

Carbohydrate-binding Agents Inhibit HIV-1 Infection of Human Primary Monocyte-derived Macrophages and Prevent MDM-directed Viral Capture and Subsequent Transmission to CD4+ T Lymphocytes

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ABSTRACT

Background: Carbohydrate-binding agents (CBAs) represent innovative anti-HIV compounds selectively targeting the glycans of the HIV-1 envelope glycoprotein gp120 and preventing DC-SIGN-directed HIV capture by dendritic cells (DC) and transmission to CD4+ T-lymphocytes. We wanted to investigate the ability of CBAs to inhibit HIV-1 capture by human primary monocyte-derived macrophages (MDM) and to inhibit the subsequent virus transmission from HIV-1-captured MDM to CD4+ T-lymphocytes.

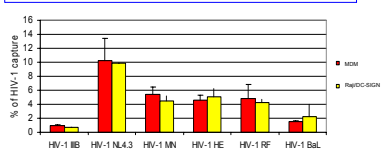
Methods: HIV-1 capture was assessed by p24 Ag ELISA test. In MDM infected by several HIV-1 strains and exposed to various doses of mannose-specific plant lectins: HHA, GNA, NPA and CA, procarcary cyanovirin-N (CV-N), or GlcNAc-specific plant lectin UDA. CBAs. HIV-1 transmission between MDM infected with CBAs-exposed HIV-1 and CD4+ T-lymphocyte cell line (C8166) was analyzed by p24 Ag production and by syncytia formation. Virus production was analyzed in MDM at day 14 after CBAs-exposed R5 HIV-1 infection. MDM were pre-incubated with different antibodies against macrophage mannose receptor (MMR), DC-SIGN and CD4, or with mannin and then analyzed for both HIV-1 capture and virus transmission to C8166 cells by measuring p24 Ag. The experiments were carried out in triplicate and the results are presented as mean values with standard deviation (s.d.).

Results: We demonstrated that the CBAs efficiently prevent R5 HIV-1 infection of MDM in the nanomolar range with a dose-dependent effect. The revealed values of EC50 were 0.4, 0.038, 0.026, 0.01, 0.207, 0.0005, 3.8 nM, for HHA, GNA, NPA, CA, UDA, CV-N, PRRA-A, respectively. Same order of magnitude was observed in C8166. Interestingly, both R5 and X4 HIV-1 strains were efficiently captured by MMR-expressing MDM. HIV-1 capture by MDM was dose-dependently inhibited by short pre-exposure of X4 HIV-1 to CBAs, by MMR antibody (95% of capture inhibition, s.d. 0.01, compared to control), and by the mannin (55% of capture inhibition, s.d. 0.2, compared to control). Short pre-exposure of X4 HIV-1 to CBAs was also able to dose-dependently prevent virus transmission, and subsequent syncytia formation, in co-cultures of CBAs-exposed HIV-1-captured MDM and uninfected C8166.

Conclusions: The potential of CBAs to impair MDM in their capacity to capture and to transmit HIV to CD4+ T-lymphocytes might be an important property to be taken into consideration in the eventual choice to select microbicide candidate drugs for clinical investigation.

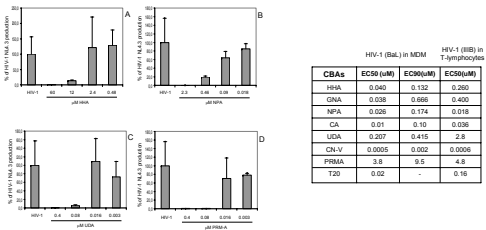
RESULTS

Raji/DC-SIGN and MDM Differently Capture Various HIV-1 Strains



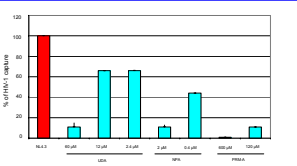
Exponentially growing B-lymphoblastoid DC-SIGN Raji cells (Raji/DC-SIGN cells) were suspended in cell culture medium at 6×10^6 cells/400 μ l. 0.4 ml-suspensions of Raji cells were exposed to 0.6 ml HIV-1 for 60 min, after which 39 ml culture medium was added to the virus-infected cell culture. The cells were centrifuged at 1250 rpm for 10 min, supernatant carefully removed and the virus-exposed cells were resuspended in 40 ml medium. After a second centrifugation step, 39.9 ml supernatant was again removed, and the remaining 0.1 ml cell suspension was 10-fold diluted in cell culture medium to 1 ml. Under these experimental (washing) conditions, a maximum of 8 pg HIV-1 p24 could have remained in the 1 ml supernatant (or 0.4 pg in 50 μ l). The Raji/DC-SIGN cell cultures were then analyzed for p24 content by a p24 ELISA (Perkin Elmer, Boston, MA). MDM were obtained by Ficoll-Hypaque and were exposed to 100 μ l of HIV-1 for two hours after which 4 washes with RPMI 20% were done to remove the unbound virus, to confirm that we retrieved the HIV-1 p24 remained in the supernatant we collected 100 μ l from the last wash to be analyzed by ELISA test. The results represented in the figure represent the percentage of captured HIV-1 p24 calculated compared with the input of HIV-1. 24. HIV-1 p24 associated with Raji/DC-SIGN cells and MDM could be reliably measured: HIV-1 N.4.3 was most efficiently captured (10% of input virus), whereas HIV-1 IB was least efficiently captured (1% of input virus). For each individual strain, similar capture efficiency was observed for DC-SIGN-expressing cells and for MDM.

CBAs Show Antiviral Activity on HIV-1 Ba1 Production



MDM were pre-incubated for 15 min with several doses of CBAs (Panel A: HHA, Panel B: NPA, Panel C: UDA, Panel D: PRRA-A) and then infected with HIV-1 Ba1 (3000 pg/ml). After two hours of infection, we removed the medium and washed the cells for four times with RPMI 20%, and cultured with CBAs, when requested. At day 7, the supernatants were collected and the CBAs were replaced to the new medium. At 14 days, the supernatants were collected and tested for p24 antigen production by p24 ELISA (Perkin Elmer, Boston, MA). The HIV-1 p24 analysis by ELISA at day 14 revealed that CBAs are able to prevent the HIV-1 Ba1 replication in MDM, perhaps blocking the virus glycoprotein sites responsible of the binding and, consequently, the virus entry and infection of MDM. Results are expressed as a percent of the value of the positive control, in which MDM were incubated with HIV-1 Ba1 without the CBAs. Each experiment was run in triplicate. The geometric mean of p24 production of replicates in each experiment was used to determine the effective drug concentration where 50% and 90% of viral replication is inhibited (EC50 and EC90, respectively), by linear regression of the log of the percent HIV-1 p24 production (compared to uninfected controls) versus the log of the drug concentration. The values of EC50 (pg/ml) and EC90 (pg/ml) are shown in the table. For T-lymphocytes the EC50 is considered the 50% effective concentration required to inhibit HIV-1 (IB) induced cytopathicity in C8166 at day 4 after infection (data taken from Balzarini et al., 2007).

CBAs Dose-Dependently Inhibit the MDM Ability to Capture HIV-1 Particles



High amounts of HIV-1 N.4.3 particles (100 μ l, 67×10^7 pg/100 μ l) were exposed to serial dilutions of the test compounds for 15 min at 37 $^{\circ}$ C. Then, the drug-exposed virus suspensions were added to MDM (100 μ l/well) for 2 hours at 37 $^{\circ}$ C. Then, the virus dilution were removed and the cells were carefully washed for four times with RPMI 20%, to be sure to remove all the virus in the well, to confirm this we collected 100 μ l from the last wash to be analyzed by p24 ELISA (Perkin Elmer, Boston, MA). MDM were detached and then analyzed by ELISA test for p24 content. The value of HIV-1 p24 from the last wash was negative and this revealed that there were not virus particles in the medium and that the HIV-1 p24 revealed only by the virus captured from MDM. Results are expressed as a percent of the value of the positive control (916 pg/ml), in which MDM were incubated with HIV-1 N.4.3 without the CBAs. The results show that the CBAs dose-dependently inhibited capture of HIV-1 N.4.3 by the MDM.

BACKGROUND

The important role of Antigen-Presenting Cells (APC) [primary monocytes-derived macrophages (MDM) dendritic cells (DC)], as targets for HIV infection and their ability to serve as reservoirs of virus, particularly in tissues, is recognized (Chougret C et al., *Curr Infect Dis Rep*, 2002). MDM and DC are known to express the CD4 receptor and may be the first cells in the subepithelial vaginal mucosa to be bound by or infected with HIV-1 during heterosexual transmission (Greenhead P et al., *J Virol* 2000; Hu J et al., *J Virol* 2000) and are able to disseminate HIV in all body compartments due to their migratory capacity (Cameron P et al., *J Leuk Biol* 1996).

It has been demonstrated that as few as 500 HIV-1-exposed MDM may cause depletion of several millions of autologous CD4+ T-lymphocytes, and may sustain HIV-viremia and spreading of HIV-1-DNA in lymphoid organs (Garaci E et al., *PNAS* 2003).

In addition, HIV-1 infected MDM may induce the apoptosis on bystander uninfected cells, such as CD4+ and CD8+ T lymphocytes, neurons and astrocytes by releasing cytotoxic factors (Aquaro S et al., *J Leuk Biol* 2000; Badley A et al., *J Exp Med* 1997; Herben G et al., *Nature* 1998; Molace V et al., *J Leuk Biol* 2002; Shi B et al., *J Clin Invest* 1996).

So MDM contribute to the transmission and the pathogenesis of HIV-1 infection through the progression of HIV-1 infection (Herbein G et al., *Curr Mol Med* 2002; Tonkovic B et al., *Blood* 2006; Williams KC et al., *Annu Rev Neurosci*, 2002).

Carbohydrate-binding agents (CBAs) have been recently proposed as innovative anti-HIV compounds selectively targeting the glycans of the HIV-1 envelope glycoprotein gp120 (Balzarini J et al., *Lancet Inf Dis* 2005).

In DC cells short pre-exposure of HIV-1 to CBAs prevents the cellular binding to HIV-1, (mediated by DC-SIGN, a dendritic cell-specific ICAM-3 grabbing HIV-transceptor able to mediate the cell-cell virus transfer), and syncytia formation by subsequent co-cultivation of DC with T-lymphocytes.

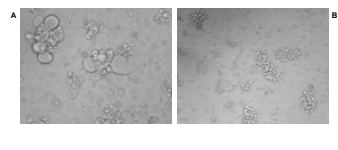
MDM lack expression of DC-SIGN, but contain a mannose-binding C-type lectin receptor (Macrophage Mannose Receptor, MMR) that can bind HIV envelope gp120, resulting in subsequent transmission to T cells (Nguyen Da and Hildner JE, *Eur J Immunol* 2003).

The MMR is a 175-kDa transmembrane glycoprotein containing three types of domains, two of which have distinct carbohydrate-recognizing properties. The amino-terminal cysteine-rich domain plays a critical role in binding sulphated glycoproteins.

The mannose-specific CBAs (i.e. the plant lectins HHA, GNA, NPA and CA; the procarcary cyanovirin-N (CV-N) and the GlcNAc-specific plant lectin UDA but not other entry inhibitors, or polyanionic compounds, have the potential to impair the ability of DC to capture HIV and to transmit HIV to T lymphocytes (Balzarini J et al., *Mol Pharmacol* 2007).

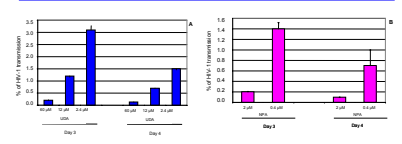
The aim of our study is to demonstrate the ability of CBAs, that were earlier shown to be able to inhibit DC-directed HIV-1 capture and subsequent virus transmission to CD4+ T-lymphocytes, also to do so in MDM. Moreover, we want to investigate the involvement of MMR in the HIV gp120/cell binding.

Syncytia Formation in Co-Culture between HIV-1 Infected MDM and T-Cells



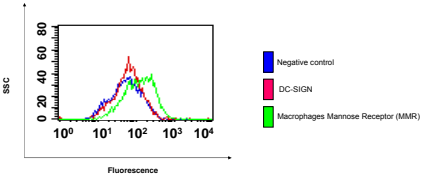
Indeed, we observed that in cocultures of uninfected C8166 cells and HIV-1 N.4.3-exposed MDM, giant cells appear at days 3 and 4 after the cocultivation (Panel A). On the contrary, no syncytia were microscopically observed to appear in the C8166 coculture with uninfected MDM (Panel B). By immunocytochemistry, uninfected C8166 cells were predominantly clustered at MDM locations.

CBAs Dose-Dependently Inhibit the MDM Ability to Transmit Captured HIV-1 Particles



Because MDM can capture HIV-1 N.4.3 particles and transmit HIV-1 to T-cells, we wanted to see if CBAs are able to prevent the transmission of the virus particles to C8166 T lymphocytes (cocultivation) by HIV-1-exposed MDM (200x10³ well, 1 ml). Like the capture procedure, 67×10^7 pg/100 μ l of HIV-1 were exposed to the serial dilutions of the test compounds for 15 min. CBA-exposed virus suspensions were added to MDM (100 μ l/well) for 2 hours at 37 $^{\circ}$ C after which the cells were thoroughly washed four times with RPMI 20% to be sure to remove unabsorbed virus particles. Then C8166 (100x10³ well, 1 ml) were added to MDM and cocultured for 3-4 days. The supernatant ELISA analysis of the HIV-1 p24 from the last wash was negative and this revealed the absence of virus particles in the medium able to infect the T cells. The coculture of the CBA virus-exposed MDM and C8166 did not result in increased p24 amount in the cell cultures at days 3 and 4 days. The percentage of HIV-1 p24 (pg/ml) transmission was expressed as a percent of the value of the positive control (100%), in which MDM were incubated with HIV-1 N.4.3 without the CBAs. As we can see in the figure CBAs, both UDA (Panel A) as NPA (Panel B), are able to prevent the transmission of HIV-1 from MDM to T cells at 3 and 4 days.

MDM Express MMR but not DC-SIGN on their Membrane Surface



To analyze the dendritic cell-specific ICAM-3 grabbing noninfecting (DC-SIGN) and Macrophage Mannose Receptor (MMR) expression on MDM we stained the cells with anti-DC-SIGN (CD208-FITC, BD Biosciences) and anti-MMR (CD206-FITC, BD Biosciences) and we processed for flow cytometry as described previously (Vermeire K et al., *Mol Pharmacol* 2003). We observed that MDM don't express DC-SIGN (0.8% positive), while MDM express MMR (11.5% positive), indicating that probably MMR and not DC-SIGN contribute to HIV-1 MDM binding.

CONCLUSIONS

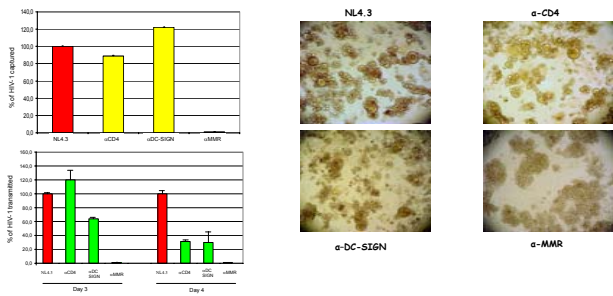
The mannose-specific plant lectins from Hippocastanum hybrid (HHA), Narcissus pseudonarcissus (NPA) and the GlcNAc-specific plant lectin from Urtica dioica (UDA) were derived and purified from these plants, as described before (Van Damme et al., 1987, 1988, 1991). Pradimicin A (PRMA) (M.W. 838) was isolated and purified from the fermentation broth of *Actinomyces* *Cyanovirin* (CV-N) was kindly provided by Dr. J.-B. McLaman (National Institutes of Health, Bethesda, MD) and Dr. A. Bolmstedt (Göteborg, Sweden). Mannan (from *Saccharomyces cerevisiae*) was from Sigma (M7504).

Cells
Human T-lymphocyte C8166 cells were obtained from the American Type Culture Collection (Manassas, VA). Exponentially growing B-lymphoblast DC-SIGN-expressing Raji/DC-SIGN cells (Raji/DC-SIGN) were constructed by Geijtenbeek et al. (2000) and kindly provided by Dr. L. Burshten (Institut Pasteur, Paris, France). All cell lines mentioned were cultivated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Biowittaker Europe, Verviers, Belgium), 2 mM L-glutamine and 0.075 M NaHCO₃.

Primary monocytes derived macrophages (MDM) were obtained from the blood of healthy seronegative donors by separation over Ficoll-Hypaque gradient (Aquaro S and Perno CF, in *Human Retrovirus Protocols* 2005). Briefly, peripheral blood mononuclear cells (PBMC) were selected in 48-well plates with 1.8 x 10⁶ cells/well in 1 ml of RPMI 1640 containing 20% heat-inactivated, endotoxin and mycoplasma-free fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 4 mM L-glutamine (Life Technologies), 50 U/ml penicillin and 50 μ g/ml streptomycin (Life Technologies). Six days after plating and culturing the PBMC at 37 $^{\circ}$ C in a humidified atmosphere enriched with 5% CO₂, non-adherent cells were carefully removed by repeated washings with warm RPMI 1640, leaving a monolayer of adherent cells which were finally incubated in complete medium. Cells treated under these conditions have been shown to be >97% MDM, as determined in detail by cytofluorimetric analysis (Aquaro S et al., *J Virol* 2001).

Viruses
HIV-1 (IB and Ba1) was provided by RC Gallo and M Popovic (at that time at the National Cancer Institute, National Institutes of Health, Bethesda, MD). HIV-1 (HE) is a clinical isolate, derived from a Belgian AIDS patient in 1987 and later propagated in MT-2 cells. HIV-1 N.4.3, MN, RF, were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) (Adachi et al., 1986).

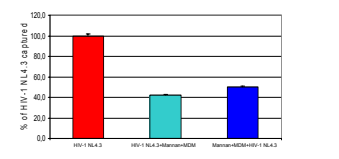
MMR, but not DC-SIGN or CD4, Inhibits HIV-1 Capture in MDM and Virus Transmission to T Cells



To study the role of MMR in the capture and transmission of HIV-1, we compare the effect of MMR antibody with that of several receptors inhibitors. MDM were incubated with MMR antibody (CD206 BD Pharmingen 10 μ g/ml), DC-SIGN antibody (BD Pharmingen 10 μ g/ml), for 15 min at 37 $^{\circ}$ C. HIV N.4.3 (67×10^7 pg/100 μ l) was then added and incubated 2 hours at 37 $^{\circ}$ C. Cells were then washed four times to be sure to remove all the virus in the well, to confirm this we collected 100 μ l from the last wash to be analyzed by p24 ELISA (Perkin Elmer, Boston, MA). Then, the cells were detached from the wells and collected to analyze the HIV-1 p24 by ELISA (capture analysis) (Panel A). For the transmission experiments C8166 (100x10³ well, 1 ml) were added to MDM and the analysis of HIV-1 p24 was done at day 3 and 4 after coculture (Panel B). The percentage of captured or transmitted p24 HIV-1 was expressed as a percent of the value of the positive control, in which MDM were incubated with and without CBAs. The results show that MMR antibody is able to prevent in MDM both the HIV-1 particles capture as the transmission to C8166, while CD4 and DC-SIGN antibodies don't prevent these effects. This shows the relevance of MMR and not of DC-SIGN or CD4 in the capture and in the consequently transmission of HIV-1 particles from MDM to T cells.

The ability of anti-MMR antibody to inhibit the capture and the transmission to T cells further supported the idea that the mannose-dependent association of HIV-1 with MDM is MMR mediated. Same results are shown in the figure below. In the presence of the MMR antibody, in fact, the syncytia formation induced by HIV-1 to C8166 after 4 days of coculture, is prevented, while the presence of CD4 or DC-SIGN antibodies do not prevent this cytopathic effect.

Mannan Inhibit the Ability of MDM to Capture HIV-1



To confirm the role of MMR, we used the mannin (MMR inhibitor) (at 2.5 μ g/ml). In this experiment we used two different procedures:
1) Mannan 2.5 mg/ml was added to MDM for 15 min at 37 $^{\circ}$ C and then the virus (67×10^7 pg/100 μ l) was added for two hours at 37 $^{\circ}$ C.
2) Mannan 2.5 mg/ml was mixed with virus (67×10^7 pg/100 μ l) for 15 min at 37 $^{\circ}$ C and then the MDM was put on MDM for two hours at 37 $^{\circ}$ C.
The cells were thoroughly washed four times with RPMI 20% as described above. MDM were then detached for ELISA p24 analysis. The analysis of the HIV-1 p24 from the last wash was negative and this revealed that there were not virus particles in the medium and that the HIV-1 p24 revealed was only by the virus captured from MDM. As evident from the figure, mannin was able to partially prevent the HIV-1 N.4.3 capture by MDM under both experimental conditions.

CONCLUSIONS

Our data shown that the HIV-1-capture by MDM, that lack expression of DC-SIGN, is mainly MMR mediated, as evidenced by inhibition with several soluble mannose-binding lectins, with mannin and with a specific anti-MMR antibody.
The CBAs also prevent transmission of HIV-1 captured by MDM to T-lymphocytes.
The outcome of these studies are very helpful in further guidance to the choice of potential candidate microbicide drugs.