to the same mechanism.

Since several Ca^{2+}-dependent adhesion proteins are expressed on PBMC that have been suggested to affect HIV binding to cells, we determined if HIV binding was trypsin sensitive by pretreating cells with medium containing 2.5–25 mg/ml trypsin for 10 min before incubation with virus. HIV attachment was reduced in a dose-dependent manner by trypsin, with

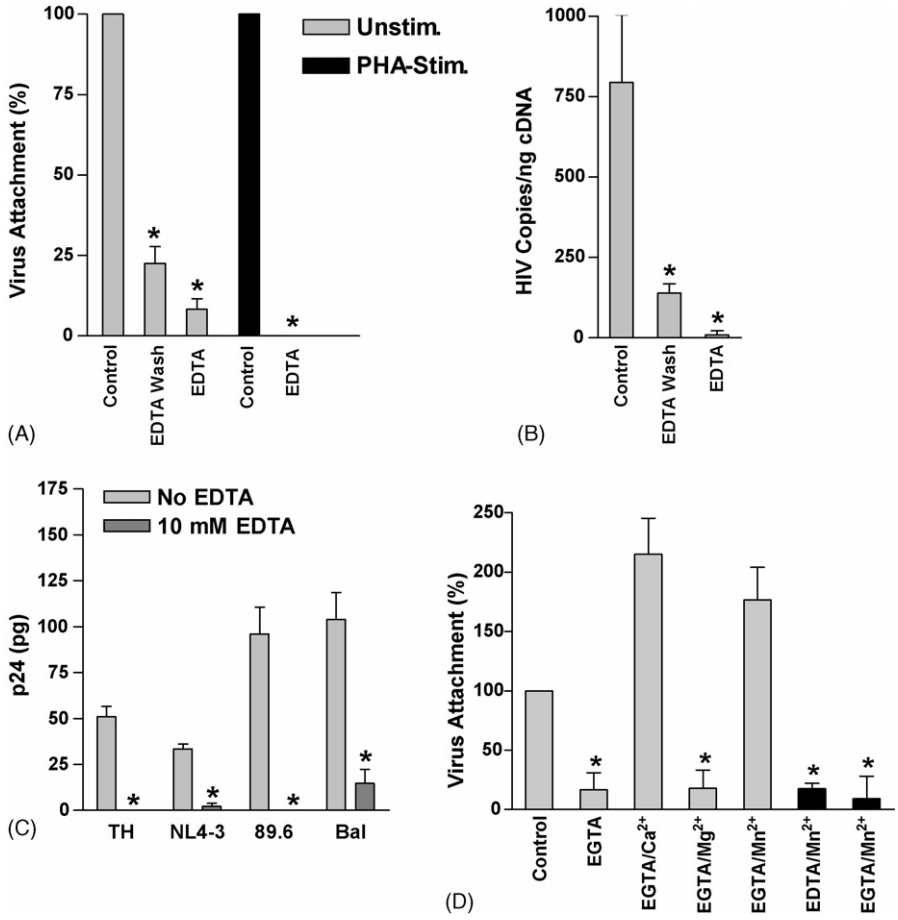

Fig. 2. Inhibition of HIV attachment to PBMC by EDTA and EGTA. (A) Unstimulated or PHA-stimulated PBMC were incubated with 2000 pg of p24 (HIV_{TH}) in serum-free medium in the absence or presence of 10 mM EDTA for 45 min on ice. After incubation with HIV, cells were washed with EDTA to remove unattached virus. For the EDTA wash group, PBMC were incubated with virus in EDTA-free medium, and then washed with 10 mM EDTA. Cells were lysed in Triton X-100 and HIV p24 was measured by ELISA. For unstimulated cells, shown are the means ± S.D. of three independent experiments. For PHA-stimulated cells, shown are triplicate determinations of one experiment. Asterisk represents a p-value < 0.05 (t-test). (B) HIV binding to PBMC was performed as in Fig. 1A. RNA was isolated from cells with bound virus and cDNA was synthesized. Twenty nanograms of total cDNA was added into each PCR reaction tube. Real-time PCR for HIV, using HIV LTR primers, was performed to assess the amount of HIV bound to cells. Results represent the means ± S.D. of triplicate determinations of three independent experiments. Asterisk represents a p-value < 0.05. (C) Experiments were performed as in (A), except that cells were incubated with 2000 pg of p24 of either HIV_{TH}, HIV_{NL4-3}, $HIV_{89.6}$ or HIV_{BaL}. Results for HIV_{TH}, $HIV_{89.6}$ and HIV_{BaL} represent the means ± S.D. of triplicate determinations representative of two independent experiments. Results for HIV_{NL4-3} represent triplicate determinations of one experiment. Asterisk represents a p-value < 0.05. (D) Experiments were performed as in (A), except PBMC were incubated with 2000 pg of p24 (HIV_{BaL}) in medium containing no EGTA, 1 mM EGTA, 1 mM EGTA + 3 mM divalent cation (either Ca^{2+}, Mg^{2+} or Mn^{2+}), 10 mM EDTA + 3 mM Mn^{2+} (black bar) or 10 mM EGTA + 3 mM Mn^{2+} (black bar). Results are the means ± S.D. of triplicate determinations of one experiment representative of two independent experiments. Asterisk represents a p-value < 0.05.

8 mg/ml inhibiting binding by 91% (Fig. 3). To confirm that cell-surface proteins were degraded by trypsin treatment, CD4 expression on PBMC was measured by flow cytometry. CD4 was detected on 44% of PBMC without trypsin treatment but was reduced to 5% with 2.5 mg/ml trypsin (Fig. 3). Cell number and viability, as monitored by trypan blue, were not affected by trypsin treatment (data not shown). These data collectively indicate that HIV attachment to PBMCs requires a cell-surface protein that is Ca^{2+}-dependent. Previous studies demonstrated that HIV binding to macrophages and dendritic cells is Ca^{2+}-dependent. Macrophages bind HIV through the mannose receptor, and attachment of HIV to macrophages is prevented in the presence of EDTA (Nguyen and Hildreth, 2003), since the mannose receptor is a C-type lectin. DC-SIGN, another C-type lectin, has been extensively studied as it has been implicated in HIV attachment and transfer to infectable cells (van Kooyk and Geijtenbeek, 2003). Chelation of Ca^{2+} by EGTA prevents HIV binding to DC-SIGN on dendritic cells (Geijtenbeek et al., 2000). We confirmed that PBMC do not express DC-SIGN as measured by flow cytometry (data not shown). However, other C-type lectin molecules have the potential for binding HIV (Turville et al., 2003) and may act as attachment factors for PBMC. Therefore, we tested the ability of mannan to inhibit binding and infection of PBMC by HIV. HIV binding and infection of PBMC was not significantly affected by treatment with mannan (data not shown). In parallel control experiments for HIV binding to PBMC, 10 mM EDTA inhibited HIV binding to PBMC by 96%. In additional control experiment, mannan inhibited by 85% HIV *trans* infection of PBMC mediated by THP-1/DC-SIGN cells ($p < 0.001$) (data not shown), similar to what

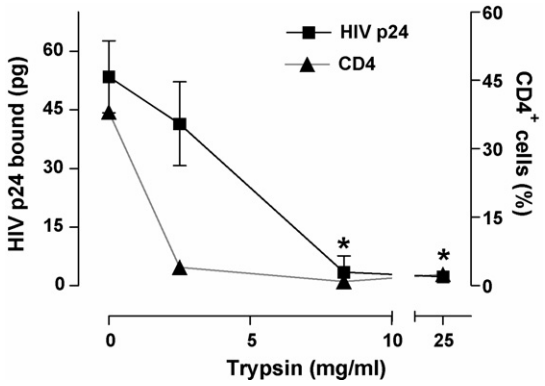

Fig. 3. Inhibition of HIV attachment to PBMC by trypsin. PBMC were pretreated with or without trypsin for 10 min at 37 °C, washed and incubated with HIV_{TH} (2000 pg of p24) for 45 min on ice. Cells were washed, lysed in 0.5% Triton X-100, and p24 measured by ELISA. Results represent the means ± S.E. of triplicate determinations of three independent experiments. Asterisk represents a p-value < 0.05. For measurement of CD4, PBMC were pretreated with or without trypsin as above, then incubated with PE-conjugated anti-CD4 and analyzed by flow cytometry.

has been previously reported (Geijtenbeek et al., 2000). Treatment of either PBMC or THP-1/DC-SIGN cells with 10 mM EDTA also substantially reduced infection ($p < 0.05$). Thus, these results suggest a mannose-binding lectin is not involved in binding of HIV to PBMC.

It has also been observed that the integrin LFA-1 on cells can act as an attachment factor for ICAM-1 expressing virions (Fortin et al., 1998). That study showed that an activating antibody to LFA-1 greatly increased HIV attachment to T-lymphoid cell lines as well as PBMC. We bound HIV to PBMC in the presence of soluble ICAM-1, the ligand for LFA-1. Soluble ICAM-1 at 60 or 600 nM did not significantly affect HIV binding (data not shown). In these experiments, 10 mM EDTA, run as a control, blocked HIV binding to PBMC. Thus, these results suggest that the β2-integrins are not involved in binding of HIV to PBMC.

While none of the inhibitors of divalent cation-dependent molecules reduced HIV binding to PBMC in the above experiments, it is possible that these molecules have a cumulative effect on HIV binding. To test this possibility, we treated PBMC and HIV with a mixture of blocking antibodies to LFA-1, ICAM-1 and DC-SIGN, as well as cRGD peptide, lewis-a tetrasaccharide and mannan. Combining these inhibitors had no effect on HIV binding to PBMC (data not shown).

Numerous Ca^{2+}-dependent adhesion proteins are expressed on PBMC. We determined that HIV binding to PBMC was trypsin-sensitive, consistent with a role for a Ca^{2+}-dependent cell-surface adhesion protein. HIV attachment to PBMC may be mediated by a Ca^{2+}-dependent molecule either on cells or on the virus surface. The reduction in infection by EDTA and EGTA were likely to be due to an effect on binding, since in all experiments binding was performed at 4 °C, a condition where fusion of HIV to cell membrane does not occur (Melikyan et al., 2000). We hypothesize that HIV binding to PBMC occurs through interaction of molecules that are both derived from host cells. HIV acquires a large number of host molecules when budding occurs, and some of these molecules can mediate binding to certain types of cells (Ugolini et al., 1999). Our data show that Ca^{2+}-dependent HIV binding accounts for greater than 85% of adhesion of HIV to PBMC, and is not strain specific. Further investigation of the molecular interactions leading to HIV tethering to PBMC is likely to present a novel target in preventing HIV infection of cells. These results suggest that identification of the molecular interactions responsible for HIV attachment to PBMC will provide useful insight into the development of an effective microbicide.

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