Alterations in Human Fetal Hematopoiesis Are Associated with Maternal HIV Infection

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**ABSTRACT. In the majority of adult and pediatric patients
with AIDS, hematologic abnormalities including leuko-
penia, anemia, and thrombocytopenia are commonly ob-
served. In addition to these findings, changes in hemato-
poietic progenitor cells occur, including a reduction of
multipotential-forming units, granulocyte-macrophages,
macrophage as well as eosinophil colony-forming units,
and bone marrow erythyroid burst-forming units. This
study examined alterations in human fetal liver hemato-
poiesis in 2nd trimester abortuses from human immuno-
deficiency virus (HIV)-seropositive women. The differen-
tiation and growth potential of hematopoietic cells *in vitro*
were monitored. Upon initial isolation, some populations
of liver hematopoietic cells from abortuses of HIV-sero-
positive women were significantly decreased when com-
pared to age-matched samples from fetuses of normal
females including the percentage of early T cells [cluster
of differentiation (CD)2], B cells (CD19), and early mono-
cytes (CD14). A decrease in multipotent progenitors
(CD34), myelomonocytes (CD33), and panleukocytes
(CD45) was also observed. In contrast, after 21 d in culture,
cells from HIV abortuses demonstrated an increase in the
percentage of CD14 cells when stimulated with erythro-
poietin and granulocyte-monocyte colony-stimulating fac-
tor, as well as an increase in CD45 phenotype after expo-
sure to granulocyte-monocyte colony-stimulating factor
alone. These samples showed a persistence of erythropoi-
etic elements (transferrin and CD36 phenotype) when com-
pared to normal controls. No significant difference in the
in vitro growth of hematopoietic progenitors (bone marrow
erythroid burst-forming units, granulocyte-macrophage
colony-forming units, and multipotential forming units)
between these samples and normal controls was found.
These findings may be the result of transplacental hema-
topoietic inhibitors produced as a consequence of maternal
HIV infection that result in similar hematologic abnor-
malities in AIDS patients. (*Pediatr Res* 32: 155–159, 1992)**

Abbreviations

BFU-E, bone marrow erythroid burst-forming unit
CD, cluster of differentiation
CFU-GEMM, multipotential-forming unit
CFU-GM, granulocyte-macrophage colony-forming unit
CFU-MK, megakaryocyte colony-forming unit
Ep, erythropoietin
GM-CSF, granulocyte-monocyte colony-stimulating factor

HIV, human immunodeficiency virus
HIV-1, human immunodeficiency virus-type 1
PE, phycoerythrin
PCR, polymerase chain reaction
**rhGM-CSF, recombinant human granulocyte-macrophage
colony-stimulating factor**
FL, fluorescence

AIDS is the result of infection of susceptible cells by HIV-1 (1,
2). Although the tropism of HIV-1 for CD4+ lymphocytes and
monocytes appears to be central in the pathogenesis of disease
(3), abnormalities in hematopoiesis with resultant peripheral
blood cytopenias also occur (4, 5). Granulocytopenia has been
reported in most patients with AIDS or AIDS-related complex
(6). An even higher incidence of anemia has been noted in a
series of individuals with AIDS (7). Similar abnormalities are
observed in HIV-infected infants and in others who show only
transient HIV seropositivity due to transplacentally acquired
anti-HIV antibody (8).

The bone marrow and hematopoietic progenitor cell compart-
ments are defective in HIV-1 patients (9, 10). Dysregulation of
hematopoiesis by alteration in the T cell subpopulations (11),
production of inhibiting glycoproteins (12), antibody-mediated
cytotoxicity (13), and infection of hematopoietic progenitor cells
by HIV-1 (14) have all been implicated as pathogenetic mecha-
nisms. Bone marrow specimens taken from patients with AIDS
exhibit myelodysplastic changes (15). A reduced proliferative
capacity *in vitro* of CFU-GM from the bone marrow of 16 out
of 18 patients with AIDS and concurrent neutropenia has been
described. Significant reduction of CFU-GEMM, BFU-E, and
CFU-MK has also been observed (11).

Maternofetal transmission of HIV-1 resulting in children with
AIDS is emerging as a major manifestation of this disease. There
is a high risk (about 30%) of HIV-1 infection in children born
to seropositive mothers (16, 17). Because the majority of children
with AIDS are congenitally infected by HIV-1, hematopoietic
organs other than the bone marrow may have a direct relation-
ship with the hematologic abnormalities associated with pediatric
AIDS. Because the liver represents the major organ of extramed-
ullary hematopoiesis during fetal life and HIV-infected children
are believed to be infected *in utero* (18), some of the hematologic
manifestations related to pediatric AIDS may be the product of
infection of hematopoietic stem cells in the human fetal liver.
To investigate these possibilities, fetal liver hematopoiesis in
abortuses of women infected by HIV was studied *in vitro* and
compared to that in normal control fetuses.

MATERIALS AND METHODS

Source of fetal tissue. The present study is part of an ongoing
research protocol that has been approved by the Albert Einstein

College of Medicine Committee on Clinical Investigation and the Health and Hospital Corporation of the City of New York. Informed consent was obtained from the participants. Fetal tissues were obtained at the time of elective pregnancy termination from normal and from HIV-seropositive women. The gestational ages of the abortuses ranged from 15 to 18 wk. Each sample was compared with age-matched controls in the following analysis.

Maternal and fetal blood studies. Five HIV-seropositive and eight normal control 2nd trimester pregnant women were studied. Routine blood studies included complete blood count [using the Coulter S plus IV (Hialeah, FL)], total lymphocyte count, T4 and T8 subsets, and coagulation profile. Sera from all patients were tested for antibodies to HIV using the ELISA and Western blot techniques. Fetal cord blood and amniotic fluid were analyzed for anti-HIV antibodies by ELISA and Western blot techniques.

Analysis of HIV-1 infection of fetal tissues. The potential of HIV-1 infection of fetal liver and other tissues was determined by autoradiography after PCR of potential HIV-1 DNA sequences.

Abortuses were obtained after dilation and extraction. Within 10 min of fetal demise, the external surface of the abortus was thoroughly rinsed with saline and the fetus surgically dissected to yield tissue samples including, but not restricted to, CNS, liver, and lung. The tissues were snap frozen in liquid nitrogen and maintained at $-70°C$ until analyzed. For DNA analysis, tissues were thawed on ice and mechanically dissociated into cell suspensions by passage through a sterile stainless steel mesh screen, cells were washed by sedimentation through PBS, and nucleic acids were deproteinized by standard phenol, chloroform-isoamyl alcohol purification.

One μg of tissue DNA was amplified for 30 cycles using the "gag" oligonucleotide primers SK38 and 39, the thermostable "tac" polymerase, and the salt and Mg optimization as described by the manufacturer (Cetus, San Francisco, CA). Detection of HIV-1 DNA was accomplished using the radiolabeled SK19 probe. In each case, a negative control DNA was obtained from a blood donor who was without risk factors for HIV-1 infection and who had been determined to be seronegative. Positive control DNA was composed of 1 μg of HIV-1-negative DNA to which had been added measured quantities of HIV-1 plasmid DNA genome copies or measured quantities of whole cell DNA from the 8E5-LAV cell. This cell line is known to contain a single proviral copy of HIV-1. All amplifications included both negative and positive controls and were done at least in duplicate.

Tissue culture. Fetal livers were obtained within 10 min of delivery. The organs were placed in Hanks' balanced salt solution containing 0.1% heparin. The livers were dissected sterilely and cells collected into 50-mL centrifuge tubes, and tissue debris and aggregates were allowed to settle by gravity. A mononuclear cell preparation was prepared by centrifugation over Ficoll Hypaque. The cells at the interface were washed twice in Hanks' balanced salt solution and the number of viable cells determined using trypan blue.

Cell cultures. In each experiment, HIV-positive and control cells were simultaneously cultured using the same conditions. RPMI-1640 (GIBCO Laboratories, St. Lawrence, MA) supplemented with 10% FCS (GIBCO), 100 units of penicillin, 5 mg streptomycin, and 0.9% methyl cellulose was used (wt/vol). Part of each sample was cultured in liquid suspension without addition of methylcellulose. The cells were resuspended in the medium and adjusted to 5×10^5 cells/mL. One mL of cell suspension was cultured in either 24-well or 6-well tissue culture plates. Cells were cultured in the presence of 1 IU/mL Ep (purified human urine ICN Biochemicals, Cleveland, OH) and rhGM-CSF (Genzyme, Boston, MA) at 5 IU/mL. In some wells, only Ep or GM-CSF was added to the cell suspension.

After 5, 10, and 21 d of incubation at 37°C and 5% CO_2 in a moist atmosphere, cell colonies were examined, classified, and counted in the 6-well plates using an inverted microscope. Red colonies usually containing more than 1000 cells were scored as BFU-E; although after 5 d in culture some investigators would call them erythroid colony-forming units, we use BFU-E to maintain internal consistency. Colonies containing nonhemoglobinized cells (primarily granulocytic precursors and macrophages) were scored as CFU-GM, whereas colonies containing both erythroid cells and one or more classes of phagocytic or megakaryocytic cells were scored as CFU-GEMM. Colony-forming units-megakaryocyte were defined by the relative size of the cells. Colonies are expressed as number of colonies per 10^5 cells.

Flow cytometry. The reagents used for immunofluorescent staining and flow cytometric analysis are shown in Table 1.

Phenotypic analysis of isolated fetal cells was carried out by direct and indirect staining with MAb. Briefly, cells were washed three times with PBS containing 2% FCS, and all samples were incubated on ice with purified human IgG for 30 min to block Fc receptors. Direct, dual-color staining was carried out by incubation with the following FITC and PE-conjugated MAb on ice for 30 min: HLe1FITC/Leu-M3-PE. [anti-CD45/CD14: Leukogate, Becton-Dickinson, Los Angeles, CA (Simultest), Becton-Dickinson, San Jose, CA)]; Leu-2a-FITC/Leu-4-PE (anti-CD8/CD3); Leu-3a+3b-FITC/Leu-16-PE (anti-CD4/CD20); and T4-FITC/4B4-RD1 (anti-CD4/CD29). Indirect staining was carried out by incubation on ice for 30 min with Leu-20 (anti-CD23), Leu-M9 (anti-CD33), My10 (anti-CD34), IOP36 (anti-CD36), or IOP61 (anti-CD61) at predetermined optimal concentrations. Cells were washed three times with PBS containing 2% FCS and 1% NaN_3 and incubated with goat anti-mouse IgG conjugated with FITC (GAM-FITC, Becton-Dickinson, Los Angeles, CA). After three additional washes, both directly and indirectly stained samples were fixed overnight in 1% paraformaldehyde at 4°C, washed, and resuspended in PBS for flow analysis. For direct immunofluorescent staining, FITC- and PE-conjugated mouse

Table 1. *Panel of antibodies used for phenotypic analysis*

Antigen	CD designation	Normal cellular expression	Source*
Leu-1	CD5	Thymocytes; activated T cells; subset of normal B cells	BD
Leu-3a,b	CD4	Normal thymocytes; T helper/inducer cells; monocyte/macrophages	BD
Leu-4	CD3	Thomocytes; mature, mitogenic T cells	BD
Leu-2a	CD8	Suppressor/cytotoxic T lymphocytes; NK cells	BD
Leu-1b	CD20	Mature cells	BD
Leu-20	CD23	B cell differentiation antigen activated monocytes (low affinity IgE Fc receptor)	BD
4B4	CD29	Helper-inducer CD4 and T cell; subset expressed on hematopoietic cells, T cells, and monocytes	C
B4	CD19	Early and mature B cells (expressed before CD20)	C
T11	CD2	All T cells (E rosette receptor)	C
Leu-M3	CD14	Monocyte/macrophage antigen	BD
HLE 1	CD45	Panleukocyte antigen	BD
Leu-M9	CD33	Human myelomonocytic antigen; promyelocytes and metamyelocytes	BD
My10	CD34	Uni- and multipotent progenitor cells	BD
IOP36	CD36	Very early erythroid differentiation antigen	BD
IOP61	CD61	Platelet glycoprotein, GP IIIa; platelet and megakaryocytes	A

* BD, Becton Dickinson; C, Coulter; and A, Amac (San Jose, CA).

IgG isotypic controls were processed for each sample. For indirect staining, a GAM-FITC control was processed for each sample.

Cells were analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). All results were based on acquisition of 10 000 cells per sample using Consort 30 software and analysis with Consort 30 and LYSYS software (Becton-Dickinson, Los Angeles, CA). The percentage of positive cells for each MAb was determined by single (FL1) or dual (FL1 × FL2) parameter list-mode analysis of events gated to exclude debris on a forward × side scattergram.

Statistical analysis. The results were analyzed using the "Statistics" program (version 5.1), produced by Basic Business Software, Inc. (New York, NY). The colony counts were analyzed by the *t* test and the flow cytometric data by the *t* test after conversion by two-way analysis of variance.

RESULTS

Maternal blood studies. Maternal blood studies including Hb, total white cell count, total lymphocytes, T4:T8 ratio, platelet count, and mean platelet volume are shown in Table 2. Of the five HIV-seropositive females, one woman suffered from a microcytic hypochromic anemia and another had leukopenia. All women had abnormal T4:T8 ratios. Two women presented with thrombocytopenia; both showed an increase in mean platelet volume that probably is due to an immune process. There were no abnormal values in the blood samples for the control females.

Potential HIV-1 infection of fetal tissue. There was no evidence by PCR to support the conclusion that any of the fetal tissues examined as part of this study were infected by HIV.

Fetal liver hematopoietic cultures. Table 3 demonstrates colony formation of fetal liver hematopoietic cells from abortuses of normal and HIV-seropositive females. Cultures were stimulated with Ep+ GM-CSF or GM-CSF alone and counted on d 5 and 10. Although fetuses of HIV-seropositive women showed a normal number and size of colonies of GFU-GEMM and CFU-GM, there was a significant ($p < 0.01$) difference between the normal and HIV samples with respect to colony-forming units-megakaryocyte on d 5. However, by d 10 there were no differences between the cultures.

Flow cytometric analysis. The relative percentages of lymphoid and nonlymphoid lineage MAb-positive fetal hematopoietic cells before culture are shown in Table 4, in which normal tissue is compared with tissue derived from abortuses of HIV-seropositive women. Normal cultures contained 6% of CD-19-positive cells (early B cell antigen) and 4% of cells expressed CD2 (an early T cell marker). Fetal liver hematopoietic cells from abortuses of HIV-seropositive women demonstrated a significantly lower number ($p < 0.01$) of CD19- and CD2-positive cells (2 and 1%, respectively), as well as fewer cells with CD14 phenotype ($p < 0.02$).

A nonsignificant decrease in multipotent progenitors (CD34) and myelomonocytic (CD33) and panleukocyte (CD45) antigens was also demonstrated. No difference was observed in the erythroid lineage (transferrin and CD36) either.

The relative percentages of erythroid and monomyelocytic

phenotypes of cultured hematopoietic cells stimulated with Ep+GM-CSF or GM-CSF on d 10 and 21 are shown in Tables 5 and 6, respectively. There is a significant increase ($p < 0.02$) in the number of CD14-positive cells in the fetal cultures for HIV-positive females on d 21 when stimulated with Ep+rhGM-CSF. The same samples showed a significant increase ($p < 0.01$) in the number of CD33-positive cells in cultures of HIV fetuses stimulated with Ep+rhGM-CSF on d 21 compared to normal controls. Transferrin- and CD36-positive cells, which are markers for the erythropoietic compartment, showed a 40% reduction between d 10 and d 21 (84 to 51% and 73 to 49%, respectively), whereas no changes were observed in transferrin- and CD36-positive cells originating from HIV fetuses (71 to 68% and 68 to 62%, respectively). No significant changes were observed in the megakaryocytic lineage (CD61).

DISCUSSION

The results of the present *in vitro* analysis of fetal liver cells from abortuses of HIV-seropositive women demonstrated significant changes in the phenotypes of some cells and hematopoietic progenitors when compared with normal control values. These changes were similar to those observed in bone marrow specimens of patients with AIDS (9, 10).

The major difference noted in this study was in the early T and B lineages, which showed a significant decrease in the percentages of cells originating from the liver of fetuses of HIV-seropositive women. One can speculate that the deficiency of immature lymphoid elements could be related to a maternal suppressor factor or factors secreted by T cell subpopulations. This may be the same mechanism that is responsible for lymphopenia observed in peripheral blood of patients with AIDS. Leiderman *et al.* (19) were the first to describe a reduced *in vitro* proliferative capacity of CFU-GM from the bone marrow of 16 out of 18 patients with AIDS and concurrent neutropenia. It was concluded that bone marrow cells from patients with AIDS released a factor inhibitory for normal granulopoietic progenitor cells. It was further reported (20) that bone marrow progenitors isolated from AIDS patients are responsive to rhGM-CSF and recombinant Ep and that antibodies present in the serum of patients infected with HIV could suppress the growth of these progenitors. A component of this immune-mediated suppression appears to be antibodies directed toward the envelope glycoprotein (*gp* 120) of HIV-1.

A decrease in CFU-GEMM, CFU-MK, BFU-E, and CFU-GM from bone marrow of patients with AIDS was found to be inversely correlated with the number of circulating CD4 cells and the T4:T8 ratio (21). This observation leads to the conclusion that an imbalance of T cell subpopulations appears to be mainly responsible for the progressive impairment of proliferation and differentiation of bone marrow progenitor cells observed in HIV-infected individuals. The present report demonstrates a decrease in myelomonocytes (CD33), panleukocytes (CD45), and early monocytes (CD14), but we failed to demonstrate any suppression of BFU-E, CFU-GM, CFU-GEMM, and CFU-MK. There was some evidence that those hematopoietic cells are more responsive

Table 2. *Hematologic profile of HIV-seropositive women*

	Hb*	WBC†	Total lymphocytes	Total T4:T8	Ratio T4:T8	Platelets‡	MPV§
1	11.8	3.3	924	92/675	0.14	131	11.0
2	11.8	7.6	1748	350/1013	0.34	272	7.9
3.	9.4	6.4	1088	239/631	0.38	274	7.8
4.	13.7	4.9	1786	319/1386	0.23	88	12.2
5.	11.0	7.4	1874	687/850	0.73	157	10.4

* g/dL.
† WBC, total per μL × 10³.
‡ Total per μL × 10³.
§ MPV, mean platelet volume in fL.

Table 3. *Hematopoietic cell colonies after addition of growth factors to cultures (no. of colonies ± SEM/5 × 10⁵ cells)**

	BFU-E		CFU-GM		CFU-GEMM		CFU-MEG	
	Normal	HIV	Normal	HIV	Normal	HIV	Normal	HIV
Day 5								
Ep + GM-CSF	490 ± 39	630 ± 230	244 ± 10	280 ± 130	63 ± 6	45 ± 5	22 ± 1	4 ± 1†
GM-CSF	334 ± 53	530 ± 340	214 ± 30	235 ± 75	57 ± 11	50 ± 10	21 ± 6	8 ± 1†
Day 10								
Ep-GM-CSF	627 ± 92	697 ± 53	164 ± 28	217 ± 12	109 ± 20	110 ± 59	19 ± 5	12 ± 4
GM-CSF	58 ± 27	220 ± 190	130 ± 15	200 ± 90	163 ± 30	185 ± 75	23 ± 3	28 ± 3

* Colony count d 5 and 10 in cultures supplemented with Ep + GM-CSF or GM-CSF alone. Data were obtained from studying the responses of cell colonies from eight normal fetuses and five fetuses from HIV-seropositive females. CFU-MEG, colony-forming units-megakaryocyte.
† $p < 0.01$.

Table 4. *Flow cytometric analysis of fetal liver hematopoietic cell phenotypes (% of total ± SEM)*

MAb	HIV fetuses	Normal controls	p value
CD2	1 ± 0	4 ± 1	<0.01
CD19	2 ± 1	6 ± 1	<0.01
CD14	0	3 ± 1	<0.02
CD61	5 ± 1	10 ± 2	<0.06
CD45	25 ± 1	32 ± 4	NS
CD34	6 ± 2	16 ± 3	NS
CD33	9 ± 3	21 ± 7	NS
Transferrin	70 ± 6	69 ± 8	NS
CD36	65 ± 12	55 ± 3	NS
CD4	2 ± 0	2 ± 0	NS

Table 5. *Flow cytometric analysis of cells after 10 d in cultures**

	CD45	CD14	Transferrin	CD36	CD61	CD33
NC Ep + GM-CSF	13 ± 2	6 ± 1	84 ± 6	73 ± 7	6 ± 1	14 ± 3
HIV fetus Ep + GM-CSF	11 ± 2	11 ± 3	71 ± 9	68 ± 4	8 ± 1	26 ± 4
NC GM-CSF	34 ± 7	20 ± 3	29 ± 6	34 ± 7	24 ± 6	45 ± 9
HIV fetus GM-CSF	27 ± 10	27 ± 6	29 ± 3	35 ± 4	11 ± 3	55 ± 10

* Percentage of different phenotypes after 10 d in culture (mean ± SEM). NC, normal control.

Table 6. *Flow cytometric analysis of cells after 21 d in culture**

	CD45	CD14	Transferrin	CD36	CD61	CD33
NC Ep + GM-CSF	38 ± 11	9 ± 2†	51 ± 9	49 ± 10	14 ± 1	4 ± 1†
HIV fetus Ep + GM-CSF	30 ± 5	22 ± 4†	68 ± 13	62 ± 6	17 ± 7	14 ± 3†
NC GM-CSF	37 ± 16	29 ± 6	9 ± 3	21 ± 8	20 ± 4	25 ± 6
HIV fetus GM-CSF	40 ± 7	39 ± 7	16 ± 3	26 ± 4	16 ± 8	16 ± 4

* Percentage of different phenotypes (gated) after 21 d in culture. NC, normal control.
† $p < 0.02$.
‡ $p < 0.01$.

to stimulation with Ep+GM-CSF or GM-CSF alone. There was an increased number of BFU-E on d 5 and 10 in HIV-associated cultures when compared to normal controls. Furthermore, phenotype analysis of cultured hematopoietic cells showed a significant increase in the number of CD14-positive cells on d 10, as well as a significant increase in the number of CD45-positive cells on d 21.

Although there remains a possibility that the observed changes are the product of HIV-1 infection of hematopoietic precursor cells, the data obtained by PCR amplification of HIV-1 DNA sequences were negative for all liver tissues. Under conditions of

PCR that demonstrated the presence of 10–25 HIV-1 genome copies of control positive samples, no positive signal was detected. Therefore, other mechanisms are suspected to be responsible for the observed results.

These findings could be due to a rebound phenomenon. Because T cells can inhibit the proliferation and differentiation of normal hematopoietic progenitor cells (by cell to cell interaction as well as by production of inhibitor factor), the separation of these progenitor cells from the endogenous inhibiting effect and the exposure to stimulating growth factor could produce the rebound effect.

The increase in CD14 phenotype in hematopoietic fetal liver cultures of abortuses from HIV-seropositive mothers could be similarly explained because most of the patients with AIDS have diminished numbers of circulating monocytes (6). All liver tissues were analyzed by PCR for HIV-1 DNA sequences and found to be negative. This fact supports our hypothesis that changes observed in the *in vitro* hematopoiesis of abortuses of HIV-seropositive women might be a rebound effect caused by inhibitors produced by the maternal T cell subpopulations or a lack of inhibitors due to a significantly diminished number of early T cells observed in liver tissue of those abortuses. Infection of a small subpopulation of hematopoietic progenitor cells or macrophages could not be excluded in our study, but it is unlikely because infected progenitor cells lose their capacity to proliferate (11).

In conclusion, the present data demonstrate that fetuses of HIV-seropositive women exhibit a normal or even increased ability of their hematopoietic cells to proliferate and differentiate in tissue culture regardless of a direct or indirect HIV effect on the hematopoietic system of the host. Infection of a small subpopulation of hematopoietic progenitor cells below the level of detectability of the PCR, however, cannot be excluded. Whatever the mechanism causing bone marrow alterations in patients with AIDS, it appears not to have a nonreversible impact on the potential of fetal hematopoietic cells to differentiate.

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