RAPID COMMUNICATION

Myeloid Malignancies Induced by Alkylating Agents in *Nf1* Mice

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Therapy-related acute myeloid leukemia and myelodysplastic syndrome (t-AML and MDS) are severe late complications of treatment with genotoxic chemotherapeutic agents. Children with neurofibromatosis type 1 (NF1) are predisposed to malignant myeloid disorders that are associated with inactivation of the *NF1* tumor suppressor gene in the leukemic clone. Recent clinical data suggest that NF1 might be also associated with an increased risk of t-AML after treatment with alkyating agents. To test this hypothesis, we administered cyclophosphamide or etoposide to cohorts of wild-type and heterozygous *Nf1* knockout mice. Cyclophosphamide exposure cooperated strongly with heterozygous inactivation of *Nf1* in myeloid leukemogenesis, while etoposide did not. Somatic loss of the normal *Nf1* allele correlated with clinical disease and was more common in 129/Sv mice than in 129/Sv × C57BL/6 animals. Leukemic cells showing loss of heterozygosity at *Nf1* retained a structural allele on each chromosome 11 homolog. These studies establish a novel in vivo model of alkylator-induced myeloid malignancy that will facilitate mechanistic and translational studies.

IN 1977, ROWLEY ET AL[1] described a group of patients who developed a myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) as a second malignant neoplasm after treatment for another cancer. Two forms of therapy-related (t) myeloid malignancies are now recognized; these subtypes are associated with distinct clinical and biologic features and differ with respect to the prior anticancer treatment. Most patients with t-AML and t-MDS previously received chemotherapeutic agents that alkylate DNA. This subtype typically involves a latency of 3 to 7 years between genotoxin exposure and disease onset, a myelodysplastic prodrome, and frequent loss of chromosomes 5 and/or 7 (−5 and/or −7) or deletions involving the long arms of these chromosomes [del(5q)/del(7q)].[1-4] The second subtype of t-AML develops after therapy with drugs that inhibit topoisomerase II. These cases are characterized by a shorter interval between cytotoxic therapy and clinical signs, overt leukemia at presentation, and balanced translocations that usually involve the *MLL* gene located on chromosome 11, band q23.[5-8] The prognosis is poor for patients with t-AML or t-MDS. Importantly, as aggressive multi-agent regimens are used increasingly to treat many primary cancers, the incidence of therapy-related myeloid malignancies is expected to increase over the next few years.

Individuals with neurofibromatosis type 1 (NF1) are predisposed to specific benign and malignant neoplasms, which arise primarily in cells derived from the embryonic neural crest.[9] In addition, children (but not adults) with NF1 show a 200 to 500-fold increase in the incidence of de novo malignant myeloid disorders, particularly juvenile myelomonocytic leukemia (JMML).[10-12] The *NF1* gene encodes neurofibromin, a guanosine triphosphatase (GTPase) activating protein that accelerates the slow intrinsic rate of GTP hydrolysis on $p21^{ras}$ (Ras) proteins.[13] Genetic and biochemical data strongly support the hypothesis that *NF1* functions as a tumor suppressor gene in human and murine hematopoietic cells by negatively regulating Ras output.[13-20] For example, approximately 10% of heterozygous *Nf1* knockout mice (*Nf1*+/−) spontaneously develop myeloid leukemia beginning around age 15 months, with tumor cells exhibiting loss of the wild-type *Nf1* allele.[16]

A few cases of t-AML associated with monosomy 7 were recently reported in children with NF1, all of whom received alkylating agents to treat primary cancers including anaplastic astrocytoma, glioblastoma, Wilms' tumor, or acute lymphoblastic leukemia.[21] However, because these patients were not ascertained in a systematic way, it is uncertain if the risk of therapy-related myeloid malignancies is increased over children without NF1 who received similar therapies. Moreover, loss of heterozygosity (LOH) at *NF1* was not detected in the leukemic cells of children with NF1 who developed t-AML.[21] If germline inactivation of *NF1* cooperates with genotoxic agents that are used to treat human cancers in leukemogenesis, we reasoned that exposing *Nf1*+/− mice to these drugs might produce an in vivo model of therapy-induced myeloid disease. To test this hypothesis, we administered two chemotherapeutic agents that have been implicated in human t-AML and t-MDS to *Nf1* mice. Here we show that the alkylating agent cyclophosphamide, but not the toposiomerase II inhibitor etoposide, efficiently induces a myeloproliferative disorder (MPD) in *Nf1*+/− mice, and we present correlative cytogenetic and molecular data.

MATERIALS AND METHODS

Animal care. Mice were housed in the University of California, San Francisco (UCSF) Animal Care facility and were examined regularly by one of the investigators. Cyclophosphamide and etoposide were prepared by the UCSF pharmacy and were administered by one of the

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investigators. Mice were weighed at the beginning of the study and weekly thereafter to adjust the drug doses. Complete blood counts (CBCs) were performed on blood samples collected from tail veins in an automated cell counter. The accuracy of abnormal blood counts was verified by direct examination of stained smears. The study procedures were reviewed and approved by the UCSF Committee for Animal Research.

Treatment and monitoring. We mated *Nf*+/− and wild-type (*Nf*+/+) mice and genotyped the offspring by Southern blot analysis of tail DNA. The initial group of mice were from the inbred 129/Sv strain in which the *Nf1* mutation was created.[16] To perform LOH analysis at loci other than *Nf1* in alkylator-treated mice, F1 offspring of a cross between the 129/Sv and C57BL/6 strains were used in the latter part of the study. *Nf1*+/− and *Nf1*+/+ littermates were assigned to observation (control group) or to receive treatment with either etoposide or cyclophosphamide beginning at 6 to 10 weeks of age. These agents were selected because they are used widely in human cancer therapy. Treated mice received a single 6-week course of 100 mg/kg/wk of either agent, a schedule which approaches the maximally tolerated doses. Cyclophosphamide (CY) was administered by intraperitoneal injection whereas etoposide was administered through an orogastric tube.

CBCs with white blood cell (WBC) differentials were performed every 3 months in mice that appeared well, and whenever a mouse showed signs of systemic illness. The CBC was repeated immediately whenever the WBC count was >20,000/μL. All mice that appeared moribund and animals with WBC counts >20,000/μL on two consecutive determinations were killed, the spleens were weighed, and hematopoietic tissues were collected for morphologic and genetic analysis.

Nf1 genotyping and LOH analysis. Genomic DNA was prepared from tail clippings or from hematopoietic tissues (spleen or bone marrow) by standard procedures.[22] *Nf1* genotypes and loss of heterozygosity were determined by digesting DNA samples with *Nco*I + *Hind*III followed by gel electrophoresis, blotting to nylon membranes, and hybridization with an *Nco*I-*Pst*I fragment from intron 31 of *Nf1* as described previously.[16] LOH was scored by comparing the relative intensities of restriction fragments derived from paired normal and leukemic tissues.

LOH analysis with microsatellite markers. These procedures have been described in detail.[14] Briefly, DNA samples were amplified in a DNA Thermocycle Machine (Perkin Elmer Cetus, Norwalk, CT). Polymerase chain reaction (PCR) was performed in reaction mixtures that include 0.66 μmol/L of respective 3′ and 5′ primers, 100 ng of target genomic DNA, 1 U of Taq polymerase (AmpliTaq; PE Applied Biosystems, Foster City, CA), and 0.4 μmol/L final concentrations of deoxynucleotides in a final reaction volume of 25 μL. The forward primer was kinase-labeled with γ-^{33}P adenosine triphosphate (ATP). Labeled PCR products were separated on (6 mol/L urea, 8% polyacylamide) sequencing-type gels and run at 60 to 80 W constant power for 2 to 4 hours. The gels were dried, placed in Saran wrap (Dow Brands L.P., Indianapolis, IN), and exposed to x-ray film at −70°C. The polymorphic markers tested included D18Mit55, D18Mit13, and D13Mit13, which are syntenic to human 5q31 and D6Mit48, D5Mit40, and D12Mit64, which are syntenic to genes within human 7q22-31.

Cytogenetic analysis and fluorescence in situ hybridization (FISH). A trypsin-Giemsa banding technique was used to analyze cells from bone marrow and spleen. Metaphase cells from short-term (24 to 72 hours) unstimulated cultures were examined. Ten metaphase cells were examined each from the bone marrow and spleen cultures for each mouse. Chromosomes were identified using the standardized mouse karyotype as described by Cowell.[23] FISH was performed as described previously.[24] Briefly, a biotin-labeled *Nf1* probe was prepared by nick-translation using Bio-16-dUTP (Enzo Diagnostics). Hybridization was detected with fluorescein-conjugated avidin (Vector Laboratories), and chromosomes were identified by staining with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI). Images were obtained using a Zeiss Axiophot microscope coupled to a cooled charge coupled device (CCD) camera. Separate images of DAPI-stained chromosomes and the hybridization signal were merged using image analysis software (NU200, Photometrics Inc, Phoenix, AZ and NIH Image 1.57, National Institutes of Health, Bethesda, MD). The *Nf1* probe used for FISH was a 10.6 kb genomic lambda clone containing exon 31 and flanking intron sequences.

RESULTS

Leukemia in Nf1+/+ *and* Nf1+/− *mice.* Myeloid disorders developed in 4 of 101 *Nf1*+/+ mice, 2 of which received CY (Table 1). In contrast, myeloid malignancies were diagnosed in 14% of the untreated *Nf1*+/− mice (8 of 58), in 25% of the etoposide-treated animals (8 of 32), and in 38% (14 of 37) of the mice assigned to the CY group (Table 1). Kaplan-Meier plots comparing disease incidence over time in *Nf1*+/+ and *Nf1*+/− mice that received no treatment, etoposide, or CY are shown in Fig 1. *Nf1*+/− mice that received either drug had a significantly higher rate of disease than wild-type animals treated in parallel (Fig 1). Treated and untreated *Nf1*+/− mice were also compared to ascertain the relative contributions of *Nf1* genotype and chemotherapy exposure to leukemia susceptibility. This analysis showed that the incidence of disease was significantly higher, and the latency period shorter, in the *Nf1*+/− mice that received CY (0.004 *v* untreated *Nf1*+/− mice by pairwise logrank statistics), but not in the etoposide group ($P = .2$ *v* the untreated group). The in vivo leukemogenic effect of CY was restricted to *Nf1*+/− mice as *Nf1*+/+ animals in the control and CY-treated groups had similar rates of leukemia (Table 1). The incidence of leukemia was higher in *Nf1*+/− mice from the inbred 129/Sv background than in 129/Sv × C57BL/6 animals (Table 1), although these differences did not achieve statistical significance.

A myeloproliferative phenotype was observed in most diseased mice that was similar in control and chemotherapy-treated animals. This MPD was characterized by elevated peripheral blood leukocyte counts with a high percentage of mature neutrophils and monocytes (Fig 2). The mean WBC count was 31,000/μL (range, 20,000 to 98,000), and the mean myeloid cell count was 28,000/μL (range, 14,000 to 88,000). Blood smears showed a variable degree of myeloid differentiation with some containing greater than 80% mature neutrophils and others showing 30% to 40% monocytes and monocytoid

Table 1. Incidence of Leukemia in *Nf1* Mice

Genotype and Treatment	Genotype	No. of Mice	No. (%) with Leukemia
129/Sv			
None	*Nf1*+/+	31	2 (6%)
	Nf1+/−	46	8 (17%)
Etoposide	*Nf1*+/+	26	0 (0%)
	Nf1+/−	32	8 (25%)
CY	*Nf1*+/+	5	0 (0%)
	Nf1+/−	12	7 (58%)
129/Sv × C57BL/6			
None	*Nf1*+/+	14	0 (0%)
	Nf1+/−	12	0 (0%)
CY	*Nf1*+/+	25	2 (8%)
	Nf1+/−	25	7 (28%)

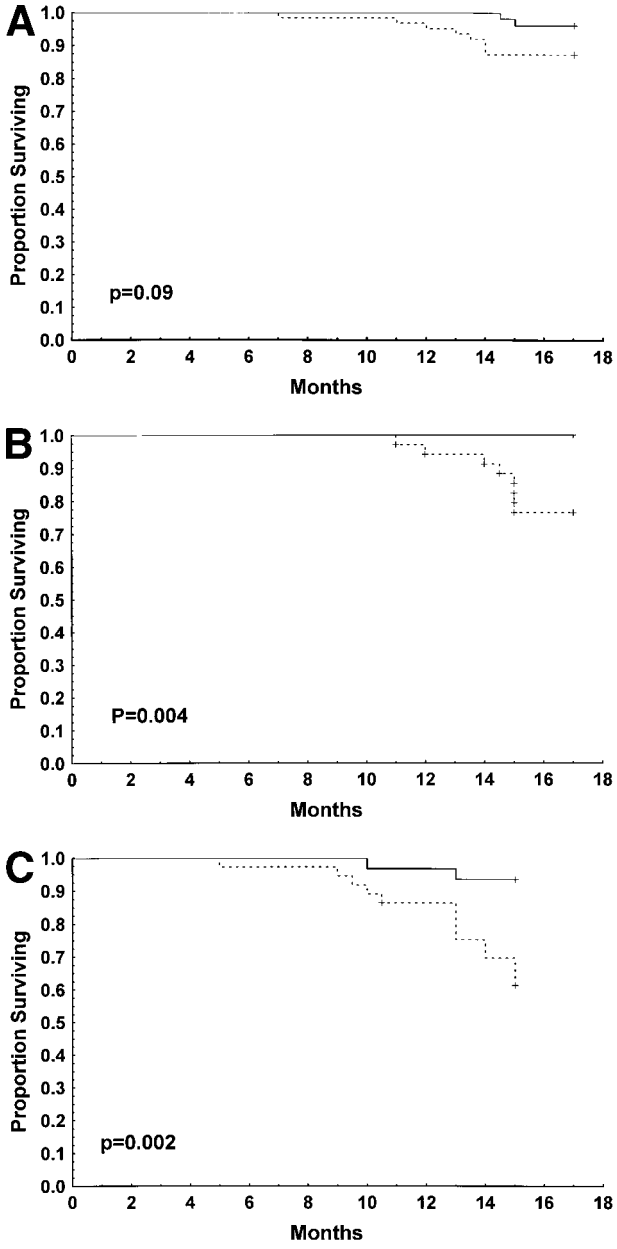

Fig 1. Kaplan-Meier plots showing the proportion of *Nf1*+/+ and *Nf1*+/− mice surviving without leukemia. *Nf1*+/+ mice are shown as an unbroken line and *Nf1*+/− mice as a dotted line. (A) Data from untreated mice; (B) data from the etoposide group; (C) data from the CY group.

cells. Some smears showed rare blasts. Platelet counts and hemoglobin values were normal in mice with MPD and immature erythroid lineage cells were not seen in the peripheral blood. There was no consistent relationship between treatment group, WBC count, and the degree of myeloid maturation visible on blood smears. The bone marrows of mice with MPD showed an overwhelming predominance of myeloid cells with a shift toward immature elements, and sections of the spleen showed expansion of red pulp with infiltration of myeloid cells at various stages of differentiation admixed with areas of erythropoiesis (Fig 2). This MPD is similar to the JMML-like disorder that arises after adoptive transfer of *Nf1*−/− fetal liver cells into irradiated recipient mice.[17] A disease phenotype more consistent with acute leukemia was seen in one CY-treated *Nf1*+/− mouse and in one mouse that received etoposide. Both animals had WBC counts >150,000/μL with large numbers of blasts and few mature neutrophils in the peripheral blood. The CY-treated mouse also had anemia (hemoglobin level, 5.7 g/dL) and thrombocytopenia.

Laboratory investigation of murine leukemias. LOH at *Nf1* correlated with clinical evidence of leukemia in *Nf1*+/− mice (Table 2) and this invariably involved loss of the wild-type *Nf1* allele (Fig 3). Within the CY-treated group, leukemic cells from 129/Sv × C57BL/6 mice showed a much lower incidence of LOH than cells from 129/Sv animals (Table 2). Both animals with evidence of acute leukemia had LOH in hematopoietic tissues. In mice with MPD, LOH was not consistently associated with higher leukocyte counts or with increased numbers of immature myeloid cells. Unexpectedly, we detected LOH at sacrifice in the hematopoietic tissues of 18% of mice that did not fulfill the criteria used to diagnose leukemia. Most of these animals appeared well and WBC counts <10,000/μL and absence of prominent myeloid infiltrates in splenic sections. These results implicate inactivation of *Nf1* as an early event that confers an in vivo proliferative advantage upon a clone of cells, but also suggest that additional mutations are required to produce the characteristic MPD. LOH in the absence of leukemia was much more common in 129/Sv mice than in 129/Sv × C57BL/6 animals (Table 2). Among mice without leukemia, LOH was relatively common in control animals but infrequent in the etoposide-treated cohort (Table 2).

Cytogenetic analysis of bone marrow and spleen cells from 6 mice with MPD (5 CY-treated mice and 1 from the etoposide group) revealed a normal karyotype (Fig 4A). To ascertain if LOH on Southern blots was associated with submicroscopic deletions of *Nf1* or with duplication of the mutant allele, we used a genomic *Nf1* probe from the disrupted segment of the gene to perform FISH analysis of hematopoietic cells from 3 of these 6 mice. FISH showed 2 structural copies of the *Nf1* gene in each case (Fig 4B). We also used six polymorphic microsatellite markers to examine bone marrow DNA from mice with t-ML for LOH at loci syntenic to regions of human chromosomes 5 and 7 that are frequently deleted in humans with t-MDS and t-AML, but found none (data not shown). Similarly, Southern blot analysis of specimens from etoposide-treated mice did not show rearrangements of *Mll* when hybridized with a probe from the human *MLL* breakpoint cluster region that detects virtually all of the breakpoints in human leukemias (data not shown).

DISCUSSION

This study establishes an in vivo model of therapy-induced myeloid malgnancies in *Nf1*+/− mice that will facilitate basic and translational research studies of this important clinical disorder. In human t-MDS/t-AML, frequent deletions involving chromosomes 5 and 7 have implicated loss of gene function in genotoxin-induced leukemogenesis. How alkylating agents actually cause leukemia is unknown; however, CY increases the frequency of somatic inactivation of target genes in a variety of

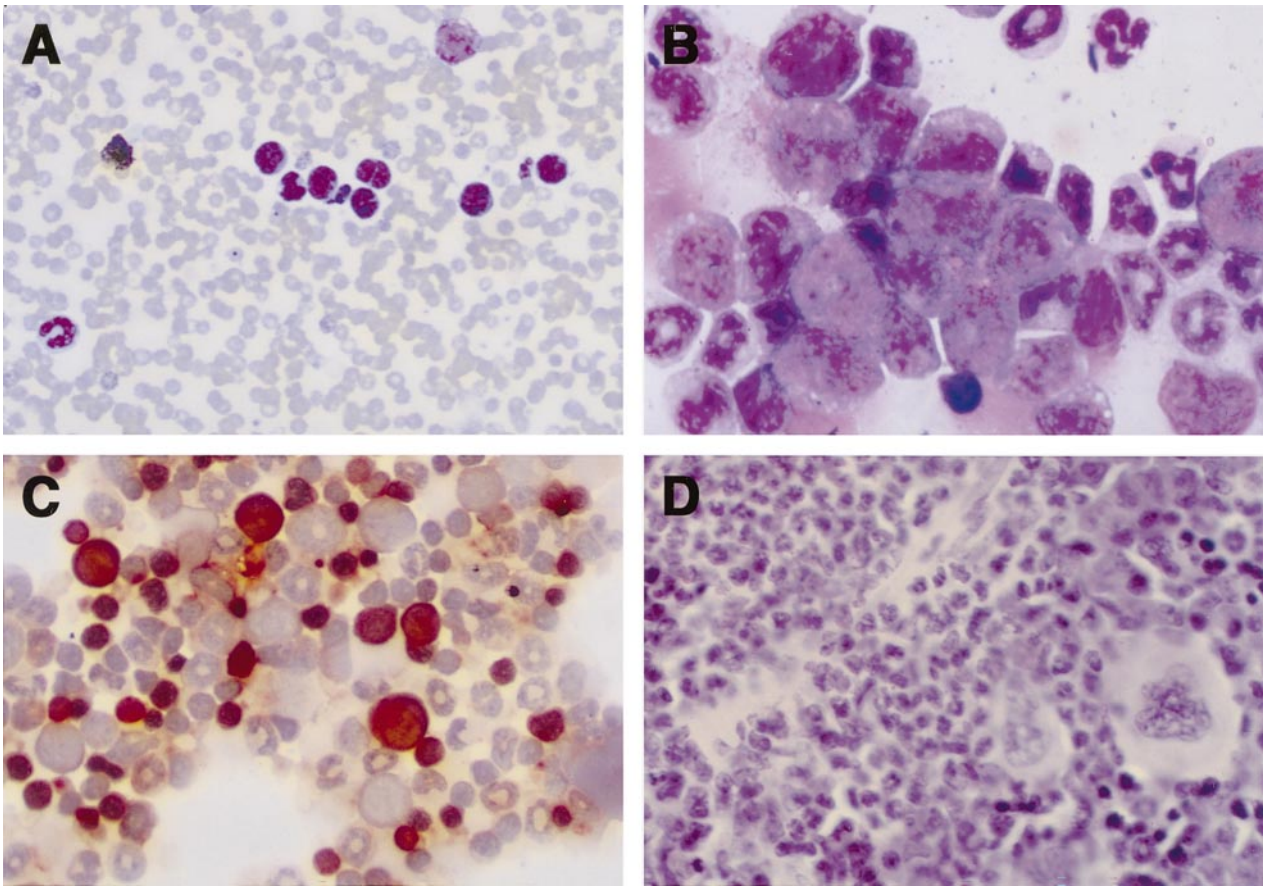

Fig 2. Tissue sections from CY-treated *Nf1*+/− mice with MPD. (A and B) Blood and bone marrow smears showing immature and well differentiated myeloid cells. (C) A cytocentrifuge preparation of spleen cells stained with the myeloid lineage marker nonspecific esterase demonstrates many positive cells (brown stain). (D) A spleen section shows a dense infiltrate of myeloid cells within the red pulp.

assays.[25] In contrast, leukemias that arise after exposure to topoisomerase II inhibitors are associated with recurring chromosomal translocations involving the *MLL* gene that result in the production of dominantly acting chimeric proteins. If the leukemias that develop after treatment with alkylating agents predominately involves the inactivation of specific target genes, it is possible that some human patients who develop t-ML after alkylator-based chemotherapeutic regimens are highly susceptible because of germline mutations of undiscovered tumor-suppressor genes that, like *NF1,* restrain the growth of immature myeloid cells.

We used clinical criteria to diagnose leukemia because the bone marrows of some children with NF1 who develop malignant myeloid disorders do not show LOH at *NF1*.[14,19,21] In this study, mice with clinical evidence of MPD or AML had a threefold higher rate of LOH at *Nf1* than mice without these findings. The presence of somatic LOH in hematopoietic tissues supports the clonal nature of these myeloid disorders. MPDs

Table 2. Loss of Heterozygosity in *Nf1*+/− Mice

Genotype and Treatment	No. of Mice	No. With LOH/ No. With Leukemia	No. With LOH/ No. Without Leukemia
129/Sv			
None	46	5/8 (62%)	10/38 (26%)
Etoposide	32	7/8 (87%)	1/24 (4%)
CY	12	5/7 (71%)	4/5 (80%)
129/Sv × C57BL/6			
None	12	0/0	1/12 (8%)
CY	25	2/7 (28%)	1/18 (5%)
All mice		19/30 (63%)	17/97 (17%)

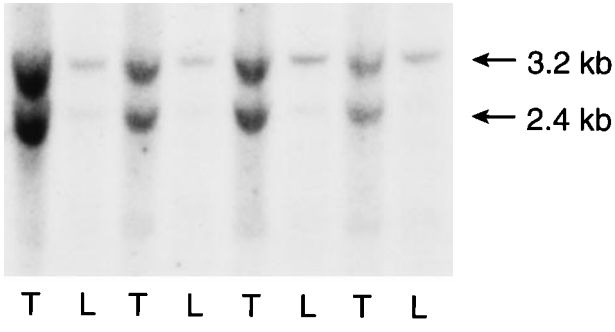

Fig 3. Southern blot analysis of tissues from *Nf1*+/− mice with MPD. The 3.2-kb restriction fragment corresponds to the targeted *Nf1* allele, and the 2.4-kb band is derived from the wild-type allele. DNA extracted from the bone marrows or spleens of five mice with leukemia (L) show absence or a marked reduction in the wild-type *Nf1* allele compared to paired tail (T) DNA specimens.

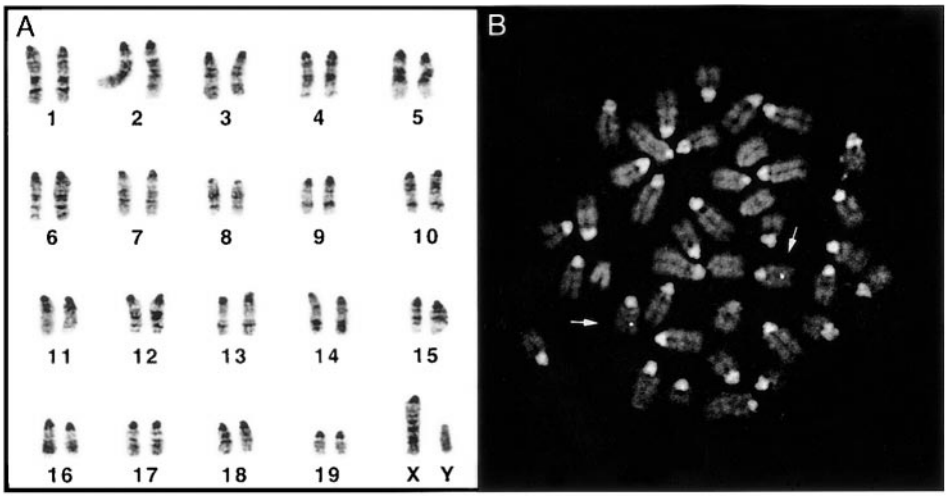

Fig 4. (A) Cytogenetic analysis of spleen cells from a CY-treated mouse with LOH at *Nf1* shows a normal diploid karyotype. (B) FISH of the same specimen shown in (A) with a genomic *Nf1* probe shows 1 copy of the gene on each chromosome 11 homolog.

with and without LOH had similar features to the myeloid disorder that emerges after adoptive transfer of *Nf1*−/− fetal liver cells into irradiated recipient mice.[17] Together with the extraordinary increase in the incidence of leukemia in *Nf1*+/− versus *Nf1*+/+ animals, these observations provide evidence that inactivation of *Nf1* is a central event in leukemogenesis even in the absence of LOH. If this is true, it is likely the wild-type *Nf1* allele is inactivated in bone marrows without LOH by subtle somatic mutations. An alternative consideration is that some *Nf1*+/− mice develop myeloid malignancies through a genetic pathway that does not involve biallelic inactivation of *Nf1*, as has recently been shown for a subset of tumors from heterozygous *p53* knockout mice.[26] Experiments using techniques that can identify point mutations will be required to distinguish between these possibilities. *Nf1* is a very large gene, and protein truncation has proven to be the most efficient method for detecting subtle mutations in normal and leukemic cells from NF1 patients.[20,27,28]

Adoptive transfer of *Nf1*−/− fetal liver cells into irradiated mice consistently induces a MPD with features of JMML.[17] Inasmuch as these data suggested that inactivation of *Nf1* in early hematopoietic cells might be both necessary and sufficient to induce clinical disease, we were surprised to detect LOH at *Nf1* in hematopoietic tissues from 18% of *Nf1*+/− mice with normal WBC counts. This idea that genetic alterations in addition to inactivation of *Nf1* are required for clinical disease is consistent with the relatively long latency between CY exposure and the onset of t-ML in Nf1+/− mice (Fig 1). Cooperating somatic mutations such as bone marrow monosomy 7 and epigenetic events have also been identified in human NF1-associated myeloid disorders.[21,29] It will be of interest to determine if LOH can be detected in circulating blood cells some months before the onset of leukocytosis and splenomegaly in *Nf1*+/− mice.

We did not inject bone marrow cells from *Nf1*+/− mice that acquired myeloid disorders associated with LOH into secondary hosts. In our hands, transferring marrow from recipients previously engrafted with *Nf1*−/− fetal liver cells consistently induces MPD in irradiated, but not in unirradiated, mice (data not shown). In an interesting experiment, Largaespada et al[17] crossed a mutant *Nf1* allele into the BXH2 line of mice in which a leukemogenic retrovirus is transmitted vertically from mother to pups. They observed preferential viral integration into the wild-type *Nf1* allele, shortened latency, and a change in disease phenotype from MPD to AML.[17] Their finding of other somatically acquired leukemia-specific viral integrations within these clones implicated alterations in addition to inactivation of *Nf1* in progression from MPD to AML. Adoptive transfer into secondary recipients provides a way of further characterizing therapy-induced myeloid disorders arising in *Nf1* mice and may be especially informative in rare cases that show features of acute leukemia.

LOH at *Nf1* and clinical leukemia were more common in homozygous 129/Sv mice than in 129/Sv × C57BL/6 animals. This was true in both control and CY-treated mice. Thus, 129/Sv hematopoietic cells are unexpectedly prone to spontaneously undergo LOH at *Nf1* followed by clonal expansion. Rates of cancer in the F1 progeny of crosses between two inbred mouse strains often correlate poorly with parental rates and may be higher, lower, or unchanged.[30] The net effect of our having assigned disproportionate numbers of 129/Sv × C57BL/6 mice to the CY group is to understate the magnitude of the leukemogenic effect of this agent. CY-treated mice showed a higher incidence of clinical leukemia than the control group irrespective of genotype (58% *v* 17% in strain 129/Sv and 28% *v* 0% in strain 129/Sv × C57BL/6; Table 1).

LOH was less frequent in CY-treated 129/Sv × C57BL/6 mice with MPD than in any of the 129/Sv cohorts. This low incidence suggests that the mechanism of *Nf1* inactivation in 129/Sv × C57BL/6 hematopoietic cells involves subtle alkylator-induced mutations rather than loss of the wild-type allele. Consistent with this, Shoemaker et al[31] recently identified somatic *Apc* point mutations caused by transitions or transversions in intestinal tumors from multiple intestinal neoplasia (Min) mice that had been exposed to the alkylating agent N-ethyl-N-nitrosourea (ENU). Interestingly, other tumors from this ENU-exposed cohort showed LOH at *Apc*. Taken together with our data from CY-treated mice, these data suggest that mechanisms of alkylator-induced tumor suppressor gene inactivation in colonic and hematopoietic cells include somatic rearrangements that result in LOH as well as subtle intragenic events.

Intestinal tumors that spontaneously arise in Min mice show LOH at *Apc* with apparent loss of an entire chromosome 18 homolog.[32] However, in a line of mice that carried mutations of the *Apc* and *Dpc4* tumor suppressor genes in *cis*, intestinal tumorigenesis was associated with apparent loss of one entire chromosome homolog with duplication of the mutant chromosome.[33] Consistent with this, FISH analysis of murine leukemias with LOH showed an *Nf1* allele on each chromosome 11 homolog. Although deletion of the chromosome containing the normal tumor suppressor gene allele followed by duplication of the mutant homolog has been proposed as a likely underlying mechanism,[33] other models are also plausible. Mitotic nondisjunction resulting in two copies of the mutant homolog might occur first, with subsequent loss of the normal chromosome. Alternatively, the DNA segment that contains the normal allele might be replaced by a homologous segment from the mutant chromosome by a double mitotic recombination event, as has been reported in a human NF1-associated leukemia.[34]

Haran-Ghera et al[35] previously observed a weak leukemogenic effect of multiple doses of CY when this agent was administered with radiation and dexamethasone to SJL/J mice, a strain that is susceptible to radiation-induced AML. However, CY did not induce leukemia in the absence of radiation, and only cooperated with radiation when it was combined with dexamethasone.[35] In contrast, we have developed a murine model of t-ML based on clinical observations in NF1 patients[21] in which CY alone efficiently induces myeloid leukemia in *Nf1*+/− mice.

Our data provide direct experimental evidence that exposure to a commonly used cancer chemotherapeutic agent can cooperate with a genetic predisposition in the development of myeloid malignancies. Although human patients with t-MDS/t-AML frequently show peripheral blood cytopenias when they seek medical attention, their bone marrows are hypercellular and the disease typically evolves into a frankly proliferative phase with time. Similarly, *Nf1*+/− mice only exhibit overproliferation of myeloid cells months after exposure to CY. As in humans, LOH in murine hematopoietic cells is associated with a copy of the mutant *Nf1* allele on each chromosomal homolog. The relevance of this model to human leukemia is further suggested by the presence of genetic alterations that deregulate Ras signaling in many human myeloid leukemias,[36,37] and by the finding of activating *RAS* mutations in the bone marrows of some patients with t-AML.[38,39] Although our data implicating mutations of genes in addition to *Nf1* in murine leukemogenesis are also consistent with observations in human patients, we did not detect LOH with polymorphic markers from regions of the murine genome that are syntenic to human 5q31 and 7q22. There are a number of potential explanations for these findings, including: (1) the probes used might be some distance from the critical murine loci, (2) the relevant murine genes may be inactivated by somatic mutations which do not result in LOH, (3) loss of DNA sequences synteic to human 5q31 and 7q22 could be late events in progression of MPD to AML that had not occurred by the time of sacrifice, and/or (4) a different spectrum of cooperating genes might be mutated in human and murine leukemias. The nature of the alterations that are involved in alkylator-related leukemias awaits identification of additional target genes in both species.

This novel model provides a rigorous in vivo system to address a number of important (and in some cases controversial) issues in therapy-related myeloid disorders including the relative leukemogenic potential of different alkylating agents, the role of dose intensity, and the additive effects (if any) of alkylating agents and external beam radiotherapy. Furthermore, molecular analysis at *Nf1* may elucidate the mechanistic basis of genetic damage induced by specific alkylating agents in immature hematopoietic cells. *Nf1*+/− knockout mice will also be useful for testing the utility of surrogate markers of gene mutation such as inactivation of *Hprt* to ascertain if exposure to specific mutagens portends an elevated risk of leukemia and to investigate chemopreventive strategies. Finally, these results have implications for the care of individuals with NF1 who develop neoplasms, because they suggest that alkylator-based regimens should be avoided whenever possible.

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