Stromal Cell Lines Derived From LP-BM5 Murine Leukemia Virus-Infected Long-Term Bone Marrow Cultures Impair Hematopoiesis In Vitro

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Murine acquired immunodeficiency syndrome (MAIDS) induced by defective LP-BM5 murine leukemia virus is a disease with many similarities to human AIDS. Previous studies indicated that the depressed hematopoiesis observed in LP-BM5-infected marrow cultures may be attributable to a defect of hematopoietic stroma. We report here the generation of permanent stromal cell lines from noninfected and LP-BM5-infected marrow cultures. Retrovirus infection was confirmed by polymerase chain reaction for viral genome. The ability of these cell lines to support in vitro hematopoiesis was studied. Results indicated that, when cocultured with normal or infected nonadherent mononuclear cells, noninfected cell lines efficiently supported the production of hematopoietic precursors, whereas viral-infected cell lines exhibited a defective function for normal hematopoiesis. The similarity to human AIDS is polyclonal B-lymphocyte activation, hypergammaglobulinemia, lymphadenopathy, BM abnormalities, and profound immunodeficiency. However, MAIDS differs from human AIDS in that MAIDS mice die of respiratory failure caused by mediastinal lymph node enlargement and not of recurrent infections, which is commonly associated with human AIDS. LP-BM5 MuLV is a mixture of viruses, consisting of a nonpathogenic replication competent B-tropic ecotropic virus, a mink cell focus-inducing virus, and a replication-defective virus. Recent studies have identified the replication-defective virus as the etiologic agent of MAIDS. Currently, the cellular and molecular events involved in the pathogenesis of MAIDS remain unknown. Our recent study indicated that LTBMC derived from LP-BM5-infected mice exhibited a defective function for normal hematopoiesis compared with marrow cultures from noninfected mice. The addition of zidovudine (AZT) did not reverse this defect in infected cultures.

The mechanism(s) inducing the stromal cell defect of viral-infected cultures is unknown. Virus infection might alter BM stromal cell functions, resulting in reduced support capacity for multilineage and committed progenitor cells in culture. Furthermore, an imbalance or a defect in production of cytokines by infected stroma cells might have a major effect on the development of the deficient state. To further characterize the defective nature of virus-infected stroma, we generated permanent stromal cell lines from LP-BM5-infected and normal noninfected LTBMCs. Our results showed the establishment of stromal cell lines of primarily fibroblastic morphology. Retrovirus infection of infected stromal cell lines has been confirmed and these cell lines, as well as the noninfected control, were then tested for their ability to support and maintain reconstituting hematopoietic cells harvested from normal and MAIDS mice. The results indicated an alteration in support and maintenance of hematopoietic progenitors from normal as well as viral-infected cells when cocultured with the infected stromal cell lines. Finally, studies were undertaken to analyze the expression of cytokine genes in established stromal cell lines. Infection with LP-BM5 MuLV was found to associate with higher induced suppression of both normal and viral-infected progenitors. Expression of cytokine genes in stromal cell lines was also examined. All cell lines expressed equivalent levels of transcripts for stem cell factor and tumor necrosis factor α. However, infection was associated with higher levels of interleukin-4 and transforming growth factor β1 transcript expression. These findings suggest that infected stromal cell lines exhibit a defective hematopoietic microenvironment that produced altered cytokine expression resulting in faulty hematopoiesis. Further characterization of the defective cell lines should prove valuable for studies of the pathogenesis of murine AIDS.

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levels of interleukin-4 (IL-4) transcript and exclusive transforming growth factor β1 (TGFβ1) expression in infected stromal cell lines.

MATERIALS AND METHODS

Mice

C57BL/6 mice were obtained from Harlan (Indianapolis, IN). Animals were housed in a temperature-humidity–controlled environment and fed Purina Lab Chow (St Louis, MO) and water ad libitum.

Infection With LP-BMS Murine Immunodeficiency Virus

The LP-BM5 MuLV stock used for inoculation consisted of a mixture of mink cell focus-forming (BM5) virus, ecotropic helper (BM5e) virus, and replication-defective (BM5d) virus that were harvested from chronically infected SC-1 (clone G6) cultures, kindly supplied by Dr Donald A. Cohen (University of Kentucky Medical Center, Lexington). Normal 5-week-old C57BL/6 mice were inoculated intraperitoneally with 0.2 mL of cell-free virus stock containing 10^5 μg total protein. This is the recommended standard infection procedure for induction of MAIDS in vivo.14 Disease progression was characterized by extensive lymphadenopathy and splenomegaly, hypergammaglobulinemia, development of high-grade B-cell lymphoma at late stages of the disease, and death as the result of acute respiratory failure 16 to 20 weeks post-viral infection.

LTBMCs and Establishment of Permanent Stromal Cell Lines

LTBMCs were established as described previously.15 Briefly, normal noninfected and 4-week post-infected mice were killed by cervical dislocation. The contents of a tibia and femur were flushed with growth medium containing Fischer’s medium (GIBCO, Grand Island, NY) supplemented with 20% horse serum (Hyclone, Logan, UT), 17 mmol/L sodium bicarbonate (Sigma, St Louis, MO), 5 × 10^−3 mol/L hydrocortisone sodium succinate (Upjohn, Kalamazoo, MI), and 80 μg/mL penicillin-streptomycin (GIBCO). Single-cell suspensions of pooled BM were seeded into 25-cm² flasks (Corning, Corning, NY) and incubated at 33°C in humidified 5% CO₂. The cultures were maintained for a period of 6 months by weekly removal of half of the medium from the flask and replenishment with fresh growth medium.

Six months after the establishment of LTBMCs, the adherent cells from representative noninfected and LP-BM5–infected culture flasks were removed by treatment with 0.25% trypsin-EDTA (GIBCO) and were passed in growth medium supplemented with 5 × 10^−3 mol/L hydrocortisone sodium succinate. Cultures were then passed weekly at a 1:2 split for a period of 5 weeks to select highly proliferative cells. After further passage and selection, two noninfected stromal cell lines, designated KLT1 and KLT2, and three viral–infected stromal lines, designated KLTM1, KLTM2, and KLTM3, were successfully established from three separate attempts. All of the stromal cell lines, whether infected or noninfected, isolated from these attempts were morphologically similar with an appearance of fibroblast, macrophage, and adipocyte morphology. However, fibroblast-like cells became the dominant cell morphology after subsequent repeated passage. Cell lines KLT2 and KLTM1 proliferated roughly at the same rate and exhibited similar cell densities. All the cell lines grew as monolayers and were contact inhibited when they reached confluency. These stromal lines were completely depleted of supernatant-derived mononuclear cells, and the observation was confirmed by the absence of detectable hematopoietic progenitors colony-forming unit–granulocyte-macrophage (CFU-GM), CFU-megakaryocyte (CFU-Meg), and burst-forming unit-erythrocyte (BFU-E) after progenitor cell assays.

Characterization of Noninfected and Infected Stromal Cell Lines

Cytochemical and histochemical stains were used to characterize established stromal cell lines. Staining for alkaline phosphatase, acid phosphatase, myeloperoxidase, naphthol AS-D chloroacetate, and α-naphthyl acetate were performed using commercially available diagnostic kits (Sigma) after collection of cells on slides by cytopsin and appropriate fixation, according to the specifications recommended by the manufacturer. Smears of fixed normal mouse BM were used as positive controls for these stains. Slides were scored according to the method described by the manufacturer.

Determination of Retrovirus Production in BM-Derived Stromal Cell Lines

To determine viral particle production in infected stromal cell lines, cells from both normal and infected cell lines were removed with a rubber policeman and centrifuged (400g; model TJ-6R; Beckman Instruments, Palo Alto, CA). At least 1 × 10⁶ cells were pelleted and prepared for examination by electron microscopy. The pelleted cells were minced into less than 1-mm cubes, fixed in 3% glutaraldehyde in buffer, postfixed in 1% osmium tetroxide in buffer, dehydrated, and embedded in epon. After selecting appropriate blocks, thin sections were cut and stained with Reynold’s lead citrate and m an y cate acetate and examined with a Phillips 400 transmission electron microscope.

Preparation of Nonadherent Mononuclear Cells From Normal and LP-BM5–Infected Mice

Normal noninfected mice and LP-BM5–infected mice were killed 4 weeks postinfection by cervical dislocation. Femoral marrow was flushed and single-cell suspensions of BM were prepared in RPMI (Whittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (ICN, Costa Mesa, CA), 2 mmol/L L-glutamine (GIBCO), 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO). After centrifugation at 400g for 10 minutes at 4°C, cells were resuspended in RPMI medium and then overlayed on an equal volume of Ficoll-Paque (specific gravity, 1.077 g/mL; Pharmacia, Piscataway, NJ). Density centrifugation was performed at 400g for 25 minutes at 4°C and the interface layer of low-density mononuclear cells was collected, washed, and resuspended in fresh medium before adherent cell depletion. The low-density mononuclear cells were further separated into adherent and nonadherent populations after incubation on culture flasks for 90 minutes at 37°C. The nonadherent cell fraction was then collected, centrifuged, and resuspended in medium for cell counting with a Coulter Counter (Model ZBI; Coulter Electronics, Hialeah, FL). To confirm the efficacy of the separation, aliquots of this nonadherent progenitor-enriched fraction were cultured alone in tissue culture flasks and were observed for growth of adherent stromal cell layers. These nonadherent normal and viral-infected low-density mononuclear cells were used as the source of marrow progenitors in the following reconstitution studies.

Stromal Cell Lines Reconstituted With Hematopoietic Cells

To assess the ability of established stromal cell lines to support hematopoiesis, stromal cells from noninfected and viral-infected cell lines were simultaneously seeded at 5 × 10⁵ cells per 25-cm² flasks. The subconfluent stromal layers were then reconstituted with 2.5 × 10⁵ fresh nonadherent-enriched marrow mononuclear cells (MNCs) harvested from both virus-free and LP-BM5–infected mice as de-
scribed above. In control experiments, enriched MNCs were added to 25-cm² flasks in the absence of preformed stromal cell layers to assess the survival of these progenitors in culture flasks. These control MCC cultures failed to produce detectable stromal cells after up to 3 weeks in culture and no viable MNCs were detected after 1 week. Stromal cell cultures incubated without reconstituting MCC were also established. No supernantant-derived nonadherent cells could be collected after 3 weeks in culture. The reconstituted flasks were maintained at 33°C in 5% CO₂ with weekly one-half volume media change with LTBM medium supplemented with 5 × 10⁻⁷ mol/L hydrocortisone sodium succinate. The reconstituted MCCs attached and formed "cobblestone islands" on each stromal cell layer. At weekly intervals after reconstitution for up to 3 weeks, culture supernatants were removed from reconstituting flasks and nonadherent cells were harvested and enumerated for total nonadherent cell production. These collected nonadherent cells were used to assay for progenitor cells giving rise to CFU-GM, CFU-Meg, and BFU-E in hematopoietic progenitor cell assays. The reconstitution experiments reported here were performed with noninfected line KLT2 and infected line KLM1 pair, although essentially similar findings were observed in other cell line pairs.

**Hematopoietic Progenitor Cell Assay**

Assay for granulocyte-macrophage (CFU-GM) and megakaryocyte (CFU-Meg) progenitors. CFU-GM and CFU-Meg were cultured as previously described. Briefly, nonadherent cells harvested from reconstitution medium were plated at 5 × 10⁴ cells/mL in 1 mL of semisolid plating medium consisting of RPMI (Whittaker), 20% fetal bovine serum (ICN), 40 mM sodium bicarbonate (Sigma), 10 mM sodium pyruvate (GIBCO), 0.1 mM/minimum essential medium (MEM) nonessential amino acids (GIBCO), 0.4% MEM essential amino acids (GIBCO), 0.2% MEM vitamin (GIBCO), 2 mM/L L-glutamine (GIBCO), 100 U/mL penicillin/100 μg streptomycin/0.25 μg fungizone (Hazleton), 1 × 10⁻⁷ mol/L β-mercaptoethanol (Sigma), and 0.6% agarose (Sigma) in 6-well plates (Falcon) in the presence of recombinant murine granulocyte-macrophage colony-stimulating factor (MuGM-CSF; 25 U; specific activity, 1 μg of total RNA was transferred to a fresh tube. An equal volume of isopropanol was added to the tube and the RNA was precipitated at -20°C for 1 hour. The precipitated RNA was pelleted at 11,000g for 20 minutes at 4°C and washed twice with 70% ethanol. The pellet was dried and dissolved in 100 μL diethylpyrocarbonate (DEPC)-H₂O. The yield and purity of the RNA was determined by spectrophotometric analysis at 260 and 280 nm. The quality of each individual preparation of RNA was assessed by electrophoresis on 1% agarose-formaldehyde gels and staining with ethidium bromide.

**Defective Virus and Cytokine Gene Expression**

Reverse transcription-polymerase chain reactions (RT-PCR) were performed to analyze the expression of the LP-BM5 MuLV defective virus genome and several cytokine genes in both infected and noninfected stromal cell lines. For first-strand cDNA synthesis, 1 μg of total RNA was reverse transcribed in a 20 μL reaction volume containing reaction buffer (50 mM/L KCl, 10 mM/L Tris-HCl, pH 8.3), 5 mM/L MgCl₂; 1 mM/L each of dGTP, dATP, dCTP, and dTTP; 20 U RNase inhibitor; 50 U MuLV reverse transcriptase; and 2.5 μmol/L Oligo d(T) primer (all reagents were supplied by Perkin Elmer Corp, Norwalk, CT) for 15 minutes of incubation at 42°C. The reaction was terminated by 5 minutes of incubation at 99°C. The resulting first-strand cDNA was used as template in the PCR. After first-strand synthesis, PCR was performed by adding 20 μL of the cDNA solution to a 80 μL master mix containing 4 μL of 25 mM/L MgCl₂, 8 μL of 10x buffer (500 mM/L KCl, 100 mM/L Tris-HCl, pH 8.3), 65.5 μL DEPC-H₂O, 2.5 U AmpliTaq DNA polymerase (Perkin Elmer), and 0.15 μmol/L of each specific primer. The samples were amplified in a Model 480 thermal cycler (Perkin Elmer Corp) programmed for the following cycle parameters: stem cell factor (SCF), tumor necrosis factor α (TNFα), and p12 gag; 95°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes for 30 cycles; TGFβ1: initial denaturation at 94°C for 1 minute, 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 3 minutes for 35 cycles, followed by a final extension at 72°C for 7 minutes; IL-3 and IL-4: 94°C for 5 minutes, 60°C for 1 minute, and 70°C for 1 minute and 30 seconds for 1 cycle followed by 94°C for 45 seconds, 60°C for 1 minute, and 70°C for 1 minute and 30 seconds for 39 cycles.

The primers specific for murine SCF, IL-3, IL-4, TNFα, TGFβ1, β-actin, and LP-BM5 MuLV defective virus p12 gag were synthesized using an Applied Biosystems 394 DNA synthesizer (Foster City, CA): SCF: 5’ primer, GCTTGACTACTCTTCCTGGGAC, and 3’ primer, CTGCTGTATCTCCTAAAGGG, with an expected product size of 216 bp; gag: 5’ primer, CACCAACTCTCAGTGGAGG, and 3’ primer, CCWATCGACACTTCCCIT, and 3’ primer, CCGCCCTCITCITCAGG, with an expected product size of 216 bp; IL-3: 5’ primer, GCAAGCTCATTGCCAGAGATGG, and 3’ primer, TGGCCITAGGGTTCAGGGGG, with an expected product size of 209 bp, and 3’ primer, GCAGAGTCATTCGCAGATGTAG, with an expected product size of 209 bp; TGFβ1: 5’ primer, GACTGGTGAGGAGCGACCAACCG, and 3’ primer, GAGCGGTGGAACCTGGCAGGAG, with an expected product size of 383 bp; β-actin: 5’ primer, CTGCGCTGACTCTGCTGGT, with an expected product size of 681 bp; TGFβ1: 5’ primer, GCTAATGGTGGAGACCGGACAAACGG, and 3’ primer, CTCTTGCCTACTGCTGGTCGGCA, with an expected product size of 240 bp; p12 gag: 5’ primer, CCTTGTCGACTTCCTCCCTT, and 3’ primer, TGCCCTTAAGGGTCCAGGGG, with an expected product size of 240 bp; Primers specific for murine β-actin were used as an internal control in all PCR reactions. All primer pairs produced products of the predicted size. All PCR analyses included a negative (reagent) control to rule out reagent contamination. Total RNA extracted from concanavalin A (Con A)- or lipopolysaccharide (LPS)-stimulated spleen and...
VIRUS-INFECTED STROMA AND HEMATOPOIESIS

Fig 1. Microscopic appearance of representative LTBMC-derived stromal cell lines. (A) Noninfected KLT2 and (B) viral-infected KLTM1 (original magnification ×100).

RESULTS

Morphology and Characteristics of LTBMC-Derived Stromal Cell Lines

In an attempt to elucidate possible mechanism(s) responsible for the hematopoietic microenvironmental defect observed in LP-BM5–infected primary cultures, permanent stromal cell lines were established from adherent cells of LP-BM5 infected and noninfected control. Two noninfected and three viral-infected stromal cell lines were isolated. These five stromal cell lines were all morphologically similar. When cultured at 33°C and examined by phase contrast microscopy, these cell lines resembled fibroblastoid cells with a pleomorphic appearance. Phaseluent lipid vacuoles were occasionally seen in the cultures (Fig 1). All cell lines

Wehi-3 cells was reverse transcribed and PCR amplified as positive controls for SCF, IL-3, IL-4, TNFα, and TGFβ1 primers. In addition, a SC-1 cell line chronically infected with LP-BM5 virus was also used as the positive control for p12 gag primers. The resulting PCR products were analyzed by electrophoresis of 20 μL of each reaction through 2% agarose gels containing 0.5 μg/mL of ethidium bromide and visualized with UV fluorescence. The gels were photographed using Polaroid 667 film (Polaroid Corp, Cambridge, MA). The negatives were scanned using the BioImage System (Millipore Corp, Ann Arbor, MI) and the density of the bands was determined using BioImage whole band analysis protocol for 1-D electrophoresis image.

Statistical Analysis

Student’s t-test was used to analyze the difference between the means of the experimental and control data. A P value < .05 was used to determine the level of significance.
grew as monolayers and were contact inhibited when reaching confluency. These cell lines proliferated with a doubling time ranging from 28 ± 6 to 32 ± 5 hours and with a plateau phase cell density ranging from 1.5 × 10^5 cells/cm^2 to 2.0 × 10^5 cells/cm^2 (data not shown). Histochemical and cytochemical studies showed that all the cell lines were acid phosphatase and α-naphthyl acetate esterase (nonspecific esterase) positive, findings consistent with a macrophage histiocyte cell lineage (Table 1). In contrast, all cell lines were negative for alkaline phosphatase and myeloperoxidase, suggesting that stromal-associated myeloid precursors were absent. Naphthol AS-D chloroacetate esterase activity was inconclusive for all cell lines (Table 1).

**LP-BM5 Virus Production in Established Stromal Cell Lines**

To confirm retrovirus infection, established stromal cell lines were examined for the presence of viral particles. Electron microscopic examination of noninfected and viral-infected cell lines showed that only the latter demonstrated the presence of viral particles that were budding from the cell membrane. Representative results are shown in Fig. 2. Retrovirus infection has been maintained in infected cell lines despite repeated passages.

**Detection of Defective Virus p12 gag in Infected Stromal Cell Lines by RT-PCR**

LP-BM5 MuLV is a mixture of viruses, consisting of nonpathogenic replication-competent B-tropic ecotropic virus, a mink cell focus-inducing virus, and an etiologic, replication-defective virus. The demonstration of viral particles production may not necessarily reflect the presence of defective virus in infected stromal cell lines. Thus, analysis for defective virus genome was performed in established stromal cell lines. Southern and Northern blot hybridization have been previously used by several research groups to detect defective virus in infected cell lines. Despite repeated passages.

**Table 1. Characterization of LTBM-Derived Stromal Cell Lines**

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<tr>
<th>Cell Line</th>
<th>Alkaline Phosphatase</th>
<th>Acid Phosphatase</th>
<th>Chloroacetate Esterase</th>
<th>Nonspecific Esterase</th>
<th>Myeloperoxidase</th>
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<tr>
<td>Noninfected</td>
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Cells were collected on slides and fixed according to the specifications recommended by the manufacturer in the diagnostic kits. Smears of fixed normal mouse BM were used as positive controls. Nonspecific esterase is α-naphthyl acetate esterase, and chloroacetate esterase is naphthol AS-D chloroacetate esterase.

**Abbreviations:** +, present in cytoplasm; −, absent in cytoplasm.

The specificity of this primer pair was shown by the amplification of total RNA extracted from SC-1 cells chronically infected with the LP-BM5 MuLV mixture (Fig 3, lane 4). Using this primer pair, we were able to detect the presence of defective viral genomes in established infected stromal cell lines. In contrast, PCR analysis was unable to detect defective virus expression in all the noninfected stromal cell lines. A representative of PCR analysis is shown in Fig 3. β-Actin was used as an internal control in all PCR analyses.

**In Vitro Support of Reconstituted Hematopoietic Cells by Noninfected and Infected Cell Lines**

Developing hematopoietic cells in the BM are closely associated with the underlying supportive stroma. Thus, the ability of established stromal cell lines to maintain and support reconstituted nonadherent hematopoietic cells was tested. Representative results are reported here and were obtained from the noninfected (KLT2) and the infected (KLTM1) stromal cell pair. Subconfluent stromal cultures of noninfected and infected cell lines were simultaneously reconstituted with identical inocula of enriched MNCs obtained from virus-free and MAIDS mice. To ensure that the MNCs reconstituted into the stromal cell lines not be contaminated with stromal cells, enriched MNCs were plated alone and incubated for 3 weeks. No adherent layer was detected and MNCs in the cultures did not survive past 1 week. In addition, endogenous hematopoiesis was also tested in each cell line when cultured alone. Nonadherent hematopoietic cells were not detected in the stromal culture media throughout the study period.

At 1 week after reconstitution, small “hematopoietic islands” indicating the presence of hematopoietic activity became visible within the stromal cell layer derived from KLT2. These islands enlarged over the next 2 weeks and eventually became indistinguishable. In contrast, hematopoietic islands were less visible within the stromal layer derived from KLTM1 throughout the duration of the experiments. Hematopoietic cells reconstituted on each stromal cell line generated nonadherent cells, which were harvested weekly by removing half of media and refeeding the cultures with an equal volume of fresh medium. As shown in Fig 4, the control cell line KLT2, when reconstituted with normal MNCs, supported nonadherent cell production for more than 3 weeks in culture (9.5 ± 0.71 × 10^5 cells/flask). In contrast, the production of nonadherent cells in KLTM1 reconstitution...
was significantly reduced for more than 3 weeks (6.9 ± 0.21 x 10^5 cells/flask, P < .05). A similar pattern was observed when these stromal cell lines were cocultured with MNCs derived from MAIDS mice. Production of reconstituted nonadherent cells was significantly lower in the KLTM1-derived underlayer (P < .05). The depressed nonadherent cell production in KLTM1 cell line was not caused by cell growth deficiency because both KLT2 and KLTM1 proliferated at approximately the same rate. By week 4, underlayers from both stromal cell lines were overgrown in all culture flasks, resulting in cell detachment and termination of the study.

**Maintenance of Reconstituted Hematopoietic Progenitors by Noninfected and Viral-Infected Stromal Cell Underlayers**

To determine the ability of noninfected and viral-infected cell lines to support hematopoietic progenitors, identical inocula of noninfected and viral-infected MNCs were reconstituted onto KLT2 or KLTM1 stromal layers. Nonadherent cells harvested weekly from reconstituted cultures were assayed for CFU-GM, CFU-Meg, and BFU-E as described. As shown in Fig 5A, KLT2 positively supported CFU-GM progenitor cells derived from normal and virus-infected BM at week 3 after reconstitution. In contrast, the number of CFU-GM progenitor cells produced at week 3 by KLTM1 was at a level of only 2% to 4% that of the control culture (P < .01). When CFU-Meg progenitor cell production was assayed, KLTM1 supported megakaryocyte progenitor cells at only 3% to 4% compared with the KLT2 control line (P < .03, Fig 5B). KLTM1 also showed a significant reduced support capacity for erythroid progenitors. Erythroid progenitor colonies were rarely detected from cells harvested after cocultivation with the KLTM1 line. Only 8% to 14% of BFU-E progenitor cells at week 3 were maintained compared with the control cell line (P < .03, Fig 5C). Thus, infected cell line KLTM1 showed a significant decreased support capacity for either normal or infected hematopoietic progenitors compared with the control cell line (P < .03, Fig 5C). Thus, infected cell line KLTM1 showed a significant decreased support capacity for either normal or infected hematopoietic progenitors compared with the normal control KLT2.

**Cytokine Gene Expression in Noninfected and LP-BM5–Infected Stromal Cell Lines**

BM-derived noninfected and viral-infected stromal cell lines have been analyzed for the in vitro hematopoiesis supporting ability. These stromal cell lines differ in their ability to support reconstituting hematopoietic cells, although they have similar growth characteristics and morphology. Altered patterns of cytokine expression and/or different cytokine gene regulation could contribute to these functional differences. To assess whether LP-BM5 infection led to general alteration in cytokine gene expression in infected stromal cells, mRNA expression for SCF, IL-3, IL-4, TNFα, and TGFβ1 were examined using RT-PCR. The PCR method was chosen for its high specificity and sensitivity, which
allows detection of both small amounts of mRNA and minor changes in mRNA levels.

First-strand cDNA templates for PCR amplification were synthesized using oligo-dT priming in the reverse transcription reaction to exclude immature cytokine transcripts in the subsequent PCR amplification. Irrespective of the primers used, no PCR products were detected in the reagent controls in which no RNA was included in the first-strand reverse transcription reaction. To determine the relative levels of cytokine transcripts present in noninfected and infected cell lines, equivalent amounts of cDNA from both cell lines were used and β-actin cDNA was also amplified as an internal control in each RT-PCR analysis. Because the same concentration of total cDNA was used for each stromal cell sample, differences in the amount of amplified product produced per sample reflect differences in the expression level of the mRNA in question. However, because PCR analyses are not truly quantitative, no comparison can be made for the levels of expression among different cytokines. To test the specificity of each primer pair, total RNA from spleen cells stimulated with mitogens and from Wehi-3 cells was reverse transcribed and the resulting cDNA was amplified with specific primer pairs. In each case, the appropriate size fragment was amplified by RT-PCR and detected by electrophoresis through an agarose gel (data not shown).

The results from representative RT-PCR are shown in Figs 6 and 7. Based on densitometric analysis, results indicated that mRNA transcripts for SCF were expressed in equivalent levels in both noninfected and infected stromal cell lines (Fig 6A). However, the transcripts for IL-3 could not be detected in either infected or noninfected cell lines (Fig 6B). To ensure that the conditions for the IL-3 cDNA-PCR reactions were correct, total RNA extracted from Wehi-3 RNA was also reverse transcribed and amplified by PCR, in parallel with other samples tested. The results showed that IL-3 transcripts were readily detected in Wehi-3 cells under these conditions (data not shown), further suggesting that neither noninfected nor infected stromal cell lines synthesized detectable mRNA for IL-3.

Both noninfected and infected stromal cell lines expressed IL-4 transcripts. However, the densitometric analysis showed that the levels of expression were found to be five times higher in infected stromal lines (Fig 6C). We next examined the levels of TNFα and TGFβ1 expression in these stromal cell lines. On conversion of total RNA to cDNA and amplification by PCR, although faintly detectable, both stromal cell lines expressed transcripts for TNFα and the expressions were equivalent (Fig 7A). In contrast, transcripts

![Fig 2. Detection of defective virus in established stromal cell lines by RT-PCR. Total RNA was isolated from noninfected stromal cell lines, LP-BM5 MuLV-infected stromal cell lines, and SC-1 cells chronically infected with LP-MBI virus. Defective viral genome was amplified with p12 gag-specific primers as described in Materials and Methods. Amplified products were separated on a 2% agarose gel containing ethidium bromide. The size for amplified products was 209 bp. Lane 1, molecular weight marker (MspI-digested pBR322 DNA); lane 2, 100-bp ladder molecular weight marker; lane 3, reagent control; lane 4, SC-1 control; lanes 5 and 7, noninfected cell line (KLT2); lanes 6 and 8, LP-BM5-infected cell line (KLTM1). β-Actin was used as the internal control.](image-url)
for TGFβ1 were not detected in noninfected stromal cell lines, whereas it was exclusively expressed in infected stromal lines (Fig 7B).

**DISCUSSION**

The BM in MAIDS mice has many similarities when compared with the marrow from AIDS patients, which is characterized by a decrease in cellularity and fat atrophy.25,26 The mechanism inducing such abnormalities is currently unknown. Previous studies in LTBMCs using marrow harvested from MAIDS mice suggested that there was a defect in the support and maintenance of the hematopoietic microenvironment required for normal hematopoiesis. The production of several progenitor cell lineages in MAIDS-LTBMCs was all depressed compared with noninfected controls.13 The present studies report that permanent marrow stromal cell lines were established from MAIDS-LTBMCs and from normal noninfected cultures as controls. Cytochemical and histochemical characterizations of the established stromal cell lines showed a heterogeneous stromal cell population in these cultures. The viral-infected cell lines were morphologically equivalent to the noninfected lines. Spindle-shaped fibroblastic cells and macrophage-like cells that stained positively for acid phosphatase and α-naphthyl acetate esterase were the predominant cell types found in all cell lines. Production of viral particles has been demonstrated using electron microscopy and this was correlated with the detection of defective virus expression by RT-PCR only in viral-infected but not in noninfected stromal cell lines.

The ability of these cell lines to support in vitro hematopoiesis was examined. After reconstitution with virus-free or viral-infected hematopoietic progenitor cells, altered patterns of hematopoiesis were repeatedly observed when the viral-infected cell lines were used as the support underlayer compared with the reconstituted, noninfected cell lines. Production of nonadherent cells was detected at a significantly lower level in the infected cell lines grown for 3 weeks. The numbers of CFU-GM, CFU-Meg, and BFU-E progenitors were also significantly reduced when cocultured with the infected cell lines compared with controls. It is unlikely that hematopoietic progenitor cell infection by virus could explain the altered patterns of growth as marrow cells derived from virally infected mice proliferated and differentiated normally when cocultured with the noninfected stromal cell lines. On the contrary, virus-free progenitor cell growth was significantly depressed when the infected cell lines were used as the supportive underlayer. These results further suggest that, as the result of virus infection, the marrow microenvironment is altered such that a defective stroma is incapable of supporting normal hematopoiesis. Furthermore, these data show that progenitor cells derived from LP-BM5 virus-infected mice are capable of responding to the systems that regulate hematopoiesis in LTBMCs because their support was not reduced when overlayed on normal, non–virus-infected stromal cells. Whereas these and other results presented in this report were generated from stromal cell lines derived from in vivo infected BM, results from subsequent studies using normal stromal cultures transfected in vitro also generated similar results, thus supporting the conclusions presented here (manuscript in preparation). Therefore, the defect observed in this viral system lies within the stroma rather than at the progenitor cell level.

The mechanism inducing the infected stromal cell defect is unclear. An imbalance in production or regulation of cytokines in infected stromal cells might have a major consequence on the support and maintenance of hematopoietic
progenitor cells. Cytokine genes that are constitutively expressed in marrow-derived stromal cells and mediate positive and negative regulation of hematopoiesis have been reported. \textsuperscript{27,28} In the present study, the levels of cytokine gene expression in noninfected and viral-infected stromal cell lines were examined using RT-PCR. With the exception of mRNA for IL-3, all other four cytokine genes were expressed, although some at different levels, by the established stromal cell lines. The absence of mRNA for IL-3 in our analysis does not necessarily mean that there is no expression of the IL-3 transcripts, but merely that levels of expression are too low to be detected under our conditions, because many groups have reported low levels of IL-3 expression in stromal cells.\textsuperscript{27,28}

The constitutive synthesis of SCF transcripts, the ligand for the c-kit receptor, in both cell lines described here is of considerable interest because of the well-documented activity of this cytokine in supporting proliferation and differentiation of a broad spectrum of cell types in the hematopoietic progenitor and stem cell hierarchy.\textsuperscript{29} Thus, the depressed hematopoiesis in infected stromal cell lines reported here could not be correlated with a low level of SCF gene expression. The levels of TNF\textalpha were found to be very low and were equivalent between noninfected and infected stromal cell lines. In this respect, our results correlate with the recent findings of Cheung et al.\textsuperscript{30} and Munis et al.\textsuperscript{31} who have demonstrated low levels of TNF\textalpha expression in retrovirus-infected cells.
Another finding in the cytokine analysis was the elevated levels of IL-4 expression in viral-infected stromal cell lines compared with those in the noninfected lines. Elevation of IL-4 expression has been demonstrated and correlated with virus-specific gene expression in MAIDS mice. A recent report by Kanagawa et al using IL-4 deficient (IL-4−/−) mice showed that IL-4 is critical to the development of retrovirus-induced immunodeficiency in mice. In addition, several previous reports showed that stimulation with IL-4 produced growth inhibitory activity against either myeloid or lymphoid lineage cell proliferation in BM stromal cells and rapid disappearance of pluripotential and committed myeloid hematopoietic progenitors on addition of IL-4 to coculture. These data provide evidence to suggest that a high level of IL-4 expression in infected stromal cell lines might contribute to the depressed hematopoiesis in the reconstitution studies reported here.

An additional significant finding from the current study is that TGFβ1 was expressed only by stromal cell line infected with LP-BM5. TGFβ1 has been shown to be a potent negative regulator of growth and differentiation of early hematopoietic progenitor cells. Thus, the upregulated expression of TGFβ1 from infected stromal cells may have contributed to the early arrest of progenitors, resulting in the impaired hematopoiesis that we observed.

Based on the cytokine analyses, it can be postulated that LP-BM5 infection of stromal cells led to deregulation of two of five cytokines examined. The change in cytokine gene expression might exert cytokine imbalance and thus induce a negative regulation of hematopoiesis by the infected stroma. As a result, both virus-free and infected reconstituting progenitors could only be supported by noninfected but not by viral-infected stromal underlayer, as observed in the coculture studies.

Infection of the nonhematopoietic microenvironment has been reported by many research groups. The recent study by Scadden et al of HIV infection and replication in the human stromal cell line and the consequent suppression of hematopoietic support function suggests an effect of viral infection on the stromal cells in the marrow microenvironment. Such an effect of viral infection on the ability of marrow stromal cells to support hematopoiesis in vitro has also been described for cytomegalovirus. These studies also suggest that, as the result of cytomegalovirus infection, which produces an ineffective stroma, BM failure develops.

Thus, the results reported here show that stromal cell lines generated from LP-BM5–infected marrow cultures retain the defective characteristics of the parental cells. Such defective characteristics perhaps relate to different levels of cytokine gene expression that would contribute to the impaired growth and proliferation of early hematopoietic progenitors. Further characterization of this defective cell line should prove valuable for studies of the defective hematopoietic microenvironment in and determining whether infected stromal cells may play a role in the pathogenesis of MAIDS.

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REFERENCES


Stromal cell lines derived from LP-BM5 murine leukemia virus-infected long-term bone marrow cultures impair hematopoiesis in vitro

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