Infection of Multipotent IL-3-dependent Stem Cells With a Retroviral Vector Containing the IL-3 Gene Confers Density-dependent Growth Autonomy Without Blocking Differentiation*

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A. Introduction

It is widely accepted that most naturally occurring leukemias are monoclonally derived from multipotent stem cells [5–7, 17], but the genetic changes leading to their transformation are poorly understood. A useful system in which to study the various processes occurring during leukemogenesis is offered by non-leukemic, multipotent stem cell lines (FDCPmix) established from murine long-term marrow cultures [20]. These cells grow continuously in vitro in the presence of Il-3, but they can also be induced to differentiate into mature granulocytes, macrophages, erythrocytes, and occasionally megakaryocytes, eosinophils, and mast cells by serum factors [20] or in association with marrow stromal cells [20] or certain embryonic mesenchymal cell lines [18]. Recent data have shown that hematopoietic growth-factor-dependent progenitor cell lines acquire growth-factor-independent growth and tumorigenicity when they are infected with retroviral vectors containing genes coding for Il-3 or GM-CSF [10, 11]. However, these studies have been restricted to cell lines which are blocked in differentiation and may therefore not reflect the alterations that occur in stem cells during leukemogenesis. To determine the effects of aberrant expression of Il-3 in differentiation-inducible stem cells we infected FDCPmix cells with a selectable retroviral vector carrying the cDNA of Il-3.

B. Materials and Methods

I. Vector Construction and Virus-producing Cell Lines

A cDNA clone of Il-3 (kindly provided by N. Gough, Melbourne) was subcloned into the MPSV-based M3neo vector [9, 10] and used for transfections into the amphotropic helper cell line, PA 317 [16], to produce infectious M3 MuV particles. Cell clone psi2 mos^{-1} no. 4 containing the neo MPSV *mos* deletion vector [22] was used to infect PA 317 in order to obtain amphotropic pseudotypes necessary for the infection of the ecotropic-virus producing FDCPmix cell lines [23]. Cell clones with titres of 10^3–10^5 for MuV and 10^5–10^8 GTU for mos^{-1} and with intact proviral genomes were used for co-cultivation experiments.

II. Cells

Virus-producing cell lines were kept in minimal essential medium supplemented

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with 10% fetal calf serum. Hematopoietic cell lines were maintained in Iscove's modified Dulbecco's medium, supplemented with 20% horse serum and Wehi 3BD-conditioned medium (WEHI CM) as a source of multi-CSF (Il-3) at a concentration that stimulated optimal cell growth.

III. Viral Infection and Selection Procedure

10^5 FDCPmix cells were inoculated onto subconfluent irradiated (20 Gy) virus-producer cell lines. Various FDCPmix cell lines were used. Two days later, the loosely adherent cells and cells in suspension were harvested, washed, and resuspended at about 10^5 cells/ml. After 2 days of culture, G418 was added to a final concentration of 1 mg/ml and the cells were subcultured as appropriate. Non-virus-infected cells died within 7 days but cells which had been co-cultured on the M3MuV and M3neo-producer cell lines continued to proliferate in the presence of the G418. About 2 weeks after selection with G418, the cells were cloned in soft agar in the presence of Il-3, and individual colonies were isolated and expanded from three different FDCPmix cell lines.

IV. Determination of Il-3 Activity

M3-MuV infected cells (10^6) were washed twice to remove residual Il-3 and incubated without WEHI-CM for 48 h. The supernatant was used as such or concentrated tenfold via Amicon filtration (exclusion mol. wt. <10000), dialyzed, and tested for stimulatory activity on indicator cell lines by determining [^{3}H] thymidine incorporation. Half-maximal stimulation of FDCP2 cells by either WEHI-CM or recombinant murine Il-3 was defined as 50 U/ml.

V. Growth Inhibition Assay

One of the clones of FDCPmix infected with M3MuV was grown at high density in the absence of Il-3. Cells were washed twice in medium without Il-3 and plated, 1×10^4 cells/well, into 96-well plates. Dilutions of the antiserum of preimmune rabbit serum ranged from 1:20 to 1:10240 final. 30 h after initiation, 0.5 µCi [^{3}H] thymidine was added for 14 h. Cells were harvested onto filters, using a cell harvester (Titertec), and counted.

VI. Colony Assay

10^3 control uninfected cells and cells infected with M3neo virus alone or M3MuV were plated in soft agar in culture conditions which allow the expression of multiple hematopoietic lineages [19]. Individual colonies were isolated after 10 days of growth, cytospin preparations made, and the cells stained with benzidine plus May-Grunwald Giemsa. At least 30 colonies were examined from each group.

VII. Marrow Stromal Cell Culture

Stromal cell cultures derived from bone marrow were irradiated [20, 21] and used as a supportive stroma for the growth of the FDCPmix cells, either uninfected, or M3MuV or M3neo infected. Between 2×10^6 and 10^7 FDCPmix cells were co-cultured with the marrow stromal cells. At various times after seeding of marrow stroma by the FDCPmix cells, cytospin preparations of the nonadherent cells were performed and the cells stained with May-Grunwald Giemsa.

VIII. Diffusion Chamber Culture

After two washes, aliquots of 5×10^5 FDCPmix cells, infected with either M3MuV or M3Neo, were inoculated into each diffusion chamber (DC). These were then inserted intraperitoneally into male CBA mice. After 7 days of culture the animals were killed; the chambers were removed and shaken for 40 min in a 0.5% Pronase solution (Merck). The resulting cell suspensions were counted for

the total number of nucleated cells. Cytospin preparations were made and the cells were classified according to morphological criteria [12].

IX. Nucleic Acid Analysis

Cellular DNA was isolated and restricted by standard techniques and separated on agarose gels. Total RNA was isolated as previously described [2] and transferred to Gene Screen Plus (NEN) after denaturation with glyoxal and dimethylsulfoxide and electrophoresis through agarose gels [14]. Nucleic acids were transferred to Gene Screen Plus (NEN) and hybridized under the conditions recommended by the manufacturer, with probes labeled as previously described [4]. Probes used for analysis included an *Eco*RI-*Nco*I fragment of pMu21A containing the Il-3 cDNA clone (N. Gough, unpublished), the *Bgl*III-*Bam*HI fragment of pAG60 containing the coding region of the neo gene [3], and the PY80B probe specific for the murine Y-chromosome [1].

X. In Vivo Administration of FDCPmix Cells

Uninfected and A4/M3neo cells (cultured with Il-3) and A4/M3MuV cells (cultured without Il-3) were suspended in Fischer's medium at an appropriate cell concentration. The cells were injected i. v. in syngeneic B6D2Fl mice that had received 10 Gy, prior to inoculation of the cells.

C. Results

I. Virus Integration, Gene Expression, and Il-3 Secretion

Following infection and selection in liquid culture the cells were cloned in soft agar in the presence of G418. Individual clones were isolated and cultured for further analysis. Analysis of the virus insertion sites revealed that the resulting cell lines were monoclonal (data not shown). The Il-3 gene was expressed in the MuV-infected FDCPmix cells, as shown by Northern analysis (data not shown). Conditioned medium of the M3MuV-infected cells growing in the absence of Il-3 contained between 4 and 50 units of Il-3 activity per ml.

II. Density-dependent Growth Autonomy

All M3MuV infected cell lines could grow in high density without Il-3, whereas the uninfected FDCPmix cells and the M3neo-infected cells died in the absence of growth factor. Cloning of FDCPmix M3MuV-infected cells in soft agar resulted in nonlinear, density-dependent growth in the absence of Il-3 and in nearly linear growth in the presence of Il-3 (Fig. 1). Growth of the M3MuV-infected cells could be blocked by neutralizing antisera to Il-3 (Fig. 2).

III. Differentiation Induction

M3MuV-infected stem cell lines retained their capacity to undergo differentiation in response to serum factors or marrow

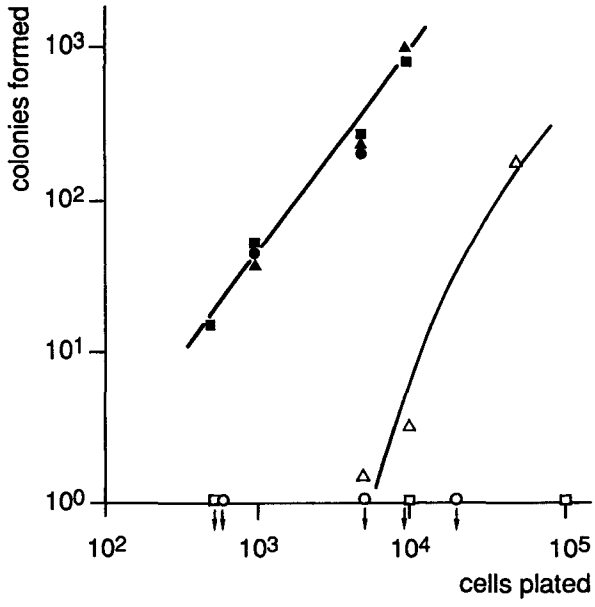


Fig. 1. Clonability of FDCPmix M3MuV-infected cells as a function of cell density. Uninfected FDCPmix cells with (■) and without (□) Il-3; M3neo-infected FDCPmix cells with (●) and without (○) Il-3; M3MuV-infected FDCPmix cells with (▲) and without (△) Il-3

Table 1. Colony formation by control and infected FDCPmix cells

	Colony morphology (%)					
	PE (%)	B	EG	LG	Mono	Mixed/Erythroid
Uninfected cells	6	9	4	63	8	16
$FDCP_{mix}$/M3neo	8	11	7	59	10	13
$FDCP_{mix}$/M3MuV	7	45	31	18	6	<1

PE (%), Plating efficiency, i.e., number of colonies formed per 100 cells plated; B, primitive blast cells; EG, promyelocytes and myelocytes; LG, metamyelocytes and mature granulocytes (including eosinophils); Mono, large mononuclear cells

Table 2. In vivo administration of FDCPmix cells

Group	Cells injected	No. of animals leukemic*	Spleen weight (mg, range)	Blood counts ($\times 10^{-3}/mm^3$, range)	Femur cellularity ($\times 10^{-7}$, range)
Uninfected	10^7	0/20	80– 120	3– 6	1.2–2.3
$FDCP_{mix}$/M3neo	10^7	0/10	70– 110	3– 7	1.3–2.2
$FDCP_{mix}$/M3MuV	5×10^6	15/15	530–1164	40–276	0.7–1.1

* Morbidity was first observed 6 weeks after injection of the cells, and the majority of the animals had developed clear evidence of hematopoietic disease (and were autopsied accordingly) within 12 weeks

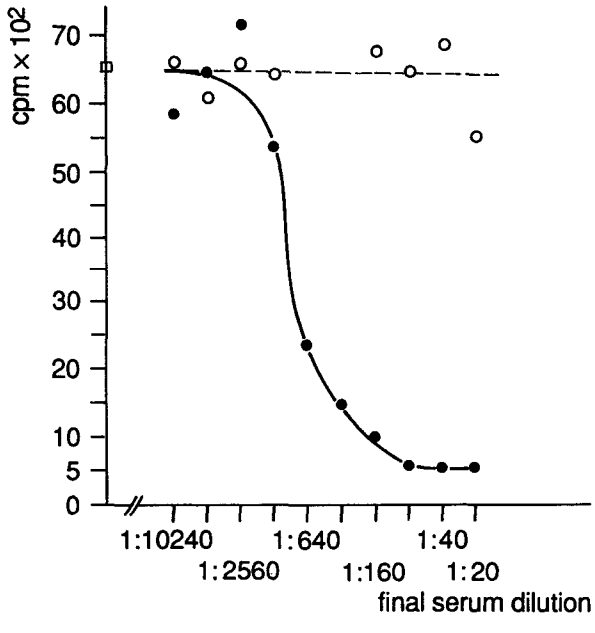


Fig. 2. Growth inhibition of FDCPmix M3-MuV-infected cells by anti-Il-3 antiserum. ● Growth medium with rabbit anti-Il-3 antiserum; o growth medium with rabbit preimmune serum; □ growth medium without antiserum or preimmune serum

stromal cells. In the mixed colony assay the plating efficiency was unaltered and the colonies produced contained maturing granulocytes and macrophages (Table 1). However, erythroid cells were rarely seen, and the balance between immature and mature granulocytes was changed in favor of immature cells (Table 1, Fig. 3). The same was true when the cells were co-cultured with marrow stromal cells (data not shown). Culture of M3MuV-infected FDCPmix cells in vivo in the DC led to an increase of immature and mature granulocytes and macrophages similar to the in vitro observations. In addition, erythroblasts were also found in the DC (data not shown).

IV. In Vivo Administration of FDCPmix MuV-infected Cells

When FDCPmix M3MuV-infected cells were injected into sublethally irradiated

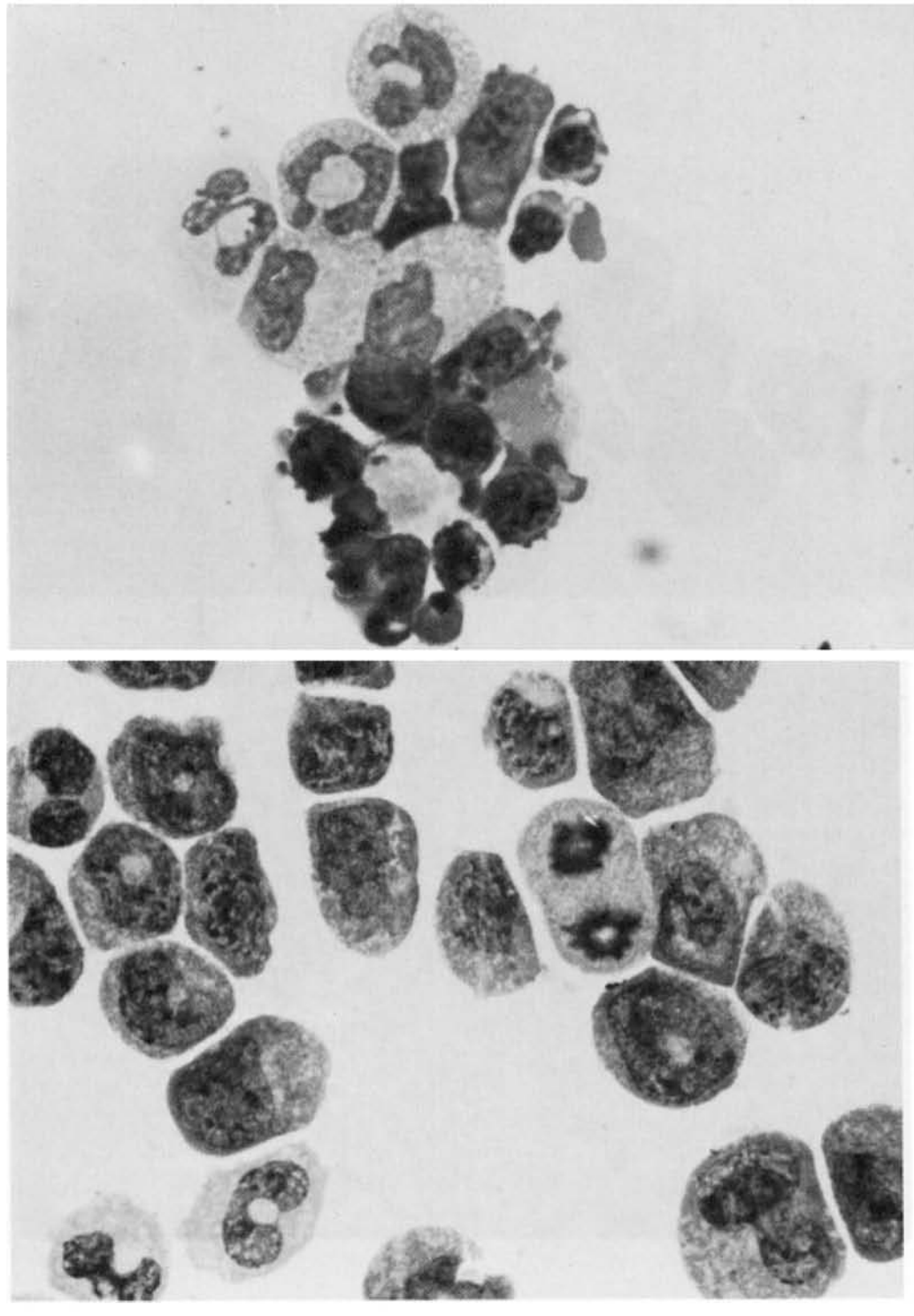
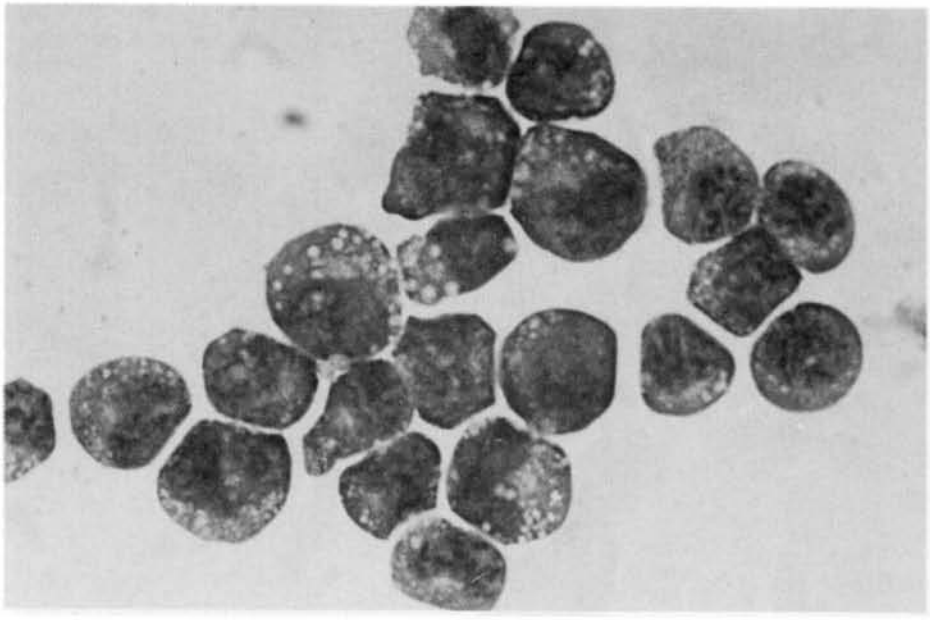
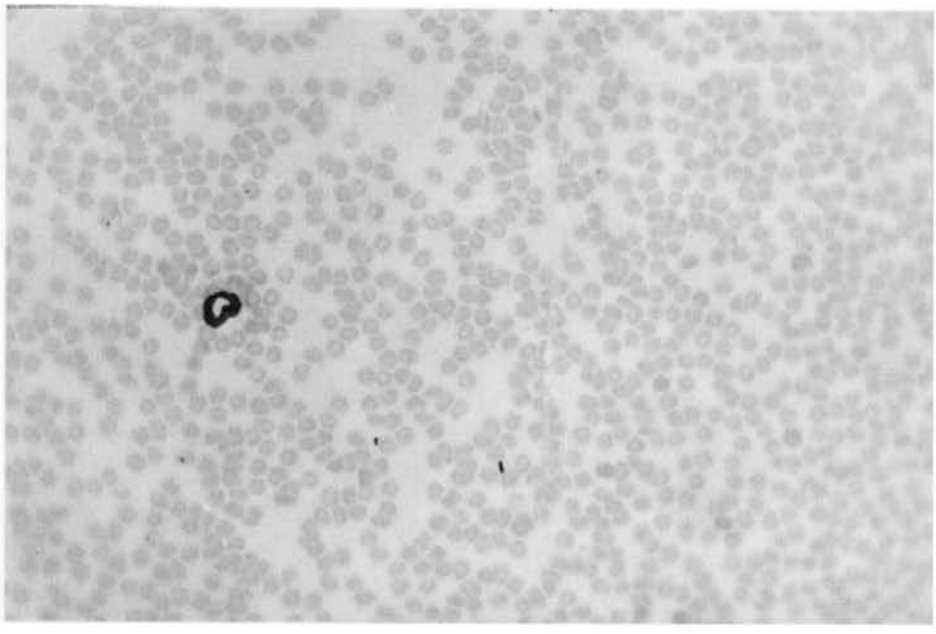


Fig. 3a, b. Mixed colony formation by control and infected FDCPmix cells. **a** Parental FDCP-mix cells; **b** FDCP-mix M3MuV-infected cells

syngeneic mice, the animals developed a five- to ten fold increase in the spleen weight, an increase in peripheral leukocytes, and a decreased hematocrit (Table 2). Morphological analysis of cells present in the spleen and peripheral blood of a representative mouse (Fig. 4) showed the following differential: spleen (blood) 16% (2)% blasts, 21% (10)% promyelocytes/myelocytes, 55% (75)% metamyelocytes and polymorphonuclear granulocytes, 5% (1)% nucleated erythroid cells, 3% (12)% other. The animals died within 2 months, whereas control mice, injected with stem cells containing the M3neo vector, showed no evidence of disease 6 months later. To determine the origin of the disease, cytogenetic analysis of cells in the spleen of the leukemic mice was performed. Initially, 80%–100% of the mitoses were of donor origin and possessed a normal (donor) male karyotype. Subsequently, however, spleen, bone marrow, and blood cells were of recipient origin, as revealed by Southern blotting (data not shown). Furthermore, the viral integration sites of cell lines recovered from leukemic animals were different



a



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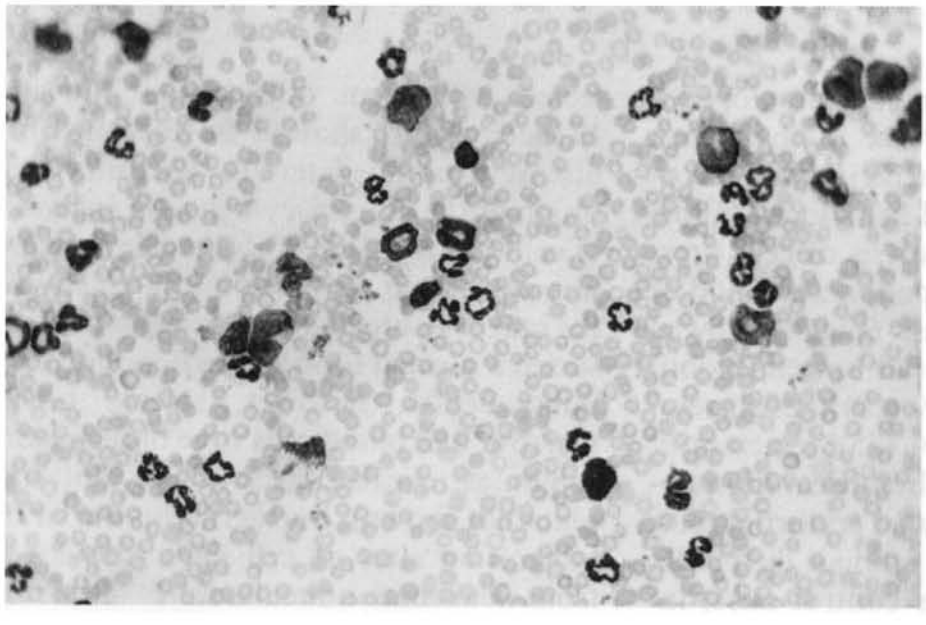

c

Fig. 4a–d. In vivo administration of FDCPmix M3MuV-infected cells. **a** FDCP-mix M3MuV-infected cells; **b** normal peripheral blood cells; **c** peripheral blood cells from leukemic mouse; **d** spleen cells from leukemic mouse

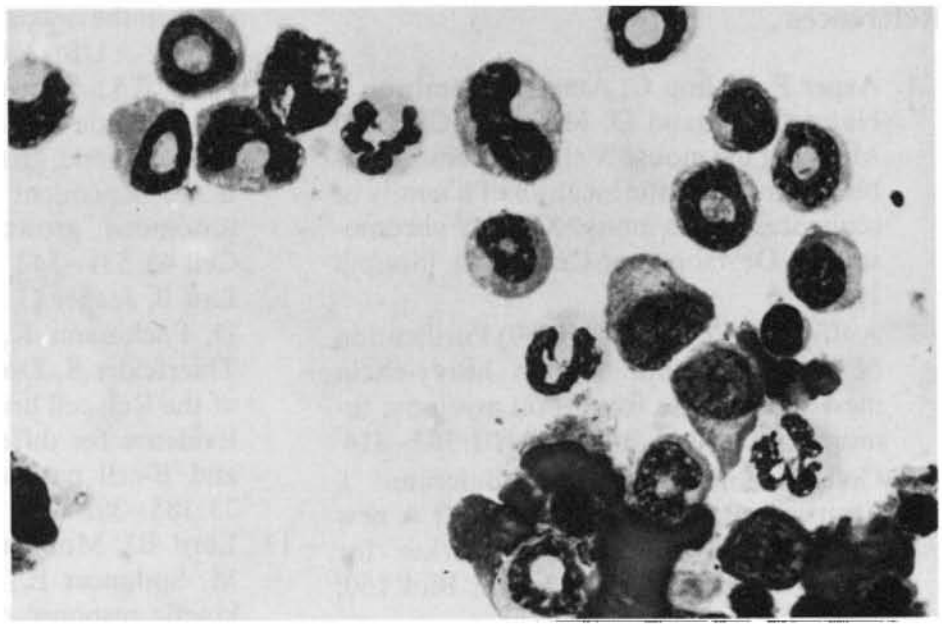

Fig. 4d

from the original viral insertion site of the donor FDCPmix cells (data not shown).

D. Discussion

The present results show that infection of multipotent Il-3-dependent stem cells with a retroviral vector containing the Il-3 gene confer density-dependent autocrine stimulation of growth without blocking differentiation, but with a change of the balance between differentiation and proliferation in favor of proliferation. From these data we conclude that inappropriate expression of Il-3 may play an important role in the multistep pathogenesis of leukemia. When the Il-3 infected cells were injected into sublethally irradiated syngeneic mice, the animals developed a myeloproliferative disease. However, the precise role of the injected cells remains to be determined. Analysis of spleen and blood cells of the leukemic mice revealed that the proliferating cells were initially derived from the transplanted stem cells but were subsequently of recipient origin. Furthermore, the viral integration sites in cell lines recovered from leukemic animals showed different bands as compared with the original injected cells, indicating infection of host cells. Since FDCPmix cells contain an ecotropic helper virus (MoMuLV) [23], it could have packaged the defective MuV vector to produce an infectious virus which may then have transformed host cells. However, when high levels of the original MuV virus are injected into mice no myeloproliferative disease is observed [9]. In the latter case, this may reflect a difficulty in the ability of the injected virus to "target" to the host cells in the sites of active hematopoiesis. This may not be the case for the MuV-infected FDCPmix cells, which can clearly lodge in the spleen and bone marrow and may be acting as "carriers" for infectious viral particles, thus facilitating infection of host hematopoietic cells. Also, it has been reported that injection of recombinant Il-3 into normal mice leads to an increase in spleen weight and content of CFU-S, as well as to an increase in progenitor cells of the myeloid lineage [8, 13, 15]. Therefore, Il-3 production by the infected cells (both donor and host) may have contributed to the disease by stimulating stem and progenitor cells from the recipient mice. Thus, the disease is probably multifactorial. Nonetheless, we have clearly shown that endogenous inappropriate expression of a growth factor gene can have profound biological effects and may well be a part of the process leading to leukemic transformation.

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