The human immunodeficiency virus (HIV) is capable of infecting certain cells of hematopoietic lineage, particularly monocyte-macrophages and T lymphocytes. Recently, the possibility that cells of megakaryocytic lineage are susceptible to HIV infection has been raised. We have characterized infection of the permanent megakaryocytic cell line CMK by HIV in vitro. CMK cells were easily infected by HIV type 2 (HIV-2), producing significant amounts of virus in culture. Infection appeared to be mediated by the CD4 surface antigen on CMK cells. Three different strains of HIV-1 were able to minimally infect CMK cells, suggesting there may be isolates of HIV tropic for megakaryocytes. Infection of CMK cells led to downregulation of the CD4 surface antigen but no discernable change in expression of megakaryocyte-associated proteins glycoprotein Ib and glycoprotein IIb/IIIa. These observations support the likelihood that megakaryocytes are susceptible to HIV infection, and cell lines of megakaryocytic origin may provide a useful model to study effects of the retrovirus on megakaryocyte function.

PROFOUND DEFECTS in hematopoiesis have been observed in some patients infected with the human immunodeficiency virus (HIV). Defining the cellular targets of HIV may yield insights into the pathogenesis of dysregulated hematopoiesis due to this retrovirus. HIV is capable of infecting hematopoietic cells that bear the CD4 surface antigen, specifically T lymphocytes and monocyte-macrophages. Recently, populations of immature bone marrow myeloid progenitors as well as nonhematopoietic bone marrow stromal fibroblasts have been reported to be susceptible to HIV infection in vitro. We were intrigued by the observations that certain patients with HIV and thrombocytopenia responded to 3'-Azido-3'-deoxythymidine (AZT) therapy with marked increases in their platelet counts. This suggested that the retrovirus might directly interfere with megakaryocytopoiesis. Zucker-Franklin and Cao, using in situ hybridization, detected HIV sequences in bone marrow megakaryocytes from HIV infected individuals. Another recent study found significant expression of the CD4 antigen on the marrow megakaryocyte. The establishment of permanent cell lines of megakaryocytic lineage allows for direct assessment of infection of these cells by HIV. Using the CMK megakaryocytic cell line, derived from a patient with megakaryoblastic leukemia, we have studied their susceptibility to infection by HIV. CMK cells can be infected with HIV, and this infection appears to occur via virus interaction with the CD4 surface receptor. This system may provide a model for exploring the consequences of HIV infection of megakaryocytes.

MATERIALS AND METHODS

Cell lines. The CMK cell line, derived from a patient with megakaryoblastic leukemia, was the generous gift of Dr T. Sato (Chiba University, Japan). Three different clones of the CMK cells were available for study. These clones were termed CMK, CMK-6, and CMK11-5. The CMK cell lines were carried in RPMI 1640 medium containing 10% fetal bovine serum (GIBCO, Grand Island, NY), L-glutamine, penicillin, and streptomycin. The cells were shown to be free of mycoplasma.

Characterization of CMK cells. The three CMK clones were characterized for expression of cell surface markers of megakaryocytic, erythroid, lymphoid, and myeloid lineage. This was done by flow cytometry using specific monoclonal antibodies (MoAbs) to platelet glycoprotein (GP) Ib (Amarc, Inc, Westbrook, ME), platelet GP IIb/IIIa (Amarc, Inc), the erythroid marker glycoporphin A (Amarc, Inc), and the myeloid marker CD15 (anti-Leu M1; Becton Dickinson Immunocytometry Systems, Mountain View, CA). The detection of the surface CD4 protein was performed using the murine MoAb Leu3a (Becton Dickinson) and of the surface CD34 antigen using the MoAB MY10 (Becton Dickinson).

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By Mamoru Sakaguchi, Takeyuki Sato, and Jerome E. Groopman


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transferred to 2 mL of media and cultured in 24-well plates (Costar, Cambridge, MA) at 37°C in 5% CO2. Control cultures consisted of mock infected CMK cells not exposed to virus. Positive control cultures used to assure the infectivity of virus within the frozen stocks consisted of H9 or CEM T cells or peripheral blood monocyte-macrophages. Cultures were periodically monitored for productive HIV infection by measuring supernatant reverse transcriptase (RT) activity, and by quantitation of cells expressing HIV specific proteins by indirect immunofluorescence (IFA), as described below. Cell viability in these cultures was assessed by trypan blue exclusion. In some experiments, expression of cell surface antigens was measured by flow cytometry before and after HIV infection.

RT assay. The assay for HIV RT activity was performed as previously described. Virus was concentrated from 1 mL of cell-free tissue culture supernatant by precipitation with 0.5 mL of polyethylene glycol overnight at 4°C.

IFA. HIV antigens on the cells were detected by IFA on methanol-fixed cells using the serum of an AIDS patient. Serum from a seronegative donor was used as a negative control.

Blocking experiments. To determine if HIV used the CD4 surface receptor to infect CMK cells, cultures were established in the presence of Leu3a, an anti-CD4 MoAb (Becton Dickinson) or with soluble recombinant CD4 (Genentech, South San Francisco, CA) as previously described. Productive HIV infection was monitored by RT and IFA.

RESULTS

Phenotypic characterization of the three different clones of CMK showed different degrees of maturity (Table 1). CMK-6 cells expressed the lowest amounts of megakaryocytic differentiation markers, particularly GPs Ib and IIb/IIIa, while CMK11-5 had the most mature surface phenotype. The parent CMK clone was intermediate in stage of maturation between the CMK-6 and CMK11-5 clones. In addition to expression of markers of megakaryocytic lineage, the CMK cells expressed the erythroid marker glycophorin A, as well as the CD34 antigen. The CD4 surface antigen was found on CMK cells at all three levels of maturation. CMK cells were strongly positive for GF-1 expression by Northern blot (data not shown).

The CMK megakaryocytic cell line proved highly susceptible to HIV-2 ROD infection. Comparable kinetics of infection of CMK-6, CMK, and CMK11-5 are shown in Fig 1. High levels of productive infection were achieved with peak RT activity greater than 10 × 10^4 cpm/mL and IFA for HIV antigens greater than 50% after 21 days of culture.

The less mature CMK-6 cells were somewhat less susceptible to HIV-2 ROD infection, with a lag in appearance of RT in the supernatant. Peak virus production was comparable among the three CMK clones. Similar kinetics of infection were seen with the HIV-2 DOU strain (Fig 1).

CMK megakaryocytic cells were significantly less susceptible to infection with the HIV-1 isolates (HIB, Ba-L, 9533) despite challenge of the three different CMK clones with high titer virus inocula (10^4 TCID50). Productive infection was barely detectable by RT or IFA. Cultures were sustained for more than 28 days without a change in HIV-1 production (data not shown). In some experiments, uninfected H9 T-lymphoid cells were added to HIV-1 IIIB infected CMK cultures. After cocultivation with H9 T cells, there was a rapid increase in detectable infection after 20 days of cocultivation (Fig 2).

To determine whether HIV entered CMK cells via the CD4 surface structure, we challenged CMK cells with HIV-2 ROD in the presence of anti-Leu3a MoAb, which binds at or near the HIV binding site on CD4. There was no detectable infection of CMK cells with HIV-2 ROD in cultures containing the anti-Leu3a MoAb (5 μg/mL). Similarly, addition of soluble recombinant CD4 at concentrations of 100 μg/mL completely inhibited productive infection of CMK cells by HIV-2 ROD (Fig 3).

Infection of CMK cells with HIV-2 ROD led to reduced expression of the surface CD4 and HLA-DR antigens, but no apparent change in expression of the megakaryocytic markers GP Ib or GP IIb/IIIa or the erythroid marker GP Ib (CD42b) or GP IIb/IIIa (CD41).

Table 1. Surface Antigen Characterization of the Three CMK Clones

<table>
<thead>
<tr>
<th>MoAb</th>
<th>CMK-6</th>
<th>CMK</th>
<th>CMK11-5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive Cells (%)</td>
<td>Positive Cells (%)</td>
<td>Positive Cells (%)</td>
</tr>
<tr>
<td>GP Ib (CD42b)</td>
<td>0.3* (0.2)</td>
<td>3.0 (1.0)</td>
<td>8.0 (0.9)</td>
</tr>
<tr>
<td>GP IIb/IIIa (CD41)</td>
<td>12.7 (3.1)</td>
<td>52.6 (3.3)</td>
<td>69.5 (6.1)</td>
</tr>
<tr>
<td>Glycophorin A</td>
<td>2.5 (0.5)</td>
<td>37.0 (7.2)</td>
<td>47.3 (4.8)</td>
</tr>
<tr>
<td>Leu3a (CD4)</td>
<td>90.5 (1.2)</td>
<td>91.1 (2.5)</td>
<td>66.8 (7.5)</td>
</tr>
<tr>
<td>My10 (CD34)</td>
<td>1.3 (0.3)</td>
<td>6.7 (0.5)</td>
<td>4.9 (0.5)</td>
</tr>
<tr>
<td>Leu M1 (CD15)</td>
<td>65.9 (6.5)</td>
<td>20.7 (4.0)</td>
<td>1.3 (0.4)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>ND</td>
<td>31.2 (5.6)</td>
<td>ND</td>
</tr>
</tbody>
</table>

IFA were performed as described in the text. Values in parenthesis represent the standard error (SE).

Abbreviation: ND, not done.

*Values represent the averages of two separate experiments.
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Fig 2. Recovery of HIV-1 IllB from CMK cells after cocultivation with noninfected H9 T cells. CMK cells (2 x 10^5/mL) were initially challenged with HIV IllB (10^5 TCID<sub>50</sub>). Three months after challenge CMK cells were cocultivated with noninfected H9 cells (2 x 10^5/mL). RT activity was monitored at 3- to 4-day intervals. Symbols: (○), CMK cells challenged with HIV-1 IllB and later cocultivated with H9 uninfected cells; (△), CMK cells challenged with HIV-1 IllB without cocultivation.

DISCUSSION

The megakaryocytic cell line, CMK, was highly susceptible to infection with the HIV-2 ROD and HIV-2 DOU strains in vitro. The CMK cells expressed specific megakaryocytic lineage markers, including the surface platelet GPs Ib and IIb/IIIa, and GF-1 RNA. Like most other permanent cell lines with megakaryocytic potential, i.e., HEL, LAMA-84, and Dami, CMK cells also expressed glycophorin A, an erythroid-specific protein. This suggests that megakaryocyte precursors and erythrocyte precursors may be derived from a common progenitor. Although the surface phenotype of the megakaryocyte and megakaryocyte progenitor have not been extensively investigated in humans, CFU-Meg (colony-forming units-megakaryocyte) bear the HLA-DR antigen and certain myeloid antigens. CMK cells appear to have the phenotype of early megakaryocyte progenitors.

Our studies with the CMK cell line provide data supporting the susceptibility of cells of megakaryocytic lineage to HIV infection. The initial study of Zucker-Franklin and Cao, which used in situ hybridization techniques, showed HIV-1 RNA in marrow megakaryocytes from HIV-infected patients. This indicated the likelihood of in vivo infection of megakaryocytic cells. The subsequent report by Basch et al of surface CD4 expression on mature bone marrow megakaryocytes also suggested that megakaryocytic cells would be susceptible to HIV infection, because the CD4 structure is the major receptor for HIV infection in T lymphocytes and monocyte-macrophages. More recently, Zucker-Franklin et al, using electron microscopy, identified HIV-1 particles in human megakaryocytes and platelets after in vitro incubation with virus. These three prior studies, and our work presented here, provide data using different methodologies and indicate HIV infection of cells of megakaryocytic lineage.

The high degree of susceptibility of CMK cells to infection with HIV-2 ROD and HIV-2 DOU compared with minimally detectable productive infection with three different HIV-1 strains suggests that there may be viral...

Fig 3. Inhibition of HIV-2 ROD infection of CMK cells by anti-Leu3a (A) and rCD4 (B). HIV-2 ROD virus, 100 TCID<sub>50</sub> in 60 μL was mixed with 60 μL of anti-Leu3a MoAb or rCD4, and incubated at 4°C for 1 hour. Then, 60 μL of CMK cells (5 x 10^5/mL) was added and incubated at 37°C for 1 hour. One hundred fifty microliters of this mixture was transferred to 2 mL of complete media and cultured in 24-well plates at 37°C in 5% CO<sub>2</sub>. RT activity was measured on the seventh day. Control cultures consisted of mock infected CMK cells. Indicated concentrations of anti-Leu3a and rCD4 represent final concentration. Each experiment was performed in duplicate.
tropism for megakaryocytic cells. HIV tropism is well described for infection of monocye-macrophages versus T lymphocytes in vitro. Of clinical interest, monocye tropic HIV isolates have frequently been derived from AIDS patients with central nervous system degeneration. HIV-1 IIIB is a T-lymphotropic isolate while HIV-1 BaL and 9533 (Groopman J: unpublished data, March 1990) are monocyte tropic isolates. HIV-2 ROD propagated in our laboratory and used in these studies can infect both T cells and monocyte-macrophages. The issue of tropism of HIV isolates for megakaryocytic cells merits further work. Our initial studies suggest that less than 1 in 10^5 CMK cells was infected by HIV-1 IIIB using the polymerase chain reaction method (M. Sakaguchi: unpublished data, January 1990). Current data indicate that early events such as virus binding, entry, and/or uncoating are important determinants of HIV tropism in T cells and monocytes. We are currently studying such early steps in HIV infection of CMK cells.

The different susceptibility of CMK cells to HIV-1 and HIV-2 could be explained by their different gene structure. For example, the VPX gene in HIV-2, not present in HIV-1, may contribute to this different susceptibility, although the effect of the VPX protein on infectivity has been controversial. Furthermore, it would be of interest to obtain isolates of HIV-1 from patients with thrombocytopenia or bone marrow failure states, and determine if they are more capable of infecting megakaryocytic cells than HIV-1 IIIB, BaL, and 9533.

The observations reported here provide evidence for HIV infection of megakaryocytic cells, a possibility suggested by the prior in situ hybridization and ultrastructural studies and the finding of surface CD4 antigen on bone marrow megakaryocytes. Because of the difficulties in obtaining highly purified populations of bone marrow megakaryocytes for in vitro study, it may be that pathologic evidence, provided by in situ hybridization or other studies, will form the major basis for determining in vivo infection.

The availability of megakaryocytic cell lines like CMK provides an in vitro model to study the effects of HIV on megakaryocyte function. Such studies may broaden our understanding of the pathogenesis of dysregulated hematopoiesis after infection with this retrovirus.

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M Sakaguchi, T Sato and JE Groopman