HIV-1 infection in peripheral blood lymphocytes (PBLs) exposed to alcohol

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Abstract

Epidemiological and in vitro studies have implied that heavy alcohol consumption may increase an individual’s risk of HIV-1 infection. To examine the role of alcohol in direct infection of T-cells, viral reverse transcripts and HIV-1 receptor expression were examined in infected peripheral blood lymphocytes (PBLs) pretreated with alcohol. PCR results showed that alcohol increased HIV-1 DNA in PBLs by at least 10-fold. Alcohol enhanced the expression of the CXCR4 chemokine co-receptor but not the major HIV-1 CD4 receptor. Pretreatment with alcohol was also associated with increased intracellular cAMP. Thus, alcohol may facilitate enhanced viral infection by increasing the availability of HIV-1 co-receptor. This effect is associated with increases in intracellular cAMP.

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Introduction

The role of alcohol consumption as a cofactor for HIV transmission remains a significant public health issue. Some evidence supports the alcohol-mediated augmentation of HIV infection. For example, pretreatment of CD4+ CEM cells with alcohol prior to HIV-1 infection results in elevated levels of p24 viral protein compared to untreated controls (Saravolatz et al., 1990). Other studies indicate that alcohol ingestion might influence HIV-1 infection of peripheral blood mononuclear cells (PBMC) in vitro. Bagasra et al. (1993) demonstrated that PBMC obtained from volunteers who had consumed alcoholic beverages supported significantly higher HIV-1 replication than PBMC not exposed to alcohol. In situ hybridization of the infected cells showed that the expression of HIV-1-specific RNA was augmented in alcohol-exposed cells. Although the above studies suggest that alcohol facilitates HIV-1 infection, further experiments are required to definitively demonstrate the role of alcohol in the HIV-1 life cycle.

The major HIV-1 receptor on T-lymphocytes and monocytes/macrophages is the CD4 glycoprotein (Dalgleish et al., 1984). However, several studies show that members of the seven-transmembrane-domain G-protein-coupled receptors are also required for HIV-1 entry into target cells (Broder and Dimitrov, 1996). Most viral strains which infect transformed T-cell lines target the CXCR4 α-chemokine receptor (Feng et al., 1996), while the macrophage-tropic viruses predominantly utilize the β-chemokine receptor, CCR5, and several other CC chemokine receptors (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). However, most primary isolates can infect both primary T-lymphocytes and monocytes/macrophages (Valentin et al., 1994; Zhu et al.,...
1996), and cell tropism dictates the clinical outcome of HIV-1 infection (McNearney et al., 1992; Zhu et al., 1996). Although no direct evidence has previously shown that alcohol facilitates HIV infection via up-regulation of HIV receptors, it is tempting to speculate that alcohol might facilitate HIV-1 entry into PBLs through up-regulation of primary or chemokine co-receptors required for HIV entry.

Alcohol has been known to increase intracellular cAMP (Swift and DePetrillo, 1990; Diamond et al., 1991). Cole et al. (1999) showed that cell surface expression of CXCR4 was increased in both resting and antigen-stimulated PBMC in response to exogenous dibutyryl cAMP or cAMP-inducing ligands. The enhanced cAMP-dependent CXCR4 expression on the cell membrane was insensitive to cyclohexamide and did not involve elevation in gene expression. This is not surprising since the cAMP response element is absent in the CXCR4 promoter (Moriuchi et al., 1997; Wegner et al., 1998). Although cAMP did not alter the total CXCR4 pool, increased intracellular cAMP did alter receptor compartmentalization. cAMP significantly reduced CXCR4 internalization and increased the re-externalization rate, thus enhancing the fraction of total CXCR4 expressed on the cell surface. To further examine the possible signal transduction mechanism for alcohol-mediated enhancement of HIV-infection and to determine the role of alcohol on regulating CXCR4 expression on PBLs, we further examine whether alcohol can increase intracellular cAMP in unexposed PBLs.

In the present study, we investigated the effect of alcohol on viral entry and HIV-1 receptor and co-receptor expression. We showed that alcohol concentration has no effect on the proliferation of PBLs under in vitro conditions. Alcohol enhanced the entry of CXCR4-tropic HIV-1 into PBLs by 10-fold compared to untreated cells. The effect is due, in part, to the expanded population of CD4+ PBLs that expressed only the CXCR4 chemokine co-receptor and not CCR5. This effect is correlated with enhanced intracellular cAMP concentration resulting from alcohol exposure. Therefore, alcohol may facilitate T-lymphotropic HIV-1 entry by increasing CXCR4 expression via a cAMP-dependent pathway.

Results

Alcohol concentrations below 1% have minimal effect on the proliferation of PBLs

Alcohol concentrations in the peripheral blood of heavy drinkers range from 20 to 100 mM (0.1% to 0.5%) (Sisson et al., 1999). To study the in vitro effect of alcohol, effects of proliferation of PBLs were initially examined by exposing unstimulated PBLs to up to 2% alcohol for either 24 or 48 hours. Cell growth was then measured by [3H]thymidine incorporation. In general, growth inhibition was not detected with alcohol concentrations below 1% (Fig. 1). Alcohol concentrations above 1% reduced proliferation concomitant with cell death as assessed by trypan blue exclusion (data not shown). At higher concentrations of alcohol, cytotoxicity was most apparent in cells treated for longer periods.

Proliferation of PBLs remains relatively constant during 48 hours of alcohol exposure

To study the effect of alcohol on cells incubated for longer periods, a time course on cell growth was also performed with unstimulated PBLs. Time-dependent proliferation of PBLs exposed to various concentrations of alcohol was again obtained by measuring [3H]thymidine incorpora-
tion. In comparison to untreated cells, alcohol concentrations ranging from 0.1% to 0.5% had minimal effect on proliferation for up to 10 days (Fig. 2). Thus, the alcohol concentrations achieved by heavy drinkers in vivo have minimal effect on lymphocyte viability.

**Alcohol facilitates viral entry into PBLs**

Previous experiments have shown that HIV-1 infection of PBLs obtained from patients with elevated blood alcohol levels resulted in higher p24 gag viral protein in culture. To determine whether alcohol facilitates viral entry into PBLs, quantitative PCR analysis was performed to assess early reverse transcription product (LTR region) in cells exposed to the CXCR4-tropic strain, NL4-3. Pretreatment of PBLs with 0.5% alcohol prior to HIV-1 infection increased the early reverse transcription product (140-bp) by 10-fold compared to control cells not exposed to alcohol (Fig. 3, lanes 2 and 3). Viral entry was not observed with heat-inactivated virus (Fig. 3, lane 1) and infection with the CCR5-tropic JR-CSF strain was not augmented (data not shown).

**Alcohol increased PBLs expressing CXCR4 chemokine co-receptor**

Infection of PBLs by T-lymphotropic HIV-1 involves the gp120 envelope protein binding to both CD4 glycoprotein and CXCR4 on the cell surface. However, macrophage-tropic strains target the CCR5 chemokine co-receptor. Since alcohol facilitated viral entry into PBLs, we examined the effect of alcohol on HIV-1 receptor and co-receptor expression. Flow cytometric studies revealed that alcohol exposure for 3 days increased the expression of CXCR4+/CCR5+ cells (Table 1) but decreased CXCR4−/CCR5+ and CXCR4+/CCR5− cells (Fig. 4). A four-color flow cytometric analyses established that CXCR4 expression increased in intensity on both CD4+ and CD4− (i.e. CD8) T-cells (Table 2).

**Alcohol fails to alter CXCR4 gene expression in PBLs**

Alcohol increased the number of PBLs that expressed the CXCR4 co-receptor and the amount of CXCR4 protein on individual CD4+ cells. To determine whether the increased CXCR4 expression was controlled at the post-transcriptional level, RT-PCR was performed to determine whether CXCR4 gene expression was altered following treatment with alcohol. The result showed that alcohol exposure up to 0.5% for 24 hours did not alter the steady state level of the CXCR4 transcript (Fig. 5).

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**Table 1**

<table>
<thead>
<tr>
<th>Alcohol concentration</th>
<th>Mean fluorescence intensity</th>
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<tr>
<td>0%</td>
<td>61.4</td>
</tr>
<tr>
<td>0.25%</td>
<td>65.4</td>
</tr>
<tr>
<td>0.5%</td>
<td>68.5</td>
</tr>
<tr>
<td>1.0%</td>
<td>72.2</td>
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*Note.* Mean fluorescence intensity (MFI), as determined by flow cytometry, of CXCR4 co-receptor expression after stimulation with alcohol for 12 hours.

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Fig. 3. Quantitative PCR analysis of alcohol-exposed PBLs. PBLs were exposed with or without alcohol for 3 days prior to infection with HIV-1 NL4-3. Cells were harvested 16 h post-infection to extract DNA for PCR amplification of the R/U5 region. A set of primers specific for the human β-globin gene (110-bp) was used to determine the DNA input. This is a representative PCR from 3 independent experiments. Lane 1, heat inactivated virus (HI) + 0% alcohol; Lane 2, virus + 0% alcohol; Lane 3, virus + 0.5% alcohol.
Alcohol increases intracellular cAMP

Alcohol has been known to up-regulate cAMP through the PKA as well as PKC dependent pathways in different cell types (Park et al., 1992). Therefore, we examined whether alcohol increases intracellular cAMP in PBLs. There was a marked increase in cAMP levels 30 min after cells were exposed to alcohol, whereas cells not exposed to alcohol maintained a basal level of approximately 50 fmol cAMP/10^6 cells (Fig. 6). In particular, cells exposed to 0.25% alcohol exhibited increased intracellular cAMP levels after 10 min and reached a level of 275 fmol/10^6 cells by 30 min or 5.5-fold higher than unexposed cells. Only a slight elevation in cAMP production was observed with other alcohol concentrations within 10 min. By 30 min, 0.1%, 0.35%, 0.5% alcohol exposed cells enhanced cAMP levels by at most 3-fold compared to unexposed cells. Although a plateau or decrease in cAMP level was observed at 60 min, some alcohol concentrations caused a secondary rise in cAMP at later times (Fig. 6).

Discussion

It is clear that alcohol enhances susceptibility to HIV infection because it has been demonstrated that chronic consumption has been known to suppress immune response and host resistance (Baker and Jerrells, 1993; MacGregor, 1986; Szabo, 1999). However, controversies surround epidemiological studies associating alcohol ingestion and HIV infection. Several published works show no correlation between alcohol and HIV infection among homosexual and bisexual men (Chandiwa et al., 1999; Penkower et al., 1995; van Griensven et al., 1990). However, other studies within the same population have generated disparate results (Penkower et al., 1991), documenting the association of heavy alcohol consumption with elevated HIV seroconversion (Israelstam and Lambert, 1986). Other reports correlate the use of alcohol with high rates of HIV infection (Jacobson et al., 1992; Mbulaiyete et al., 2000). Moreover, alcohol consumption has been associated with the increased chance of infection with HIV-1 (Chaisson et al., 1989; Lohrenz et al., 1978). Despite these conflicting findings, suggestive evidence exists to support alcohol-mediated augmentation of HIV infection.

In this study, we demonstrated that exposure to alcohol at concentrations present in the peripheral blood of heavy drinkers could enhance the synthesis of early reverse transcription product in infected PBLs, by facilitating heightened viral entry. The presence of alcohol during this time expanded the population of PBL that expressed more CXCR4 chemokine co-receptor. Flow cytometry analysis demonstrated that enhanced expression of CXCR4 was concomitant with a decrease in the population of PBLs expressing CCR5 alone or in combination with CXCR4 co-receptor. We thus speculated the alcohol-potentiated HIV infection might be partially attributed to up-regulation of the co-receptor CXCR4. The clinical implication of this observation would be enormous, since the presence of alcohol

<table>
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<tr>
<th>Alcohol concentration</th>
<th>CD4^+</th>
<th>CD4</th>
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<tbody>
<tr>
<td>0%</td>
<td>524</td>
<td>689</td>
</tr>
<tr>
<td>0.5%</td>
<td>664</td>
<td>874</td>
</tr>
</tbody>
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Note. Mean fluorescence intensity (MFI), as determined by flow cytometry, of CXCR4 on CD3^+ lymphocytes exposed to alcohol for 3 days. Quadrants were based on isotype control antibody staining. MFI of CXCR4 expression on CD3^+/CD4^+ and CD3^+/CD4^- T-cells is presented.
might accelerate disease progression by facilitating infection of CD4+ lymphocytes with the more virulent SI strains. A continued investigation on the molecular mechanism of alcohol-enhanced infection and replication will be important to elucidate the clinical relevance in HIV pathogenesis.

Alcohol has been known to influence the activity of several signal transduction systems at both the receptor and post-receptor level (Pandey, 1998; Seiler et al., 2001; Torres and Horowitz, 1996). One target is the adenyl cyclase (AC) system (Pauly et al., 1999; Rabin and Molinoff, 1983; Yoshimura and Tabakoff, 1999). AC is activated via a receptor modulated G-protein and generates cAMP. The second messenger cAMP activates the phosphorylating enzyme protein kinase A or protein kinase C (Dahmen et al., 2000; Zidovetzki et al., 1998). Our results and those of others show that acute exposure to alcohol stimulates intracellular cAMP levels (Boyadjieva and Sarkar, 1999; Gordon et al., 1986; Stenstrom and Richelson, 1982). Cole et al. (1998) have shown that increasing cAMP levels in T-lymphocytes results in increased cell surface expression of CXCR4. Thus, elevation of cAMP induced by alcohol may affect vulnerability to HIV-1 infection by increasing CXCR4 expression.

Similar to the results seen by Cole et al. (1998), we observed that the increased expression of CXCR4 on the cell surface was not mediated by increased CXCR4 gene expression, since receptor transcripts remained unaltered at various concentrations of alcohol. Thus, the increased CXCR4 expression may occur at the translation or post-translation level. We speculate that alcohol might enhance CXCR4 co-receptor expression on PBLs by inhibiting receptor internalization via modulation of cAMP levels. The result would be increased vulnerability to infection by CXCR4-tropic HIV strains. The effect of alcohol on CXCR4 trafficking is currently being investigated to determine whether the mechanism involves the cAMP-dependent pathway. It is also interesting to speculate that alcohol consumption might influence T lymphocyte trafficking by altering responses to SDF-1, the natural ligand for CXCR4. This could lead to alteration of immune responses to antigen.

Materials and methods

Cell culture and viruses

PBLs purchased from the American Red Cross were cultured in RPMI 1640 containing 10% human AB serum (Gemini Bioproducts, Inc., Calabasas, CA), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (GIBCO Life Technologies, Carlsbad, CA) in 5% CO2/95% air. CEM were obtained from ATCC (Manassas, VA). CEM cells were grown in RPMI 1640 with 10% fetal bovine serum, 100 U/ml penicillin and 1 μg/ml streptomycin (GIBCO Life Technologies).

T-lymphotropic HIV-1 NL4-3 strain (Adachi et al., 1986) was harvested from CEM cells transfected with the molecular clone (Cann et al., 1988). The JR-CSF strain was utilized for the macrophage-tropic HIV-1 (Koyanagi et al., 1987). To monitor productive infection, p24gag was detected by HIV-1 p24 Antigen ELISA kit from Beckman Coulter Inc. (Hialeah, FL). In general, 1 ng of viral p24 was equivalent to 100 infectious units as determined by limiting dilution analyses. In our experiments, we routinely infected PBLs with 0.05 to 0.1 infectious units per cell.

Proliferation assay

PBLs were incubated with various concentrations of alcohol for up to 10 days with media change on the third day. For all experiments, 95% ethanol (Molecular Biology Grade, Sigma-Aldrich Chemicals, St. Louis, MO) was used.

Fig. 5. RT-PCR analysis of CXCR4 transcripts in alcohol exposed PBLs. 10⁷ PBLs were exposed to various concentrations of alcohol for 24 h before harvesting the cells to prepare total RNA. RT-PCR was conducted with end-labeled paired primers specific for CXCR4 and β-actin genes. The labeled PCR products were fractionated on a 6% polyacrylamide gel and autoradiographed. Lane 1, 0% alcohol; Lane 2, 0.1% alcohol; Lane 3, 0.25% alcohol; Lane 4, 0.5% alcohol; and Lane 5, 100 μM dibutyryl cAMP.

Fig. 6. Intracellular cAMP concentration of PBLs exposed to alcohol. 10⁷ PBLs were exposed to various concentrations of alcohol for different times and cAMP levels measured by EIA.
as the source of alcohol. After alcohol treatment, 10⁵ PBLs were incubated for an additional 4 hours at 37°C with 200 μl fresh medium and 20 μl [³H]thymidine (10 μCi). The labeled cells were harvested onto glass fiber filters and thymidine incorporation measured by liquid scintillation counting.

Flow cytometry

10⁶ PBLs were exposed to alcohol ranging from 0% to 0.5% and then stained with phycoerythrin (PE)-conjugated CXCR4 monoclonal antibody (MAb, Becton Dickinson) and/or fluorescein isothiocyanate (FITC)-conjugated CCR5 MAb (Pharmingen, San Diego, CA). IgG₂a, served as the negative control for the antibody isotype. Data was accumulated on FACStarplus flow cytometer (Becton Dickinson Immunocytometry, Mountain View, CA) and analyzed using the Cell Quest program (Becton Dickinson Immunocytometry). For 4-color analyses in Table 2, 10⁶ PBLs were exposed to alcohol for 3 days and the cells were simultaneously stained for CXCR4-PE (Pharmingen), CD4-APC (allophycocyanin) (Beckman Coulter), CD3-ECD (phycoerythrin-texas red) (Beckman Coulter, Inc.), and CCR5-FITC (Pharmingen). Samples were analyzed on a FACSCaliber flow cytometer using the Cell Quest program as before.

HIV-1 infection

10⁶ PBLs were incubated with 500 ng of HIV-1 NL4-3 strain or heat-inactivated virus (HI) (0.05 to 0.1 infectious units/cell) for 1.5 h at 37°C in the presence of 10 μg/ml polybrene. Heat inactivation of the virus was done at 65°C for 1 h.

Polymerase chain reaction (PCR)

Infected PBLs were harvested 16 hours post-infection to prevent reinfection by the released virus. DNA was extracted from PBLs by urea lysis buffer (4.7 M urea, 1.3% SDS [w/v], 0.23 M NaCl, 0.67 mM EDTA, pH 8.0) followed by phenol-chloroform extraction and ethanol precipitation. Extracted DNA was PCR amplified with ³²P-end-labeled oligonucleotide primers (M667/AA55) to detect early reverse transcription product from the viral long terminal repeat (LTR) R/U5 region (Zack et al., 1990). A set of primers specific for the human β-globin gene was used to determine the cellular DNA input. PCR conditions consisted of 25 cycles of denaturation for 1 min at 94°C and polymerization for 2 min at 65°C (Zack et al., 1990). Amplified products were resolved on a 6% nondenaturing polyacrylamide gel and visualized by phosphorimage analysis. For PCR amplification, standard curves were established with predetermined concentrations of HIV-1 plasmid DNA for quantification (Cann et al., 1988). A serial dilution of HIV-1 plasmid was performed in carrier tRNA (4 μg/ml) and amplified with paired primers to construct a standard curve of HIV-1 copy number. HIV-1 copy number in the standard curve ranged from 10 to 1000 copies in a reaction and the individual standard curves used are indicated in each figure. In addition, we normalized for DNA loading by PCR amplification of human β-globin gene sequences. A paired oligonucleotide primer complementary to the first exon of the human β-globin gene at positions 14–33 and 123–104 (Lawn et al., 1980) was utilized in each reaction mixture to normalize the total amount of cellular DNA present. This oligonucleotide pair forms a 110-bp amplified product easily distinguished from the products of the HIV-1-specific primers. During PCR amplification, labeled β-globin-specific oligonucleotide was incorporated into the reaction at 1 × 10⁵ to 5 × 10⁶ cpm per reaction. The standard curves for β-globin DNA using total cellular DNA (300 to 3000 cell equivalents) was also included in the figure.

The signals obtained from the PCR results were quantified by phosphorimage analysis in conjunction with the standard curves. Phosphorimage analysis was performed with Storm® gel and blot imaging system (Molecular Dynamics, Sunnyvale, CA), utilizing ImageQuant® software (Molecular Dynamics) to quantify the radioanalytic signal.

For RT-PCR, total cellular RNA was extracted using RNaseasy (Qiagen Inc., Valencia, CA) treated with DNase I (Promega Corp., Madison, WI) and reverse transcribed using murine leukemia virus reverse transcriptase (MuLV; Perkin-Elmer Cetus, Norwalk, CT) and a 20-thymidine oligonucleotide primer. One-tenth of the resultant cDNA was amplified by PCR using the CXCR4-specific primers, 5'-TCATCTACACAgTCAACCTCTACA-3' and 5'-gAACA- CAACCACCACAgtCTATT-3'. As a control for RNA loading, β-actin cDNA was amplified in parallel using commercial primers (R & D Systems, Minneapolis, MN). Primers were end-labeled with [γ-³²P]ATP and samples were amplified with Taq DNA polymerase (Perkin-Elmer Cetus). The labeled PCR products were fractionated on a 6% polyacrylamide gel and autoradiographed. The negative control consisted of reaction with no reverse transcriptase to demonstrate that all amplifications were RNA specific.

cAMP measurement

Intracellular cAMP was measured using an enzyme immunoassay (EIA) kit purchased from Amersham Biosciences Corp. (Piscataway, NJ). The level of detection for the standard curve is 0 to 3200 fmol of cAMP.

Acknowledgments

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