Defresne MP_04

Mixed Phenotype Murine Leukemias

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Cell lines were derived from eight individual leukemias induced by X-rays in NFS mice. First typed as null cells (surface immunoglobulin negative, Thy-1 negative), they turned out to have a mixed phenotype with myeloid cytochemical markers, pre-B surface antigens and molecular markers of pro-B lymphocytes. They represent murine models for mixed phenotype (pro-pre-B-myeloid) leukemias.

INTRODUCTION

Murine models are available for most forms of human leukemia and have proved of great help for the study of these diseases. Recently, it was recognized that lineage infidelity and mixed phenotype human leukemias were more frequent than initially thought. Clearly, the same cell often expresses both lymphoid and myeloid markers. A number of cell lines also express at the same time, on the same cells, several markers characteristic of differentiation stages that are not currently seen as directly related, e.g. myeloid and pre-B (1). This situation is quite frequent in human leukemias (2–5). Among the questions raised by such observations are: (a) Do some normal cells also express differentiation markers from several lineages? (b) Do such leukemias require a different treatment and do they carry a different prognosis?

During studies on radiation leukemogenesis in mice, we have characterized eight leukemic lines with myeloid histochemical markers and immunological and molecular markers compatible with a pro-B origin. Our approach rests mainly on classical hematological techniques, immunophenotyping and study of the rearrangement of immunoglobulin (Ig) genes. For instance, we demonstrated the expression of the v-pre-B component of the surrogate light chain, which is not expressed after the pre-B stage (6) and has recently been recognized as a valuable marker for pre-B leukemias (4,7). A cytokine gene expression pattern was obtained to refine the typing of the cell lines. Last, a less conventional approach used by our group was the study of the cell surface receptors for neurotransmitters. The detection of such receptors may prove of diagnostic value. Thus, we describe cell lines which co-express markers of both myeloid and pro-B lymphoid cells.

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LEUKEMIA

METHODS

Mice

Inbred NFS/N mice were purchased from NIH and bred in our facilities.

Leukemia Induction

Protocol 1: intact mice irradiated during the second month of life; protocol 2: mice thymectomized at the age of 6 weeks and irradiation started 4 weeks later; within 24 hours after the last irradiation, two to four freshly collected neonatal (0–3 days) syngeneic thymuses implanted subcutaneously.

Protocol 1 results in 'direct' radiation-induced leukemias whereas protocol 2 allows the study of 'indirect' leukemogenesis. In this case, leukemia originates in the grafted, non-irradiated tissue (8). This aspect was also investigated in the present study by giving female thymuses to male, irradiated recipients (see Pathology).

Mice were exposed to 1.75 Gy total body irradiation, four times, with one-week intervals between irradiations. Irradiation details were: 0.7 Gy/min at 250 kV, filter 1 mm Cu (1.9 mm half value layer). Target distance: 60 cm.

Pathology

Mice were killed when large subcutaneous tumors or other signs of disease developed. Autopsy data for eight non-B, non-T tumors studied in this paper are given in Table 1. Leukemias were scored by palpation and histological examination. For five leukemias obtained by the indirect protocol, karyotypic analysis was performed. All five were of male origin. Thus, the thymic transplants (of female origin) did not contribute the target cell for leukemic transformation. No major chromosomal aberrations were detected. In particular, we looked for trisomy 15 or deletion of chromosome 2 (9,10).

Cell Culture

Cultures were established from leukemic tissues (spleen, lymph nodes, pancreas, brain) from eight independent leukemic mice (Table 1), according to a protocol used for growing leukemic T cells (8). Briefly, cell suspensions were cultivated in RPMI-1640 (Gibco-BRL, Ghent, Belgium), fetal bovine serum (10%), with 0.5μM 2-mercaptoethanol, sodium pyruvate (1 mM), non-essential amino acids (Gibco, 1:100) and gentamycin (Gibco, 100 mg/l) with 5% CO_2 in a humid

Table 1. Origin of the Cell Lines.

Code	Protocol[a]	Leukemia Tissues[b]	Cell Line Derived from
NFD1	TXG	Graft, lymph nodes	Mesenteric lymph node
NFD3	TXG	Graft, lymph nodes	Axillary lymph node
NFD4	TXG	Graft, lymph nodes, spleen	Mesenteric lymph node
NFD5	TXG	Graft, mesenteric lymph nodes	Mesenteric lymph node
NFF8	TXG	Spleen	Spleen
NFA24	X	Spleen	Spleen
NFN30	X	Spleen, pancreas, brain, lymph nodes	Spleen, pancreas, brain[c]
NFL32	X	Spleen	Spleen

[a] X, irradiation of intact mice; TXG, thymectomy, irradiation, thymic graft. See Methods, Leukemia Induction.
[b] Macroscopic and microscopic evaluation.
[c] Three cell lines were established. All work was done on NFN 30S, derived from the spleen. However, lines derived from pancreas and brain had the same phenotype as NFN 30S.

atmosphere, at 37°C. Culture was also attempted in the same medium without mercaptoethanol.

For several weeks, leukemic cells were dependent on adherent accessory cells (present in the original inoculum), thereafter they were autonomous and were cloned in 96-well plates.

Cytochemistry

For cytochemical analysis, cells resuspended in culture medium were centrifuged at 1000 rpm in a Beckman cytofuge. Slides were air-dried. Staining was performed as described (11,12).

Electron Microscopy

Cells were investigated either as cell pellets, after cell culture, or as solid tumors after subcutaneous passage (from cultured cells) in nude mice. Samples were fixed in 4% glutaraldehyde inphosphate-buffered saline (PBS) and after post-fixation in O_5O_4 solution and dehydration, they were embedded in Epon 812. Sections were contrasted with uranyl acetate and lead citrate and examined at the transmission electron microscope.

Immunocytochemistry

Antibodies. Most antibodes used in this study are described in ref. 13. In addition, Thy-1.2 was detected with rat monoclonal antibody HO-13-4.9 obtained from ATCC (14) and the IL-2R (CD25) was detected with rat monoclonal antibody 7D4 provided by E. Schevach (15). Further, rat anti-Sca-1 monoclonal antibody was kindly provided by B. Frangoulis and J. Klein (16) and rat monoclonal antibodies Joro 38 and Joro 75 (recognizing early lymphoid and pre-T cells respectively) were given by R. Palacios (17). Rat monoclonal anti-mouse CD45 (B220) antibodies RA3-2C2 and RA3-6B2, rat monoclonal anti-heat-stable antigen (HSA) antibody Ml/69, as well as rat anti Gr-1 granulocytic antigen monoclonal antibody RB6-8C5 were supplied by R.L. Coffman (18–20). As in ref. 13, the double antibody technique was used. The anti LFA1 GK1.5 antibody was also used biotinylated and detected with Texas Red conjugated to avidin (Tago, Burlingame, CA, USA). The anti-CD3 (145.2C11) was directly coupled with fluorescein isothiocyanate. The anti-Thy-1.2 (HO.13.4.9) and the anti-CD8 (53–6.72) were detected as direct conjugates and with the double antibody technique.

Staining Procedure. Cells from the different lines (3–5 × 10^6 cells in 0.1 ml PBS-azide) were incubated for 30 min at 4°C with 20 μl antibody. Thereafter, they were washed twice with PBS-azide and, whenever applicable, the relevant fluorescein isothiocyanate (FITC)-conjugated antibodies or $F(ab')_2$ fragments or Texas Red/avidin were added to the cell pellet for a further 30 min incubation at 4°C. Cells were examined after washing twice with PBS-azide.

Microscopy. The cells were examined under a fluorescent microscope and arbitrary scores were given according to relative brightness. Most samples were also analyzed by flow cytometry. For each sample, 20 000 cells were run on a FACS 440 cell sorter equipped with a Consort 30 management system (Becton Dickinson, Mountain View, CA, USA).

Cytoplasmic Ig. For the detection of cytoplasmic Ig, cells were centrifuged with a cytofuge (as for cytochemistry). Slides were fixed for 20 min on ice with acetic acid-ethanol (95:5, v/v) and rinsed for several minutes in running tap water. After drying, slides were incubated with 50 μl goat anti-mouse μ chains (Dako, 1:20) for 30 min at 4°C, or with 50 μl goat anti-mouse chains (Sera-Lab, 1:10) for 30 min at 4°C, followed by fluorescein (FITC)-conjugated rabbit anti-goat immunoglobulin G (IgG) serum (Sera-Lab, 1:100) and examined under a fluorescence microscope.

TdT. The terminal transferase (TdT) immunofluorescent assay kit (Sera-Lab, Crawley Down, Sussex, UK) was used as recommended by the supplier.

Analysis of Ig Genes

High molecular weight DNA was extracted from NFS liver and kidney and from cell lines as described by Maniatis *et al.* (21). 10 μg samples of DNA were cleaved with *Eco*RI restriction enzyme under the conditions recommended by the supplier (Gibco-BRL). After electrophoresis in 0.8% agarose gels, the DNA restriction fragments were blotted onto Gene Screen Plus membranes (DuPont, NEN products, Brussels, Belgium) and hybridized with a ^{32}P-labeled probe according to the Gene Screen Plus users' manual.

The J3-4 probe was a 2 kb *Eco*RI-*Bam*HI J_H specific probe (22) and the pCμ TSI probe was a 1050 bp *Eco*RI-*Hind*III Cμ specific probe (23). Both probes were kindly supplied by Dr K. Rajewsky. The probes were ^{32}P-labeled by nick translation.

Expression of Cytokine and v-Pre-B Genes

RNA Extraction. 5 × 10^6 cells were washed three times with PBS and saved as dry pellets in liquid nitrogen. For RNA extraction, pellets were resuspended in 4 M guanidine thiocyanate. This material was loaded on a caesium chloride gradient as recommended by the manufacturer (RNA extraction kit; Pharmacia). 2 μg RNA were digested with 50 units RNAse-free DNAse (Boehringer). Precipitation with 3 M sodium acetate allowed removal of oligonucleotides. For the study of v-pre-B mRNA, total RNA was isolated from 5 × 10^6 cells by the thiocyanate/pheno/chloroform method according to Chomczynski and Sacchi (24).

Reverse Transcription. 1 μg RNA (in polymerase chain reaction, PCR, buffer without gelatine, see below) was incubated with 50 units AMV reverse transcriptase (Boehringer), random

primers (Boehringer) and RNAse-free BSA in a total volume of 20 μl for 10 min at 23°C, then for 60 min at 42°C, and finally for 10 min at 95°C. Samples without reverse transcriptase were always run in parallel to control for DNA contamination. For the synthesis of cDNA corresponding to the v-pre B mRNA the Gene Amp RNA PCR kit from Perkin Elmer Cetus (Emeryville, CA, USA) was used on 1 μg total RNA of each cell line.

PCR and Agarose Gel Electrophoresis. Each reaction mixture contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatine, 100 μM each NTP, 1 μg each primer, and 0.5 units Taq DNA polymerase (Perkin-Elmer Cetus) in 25 μl. The reaction was started by adding 0.5 μl product of the reverse transcription reaction. 40 cycles were run, each cycle consisting of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C in a DNA Thermal Cycler (Perkin-Elmer, Cetus). Amplification of the v-pre B cDNA was performed, as recommended in the Gene Amp PCR Kit. PCR products were separated on a 3% agarose gel, a 2:1 mixture of Nusieve (FMC), and NA agarose (Pharmacia, Uppsala, Sweden), except for the v-pre B products which were separated on a 2% agarose gel, a 3:1 mixture of Nusieve, and ultra-pure DNA grade agarose (Bio-Rad).

All primers except those for interleukin 7 (IL-7) and v-pre-B were used as described in Murray *et al.* (25). The primers for IL-7 were used as described by Montgomery and Dallman (26). For v-pre-B, the following 21-mer primers corresponding to positions 241 to 261 and 355 to 375 were used: 5'AGCCACCATCCGCCTCTCCTG3' and 5'TGGTGCTTGTCTGAGTGTGAG3' [according to the sequence published by Kudo and Melchers (27)].

Receptors for Neurotransmitters Coupled to Adenylate Cyclase

The calcitonin-gene-related peptide (CGRP) was obtained from Sigma and used at 1 μM. All other reagents and procedures are as described (28).

Cortisone Sensitivity

Cortisone sensitivity was assessed as described (29). We used hydrocortisone and considered growth in 1 μM hydrocortisone as indicative of cortisone resistance.

Apoptosis

Cells were aliquoted (10^6 cells in 5 ml medium), irradiated (0, 1, 2, or 4 Gy), returned to normal culture conditions. Investigations included cell counting with Trypan Blue [apoptotic cells take Trypan Blue at early stages (30)] 6 h and 18 h after irradiation and DNA electrophoresis (31) 18 h after irradiation.

RESULTS

Establishment of Cell Lines

Tumors are contaminated with other cell types in various proportions. We therefore routinely grow our tumors *in vitro*. Cultures did not require exogenous growth factors other than those present in fetal bovine serum. In contrast to most T-cell lymphomas, however, they did not survive in the absence of 2-mercaptoethanol. All cell lines were cortisone-resistant. Cloning was successful, whenever attempted (all lines except NFL32).

Histochemistry and Electron Microscopy

The preliminary immunological characterization suggested either a very early lymphoid or a non-lymphoid origin. Therefore, hematological stainings were performed, after several passages *in vitro*. Cytochemistry is diagnostic for non-lymphoid leukemias and the results are summarized in Table 2 and in Figure 1. All lines expressed chloroacetate esterase, acid phosphatase, and (whenever looked for) alkaline phosphatase. Cells in culture were negative for Sudan Black, myeloperoxidase, non-specific esterase, periodic acid/Schiff and Prussian Blue. Cytochemistry, suggested a myeloid origin.

Cells were also studied by transmission electron microscopy. Diagnosis was difficult on cells in culture. Of four lines examined, two (NFD1 and NFA24) were tentatively classified as myeloid, the remaining two (NFD5 and NFF8) as lymphoid (Figure 2a, b). The latter were quite similar to the human pre-B line NALM-6 taken as a reference. Cells (after several *in vitro* passages) were also grown subcutaneously in nude mice. All five samples processed for electron microscopy were diagnosed as myeloblastic leukemia, poorly differentiated. Tumors consisted mainly of large clusters of myeloblasts. A small proportion among them contained small, clear, round granulations suggestive of basophilic differentiation. Some mature basophilic granulocytes, with large electron dense granules (Figure 2c, d) and a few lymphoblasts (but no mature lymphocytes) were also present. Taken together, the electron microscopy data favored the diagnosis of myeloid leukemia.

Immunological Characterization

All eight lines were surface Thy-1, CD3, CD4, CD8, and Joro75 (pre-T marker) negative. The early lymphoid

Table 2. Cytochemical Analysis.

	NFD1	NFD3	NFD4	NFD5	NFF8	NFA24	NFN30	NFL32
Sudan Black	–	–	–	–	–	–	ND	ND
Chloroacetate esterase	+	++	+	++	+	++	+	+
Peroxidase	–	–	–	–	–	–	ND	ND
Acid phosphatase	++	+++	+++	++	+++	+++	+	+
Non-specific esterase	–	–	(+)	(+)	–	–	–	–
Prussian Blue	–	–	–	–	–	–	ND	ND
Periodic acid/Schiff	–	–	–	–	–	–	–	–
Alkaline phosphatase	++	++	++	++	++	++	ND	ND

ND, not done.

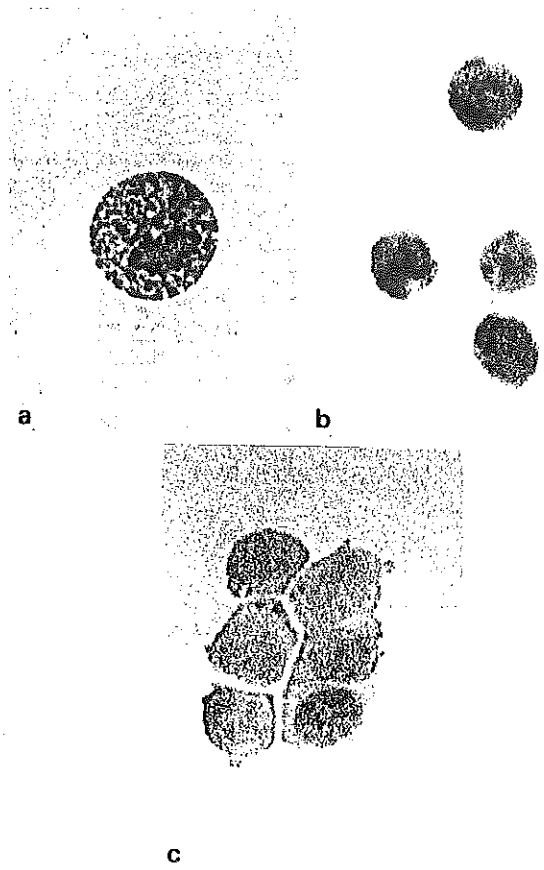

Figure 1. Histochemistry of NFD3 cells. (a) Chloroacetate esterase activity is observed as a bright red precipitate in the cell granules; (b) alkaline phosphatase activity results in a dark blue deposit in the granules of some cells only; (c) acid phosphatase activity yields a brown deposit in the cell granules. Magnification × 450 (a); × 200 (b,c,).

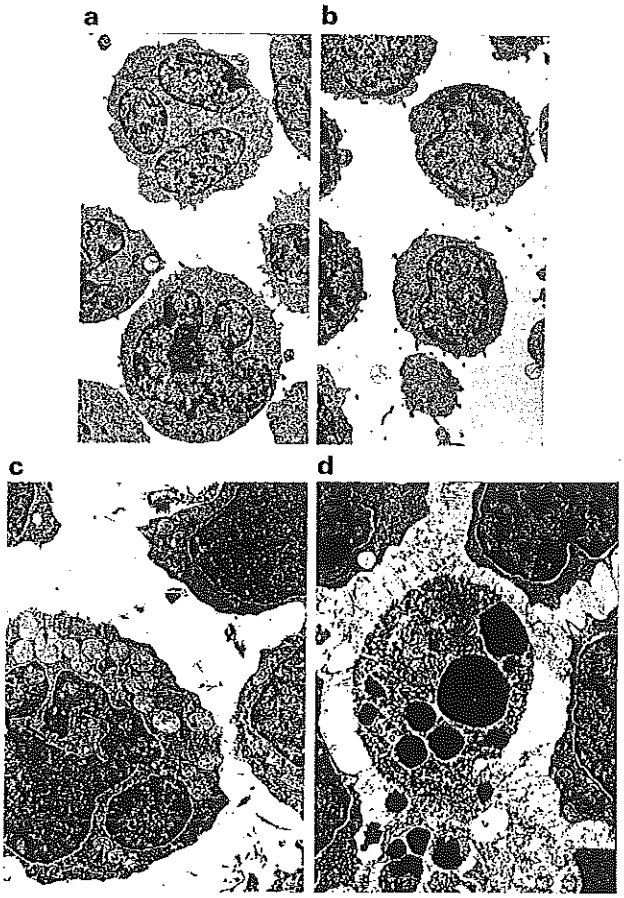

Figure 2. Electron microscopy of mixed phenotype cell lines: (a, b) *in vitro*; (c, d) *in vivo*. (a) Cell line NFD1: leukemic blasts, possibly myeloid. Magnification: × 4375. (b) Cell line NFD5: leukemic blasts, suggestive of lymphoid differentiation (more rounded nuclei, high nucleo-cytoplasmatic ratio) and very similar to the human pre-B line NALM6 (not shown). Magnification: × 4375. (c) Cell clusters in a hyaline stroma. The cytoplasm contains numerous scattered ribosomes, few mitochondria (arrowhead) and low density granules (arrow). Magnification: × 19 500. (d) Typical basophilic granules in several degenerating cells. Magnification: × 19 500.

marker Joro38 was also not expressed.

B-lineage markers were positive on all lines (6C3) or on seven out of eight (B220) (Table 3). Ly 1 (CD5) was expressed on four (out of six) lines. Repeated searches for surface Ig were negative except for the occasional detection of surface μ on NFD3 cells. The FcγRII was present on six (out of six) lines. The expression of other leucocytic markers was variable: Sca-1 (one out of eight lines), pgp-1 (CD44) (six out of six), IL-2R (CD25) (one out of five), LFA-1 (three out of five). The granulocytic marker Gr-1 (8C5) was never expressed. The heat-stable, Forsman antigen (HSA) was found six (out of six) times. Cytoplasmic Ig was always negative. TdT was detected in two (out of 5 lines). Thus the immunological characterization was indicative of pro-B origin.

Table 3. Immunological Phenotype.

Cell Line	TdT	Sca-1	pgp-1 CD44	IL-2R CD25	Lyl CD5	HSA	6C3	B220 CD45	LFA-1	FcγRII Ly17
NFD1	–	–			+		+	+		+
NFD3	+	–	+	–	+	+	+	+	+	+
NFD4	–	–	+	–	+	+	+	+	+	+
NFD5	–	+/–	+	–	+	+	+	+	+	+
NFF8	+	–	+	–		+	+	+	–	+
NFA24		–					+	+		+
NFN30		–	+	+	–	+	+	–	–	
NFL32		–	+		–	+	+	+		

The following markers were negative on all lines: Thyl, CD4, CD8, Gr-1, surface μ (except for rare detection on NFD3), cytoplasmic μ and κ.

Molecular Biology

Ig and Related Gemes. High molecular weight DNA was digested with *Eco*RI, electrophoresed on agarose gels, blotted onto nitrocellulose filters and hybridized to J_H and μ probes. An identical pattern of J_H rearrangement was seen in NFD1, NFD3, NFD4, and NFA24. Different types of J_H rearrangements were seen in NFN30 and NFN32. A fourth pattern was seen in NFD5, and this could correspond to a deletion. Only the germline configuration was seen in NFF8. All other lines had lost both germline fragments (Figure 3). We found no evidence for rearrangement of $C\mu$. Thus six out of seven lines investigated had J_H but not $C\mu$ rearrangement, compatible with a pro-B assignment. To explore this possibility further, the expression of the v-pre-B component of the surrogate light chain was looked for by PCR. All lines investigated were found positive for this probe whereas a T-cell line was negative.

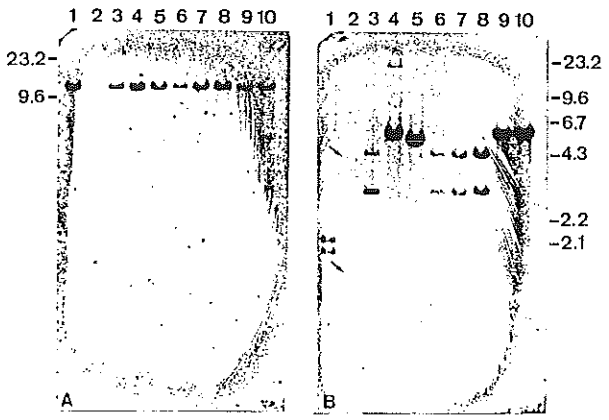

Figure 3. IgH rearrangements. (A) *Eco*RI digest of same samples hybridized to the $C\mu$ probe. No visible rearrangement. (B) *Eco*RI digest of DNA from NFS cell lines, liver, and kidney hybridized to the JH probe. One line has germline configuration, one has a shorter fragment and six have two fragments. Of these four have the same pattern. All rearranged lines have lost the germline configuration. Lanes: 1, NFL32; 2, NFN30 (arrows indicate faint bands); 3, NFA24; 4, NFF8; 5, NFD5; 6, NFD4; 7, NFD3; 8, NFD1; 9, control NFS liver; 10, control NFS kidney.

Cytokine Expression. A cytokine expression pattern was next established using the PCR technique (Table 4). The cells did not express IL-2, IL-4 or γIFN, in agreement with the lack of other T markers. No single cytokine was expressed by all cell lines. All but one (NFA24) expressed IL-6 and NFA24 was positive for IL-3.

Receptors for Neurotransmitters Coupled to Adenylate Cyclase

Each cell line tested responded to at least one of the four agonists investigated. Quite remarkable was the high number of lines (six out of eight) expressing receptors for calcitonin-gene-related peptide (CGRP). Receptors for the vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase activating peptide (PACAP) were detected on five lines, and β-adrenergic receptors on three lines (Table 5).

Apoptosis

Lymphoid cells are very susceptible to radiation. A recent report documents the death by apoptosis of B cells and preleukemic B cells after irradiation with 4 Gy (X-rays) (31). We have checked the radiation susceptibility of our cell lines. Apoptosis (documented by DNA fragmentation) was induced in all our lines (but not in WEHI-3, N122, or HL60) with 2 Gy. Trypan Blue uptake after irradiation with 2 Gy was 15 to 50% (depending on the cell line) at 6 h and 40 to 95% at 18 h, compared to less than 5% in non-irradiated cells.

DISCUSSION

Eight murine NFS leukemic cell lines have been studied in some detail in the first instance because they represent a previously unrecognized form of radiation-induced leukemia, the mixed phenotype (myeloid-pro-B) leukemia.

Leukemia was induced by split-dose irradiation, a protocol that generates a high percentage of T-cell leukemias in several mouse strains, including NFS (32). Non-T leukemias have been obtained also with this protocol in the past but were not thoroughly characterized (32). The proportion of non-T leukemias is increased in thymectomized animals reconstituted with thymic grafts after completion of the irradiation protocol (10 and our

Table 4. Cytokine Expression Pattern.

Cell Line	IL-1α	IL-3	IL-6	IL-7	IL-10	TNFα	TNFβ
NFD1	+	−	+	−	+	+	+
NFD3	+	−	+	−	−	+	+
NFD4	+	−	+	−	+	+	+
NFD5	−	−	+	−	−	+	+
NFF8	+	−	+	+	−	−	−
NFA24	−	+	−	−	−	−	+
NFN30	−	−	+	+	−	−	−
NFL32	+	−	+	−	−	−	−

The expression of β-actin, used as a positive control was detected in all lines. IL-2, IL-4 and γIFN were not expressed in any line. The expression of CD4 and CD8 were also evaluated and found negative, except for NDF4, which expressed CD8 mRNA (but not as surface protein).

Table 5. Expression of Receptors for Neurotransmitters.

Cell Line	Isoproterenol	VIP	PACAP	CGRP
NFD1	−	+	+	−
NFD3	−	+	+	+
NFD4	+	−	−	+
NFD5	+	−	−	+
NFF8	−	+	+	+
NFA24	−	+	+	+
NFN30	−	−	−	+
NFL32	+	+	+	−

An increase of at least 50% above basal cAMP production is considered significant (+). Responses to CGRP were 200 to 500% of basal, except for NFN30 (more than 1200%) and for the negative line (NFD1). Isoproterenol binds to β2-adrenergic receptors.

own unpublished results). In one study with C57BL mice, these non-T leukemias were of pre-B origin ($B220^+$, Ig^- and Ig gene rearrangement) (8). Non-T leukemias also develop in thymectomized AKR mice (35,36). These are B, null or myeloid (35,36, and E. Legrand, unpublished results). Thus, in cases where thymectomy prevents the development of thymic lymphomas, other hematological malignancies often develop.

Our own lines, derived from intact or from thymectomized mice have a combination of histochemical myeloid markers and immunological and molecular pro-B markers. All cell lines expressed chloroacetate esterase, acid phosphatase, and alkaline phosphatase. This phenotype is not found among normal hemopoietic cells, neither among leukemic cells. Despite several discrepancies, we suggest these cells are close to acute myeloblastic leukemias, on the basis of the presence of chloroacetate esterase and acid phosphatase. Most acute myeloblastic leukemias are alkaline phosphatase negative and myeloperoxidase positive. Myeloperoxidase is sometimes detected only with more sensitive techniques such as immunocytochemistry or cytochemistry at the electron microscopy level (33,34). On hematological grounds, the cell lines would thus be diagnosed as myeloid, immature. Autopsy slides from the primary lesions were diagnosed as acute leukemia, no further distinction could be made. Myeloid differentiation occurred, however, when the cell lines were reinjected *in vivo* (Figure 2).

Biochemical investigations on receptors for neurotransmitters coupled to adenylate cyclase are also compatible with a myeloid origin. Indeed, we have so far not observed receptors for CGRP on lymphoid cells (including pre-B, B, and myeloma lines). In contrast, we have identified such receptors on human HL60 (only after induction of differentiation) and on murine N122 cells (Robberecht *et al.*, unpublished results). Receptors for CGRP have been most studied on endothelial cells (37). In view of the small number of pre-B and B lines studied so far, no final conclusion, however, should be drawn from these observations. The effect of CGRP on leukemic cells should be investigated since this factor is now undergoing clinical trials for vascular diseases (38). It might stimulate or inhibit the growth of leukemic cells.

Apoptosis induced by X-rays has been described for T-cell leukemias and more recently for pre-B and B cells (31,39). Further investigations may confer diagnostic value to apoptosis. This approach also holds much promise in the field of pharmacology and our lines may prove to be valuable models in this respect.

Phenotyping the cells with immunological reagents first indicated that they were non-T, non-B: $Thy1^-$, $CD4^-$, $CD8^-$, sIg^-, cIg^-. Further investigations failed to detect the presence of pre-T or T-specific markers. The granulocytic marker Gr-1 was never found. The heat-stable, Forsman antigen was often expressed. This is compatible with B as well as myeloid lineages. Pre-B specific marker 6C3 was present in all cases and the B220 antigen (specific for the B lineage) was also often expressed. Several lines were CD5 (Ly1) positive. Surface Ig was only detected on rare occasions. The immunological phenotype was clearly indicative of a pre-B origin, B-la subtype for the CD5 positive lines (40). All our lines derived from leukemias originating in thymectomized mice were CD5 positive. Lymphomas obtained from thymectomized AKR mice were also CD5 positive (36).

Molecular studies at the DNA level indicated that the first (v to J) rearrangement of IgH had occurred in seven out of eight lines that were tested. No further rearrangement was detected in these lines. An identical pattern in four lines deserves further investigation. Cutting with different enzymes will show whether a same rearrangement was indeed present in four individual cell lines, derived from four different primary tumors. These cell lines have otherwise different phenotypes, in particular they express different sets of receptors for neurotransmitters.

At the RNA level, the v-pre-B component of the surrogate L chain was expressed. Thus, both DNA and RNA data supported the diagnosis of pre-B leukemia. The cytokine expression pattern determined by PCR was quite remarkable. Two cell lines (NFF8 and NFN30) expressed IL-7. This factor is expressed normally by stromal cells and induces proliferation of pre-B cells (41,42). Our cells are probably frozen at a $B220^+$, sIg^-, stage of differentiation. At this stage, normal cells respond to IL-7, then lose their reactivity to IL-7 upon maturation into $sIgM^+$ cells (41). The NFF8 and NFN30 could be dependent upon autocrine IL-7 for growth. Autocrine IL-7 indeed plays a role in some (but certainly not all) pre-B leukemias (43). Receptors for IL-7 were found on lymphoid (B and T) and myeloid leukemic cells (44,45). With the exception of NFA24, our cell lines could be dependent on autocrine IL-6. We failed to detect IL-6 in the supernatant (not shown) but this does not completely rule out the involvement of IL-6. Normal pro-B cells however are not known targets for IL-6. The NFA24 line, which also has pro-B features, expressed IL-3. Production of IL-3 by pro-B cells is unexpected. In contrast, IL-3-dependent pre-B clones have been reported (46) and IL-3 is a growth factor for normal B cells (42,47). An IL-3-dependent mixed lineage (myeloid-B lymphoid) leukemic line has recently been established from peripheral blood of a patient with chronic B lymphocytic leukemia in the acute phase (48).

Taken together, the cytokine data illustrate the heterogeneity of our panel of cell lines. Future research should identify those factors that are actually produced and those that play a role in the leukemogenic process. In this respect, future investigation will concentrate on receptors for growth factors. So far, we have studied only the IL-2R. This was expressed on two (out of six) lines. In human pre-B leukemic cells, IL-2R expression could be induced by exposure to lectins or phorbol ester (49).

Summarizing the observations, our cell lines cannot be unambiguously described as either myeloid or lymphoid. Each individual line expressed at the same time at least one cytochemical marker indicative of a myeloid origin and several molecular and immunological markers of the B-lymphoid lineage, the simultaneous occurrence of Ig gene rearrangement and v-pre-B surrogate light chain

expression indicates committment to the B-cell pathway and the expression of B220 and 6C3 supports this assignment.

The expression of myeloid markers in a pro-B line seems aberrant (lineage infidelity) at first sight. Quite surprisingly, however, Hara *et al.* have established an adherent clone derived from the much studied pre-B cell line 70Z/3 (50). These cells had the morphological features of macrophages, stained positive for non-specific esterase and expressed lysozyme (50). Recently, it has become clear that some well differentiated leukemias can change phenotypes when exposed to a different environment. For example, in a murine model of chronic myeloid leukemia, engraftment of leukemic cells within the thymus allows for differentiation into T-lymphocytes. This leukemia thus originates from a multipotent stem cell (51). Our situation is more analogous to that of an immature human T-ALL which can switch to myeloid or myelomonocytic phenotypes when grown in IL-3 or granulocyte-macrophage colony-stimulating factor (GM-CSF). The T-cell receptor gene rearrangements are conserved, whatever the phenotype, clearly indicating that the leukemia is not biclonal (52). We are now growing our cells in different conditions to evaluate their phenotypic repertoire. Whatever the results, pre-B or pro-pre-B leukemias with myeloid features are quite common among hematological malignancies in the human (4) and our cell lines may help understanding this clinical entity.

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