CD8$^+$ cells from asymptomatic human immunodeficiency virus-infected individuals suppress superinfection of their peripheral blood mononuclear cells

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Most human immunodeficiency virus (HIV)-infected individuals show evidence of infection by only one strain of the virus despite possible frequent contact with multiple strains. The reason(s) for the emergence of a dominant strain of virus in HIV-infected people and the mechanism(s) which prevent other strains from establishing an infection is not known. In the present study, we demonstrate that peripheral blood mononuclear cells (PBMC) of asymptomatic HIV-infected individuals can resist productive infection by HIV-1 and HIV-2 strains. Although the PBMC of these individuals are resistant to superinfection, their CD4$^+$ cells are susceptible to infection. Moreover, two weeks after infection of their PBMC in culture, the superinfecting virus can be recovered from isolated CD4$^+$ cells. When CD8$^+$ cells from asymptomatic individuals are added to the superinfected CD4$^+$ cells, replication of the exogenously introduced virus is inhibited. In contrast, PBMC from individuals who have progressed to disease (Progressors) do not resist superinfection and their CD8$^+$ cells do not show the antiviral activity which controls productive HIV infection. These findings suggest that CD8$^+$ cells suppressing HIV replication in infected individuals may be critical in preventing the establishment of infection by other strains of HIV by blocking virus replication.

Introduction

CD4$^+$ cells, following infection with human immunodeficiency virus (HIV), may be rendered resistant to subsequent infection by another strain of HIV (i.e. superinfection) (Levy, 1994). On a cellular level, this resistance usually results from down-regulation of the virus receptor (i.e. the CD4 molecule) (Hoxie et al., 1986; LeGuern & Levy, 1992; Salmon et al., 1988; Shirazi & Pitha, 1992). Other mechanisms of resistance to superinfection at the level of the infected cell involve interferon production (Shirazi & Pitha, 1993), a block at the post-entry level before down-modulation of the CD4 receptor (Volsky et al., 1996), and to some extent, competition for the host cell's metabolic machinery (Taddeo et al., 1993).

In the host, during the asymptomatic phase of infection, as few as 1/1000 to 1/100 000 CD4$^+$ cells in peripheral blood are infected by HIV (Brinchmann et al., 1991; Harper et al., 1986; Psallidopoulos et al., 1989). Therefore, many uninfected CD4$^+$ cells are available for infection by another HIV strain. However, the incidence of dual infection in an infected individual is rare (Levy, 1994), and in most instances one dominant replicating virus strain is recovered from the infected person (McNearney et al., 1992; Meyerhans et al., 1994).

The establishment of immune responses against the virus following initial infection may explain the presence of only one strain of HIV in the host. Antiviral antibodies (Tremblay et al., 1990) and CD8$^+$ cell anti-HIV activity (Mackewicz & Levy, 1992) could prevent further virus strains from replicating in the host. The latter explanation is suggested by studies in which peripheral blood mononuclear cells (PBMC) from asymptomatic individuals were found to be resistant to superinfection by another HIV-1 strain (Kannagi et al., 1990). Superinfection of PBMC of these subjects was achieved only after removal of the CD8$^+$ cells prior to infection. Whether CD8$^+$ cells controlled this HIV-1 superinfection by killing the infected cells (Walker & Plata, 1990) or by suppressing virus replication in a non-cytotoxic manner (Levy et al., 1996) has not been determined.
In the present study we demonstrate that despite in vitro infection of CD4+ cells from asymptomatic HIV-infected people by an exogenously introduced virus, CD8+ cells from these individuals can inhibit replication of the superinfecting virus. The data suggest that CD8+ cells can prevent the establishment of a second HIV infection in previously infected individuals.

Methods

Subjects. Peripheral blood from HIV-1-infected individuals was collected by venipuncture in Vacutainer tubes containing sodium heparin (Becton Dickinson). The HIV-infected individuals in this study were classified as asymptomatic subjects or Progressors (those individuals who have progressed to disease) based on the 1993 Centers for Disease Control (USA) criteria for AIDS diagnosis. The asymptomatic subjects (n = 11) had a mean CD4+ cell count (±SD) of 865 ± 355 cells/μl and a mean CD8+ cell count (±SD) of 1607 ± 1061 cells/μl; those subjects progressing to disease (n = 5) had a mean CD4+ cell count (±SD) of 270 ± 91 cells/μl and a mean CD8+ cell count (±SD) of 1114 ± 744 cells/μl. PBMC from healthy seronegative volunteers were used as controls and were provided by Irwin Memorial Blood Centers, San Francisco, Calif., USA. This study was approved by the Committee for Human Research, University of California, San Francisco.

Isolation of PBMC. PBMC from blood donors were prepared by Ficoll–Hyphaque (Sigma) gradient centrifugation (Levy & Shimabukuro, 1985). The PBMC were stimulated at a concentration of 3 x 10^6 cells/ml with 3 μg/ml phytohaemagglutinin (PHA) (Sigma) for 3 days in complete growth medium RPMI 1640 medium (BioWhitaker, Walkersville, Md., USA) containing 10% heat-inactivated (56 °C, 30 min) foetal bovine serum (FBS) (GIBCO), 10% natural interleukin (IL)-2 (T-stim; Collaborative Research, Bedford, Mass., USA), 2 mM glutamine (BioWhitaker), 100 U/ml penicillin and 100 μg/ml streptomycin (BioWhitaker). Prior to infection, the cells were washed three times in calcium and magnesium-free Hanks' balanced salts solution (BioWhitaker) and plated at 3 x 10^6 cells/ml in complete growth medium.

Isolation of CD4+ and CD8+ cells. CD4+ and CD8+ cells were isolated from the PBMC by positive selection using anti-CD4+ or anti-CD8 antibodies coupled to magnetic beads (Dynal) as described (Mackewicz et al., 1991). Beads were removed from the cells by Detachabead (Dynal). The purity of the CD4+ and CD8+ cell populations isolated by this technique was > 95% CD4+, < 1% CD8+, CD19+, CD56+ and CD14+ cells, and > 95% CD8+ and < 1% CD4+, CD19+, CD56+ and CD14+ cells as determined by flow cytometry (FACSort: Becton Dickinson) (Levy et al., 1985).

Acute infection of PBMC and CD4+ cells with HIV. PHA-stimulated PBMC or CD4+ cells (3 x 10^6 cells/ml) were pretreated for 30 min with 2 μg/ml polybrene (Sigma), washed, and infected in complete growth medium with 100 TCID50 per 10^6 cells of HIV-1 (F-82, HIV-2_RoC or HIV-2_cm5 (Castro et al., 1988). For the passage of the viruses and determination of their TCID50, PHA-stimulated PBMC were used. Virus-cell suspensions were incubated for 2 h at 37 °C in a cell concentration of 3 x 10^6 cells/ml. Cells were then washed and resuspended in the complete growth medium at a final concentration of 3 x 10^6 cells/ml. Culture fluids were removed every 3–4 days for 30 days and monitored for reverse transcriptase (RT) activity. RT activity of ≥ 10^4 c.p.m./ml culture fluid was considered positive for virus production.

Endogenous virus assay. Two cell culture systems (A and B) were used to detect production of the naturally infected (endogenous) HIV-1 from PBMC of HIV-infected individuals (Castro et al., 1988). For the A cultures, 6 x 10^6 PBMC from HIV-seropositive subjects at 3 x 10^6 cells/ml were incubated for 3 days with 3 μg/ml PHA. Seven days later, PHA-stimulated PBMC (3 x 10^6) from HIV-seronegative individuals were added to these cultures. For the B cultures, PHA-stimulated PBMC (3 x 10^6) from HIV-seronegative individuals were added to non-PHA-treated PBMC (3 x 10^6) from the HIV-infected subject at the initiation of the culture. Fresh PHA-stimulated PBMC from seronegative donors were added every 7 days. The final cell concentration for both cultures was maintained at 3 x 10^6 cells/ml. Culture fluids were removed every 3–4 days for 30 days and monitored for RT activity (Hoffman et al., 1985), since most B cultures yield virus by this period of time (Castro et al., 1988).

The A and B cultures can be used to provide an indication of the relative ability of CD8+ cells within the PBMC to suppress endogenous HIV replication. In the A culture, CD8+ cells are activated by PHA which enhances their ability to suppress endogenous virus replication in CD4+ cells (Landay et al., 1993). In contrast, in the B culture, the subject's CD8+ cells are less effective at suppressing virus replication because of the absence of PHA stimulation and the early addition of fresh target cells to the culture (Castro et al., 1988).

Immunoblot procedure. Virus was isolated from the culture fluids of superinfected cells and concentrated as described previously (Werner & Levy, 1993). The viral proteins were separated by SDS-PAGE (Laemmli, 1970) under reducing conditions using a 12.5% gel and electrophoretically transferred to PVDF membranes (Millipore) for immunoblot analysis (Towbin et al., 1979) with minor modifications (Pan et al., 1987). Immunoblots were assayed for 2 h on a rocker platform with anti-sera (diluted to 1:200 in 5% milk, Tris buffer) containing anti-actin, anti-actin (21.5 kDa) and lysozyme (14.3 kDa) (Amersham). After washing, the reaction was developed with nitroblue tetrazolium-5-bromo-4-chloro-3-indoyl phosphate (Kirkegaard and Perry). The following molecular mass standards were used: myosin (heavy chain, 200 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa) (Amersham).

PCR analysis. Total DNA was prepared by using TriReagent (Molecular Research, Inc.) according to the manufacturer's instructions. Each sample was amplified with β-actin primers and an internally conserved fluorescent probe (Taqman β-actin detection kit; Perkin-Elmer) to verify the presence of amplifiable DNA. To separate high molecular weight (HMW) DNA from low molecular weight (LMW) DNA, 5 μg of each DNA sample was resolved on a 0.7% agarose gel prepared with 1 x Tris-acetate–EDTA buffer. High and low molecular weight bands were excised with Gene Capsules and the DNA was purified according to the manufacturer's instructions. An equivalent amount of DNA from each HMW and LMW sample was amplified using the SK100/SK104 primer set which amplifies the gag gene from HIV-1 and HIV-2 (Tang et al., 1995). Product DNA was resolved and detected as previously described (Tang et al., 1995) using HIV-1-(SK19) or HIV-2-(SK109) specific oligonucleotide probes.
Results

HIV infection of PBMC from HIV-1-infected asymptomatic individuals

In initial studies we determined whether the PBMC from asymptomatic HIV-1-infected individuals are susceptible to acute infection with HIV-1 and/or HIV-2.PHA-stimulated PBMC from asymptomatic subjects were inoculated with 100 TCID_{50} per 10^6 cells of a cytopathic strain of HIV-1 (HIV-1_{SF3}) (Tateno & Levy, 1988) or two different HIV-2 strains: HIV-2_{UC2} and HIV-2_{UC3} (Castro et al., 1990). Virus production was evaluated by monitoring RT activity in the culture fluid every 3-4 days for 30 days. As a control, the same dose of the three virus strains was used to infect PHA-stimulated PBMC of HIV-seronegative individuals. The PBMC from seven different asymptomatic individuals did not show detectable infection after inoculation of the cytopathic HIV-1_{SF3} (Fig. 1) whereas the PBMC of all seven seronegative donors released high levels of virus. The ability to resist superinfection was not limited to HIV-1 strains as reported by others (Kannagi et al., 1990) since PBMC from eight asymptomatic individuals were able to resist superinfection by HIV-2_{UC2}. Similar results were obtained with HIV-2_{UC3} infection of PBMC from eleven asymptomatic subjects. In contrast, the average peak RT activity of culture fluids obtained from HIV-2_{UC3} and HIV-2_{UC3} infected PBMC of eight seronegative individuals was over 2 x 10^5 c.p.m./ml of culture fluid. The lack of virus replication was not affected by the dose of virus used since PBMC from two asymptomatic subjects resisted productive infection with HIV-2_{UC3} at a dose as high as 10000 TCID_{50} per 10^6 cells (data not shown).

As a measure of endogenous HIV-1 production, fluids from A and B cultures (see Methods) of the subject's PBMC were monitored for the presence of RT activity. None of the PBMC in the A cultures from the eleven different asymptomatic subjects studied produced virus (RT activity < 10^4 c.p.m./ml of culture fluid). With B cultures, the PBMC from six of eleven of these individuals produced virus (RT activity ≥ 10^4 c.p.m./ml of culture fluid) during the 30 day period of study. Despite the presence of endogenous virus in the B cultures of the six individuals the ability of their PBMC to resist superinfection did not differ from the five individuals who were negative for endogenous virus production in the B cultures (data not shown). As noted in Methods, these results most likely reflect the relative extent of antiviral activity of the subjects' CD8^+ cells.

Susceptibility of CD4^+ cells from asymptomatic infected individuals to acute infection by HIV

Infection of CD4^+ lymphocytes renders these cells resistant to superinfection (LeGuern & Levy, 1992; Levy, 1994). This finding could account for the inability to acutely infect the PBMC of asymptomatic individuals in this study. To examine this possibility, CD4^+ cells were removed from the PBMC of three asymptomatic HIV-infected subjects using anti-CD4 antibodies coupled to magnetic beads, and stimulated with PHA 3 days prior to virus inoculation. Both the PHA-stimulated PBMC and CD4^+ cells from these subjects received 100 TCID_{50} per 10^6 cells of HIV-2_{UC3}. CD4^+ cells from two of the three individuals were also cultured for 30 days without acute infection to determine the level of endogenous virus production. Following acute infection with HIV-2_{UC3}, HIV was recovered from the CD4^+ cells (Fig. 2a) at levels similar to those observed in the acutely infected PBMC from HIV-seronegative donors. The level of virus in the culture fluids of all individuals tested peaked 7-10 days post-superinfection. The variability in virus replication in the PBMC and CD4^+ cells from different donors has been described (Evans et al., 1987). Growth of HIV in the CD4^+ cells of the infected subjects showed kinetics of replication similar to those observed with PBMC from HIV-seronegative donors infected with the same dose of virus (Fig. 2b).
Endogenous HIV-1 was recovered from culture fluids of CD4+ cells not receiving HIV-2uc3 in one of two subjects studied but only after adding PHA-stimulated PBMC from HIV-seronegative individuals 10 days after initiation of the culture. Similar to observations with other asymptomatic subjects (Fig. 1), the PBMC from the three HIV-infected

Fig. 2. HIV-2 infection of CD4+ cells from HIV-1-infected asymptomatic individuals. PHA-stimulated PBMC (A) and CD4+ cells (B) from three different asymptomatic individuals and PHA-stimulated PBMC (C) from three different HIV-seronegative donors were infected with 100 TCID_50 per 10^6 cells of HIV-2uc3. HIV-particle associated RT activity was monitored in the culture fluids. Data presented in (a) are the highest RT activity detected in the culture fluids. Each bar represents results from a different subject. In (b) the kinetics of HIV replication over a 30 day period in PBMC and CD4+ cells from one of the individuals tested (■) are shown. Similar kinetics were observed with the other two subjects. A, PBMC from the asymptomatic individual; ▲, PBMC from a HIV-seronegative donor; O, CD4+ cells isolated from the asymptomatic individual's PBMC prior to infection by HIV-2uc3.

Fig. 3. HIV replication in CD4+ cells obtained from the superinfected PBMC from HIV-1-infected asymptomatic individuals. PHA-stimulated PBMC from six different asymptomatic individuals (A) and six HIV-seronegative donors (B) were infected with 100 TCID_50 per 10^6 cells of HIV-2uc3. Ten to fourteen days post-infection, CD4+ cells were isolated from the PBMC of the asymptomatic individuals and cultured (C). HIV-particle associated RT activity was monitored in the culture supernatants. The highest RT activity in the culture fluids is presented in (a). Each bar represents results from a different subject. In (b) the kinetics of virus replication over a 40 day period in PBMC and CD4+ cells from one of the individuals tested (■) are shown. Similar kinetics were observed with superinfected cells from the other subjects. ■, PBMC from the asymptomatic individual; ▲, PBMC from an HIV-seronegative donor; O, CD4+ cells isolated from the HIV-2uc3-infected PBMC of the asymptomatic individual.
Fig. 4. Immunoblot analyses of viral proteins in infected cell culture fluids obtained at peak HIV replication. HIV antigens were detected by human sera containing antibodies specific for HIV-1 or HIV-2. (a) Pelleted virus from culture fluids of PBMC of an HIV-seronegative donor infected with HIV-1sf33 (lanes 1 and 2) or HIV-2uc2 (lanes 3 and 4) were evaluated with HIV-1- (lanes 1 and 3) and HIV-2- (lanes 2 and 4) specific antisera. The specificity of the sera to gp120 can be observed. (b) Lanes 1 and 2 contain pelleted virus from culture fluids of CD4+ cells isolated from HIV-2uc2-superinfected PBMC of an asymptomatic individual. Lanes 3 and 4 contain pelleted virus from culture fluids from HIV-2uc2-superinfected PBMC from a Progressor. Immunoblots were done with HIV-1- (lanes 1 and 3) or HIV-2- (lanes 2 and 4) specific antisera. Reaction with HIV-2 antigens is noted. In particular, HIV-2 envelope protein (gp120) is demonstrated in lanes 2 and 4. (c) Pelleted virus from culture fluids from CD4+ cells isolated from an asymptomatic individual’s PBMC infected with both HIV-1sf33 and HIV-2uc2. Immunoblots were done with HIV-1- (lane 1) and HIV-2- (lane 2) specific sera. Reaction with gp120 from both virus types is observed.

individuals, whose isolated CD4+ cells could be superinfected, showed no detectable virus in the culture fluids during the 30 days post-superinfection.

**Recovery of superinfecting virus from the CD4+ cell cultures**

To determine if CD4+ cells within the superinfected PBMC of asymptomatic individuals contain the exogenously introduced virus, we isolated CD4+ cells from six subjects’ PBMC 10–14 days post-virus inoculation (Fig. 3a). Despite the continued absence of virus production from the PBMC after 30 days in culture, the isolated CD4+ cells showed the presence of virus in the culture fluids within 7–10 days of culture (Fig. 3b).

HIV-1- and HIV-2-specific antisera were used to ascertain the virus type released by the superinfected CD4+ cells from the asymptomatic individuals. Approximately 50 different antisera from HIV-1 and HIV-2 individuals were screened by immunoblot to identify the sera with minimal cross-reactivity (Fig. 4a). The antisera did not bind to proteins from uninfected PBMC from any subjects tested (data not shown). Although cross-reactivity with p24 is evident in Fig. 4(a), specific bands for gp120 for HIV-1 are demonstrated in lane 1 but not lane 3. In addition, lanes 2 and 4 show specificity for HIV-2 gp120 antigens. The immunoblot of virus isolated from the culture fluids of CD4+ cells removed from the HIV-2-superinfected PBMC of one study subject showed the presence of HIV-2, specifically gp120 after 14 days (Fig. 4b). We have evaluated cell lysates from this asymptomatic individual’s PBMC which were superinfected in culture; viral proteins as evaluated by immunoblots were not detected (data not shown). Endogenous HIV-1 was not recovered from the A or B cultures of the PBMC or the CD4+ cells of this individual. This fact can explain the lack of HIV-1 viral proteins detected in the culture fluids of the superinfected CD4+ cells by immunoblot analysis (Fig. 4b).

Isolation of CD4+ cells from asymptomatic individuals’ PBMC superinfected with the highly cytopathic strain HIV-1sf33 also showed recovery of the exogenously added virus. Whereas the PBMC culture fluids of these subjects did not contain detectable virus, the isolated CD4+ cells released a virus that induced syncytia formation (data not shown). This cytopathic effect was absent in CD4+ cells from the same subject’s PBMC, which were not superinfected with HIV-1sf33.

Finally, we examined whether superinfection of PBMC from asymptomatic individuals with both HIV-1 and HIV-2 would lead to simultaneous infection by both subtypes of HIV or whether one strain dominates in this superinfection. The PBMC from an asymptomatic individual were infected with 100 TCID50 of both HIV-2uc2 and HIV-1sf33. No virus production was detected in the PBMC during 30 days in culture. After 2 weeks, CD4+ cells were removed from an aliquot of the cultured PBMC and the presence of either virus in the fluid was evaluated by immunoblot. Fig. 4(c) demon-
Table 1. Detection of viral DNA in PBMC infected with HIV-1 or HIV-2

Uninfected and HIV-1sF33- or HIV-2uc3-infected PBMC from an HIV-seronegative individual and three asymptomatic HIV-1-infected subjects were analysed by PCR for HIV-1- and HIV-2-specific sequences in HMW and LMW DNA (see Methods). The extent of viral DNA production was measured by densitometry and ranged from a strong reaction (+) to no reaction (−). Particle-associated RT activity was detected only in the culture fluids from PBMC of seronegative donors infected with HIV-1sF33 or HIV-2uc3.

<table>
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<tr>
<th>Donor</th>
<th>Acute infection</th>
<th>HIV-1</th>
<th>HIV-2</th>
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<tbody>
<tr>
<td>Seronegative</td>
<td>None</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV-infected</td>
<td>HIV-1sF33</td>
<td>2+</td>
<td>3+</td>
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<tr>
<td>HIV-infected</td>
<td>HIV-2uc3</td>
<td>1</td>
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<td>None</td>
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The identity of HIV-1sF33 was confirmed by its highly cytopathic activity, which is not a characteristic of the endogenous virus in this subject (data not shown).

Role of CD8+ cells in suppressing virus production following acute superinfection of PBMC

CD8+ cells can suppress HIV replication in CD4+ cells by a non-cytotoxic mechanism (Levy et al., 1996). We therefore evaluated whether this CD8+ cell activity is responsible for the lack of virus replication in the superinfected PBMC of asymptomatic individuals. CD4+ cells were removed from the PBMC of four asymptomatic infected individuals 10–14 days post-infection, CD8+ cells were removed from the PBMC of the HIV-infected individuals and cultured alone (C) or mixed (1:1) with CD8+ cells isolated from the autologous PBMC (D). Every 3–4 days, culture fluids were monitored for RT activity. Data are presented as peak RT activity present in the culture fluids. Each bar represents results from a different subject.

HIV infection of PBMC from Progressors

Whether the PBMC from Progressors can be acutely infected by HIV-1 or HIV-2 was also examined. Progressors with CD4+ cell counts of 160–305 cells/μl were used to ensure sufficient target cells to detect virus production. PHA-stimulated PBMC from five Progressors were inoculated with 100 TCID50 of HIV-2uc3. This virus strain was used initially for superinfection in order to distinguish the exogenously introduced virus from the endogenous HIV-1 strain present in
Suppression of HIV superinfection by CD8⁺ cells

the PBMC. For controls, the same dose of virus was inoculated onto the PBMC of five different seronegative individuals. As shown in Fig. 6, after 7–10 days in culture the average peak RT activity of culture fluids from the PBMC of Progressors was 2.5 x 10⁶ c.p.m./ml, and the mean peak RT activity in the five control PBMC used in this study was 3 x 10⁵ c.p.m./ml. In contrast to the superinfected cell cultures, the A cultures from all the Progressors took 2 weeks before endogenous virus production was noted. Similar results were observed following inoculation of 100 TCID₅₀ of HIV-1SF33. In these studies, peak virus production was observed within 7 days of virus inoculation, whereas the A culture of the PBMC of the two Progressors studied released endogenous virus after 2 weeks.

Immunoblot analyses conducted on the recovered viruses from the culture fluids of HIV-2uc2-infected PBMC from one of the five Progressors showed predominantly HIV-2 proteins (Fig. 4b). The absence of HIV-1-specific proteins in the culture fluids studied by immunoblot most likely reflects the relatively low level of HIV-1 replication at the time the sample was collected (i.e. 10 days) (see above).

Since the ability of CD8⁺ cells to suppress HIV replication decreases as an infected individual progresses to disease (Landay et al., 1993; Mackewicz et al., 1991), we investigated the antiviral activity of CD8⁺ cells from the Progressors. CD4⁺ cells were removed from the PBMC of asymptomatic individuals 10–14 days post-acute infection in vitro with HIV-2uc2 and were either cultured alone or mixed together with CD8⁺ cells (CD8⁺ to CD4⁺ cell ratio of 1:1) obtained from the PBMC of Progressors and cultured for the same length of time. Since CD8⁺ cells from HIV-infected individuals are able to suppress HIV replication in a non-MHC-dependent manner (Walker et al., 1991a), the CD8⁺ cells from Progressors can be mixed with heterologous CD8⁺ cells of asymptomatic individuals. As controls, heterologous CD8⁺ cells from asymptomatic individuals were isolated and mixed with the CD4⁺ cells at the same CD8⁺ to CD4⁺ cell ratio. As shown in Fig. 7, the
CD8+ cells from Progressors were not able to suppress the productive infection of CD4+ cells, whereas CD8+ cells from the asymptomatic individuals inhibited virus replication.

To further investigate the role of CD8+ cells in preventing superinfection, we mixed CD8+ cells from asymptomatic individuals with HIV-2_Tc2-infected PBMC from Progressors. As was observed in Fig. 7, the presence of CD8+ cells from three asymptomatic individuals decreased the ability of HIV-2_Tc2 to replicate in the PBMC of Progressors. In contrast, the Progressors' PBMC cultured in the absence of these CD8+ cells from the asymptomatic individuals were not able to resist superinfection (data not shown). Thus, the inability of PBMC from Progressors to resist production of virus following infection correlates with the decreased capacity of their CD8+ cells to suppress HIV replication.

**Discussion**

In this study, we demonstrate that cultured PBMC from asymptomatic HIV-1-infected individuals are resistant to productive infection with HIV. This ability to suppress superinfection was not limited to a single virus strain, since PBMC from asymptomatic people were equally resistant to infection with a cytopathic strain of HIV-1 (HIV-1sFG3) and two different HIV-2 strains: HIV-2_Tc2 and HIV-2_UC3 (Fig. 1). In contrast to these findings with the PBMC from asymptomatic individuals, the PBMC from HIV-infected individuals progressing to disease produced the exogenous virus following superinfection (Figs 4 and 6). The resistance of PBMC from asymptomatic HIV-infected individuals to in vitro superinfection with HIV did not reflect a lack of susceptibility of the CD4+ cells to infection by HIV; the CD4+ cells purified from the PBMC were sensitive to subsequent infection by both HIV-1 and HIV-2 (Fig. 2).

A possible explanation for the ability of CD4+ cells from asymptomatic subjects to become infected while their PBMC remain resistant to superinfection could be the limited number of CD4+ cells present in the PBMC available for superinfection. However, the PBMC of asymptomatic individuals contained an average of 865 CD4+ cells/μl and were resistant to superinfection (Fig. 1), whereas the PBMC of the Progressors used in these studies had an average of 276 CD4+ cells/μl and were susceptible to productive infection with HIV (Fig. 6). Moreover, 45% of the PBMC from one asymptomatic subject studied were CD4+ cells. Since 25 x 10^6 PHA-stimulated PBMC were used in this experiment, approximately 11 x 10^6 CD4+ cells within the PBMC population were available for infection. The study showed that infection occurred in 3 x 10^6 isolated CD4+ cells from this subject (Fig. 2).

An important observation was that removal of CD4+ cells 10–14 days after superinfection of the PBMC of HIV-1-infected asymptomatic donors led to the recovery of the exogenously added HIV (Figs 3 and 4). Evidence of the superinfecting virus in the PBMC was supported by immuno-blot and PCR analyses (Fig. 4 and Table 1). When the CD8+ cells from these superinfected PBMC were added back to the CD4+ cells, HIV replication was inhibited (Fig. 5). These results indicate that virus infection of the CD4+ cells in the PBMC takes place and that CD8+ cells are associated with the resistance of PBMC to productive infection. Cytotoxic activity of the CD8+ cells is likely not involved since removal of CD4+ cells from the PBMC several weeks after infection yielded the superinfecting virus (Figs 3 and 5). Moreover, molecular studies showed the presence of superinfecting virus in the cells when virus production was absent (Table 1). Thus, the virus was in a non-replicating state in the PBMC.

Previous studies in our laboratory have shown that CD8+ cells from HIV-infected individuals can control HIV replication by a non-cytotoxic mechanism involving a novel soluble factor termed the CD8+ cell antiviral factor (CAF) (Levy et al., 1996). We conclude that this cellular antiviral activity is most likely responsible for the present findings in which replication of the superinfecting virus is inhibited. Recent reports have suggested that β-chemokines may have a role in CD8+ cell-mediated suppression of HIV replication (Cocchi et al., 1995). In this regard, current studies in our laboratory have demonstrated that CAF is not a β-chemokine (Mackewicz et al., 1996). Moreover, the mechanism involved in the antiviral effect of β-chemokines is a block of the virus interaction at the cell surface (Oravecz et al., 1996). CAF suppresses virus transcription after virus entry (Mackewicz et al., 1995). Since superinfection of CD4+ cells with HIV appears to be arrested after integration of the viral DNA into the host cell genome (Table 1), it is likely that CAF is involved in the block of HIV superinfection described in this report. Whether long-term suppression of virus replication will lead to a loss of superinfecting virus cannot be determined by these studies.

However, preliminary studies have shown no evidence of superinfecting virus in the CD4+ cells removed from the cultured PBMC of an asymptomatic individual 3 weeks after virus inoculation (data not shown); in contrast, the superinfecting virus was recovered from these CD4+ cells as early as 5 days after superinfection.

The potential role of CD8+ cells in preventing virus production after acute infection of PBMC of asymptomatic individuals is supported by previous findings that these cells have anti-HIV suppressing activity (Brinichmann et al., 1990; Tsubota et al., 1989; Walker et al., 1986, 1991a). Moreover, in an earlier study of superinfection, Kannagi et al. (1990) demonstrated in preliminary studies that CD8+ cells from asymptomatic individuals do not lyse CD4+ cells infected with an exogenous virus, thus suggesting a suppressing mechanism. In their study, the PBMC from asymptomatic HIV-1-infected subjects were not susceptible to productive infection by HIV-1 but could be superinfected with HIV-2. This observation differs from our study showing asymptomatic individuals were resistant to both HIV types. The reason for these different findings is not known. Walker et al. (1991b) demonstrated that
CD8\(^+\) cells are able to suppress HIV-2 replication as effectively as HIV-1. Thus, if the CD8\(^+\) cells are involved in suppressing HIV replication following superinfection, productive infection by both strains of virus should be prevented. The HIV-2 titre used in the Kannagi et al. study could have been higher than the level controllable by the CD8\(^+\) cells in the PBMC from the asymptomatic individuals. However, we observed that the PBMC from asymptomatic individuals were resistant to superinfection by as much as 10000 TCID\(_{50}\) of HIV-2.

This CD8\(^+\) cell-mediated suppressing activity is diminished in symptomatic individuals (Landay et al., 1993; Mackewicz et al., 1991), which probably accounts for the productive infection observed after superinfection of the PBMC from Progressors. Their CD8\(^+\) cells could not prevent productive HIV infection of CD4\(^+\) cells (Fig. 7). This reduction in CD8\(^+\) cell non-cytotoxic anti-HIV responses is also manifested by a greater recovery of endogenous virus from A cultures and higher virus loads in the PBMC of Progressors.

The relationship of these findings on superinfection in vitro to observations in vivo merit attention. Thus far, only rarely has more than one strain of HIV been found in HIV-1-infected individuals, even during disease progression (Leonard et al., 1993; Levy, 1994). When detected, the second strain is present at much lower levels. A dominant virus is usually detected which persists throughout the course of infection (McNearney et al., 1992; Meyerhans et al., 1994).

Based on the present observations, CD8\(^+\) cells may prevent the establishment of a second productive infection during the asymptomatic phase of infection, and thus the newly introduced virus remains undetected. As an infected individual progresses to disease, the CD8\(^+\) cell antiviral activity declines, and a second HIV strain either suppressed previously or recently introduced may begin to grow. However, the presence of this new strain may not be recognized because of the increasing virus titre of the previous dominant strain. This decreased ability of CD8\(^+\) cells to suppress HIV replication with disease progression may also be responsible for the recent observation of recombinant viruses (of two or more strains) present in HIV-infected individuals (Gao et al., 1994; Robertson et al., 1995). Whether these recombinant viruses are more prevalent in Progressors remains to be determined. Moreover, this suppressing activity of CD8\(^+\) cells might explain the suggested protection from HIV-1 infection in previously HIV-2-infected individuals (Travers et al., 1995). It may also reflect the lack of simian immunodeficiency virus pathogenicity in animal model systems in which prior infection with HIV-2 has occurred (Putkonen et al., 1995).

These observations of CD8\(^+\) cell antiviral activity in association with a block in HIV superinfection in asymptomatic individuals support further our conclusions that this immunological response plays an important role in long-term survival (Barker et al., 1995; Levy, 1993; Levy et al., 1996). Induction of this antiviral activity by a vaccine may provide an effective means for preventing establishment of HIV infection.

References


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