Allogeneic Leukocytes But Not Therapeutic Blood Elements Induce Reactivation and Dissemination of Latent Human Immunodeficiency Virus Type 1 Infection: Implications for Transfusion Support of Infected Patients

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Various immunologic stimuli and heterologous viral regulatory elements have been shown to increase susceptibility to, and replication of, human immunodeficiency virus type 1 (HIV-1) in lymphocytes and monocytes in vitro. Transfusion of allogeneic blood components from heterologous donors constitutes a profound immunologic stimulus to the recipient, in addition to being a potential route of transmission of lymphotropic viral infections. To investigate the hypothesis that transfusions, and particularly those containing leukocytes, activate HIV-1 replication in infected recipient cells, we cocultured peripheral blood mononuclear cells (PBMC) from three anti-HIV-1–positive individuals with allogeneic donor PBMC, as well as partially purified populations of donor lymphocytes, monocytes, granulocytes, platelets, and red blood cells (RBC) and allogeneic cell-free plasma. Allogeneic PBMC induced a dose-related activation of HIV-1 expression in vivo in infected cells, followed by dissemination of HIV-1 to previously uninfected patient cells. Activation of HIV-1 replication was observed with donor lymphocytes, monocytes, and granulocytes, whereas no effect was seen with leukocyte-depleted RBC, platelets, or plasma (ie, therapeutic blood constituents). Allogeneic donor PBMC were also shown to upregulate HIV-1 expression in a “latently” infected cell line, and to increase susceptibility of heterologous donor PBMC to acute HIV-1 infection. Studies should be performed to evaluate whether transfusions of leukocyte-containing blood components accelerate HIV-1 dissemination and disease progression in vivo. If so, HIV-1–infected patients should be transfused as infrequently as possible and leukocyte-depleted (filtered) blood components should be used to avoid this complication.

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with substantial transfusion requirements. To investigate this possibility, we characterized the kinetics of HIV-1 expression and dissemination in PBMC from HIV-1-infected persons after cocultivation with various transfused blood components. We also studied the effect of allogeneic blood components on induction of HIV-1 from a latently infected cell line, and on the kinetics of acute HIV-1 infection of PBMC in vitro. Our results indicate that both reactivation and enhanced dissemination of HIV-1 occur after heterogenous leukocyte exposure.

**MATERIALS AND METHODS**

**Preparation of blood components from normal donors.** Blood was obtained from three anti-HIV-1-negative volunteer whole blood donors at Irwin Memorial Blood Centers (IMBC) and processed according to routine blood bank protocols. Platelet-rich plasma (PRP) was prepared by centrifugation at 1,000 RPM for 10 minutes. Platelets were concentrated from PRP by centrifugation at 2,000 RPM for 10 minutes, and resuspended in RPMI-1640 media (GIBCO, Grand Island, NY) at 10^7 cells/mL. Supernatant plasma was passed through a 0.2-μm filter to remove residual cells and large cellular fragments. A portion of the packed red blood cells (RBC) was subjected to filtration (Pall RC50; Pall Biomedical Products, Glen Cove, NY) to remove leukocytes. PBMC and granulocytes were separated from the remaining packed RBC using a commercially available separation media (Sepratech Corp, Oklahoma City, OK). A portion of the PBMC were further separated into enriched lymphocyte (nonadherent) and monocyte (adherent) fractions by overnight incubation in plastic tissue culture flasks. These enriched cell preparations were determined to be composed of at least 95% granulocytes, lymphocytes, and monocytes, respectively, by flow cytometric analysis. PBMC and lymphocyte fractions were treated with mitomycin C (0.25 mg/mL) immediately before use to prevent their replication (and infection) in vitro. The inability of these cells to survive/replicate was determined by measuring their concentration and viability in separately maintained cultures of each cell preparation. Each cell fraction was washed twice in phosphate-buffered saline (PBS) and resuspended at 1 x 10^7 cells/mL in RPMI-1640 media before use in coculture experiments. In one experiment, 1 x 10^7 PBMC from one donor were stimulated for 3 days with phytohemagglutinin (PHA) before mitomycin C treatment.

**Preparation of PBMC from HIV-1-positive individuals.** Blood was obtained from three anti-HIV-1-seropositive persons, two of whom were asymptomatic, while one had lymphadenopathy, oral candidiasis, and a diffuse erythematous skin rash. PBMC were obtained using lymphocyte separation medium (Sepratech Corp) and washed twice in PBS. The number of viable cells was determined by trypan blue exclusion and hemacytometer counting. Cells were resuspended at 2 x 10^7/mL in RPMI-1640 media containing 0.5% penicillin, 1% glutamine, and 5% fetal bovine serum (no interleukin-2 [IL-2]) and placed in tube cultures at 4 x 10^6 PBMC/2 mL. Negative control cultures were maintained in this nonstimulating media. The viable cell number in the nonstimulated cultures maintained in 5% serum declined significantly to 42% to 65% of the input cell number by day 11 of culture, and to less than 10% by day 21. For this reason, experimental observations on all culture were curtailed at day 10 or 11. Positive control cultures were stimulated with PHA (0.5 μg/mL). Experimental cultures were mixed with 1 mL of each of the heterologous donor blood components prepared as described above.

**Assays for HIV-1 replication and expression.** PBMC cultures were monitored for the extent and kinetics of HIV-1 replication by determination of the proportion of cells harboring HIV-1 DNA and protein, and by quantitative p24 antigen analysis of culture supernatants. The approximate proportion of cells harboring HIV-1 cDNA or integrated provirus was determined by a semiquantitative polymerase chain reaction (PCR) technique, in which HIV-1 gag DNA was amplified from serial dilutions of fresh and cultured PBMC. PCR reaction conditions were identical to those previously described. Briefly, fresh PBMC and cultured cells were counted by a hemacytometer, serially diluted to endpoint, and lysed in PCR Solution A/B (Cell Culture Facility, University of California, San Francisco). Each dilution was separately digested with proteinase K (240 μg/mL), followed by denaturation at 95°C for 2 hours. The cell lysates were then subjected in parallel to 30 cycles of amplification using a combination of primers for the HIV-1 gag (SK38 and SK39) and HLA-DQAq (GH26 and GH27) genes. HLA coamplification served both as a qualitative internal control for amplification efficiency of each reaction and, by evaluation of HLA signal endpoint, as a semiquantitative assessment of cell input to validate hemacytometer cell counts. After amplification, the preparations were hybridized with 32P-labeled HIV-1 (SK19) and HLA-DQAq (GH64) oligonucleotide probes at 95°C for 5 minutes and 56°C for 5 minutes. Specific amplified products were detected by autoradiography after separation on 6% polyacrylamide slab gels. HIV-1 target copy number was estimated based on the endpoint dilution at which specific HIV-1 signal was no longer visible on autoradiography. Viral burden was expressed as the number of HIV-1-positive cells per 100,000 PBMC.

**Immunochemistry studies.** HIV-1 p18 and p24 antigens on smears of fixed cells was accomplished using monoclonal antibodies (MoAbs; Genetic Systems Corp, Seattle, WA) and alkaline phosphatase/anti-alanine phosphatase immunocytochemistry reagents, as detailed elsewhere. Samples were screened microscopically for the proportion of HIV-1 antigen-positive cells (indicated by bright red reaction product overlying cells) relative to the total number of PBMC on the slide (range, 100,000 to 200,000 cells). Extracellular virus production was evaluated by testing filtered (0.2 μm) culture supernatants for p24 antigen using a commercially available enzyme-linked immunoassay (Coulter Diagnostics, Hialeah, FL). For quantitation, the optical density derived from each positive supernatant was compared with that of a standard curve generated by parallel evaluation of a serial dilution of a commercially available p24 antigen standard.

**U1.1 activation studies.** The U1.1 monocytic cell line, containing two integrated HIV-1 proviral copies per cell, was obtained from Dr Tom Folks (Laboratory of Immunoregulation, NIAID, NIH, Bethesda, MD). A control (nonstimulated) culture of U1.1 cells was maintained in RPMI-640 media with antibiotics; under these conditions, HIV-1 expression was limited to less than 5% of cells, as determined by immunochemistry. As positive control, the phorbol ester phorbol myristate acetate (PMA) (1 μg/mL), which leads to induction of HIV-1 replication, was added to a culture of 2 x 10^6 U1.1 cells. A series of experimental cultures were prepared containing 2 x 10^6 U1.1 cells cocultured with either 1 x 10^6 mitomycin C-treated allogeneic PBMC, 1 x 10^7 platelets, 1 x 10^7 RBC, or 1 mL of allogeneic plasma. Cultured cells were monitored for HIV-1 DNA load and protein expression as described above.

**Cell-free virus infection studies.** A high-titer stock of cell-free HIV-1 was prepared from the supernatant of HIV-1-infected HUT-78 cells. Serial dilutions (1:100) of the supernatant were inoculated onto duplicate 1 mL cultures containing 2 x 10^6 PHA-stimulated or nonstimulated PBMC from each of two normal donors, as well as duplicate cultures containing an equal mixture of 1 x 10^6 nonstimulated cells from each normal donor. Polybrene (2 μg/mL; Sigma, St Louis, MO) was included in the media during the 12-hour virus inoculation, after which the cells were washed.
and the cultures monitored for HIV-1 replication as described above.

RESULTS

When PBMC from each of the three HIV-1-infected individuals were cultured under conditions of minimal stimulation (5% serum and no PHA), no detectable viral antigen expression was observed either associated with cultured cells or released into the supernatant fluid (Fig 1A, no stimulation). Quantitation of HIV-1-proviral DNA from nonstimulated cultures showed that HIV-1 gag DNA concentration (ie, the proportion of cells harboring provirus) remained unchanged (at 1 to 4 infected cells per 100,000 PBMC) relative to that found in uncultured PBMC for up to 2 weeks in culture (not shown). In contrast, PHA stimulation of HIV-1-infected patients' PBMC resulted in a dose-dependent activation of HIV-1 replication, as evidenced by high-level expression of gag antigen in culture supernatants (Fig 1A), and a progressive increase in the proportion of cells testing positive for HIV-1 gag antigens by immunocytochemistry and for HIV-1 DNA by dilutional PCR analysis (see below).

Coculture of PBMC from the three HIV-1-seropositive patients with mitomycin C-treated allogeneic PBMC resulted in an induction of viral expression similar to that observed after PHA stimulation (Fig 1B). Allogeneic PBMC from three different normal donors (RE, FS, and MB) were similar in their ability to induce HIV-1 expression from the three seropositive patients' PBMC. The heterologous leukocyte-induced activation was dose-dependent, with markedly enhanced viral replication occurring after coculture with $10^7$ normal donor PBMC, low-level viral stimulation with $10^5$ normal PBMC, and no detectable viral replication after coculture with $10^3$ or fewer PBMC (Fig 1B). In one experiment, prior PHA activation of normal donor PBMC enhanced their ability to induce HIV-1 reactivation at the $10^5$ cell input (not shown). Induction of viral gag protein expression was also observed after coculture of $2 \times 10^6$ PBMC from one of the infected patients with $1 \times 10^7$
mitomycin C-treated allogeneic donor lymphocytes, monocytes, or granulocytes (Fig 1C). In contrast, coculture with either $1 \times 10^7$ heterologous donor RBC or platelets showed no effect on HIV-1 expression or proviral load above that seen with the nonstimulated control cultures. Similarly, addition of heterologous donor plasma at a 5% final concentration failed to induce detectable HIV-1 expression (Fig 1D).

More detailed analysis of the allogeneic PBMC cocultures enabled us to discriminate two phases of viral replication, the first characterized by activation of viral expression in in vivo infected patient cells, and the second by dissemination of infection in vitro to previously uninfected patient cells. Data from a representative experiment are summarized in Fig 2A, with corresponding semiquantitative PCR and immunocytochemistry data presented in Fig 2B and C, respectively. Semiquantitative PCR analysis of uncultured cells from this patient showed that approximately 1 in 25,000 PBMC (4 in 100,000) harbored HIV-1 DNA (Fig 2B, day 0). However, less than 1 in 100,000 cells expressed HIV-1 p18 or p24 antigens detectable by immunocytochemical analysis (Fig 2C, day 0). Three days after exposure to allogeneic donor PBMC, the proportion of cells harboring HIV-1 DNA was essentially unchanged (at approximately 1 infected cell per 25,000 input PBMC). However, we now observed low-level expression of p24 antigen in the culture fluid, and gag antigens became detectable on the surface of a proportion of cells comparable to that determined to harbor HIV-1 DNA by PCR analysis (Fig 2A, B, and C, day 3). This early phase was thus consistent with reactivation of HIV-1 provirus expression in vivo infected cells. By day 7, and continuing through day 10, dissemination of infection was observed, as evidenced by a progressive increase in the proportion of cells harboring HIV-1 DNA and gag proteins, and an increase in the concentration of extracellular p24 antigen (virus production) (Fig 2A, B, and C, days 7 and 10). It is important to note that quantitation of the proportion of cells harboring viral antigen or DNA, as observed in this experiment, reflects viral infection and expression in PBMC derived from the HIV-1-infected patient only. Pretreatment of the normal donor leukocyte preparations with mitomycin C immediately before their addition into cocultures precluded their ability to propagate beyond 3 days. This was shown by monitoring parallel cultures of mitomycin C-treated donor PBMC alone, which showed that less than 2% of input donor cells were present and viable after 3 days of culture. Furthermore, inoculation of a control culture of these cells (both with and without internal control).
prior PHA stimulation) with a high-titer HIV-1 stock confirmed that these cells could not support HIV-1 infection, whereas virus grew readily in the stimulated donor PBMC in the absence of mitomycin C treatment. Rendering the allogeneic donor cells nonviable and incapable of supporting viral replication enabled us to conclude that the observed increase in the proportion of HIV-1–DNA and gag antigen-positive cells was the result of dissemination of infection from in vivo infected cells to previously uninfected patient cells, rather than the spread of virus to allogeneic normal donor cells.

To further study the effect of allogeneic cell interaction on the induction of expression of HIV-1 in latently infected cells (reactivation), we used the U1.1 monocytic cell line that harbors two integrated copies of HIV-1 provirus per cell. In the absence of stimulation by the phorbol ester PMA, only a small percentage (1% to 3%) of these cells express HIV-1 antigens detectable by immunocytochemistry and only a low level of virus is detectable in culture supernatant.23 After stimulation with PMA, a high proportion (>25%) of U1.1 cells was induced to express detectable viral antigen, and cell-free virus products (ie, supernatant p24 antigen levels) increased markedly (Fig 3A). When 2 × 106 U1.1 cells were cocultured with 1 × 107 mitomycin C-treated normal donor PBMC, the proportion of antigen-positive cells increased from 2% to more than 15%, and extracellular virus production (as evidenced by p24 antigen in the culture supernatant) increased markedly (Fig 3B). This effect was observed by 1 day of culture, and was not associated with any change in proviral copy number per cell (not shown), indicating that the effect was due to enhanced transcription and/or translation of latent viral sequences and not superinfection. Because this monocytic cell line lacks T-cell receptors, the induction is presumably secondary to cytokine release from PBMC rather than direct activation via cell-to-cell contact. No stimulation of viral replication was seen after the addition of allogeneic donor RBC, platelets, or plasma to U1.1 cultures.

We next investigated the effect of allogeneic leukocyte interactions on the increasing susceptibility of quiescent PBMC to initial HIV-1 infection. Serial 10-fold dilutions of cell-free virus were inoculated into replicate cultures containing 2 × 106 PHA-stimulated or nonstimulated PBMC from each of two normal donors, as well as a coculture containing a mixture of 1 × 106 mitomycin C-treated normal donor PBMC from each of these donors. Prior coculture of heterologous PBMC increased the susceptibility of these cells to HIV-1, relative to unstimulated cells, both in terms of a 1-log increase in the endpoint dilution of virus stock yielding detectable viral replication (Table 1) and the kinetics of viral replication at several infectious doses (Fig 4). The stimulatory effect of allogeneic cells, vis-a-vis enhancing susceptibility of PBMC to acute HIV-1 infection, was similar to that seen after PHA-induced activation of the individual PBMC preparations.

**DISCUSSION**

Although coculture of PBMC from seropositive patients with stimulated normal donor PBMC has become the

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**Table 1. Allogeneic Leukocyte Interaction Facilitates Acute HIV-1 Infection of PBMC**

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<tr>
<th>PBMC Source</th>
<th>PHA</th>
<th>Dilution of HIV-1 Stock Inoculum</th>
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MB and FS represent 2 × 10⁶ PBMC from two unrelated, HIV-1–negative donors. MB/FS represents a mixed culture of 1 × 10⁶ cells from donors MB and FS. Viral replication was graded as follows: –, no p24 antigen detected through 11 days of monitoring; +/-, low-level (<100 pg/mL) detection of p24 antigen at 11 days; +, high-level p24 antigen (>100 pg/mL) first detected at 11 days; ++, high levels of antigen by day 7; ++++, high levels of antigen by day 3.

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**Fig 3. Allogeneic leukocytes stimulate expression of HIV-1 in latently infected U1 cells.** In the absence of stimulation, only 1% to 3% of U1 cells express HIV-1 gag antigen detectable by immunocytochemistry or supernatant p24 antigen capture assay. By 1 day poststimulation with PMA, greater than 30% of U1.1 cells express detectable viral antigen, and cell-free virus levels increase markedly (A). Similarly, when U1.1 cells were cocultured with allogeneic PBMC, the proportion of antigen-positive cells increased from 2% to more than 20%, and cell-free p24 antigen concentrations increased (B).
LEUKOCYTE-INDUCED REACTIVATION OF HIV-1

Our findings further implicate cell activation as an important element driving HIV-1 replication and dissemination. Although numerous investigators have presented in vitro data suggesting that a variety of factors (e.g., UV irradiation, alloantigens, cytokines, and heterologous viral infections) can upregulate HIV-1 replication, validation of the clinical significance of these findings has proven difficult. In contrast, the allogeneic leukocyte-induced activation described in the current study can be tested directly by quantitative analysis of HIV-1 replication and its consequences (e.g., CD4 cell counts) in blood collected from infected persons before and after the transfusion of cellular blood components. We are now in the process of conducting this study. If a clinically significant in vivo effect is demonstrated, it would represent important evidence confirming the importance of immunologic stimulation in activating HIV-1 replication.

It is noteworthy that our experimental design, in which allogeneic cells were rendered incapable of propagation and infection before their introduction into cocultures, allowed us to discriminate reactivation of latent provirus in vivo infected cells from dissemination of infection among the patients' cells ex vivo. By documenting the spread of virus from infected to noninfected patient cells, we were able to confirm that the peripheral blood of infected individuals contains a substantial population of cells that, if activated, are highly susceptible to HIV-1 infection. The fact that these cells were as yet uninfected in vivo presumably relates to their quiescent phenotype and to viral and immunologic regulatory elements that maintain a relatively low level of viral replication and circulating free virus during the asymptomatic phase of infection. It would be interesting to characterize the relative sizes of the infected versus susceptible PBMC pools in patients at varying clinical stages, and in serial samples collected from single individuals over the course of infection or after treatment with antiviral or immunomodulating therapy. Careful evaluation of the capacity of immunologic elements (e.g., CD8+ cells, HIV-1 antibody) and antiviral agents (e.g., azidothymidine, soluble CD4) to control or block the reactivation versus the dissemination phases of infection after in vitro allogeneic cell stimulation of infected patient PBMC would also be of interest.

The finding that leukocytes expressing HLA class I and II
antigens but not therapeutic blood constituents (RBC, platelets, plasma) lacking those antigens are responsible for “turning on” HIV-1 expression in infected allogeneic cells in a dose-related fashion has practical implications. In our experiments, ratios of donor leukocytes to patient PBMC exceeding 1 to 10 resulted in HIV-1 reactivation. Efficient leukocyte depletion filters (so-called third generation filters), capable of producing RBC or platelet components with less than $5 \times 10^9$ residual leukocytes, are now widely available.\textsuperscript{56} Exclusive transfusion of components that have been leukocyte-depleted using these third generation filters prevents, or at least markedly delays, alloimmunization.\textsuperscript{3,57} This clinical observation suggests that these products may not contain the threshold number of leukocytes necessary to trigger recipient virus reactivation. These newer leukocyte-depleted products also appear to prevent CMV transmission, and may therefore reduce transmission of viruses capable of transactivating HIV-1 expression.\textsuperscript{58} Should further study of HIV-1-infected recipients substantiate the clinical importance of transfusion-induced activation of HIV-1, it would be important to rapidly determine whether the use of currently available leukocyte-depleted products prevents this complication.

In conclusion, we have shown that allogeneic donor leukocytes harbored in cellular blood components can markedly upregulate HIV-1 expression and dissemination in infected PBMC ex vivo. If these observations are corroborated in vivo, they would have important implications for transfusion therapy of HIV-1 infected persons: (1) therapies that induce anemia, and hence necessitate transfusion support, should be avoided or minimized\textsuperscript{44}; (2) use of erythropoietin and, when possible, autologous transfusions should be encouraged\textsuperscript{59}; and (3) leukocyte-depleted (filtered) blood components, if shown to be effective at preventing this complication, should be used when administering transfusions to HIV-1-infected patients. Finally, the effects of allogeneic leukocytes in transfusions on other latent or chronic viral infections (eg, herpes viruses, HTLV-I/II, hepatitis viruses, etc) should be further investigated both in vitro and in vivo. If reactivation of recipient infections proves to be a general phenomenon, the current trend toward routine implementation of filtered, leukocyte-depleted blood components should be expedited.

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LEUKOCYTE-INDUCED REACTIVATION OF HIV-1

Allogeneic leukocytes but not therapeutic blood elements induce reactivation and dissemination of latent human immunodeficiency virus type 1 infection: implications for transfusion support of infected patients

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