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Award Number: DAMD17-98-1-8608

TITLE: Therapeutic and Biologic Studies in a Murine Model of NF1

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REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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20010122 072

DTIC QUALITY INSPECTED 4

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Annual (23-Sep-98 - 22-Sep-99)

4. TITLE AND SUBTITLE
Therapeutic and Biologic Studies in a Murine Model of NF1

5. FUNDING NUMBERS
DAMD17-98-1-8608

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

This report describes progress made during the first year of support for a translational research project involving *Nf1* mutant mice with myeloid leukemia. This study has two Technical Objectives. First, examining the therapeutic efficacy of two agents (1) mycophenolate mofetiel (MM) and, (2) a fusion toxin that targets the GM-CSF receptor. These compounds represent rational new approaches for treating NF1-associated tumors. MM has been tested in the mouse model and our preliminary data indicate that it is unlikely to provide benefit to NF1 patients. We have also produced and purified the GM-CSF immunotoxin for use in preclinical studies during the next fund year. We are also performing correlative biochemical studies to elucidate the effects of these therapeutics on cellular GTP levels and Ras signaling. In aim 2, we are utilizing *Nf1* mice to extend clinical observations suggesting that individuals with NF1 are susceptible to the development of therapy-associated second cancers. These studies were initiated over the past year. We will perform other correlative molecular studies in mice that develop tumors. We anticipate that the proposed experiments will yield novel data that may be of practical value to patients with NF1 and their physicians.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Neurofibromatosis	43
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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K. Hu 10/15/99

PI - Signature Date

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(5) INTRODUCTION

Individuals with neurofibromatosis type 1 (NF1) are predisposed to specific benign and malignant neoplasms including juvenile myelomonocytic leukemia (JMML; formerly known as JCML). Clinical data also suggest that children with NF1 are at increased risk of developing leukemia as a complication of genotoxic therapies for another primary cancer. Genetic and biochemical studies of leukemia samples from children with NF1 performed in our laboratories have shown that *NF1* functions as a tumor suppressor gene in immature hematopoietic cells by negatively regulating the Ras signaling pathway (Bollag et al., 1996; Kalra et al., 1994; Miles et al., 1996; Shannon et al., 1994; Side et al., 1997). Similarly, heterozygous *Nf1* mutant *(Nf1+/-)* mice show an increased incidence of myeloid leukemia and other cancers (Jacks et al., 1994). We recently reported that treatment with the alkylating agent cyclophosphamide cooperates strongly with heterozygous inactivation of *Nf1* in murine leukemogenesis (Mahgoub et al., 1999). Homozygous *Nf1* mutant embryos *(Nf1-/-)* die *in utero.* Like human JMML cells, *Nf1-/-* fetal hematopoietic cells display a selective pattern of hypersensitivity to the cytokine growth factor GM-CSF in myeloid progenitor colony assays (Bollag et al., 1996; Largaespada et al., 1996). Adoptive transfer of these cells consistently induces a myeloproliferative disorder (MPD) that resembles JMML in irradiated recipients (Largaespada et al., 1996; Zhang, 1998). The predictable nature of this syndrome, the fact that transplanted mice survive for many months, and the well-characterized biochemical alterations in *Nf1*-deficient hematopoietic cells make this model attractive for testing novel therapeutics and for biologic studies of growth control.

The approved Statement of Work for this translational research project has two Technical Objectives which we are pursuing through two Specific Aims. Aim 1 proposes preclinical studies in recipient mice that have been transplanted with *Nf1*-deficient fetal liver cells to investigate the therapeutic efficacy of two agents (1) an inhibitor of *de novo* guanine nucleotide synthesis and, (2) a recombinant fusion toxin that targets the GM-CSF receptor. These compounds were chosen because they represent rational new approaches for treating NF1-associated tumors. We are also performing correlative biochemical studies to elucidate the effects of these therapeutics on cellular GTP levels and Ras signaling. In aim 2, we are utilizing *Nf1* mice to extend clinical observations suggesting that individuals with inactivation of one *NF1* allele are susceptible to the development of therapy-associated second cancers. We are exposing cohorts of wild type and *Nf1+/-* mice to either radiation therapy alone, or to radiation combined with cyclophosphamide to test the hypothesis that these mutagens will cooperate with each other and with inactivation of *Nf1* in tumorigenesis. We will examine tumor tissues for loss of heterozygosity (LOH) at *Nf1* and will perform other correlative molecular studies. We anticipate that the proposed experiments will yield novel data that may be of practical value to patients with NF1 and their physicians.

(6) BODY

Technical Objective (Aim) 1: Testing Rational Therapeutics in Nf1 Mice

Overview of Preclinical Therapeutic Studies. This component involves independently testing the efficacy of two rational therapeutics to inhibit the growth of *Nf1-/-* hematopoietic cells *in vivo,* and performing correlative biochemical and cell biologic assays. Our progress is described below.

Preclinical Evaluation of Mycophenolate Mofetiel (MM). As described in our proposal, MM is an inhibitor of the enzymoe inosine 5' monophosphate dehydrogenase (IMPDH), which is required for *de novo* guanine nucleotide synthesis. The rationale for investigating this agent is based upon the idea that therapeutics that lower the ratio of GTP to GDP in the cell should decrease Ras-dependent growth because the activation state of Ras depends on the selective binding of GTP. The murine transplant model is ideal for initial studies that examine the efficacy of GTP reduction in inhibiting the abnormal growth of *Nf1*-deficient cells because the biochemical consequences of gene inactivation are well characterized in hematopoietic cells and because these cells rely heavily on the *de novo* pathway for nucleotide biosynthesis. Indeed, MM has shown anti-tumor efficacy in a number of preclinical studies performed in athymic nude mice. Additional background information, including calculations which suggested that cells in which an oncogenic *RAS* mutation or loss of *Nf1* would show enhanced sensitivity to a reduction in intracellular GTP concentrations, are presented in our proposal.

In year 1, the technical objectives related to the MM component of this project were (1) to treat normal mice at three dose levels of MM, to monitor clinical responses, and to examine diseased tissues at necroscopy; (2) to breed mice and perform adoptive transfer experiments to generate recipients repopulated with wild type or *Nf1-/-* fetal liver cells; (3) to select a dose of MM for preclinical evaluation; and, (4) to undertake correlative biochemical studies.

MM has been approved by the FDA for use in organ transplant rejection under the trade name Cell Cept. Based on preclinical data showing that MM is well-tolerated in mice at doses up to 240 mg/kg/day with diarrhea and anemia as the dose-limiting toxicities, we first treated cohorts of wild type F1 mice from a cross between strains C57BL6 and 129Sv with either 100, 150, or 200 mg/kg/day of MM. The drug was given as a single daily oral gavage dose for 8 weeks. MM was well-tolerated at these doses and, as shown in Figure 1, we observed no significant changes in blood cell counts. Given the lack of hematologic or other significant toxicities in the initial cohorts of C57BL6/129Sv F1 mice, we administered 300 or 400 mg/kg/day of MM to additional animals for up to 20 weeks. These doses were generally well tolerated, although mice treated at both doses showed weight loss and anemia (Figure 2A). White blood cell counts and myeloid counts declined after 6 weeks, but rebounded and stabilized thereafter (Figure 2B).

Based on these pilot data in wild type mice, we initiated a preclinical trial in which irradiated recipients engrafted with either *Nf1+/+* or *Nf1-/-* fetal liver cells were randomly assigned to receive either MM at a dose of 400 mg/kg/day, or a vehicle control, for 8 weeks. These animals were entered at least 12 weeks after adoptive transfer. All of the recipients of *Nf1-/-* fetal liver cells had leukocytosis with elevated myeloid cell counts at entry, and engraftment with *Nf1*-deficient cells was verified by Southern blot analysis. We evaluated 4 groups (1) mice reconstituted with *Nf1+/+* cells that received MM; (2) mice reconstituted with *Nf1-/-* cells that received MM; (3) untreated mice reconstituted with *Nf1+/+* cells; and, (4) untreated mice reconstituted with *Nf1-/-* cells. The results of this experiment are shown in Figures 3 and 4. Surprisingly, treatment with MM was associated with a dramatic rise in total leukocyte and myeloid cell counts in recipients engrafted with *Nf1-/-* or wild type fetal liver cells. White blood cell counts in excess of 100,000 per mm^3 were detected in some *Nf1-/-* recipients, and bone marrow smears revealed myeloid hyperplasia. The lack of statistical significance in MM-treated mice engrafted with *Nf1-/-* fetal liver cells after 8 weeks is due to the fact that 2 animals that had white blood cell counts > 100,000 per mm^3 after 6 weeks died before the end of treatment. Spleen weights were greater in wild type recipients that received MM than in untreated controls

(mean spleen weight 0.21 gm vs 0.03 gm in the untreated mice; p = 0.004), but not in *Nf1-/-* recipients (0.39 gm vs. 0.35 gm in untreated mice with MPD). In contrast, red blood cell counts declined in both cohorts of MM-treated mice, although this difference did not achieve statistical significance in the *Nf1-/-* group (Figure 4). We are presently analyzing liver and spleen sections from these mice. It is not clear why we observed a dramatic leukocytosis in MM-treated transplant recipients, but not in the wild type mice treated in our pilot experiments. We are presently treating additional untransplanted wild type mice in an effort to address this question.

Drs. Bollag and Shannon have initiated cell biologic and biochemical studies to further elucidate the basis of this unexpected response to MM. Assays of bone marrow colony forming unit granulocyte macrophage (CFU-GM) numbers revealed a decline in MM-treated *Nf1-/-* mice that did not achieve statistical significance, and a slight increase in the wild type controls (Figure 5A). In contrast, *Nf1-/-* recipients had a slight rise in splenic CFU-GM numbers from abnormally high baseline levels, while wild type mice showed a highly significant increase (Figure 5B). Thus, accelerated myelopoiesis in MM-treated wild type mice is associated with the appearance of substantial numbers of splenic CFU-GM. Our preliminary biochemical data indicate that exposing myeloid cells to mycophenolic acid (the active metabolite of MM) *in vitro* is associated with a reduction in cellular GTP levels.

Plan for the Next Year of Funding. The paradoxical increase in leukocyte counts seen in mice treated with MM preclude the therapeutic use of this agent in JMML. However, it is important to determine if we have succeeded in reducing GTP levels in primary cells and, if this is true, how this has altered Ras activation in resting and in growth factor-stimulated cells. We have previously measured mitogen activated protein (MAP) kinase signaling in primary cells from mice treated with a farnesyltransferase inhibitor (Mahgoub et al., 1999), and have developed assays to examine activation of Akt and Ras•GTP levels. One potential explanation is that reducing cellular GTP levels has had a pronounced effect on inhibitory GTP-binding proteins, and that this has led to unrestrained Ras signaling. Because our results have implications for the general strategy of reducing GTP levels in *Nf1*-deficient cells, we will examine the biochemical effects of MM on Ras signaling in myeloid cells over the next few months.

Preclinical Evaluation if a DT_{ct}GM-CSF Recombinant Fusion Toxin. Investigations of myeloid progenitor colony growth in JMML patients and *Nf1-/-* mice have implicated hypersensitivity to GM-CSF in leukemogenesis. Studies performed in our laboratory through NIH grant RO1 CA72614 since this Army proposal was funded provide additional support for this hypothesis. We developed embryos with homozygous disruptions of both *Nf1* and *Gmcsf* genes by breeding *Nf1* and *Gmcsf* mutant mice, and transferred these cells into irradiated wild type or *Gmcsf*-deficient recipients. Development of the JMML-like MPD that follows adoptive transfer of *Nf1-/-* cells was delayed, but not eliminated, in *Gmcsf-/-* hosts that survived with a graft of *Gmcsf-/- x Nf1-/-* cells. We also performed secondary adoptive transfer experiments in which bone marrow cells from wild type recipients that had developed MPD after receiving *Gmcsf-/- x Nf1-/-* fetal liver cell grafts was transferred into wild type or *Gmcsf-/-* hosts. Although all of the wild type recipients of these cells developed MPD, the *Gmcsf-/-* hosts did not. Furthermore, GM-CSF treatment promptly induced MPD in secondary recipients of *Gmcsf-/- x Nf1-/-* cells, but had no effect in mice reconstituted with *Gmcsf-/- x Nf1+/+* cells. Together these data, which are summarized in Figure 5, implicate GM-CSF as playing a central role in the pathogenesis of JMML and suggest that inhibiting this signaling pathway might provide therapeutic benefit.

One aspect of Technical Objective 1 involves production of highly purified recombinant DT_{ct}GM-CSF fusion toxin and *in vitro* and *in vivo* murine studies in the *Nfl* mouse model system with the goal of specifically delivering the diphtheria toxin to myeloid cells via the GM-CSF receptor. Substantial progress has been achieved in accomplishing this objective. The work in the first year of support has focused upon the large-scale production and purification of the murine DT_{ct}GM-CSF fusion toxin in at a high quality clinical grade appropriate for *in vivo* murine studies. We have successfully developed a refined expression and purification method for DT_{ct}GM-CSF that overcomes problems with product aggregation and denaturation during production when standard purification methods are employed. We have also initiated *in vitro* biochemical and functional characterization of DT-mGMCSF. Work is proceeding on-schedule for the investigations proposed for this research project.

DT_{ct}GM-CSF Immunotoxin Production. The structure of the recombinant murine growth factor-toxin fusion expression vector pET11d-DT-mGMCSF is shown in comparison to native diphtheria toxin in Figure 7A, and the construction of DT_{ct}GM-CSF is depicted in Figure 7B. In summary, a synthetic cDNA encoding murine GM-CSF using *E. coli* codon preferences was obtained from R & D Systems (Minneapolis, MN). The polymerase chain reaction (PCR) was used for mutagenesis of the murine *Gmcsf* gene to add NcoI and BamHI restriction enzyme to 5' end and 3' end respectively. The PCR primers for murine *Gmcsf* included the 5' primer mGM1F (5'-CCCATGGCACCCACCCGCTCACCC-3') and the 3' primer mGM1R (5'-GGGGATCCTCATTTTTGGACTGG-3'). The cloning vector pET11:mGMCSF was constructed by the cloning of the murine *Gmcsf* gene cassette downstream of the T7 promoter into the NcoI and BamHI restriction sites of plasmid pET11. PCR mutagenesis of the diphtheria toxin gene was employed to obtain an NcoI gene cassette that encoded 385 amino terminal residues of diphtheria toxin that included the entire ADP-ribosyltransferase catalytic domain and the contiguous proximal portion of the toxin that is associated with translocation across cellular membranes. The mutagenesis of the native diphtheria toxin gene also resulted in the deletion of the coding region for the native toxin binding domain, introduction of coding sequences for a translation initiation ATG codon, a seven residue linker segment for fusion with the *Gmcsf* gene, and convenient flanking NcoI restriction enzyme sites for cloning. The diphtheria toxin gene PCR mutagenesis primers included a 5' primer (5'-GCCATGGGCGCTGATGATGTTGTTGATTC-3') introducing an NcoI restriction enzyme site and ATG codon, and a 3' primer (5' GCCATGGAGCCACCTCCACCCGATTTATGCCCCGGAGAATACGC-3') incorporating sequences encoding a linker domain for steric spacing of the murine GMCSF gene and an NcoI restriction enzyme site. The expression plasmid pET11dDT-mGMCSF was constructed by the cloning of the intact DT NcoI gene cassette into the NcoI site of pET11d-GMCSF as shown in Figure 6. Cloning strategies and other genetic manipulations were positioned to assure maintenance of the translational reading frame, and fidelity of PCR amplification and genetic constructions were confirmed by DNA sequencing. Oligonucleotide primers were synthesized with an Applied Biosystems 394 DNA synthesizer at the University of Minnesota Microchemical Facility. Plasmid DNAs were prepared by use of the Wizard DNA purification resin (Promega, Madison, WI). DNA fragments amplified by the polymerase chain reaction (PCR) were initially cloned into the pT7Blue vector as directed by the manufacturer (Novagen), with DNA sequencing confirmation by the dideoxy method of Sanger using CircumVent thermal

cycling reagents (New England Biolabs, Beverly, MA). Restriction endonucleases, Taq DNA polymerase, and T4 DNA ligase were procured from BRL-Life Technologies (Gaithersburg, MD), Promega, New England Biolabs, or Perkin Elmer (Norwalk, CT), and used according to the specifications directed by the manufacturer. Standard techniques were employed for other manipulations of DNA including agarose gel electrophoresis, isolation and purification of restriction endonuclease fragments, cloning, and plasmid transformation into bacteria.

Expression and Purification of the Recombinant Fusion Toxin DT_{ct}GM-CSF.
All manipulations of *E. coli* bearing intact recombinant fusion toxin were performed under modified Biosafety Level 3 (BL3) containment practices. Initial attempts to achieve high level expression using techniques in Dr. Perentesis' laboratory that were developed for other fusion toxins resulted in production of relatively low yields for DT_{ct}GM-CSF because of product accumulation in inclusion bodies and aggregation. He an his colleagues subsequently successfully developed a refined production and expression method to isolate the product from bacterial inclusion bodies that yields high quality purified recombinant DT_{ct}GM-CSF. These refined methods are detailed in Figure 8, and include the addition of inclusion body isolation and protein recovery and renaturation procedures. In summary, *E. coli* HMS174(de3)plysS is transformed with pET11d:DT-mGMCSF and grown at 37 °C in LB medium with carbenicillin (50 μg/ml) to an absorbance (Å595) of 0.55-0.65. Expression of the fusion gene is induced by the addition of isopropyl-B-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The bacterial cells are then collected by centrifugation after one hour of induction. The bacterial pellets are resuspended in TE buffer (50mM Tris/20mM EDTA/100mMNaCl pH 7.8). Lysis of the cells is achieved by adding 5 mg/ml of lysozyme and incubating 30 min in 40 °C. The insoluble extract containing is resuspended in Triton-X buffer (89%TE buffer/11%vol/vol Triton-X) and homogenized briefly with a tissumizer. After incubation at room temperature for 1 hour, the inclusion bodies are obtained by ultracentrifugation at 24,000 g for 50 min and solubilized in solubilization buffer (7M guanidine/0.1M Tris pH:8/2mM EDTA/65M dithioerythriotol) overnight at room temperature. The solubilized protein is collected by ultracentrifuge at 40,000g for 10 min, then diluted 100 times in refolding buffer (0.1M Tris pH:8/0.5M L-arginine/0.9mM Oxidized glutathione/2mM EDTA/0.1M Urea) for 48 hours at 100 °C. The refolded protein is diafiltrated and ultrafiltrated against 20mM Tris pH:7.8/100mM Urea, then loaded on to Q-sepharose column and eluted with 0.3M NaCl in 20mM Tris pH:7.8. The eluted protein is diluted 5 times with 20mM Tris, then loaded on to Q-sepharose column again and eluted with linear salt gradient from 0.06-0.4M NaCl in 20mM Tris pH:7.8. Final size-exclusion purification is conducted using a TSK-gel G2000 column.

In Vitro Characterization of Recombinant DT_{ct}GM-CSF. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses using equine diphtheria antitoxin (anti-DT; Bethyl) antibodies were performed by standard methods using 10% gels in a Mini-Protean II gel apparatus (Bio-Rad). Diphtheria toxin standards were obtained from Sigma. Primary antibodies were used at a dilution of 1:5000. Secondary antibodies, rabbit anti goat(Bethyl laboratories), covalently linked to horseradish peroxidase were used at a 1:5,000 dilution. SDS-polyacrylamide gel analysis of the uninduced and induced whole cell extract, Q-sepharose chromatography column fractions revealed high level production and >95% purity of a monomeric protein with a molecular mass of ~57 kDa, the expected molecular mass of DT_{ct}GM-

CSF as deduced from its nucleic acid sequence. The final product of TSK-gel G2000 purification is estimated to exceed 98% purity (Figure 8). The integrity of expression of both the diphtheria toxin and GM-CSF moieties of DT_{ct}GM-CSF was confirmed in immunoblot analysis employing antisera to diphtheria toxin (Figure 9).

Plans for the Next Year of Funding. In year 2, we plan to continue and complete the studies of DT_{ct}GM-CSF in *in vitro* models of hematopoiesis. These studies will include experiments to inhibit the CFU-GM colony formation in methylcellulose cultures established from *Nf1+/+*, *Nf1+/-*, and *Nf1-/-* fetal liver cells, and of bone marrow cells of mice previously engrafted with fetal liver cells of all three *Nf1* genotypes. We plan to also initiate the *in vivo* treatment of *Nf1* and wild-type mice with DT-GMCSF that will extend through years 2-3 of this proposal. Groups of 5 wild-type mice will be treated with daily i.p. doses for 5 treatment days for a total of 1 μg, 2 μg, 5 μg, 10 μg, or 20 μg murine DT_{ct}GMCSF. These regimens were chosen since they were well tolerated and yielded very encouraging results in the preliminary studies using human DT_{ct}GMCSF. The dose-finding study will be expanded with either lower or higher dose ranges depending on the observed toxicity. In treatment regimens involving *Nf1* mice, the same numbers of *Nf1-/-* and *Nf1+/+* transplant recipients will be enrolled in phase 1 and phase 2 experiments and the clinical and laboratory observations will be identical. However, the phase 1 experiment will also involve treating a cohort of the mice that are homozygous for a mutation in the β chain of the GM-CSF receptor (GMRβ) with DT_{ct}GMCSF (Nishinakamura et al., 1995). Cells from these mice do not express the GM-CSF receptor, and should therefore be resistant to the toxic effects of DT_{ct}GMCSF. The Shannon laboratory has obtained these mice and has crossed them with *Nf1* mice. *GMRβ-/-* mice provide an excellent model for testing the specificity of DT_{ct}GMCSF therapy, and we predict that they will show no changes in blood cell counts even at high doses of the recombinant fusion toxin.

Technical Objective (Aim) 2: Chemotherapy and Radiation Studies

Overview. These studies are based upon clinical observations which suggested that children with NF1 are at increased risk of developing myeloid and other tumors after being treated with multi-modal therapy for another cancer (Maris et al., 1997). These human data implicated exposure to alkylating agents in the development of therapy-related leukemia (t-ML). Based on these clinical findingd and on the 10% risk of leukemia in untreated *Nf1+/-* mice (Jacks et al., 1994), we exposed *Nf1+/-* mice to mutagenic agents frequently used to treat malignancies in patients with and without NF1 (Mahgoub et al., 1999). Preliminary data from these studies were presented in our original application. We have now completed our analysis of this cohort as described in the appended article (Mahgoub et al., 1999). Initially, mice were enrolled from the inbred 129Sv strain in which the *Nf1* mutation was created. In order to perform LOH analysis at loci other than *Nf1* in alkylator-treated mice, F1 offspring from a cross between the 129Sv and C57BL6 strains were used in the latter part of the experiment. *Nf1+/-* and *Nf1+/+* littermates were assigned to observation (control group) or to receive 6 weeks of treatment with either etoposide or CY beginning at 6-10 weeks of age.

Myeloid leukemia or MPD developed in 4 of 101 *Nf1+/+* mice, 2 of which received CY (Table 1). In contrast, MPD was diagnosed in 14% of the untreated *Nf1+/-* mice (8 of 58), in

25% of the etoposide-treated animals (8 of 32), and in 43% (16 of 37) of the mice assigned to the CY group (Table 1). A Kaplan-Meier analysis demonstrated that the incidence of MPD was significantly higher and the latency period was reduced in CY-treated mice ($p = 0.001$ vs. untreated *Nf1*+/- mice), but not in the etoposide group ($p = 0.2$ vs. the untreated group). Both control and CY-treated 129Sv *Nf1*+/- mice showed higher rates of MPD than the corresponding groups of 129Sv x C57BL6 mice (Table 1). Most of the treated and control mice developed MPD with large numbers of mature neutrophils and monocytes in the peripheral blood. Bone marrow examination revealed 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. Frank myeloid leukemia was diagnosed in 2 of the mice (1 etoposide-treated and 1 CY-treated). LOH at *Nf1* correlated with clinical evidence of MPD in *Nf1*+/- mice and this invariably involved loss of the wild-type *Nf1* allele. Within the CY-treated group, leukemic cells from 129Sv x C57BL6 mice showed a much lower incidence of LOH than cells from 129Sv animals (Mahgoub et al., 1999).

Table 1
Incidence of Leukemia in *Nf1*+/- Mice

Strain and Treatment	Genotype	No. of Mice	No. (and %) with Leukemia
129Sv			
None	*Nf1*+/-	46	8 (17%)
Etoposide	*Nf1*+/-	32	8 (25%)
Cyclophosphamide	*Nf1*+/-	12	7 (58%)
129Sv x C57BL6			
None	*Nf1*+/-	12	0 (0%)
Cyclophosphamide	*Nf1*+/-	25	9 (36%)

This *in vivo* model of t-ML has a number of novel features that facilitate basic and translational research studies of this important clinical disorder. First, the fact that *Nf1* mice recapitulate clinical observations made in NF1 patients suggest that this model will be highly relevant for understanding specific aspects human t-ML. Second, *Nf1* provides a genetic target to examine the mechanism(s) of alkylator-induced DNA damage in hematopoietic cells. Finally, this model allows us to undertake controlled experiments that are neither feasible nor ethical in humans. We are exploiting this system to ask if radiation therapy, alone and in combination with CY, accelerates tumorigenesis in heterozygous *Nf1* mice. This question is highly relevant to the care of individuals with NF1 because radiation therapy is used frequently to treat NF1-associated tumors. Indeed, our initial data led the Children's Cancer Group to modify the treatment of children with NF1 who develop brain tumors so that they are not assigned to alkylator-intensive regimens. In addition to treating *Nf1* mutant mice with radiation and CY, we are performing molecular analyses at the *Nf1* locus and are examining the incidence of hypoxanthine guanine phosphoribosyl transferase *(Hprt)* mutations as an *in vivo* measure of DNA damage.

In year 1, the technical objectives related of this aspect of the project were (1) to breed and genotype mice for the proposed studies, and (2) to perform pilot studies of CY + radiation to establish a dose for treating mice. We have accomplished these goals and have begun enrolling the treatment groups.

Pilot Treatment of *Nf1*+/- Mice with CY and Irradiation. The mice treated with CY in our initial study were exposed to 100 mg/kg/week for 6 weeks. This dosing schedule was based upon data provided to us by Dr. Peter Houghton (St. Jude Children's Research Hospital) and on pilot data we generated in 129Sv mice. In the course of the experiment, we noticed that many F1 129Sv/C57BL6 mice maintained normal neutrophil counts (data not shown). This resistance to neutropenia seen in the 129Sv/C57BL6 mice may explain the lower incidence of MPD seen in this genetic background (Table 1). We therefore performed a dose escalation study to define the maximally tolerated dose of CY in 129Sv/C57BL6 mice and observed significant leukopenia that was associated with neutropenia, but with minimal morbidity, at 200 mg/kg/week for 6 weeks (Figure 10).

We next examined the feasibility of combining this dose of CY with total body irradiation in 129Sv x C57BL6 mice. In these studies, mice received a single fraction of either 2 or 3 Gray (200 or 300 rads) of total body irradiation as a single fraction two weeks after the last dose of CY. The use of total body irradiation insures that all of the blood-forming marrow is exposed, and previous data have shown that 2-3 Gy is more leukemogenic than higher or lower doses in susceptible mouse strains (Major and Mole, 1978). We found that *Nf1*+/- 129Sv x C57BL6 F1 mice tolerated these treatment regimens well with no deaths occurring during or after the radiation phase of the study. White blood cell counts are shown in Figure 11. Based on these results, we will compare CY + 3 cGy, with CY alone, irradiation alone, and no treatment as shown in Table 2. In order to minimize the number of animals treated, we plan to assign fewer mice to the control and CY alone arms (groups 1, 2, 5, and 6) because we have already ascertained the expected incidence of myeloid diseases in these cohorts. We began enrolling mice in July, 1999 and have entered 42 to date. An additional 37 mice have been genotyped and will begin treatment later this month.

Table 2
Treatment Schedule for F1 C57BL6/129Sv *Nf1*+/- and *Nf1*+/+ Mice

Group	Number	Genotype	Treatment	Schedule
1	15	*Nf1*+/-	None	Untreated Control
2	15	*Nf1*+/+	None	Untreated Control
3	30	*Nf1*+/-	Radiation	300 cGy x 1 dose
4	30	*Nf1*+/+	Radiation	300 cGy x 1 dose
5	15	*Nf1*+/-	CY	200 mg/kg/week i.p. x 6 weeks
6	15	*Nf1*+/+	CY	200 mg/kg/week i.p. x 6 weeks
7	30	*Nf1*+/-	CY + Radiation	200 mg/kg/week i.p. x 6 weeks, then 300 cGy x 1 dose
8	30	*Nf1*+/+	CY + Radiation	200 mg/kg/week i.p. x 6 weeks, then 300 cGy x 1 dose

Hprt Inactivation as a Surrogate Marker for Chemotherapy-Induced DNA Damage in Myeloid Cells Hypoxanthine guanine phosphoribosyl transferase (HPRT) is a cellular enzyme crucial in the metabolism of the chemotherapeutic agent thioguanine into deoxythioguanine triphosphate which can then be incorporated into DNA and cause cell death. Lymphocytes that have

inactivated *Hprt* acquire the ability to proliferate in the presence of 6-thioguanine because they are unable to convert this drug to its active metabolite. *Hprt* mutation rate has been used as a surrogate marker for DNA damage induced by mutagenic compounds such as CY and irradiation, and for evaluating potential chemoprotective compounds (Kataoka et al., 1996; Meng et al., 1998). However, it is not known if reducing the frequency of *Hprt* inactivation will correlate with a decrease in the risk of therapy-related cancer *in vivo.* If this proves true, *Hprt* could be used as a surrogate marker to test the mutagenic potential of new chemotherapeutic agents and of specific regimens. Our *Nf1* mouse model provides the first opportunity to rigorously address this important question. *Hprt* is an attractive marker for ascertaining mutagenic damage in this model because somatic inactivation of the relevant target genes *(Hprt* or *Nf1)* contributes to clonal proliferation in both instances.

We recently established the *Hprt* assay in our laboratory using published methods (Meng et al., 1998). *Hprt* mutation frequency is measured in male mice because this locus is on the X chromosome. We initiated these studies in C57BL6/129Sv mice treated with CY at a dose of 200 mg/kg/week for either 1 week (single dose) or for 6 weeks. The mice are sacrificed and splenocytes are isolated 55-60 days after the last drug dose. Preliminary data from a cohort of mice treated for 1 week showed a greater than 10 fold increase in the mutation frequency of CY-treated mice compared to controls (mutation frequency = 4.7×10^{-5} in CY-treated mice vs. 3.3×10^{-6} in the controls; $p = 0.038$).

In Vitro Transcription Translation (IVTT) Assay to Screen Murine Tissues for *Nf1* Mutatons. Most of the mutations that cause NF1 involve nucleotide substitutions or small deletions/insertions that lead to premature termination of protein translation (Upadhyaya et al., 1994). Heim et al. (Heim et al., 1995; Heim et al., 1994) described an IVTT assay to screen human patient samples for mutations in *NF1* which we used successfully to define mutations in childhood leukemias (Side et al., 1998; Side et al., 1997). We have now developed oligonucleotide primers and amplification conditions for screening murine *Nf1* by IVTT (data not shown). This assay will allow us to correlate the incidence and spectrum of somatic mutations that occur at the *Nf1* locus in murine tumors with treatment group (i.e. CY alone, radiation alone, or CY + radiation). These studies will allow us to test the hypothesis that alkylating agents and radiation inactivate target genes by distinct mechanisms.

Plan for the Next Year of Funding. Having achieved our goals of defining a treatment schedule and establishing an active breeding colony in year 1, we will now finish enrolling and treating the cohorts of mice shown in Table 2. We will observe these mice and will begin collecting follow-up data. We will also compare *Hprt* mutation rates in untreated mice and in mice treated with CY alone, radiation alone, or CY + radiation to test the hypothesis that *Hprt* mutation rates will predict survival and cancer rates. We will also investigate any murine leukemias or solid tumors for LOH at *Nf1,* and will perform IVTT to define the incidence and nature of somatic *Nf1* mutations in samples that do not show LOH.

(7) KEY RESEARCH ACCOMPLISHMENTS

- Developed breeding stocks of mouse strains and generated recipients repopulated with *Nf1-/-* or wild-type fetal liver cells.

- Completed preclinical study of MM dosing in wild type mice and selected drug dose for preclinical efficacy study.
- Performed preclinical evaluation of MM in transplant recipients.
- Initiated correlative biochemical studies of tissues from MM-treated mice.
- Developed strategy and construct for making DT_{ct}GM-CSF, and produced sufficient amounts of this compound for *in vitro* and *in vivo* testing.
- Performed preclinical studies to determine optimal regimen for administering cyclophosphamide with irradiation, and enrolled initial cohorts of mice.

(8) REPORTABLE OUTCOMES

(a) Review Article

Weiss B, Bollag G, Shannon KM. Hyperactive Ras as a therapeutic target in neurofibromatosis type 1. *Am J Med Genet* 1999; **89:** 14-22.

(b) Model Development

The studies conducted to date have established a regimen for administering cyclophosphamide with and without radiation to F1 C56BL6/129Sv mice that should be useful for future studies of tumorigenesis and chemopreventive strategies in *Nf1* mice.

(c) Employment and Research Opportunities

Richard Chao, M.D. is a fellow in adult hematology/oncology who is supported by this award. Dr. Chao is primarily working on the radiation/cyclophosphamide studies. He is interested in pursuing a career in translational research.

Brian Weiss, M.D. is a fellow in pediatric hematology/oncology who is participating in the experimental therapeutics studies. His salary is supported by a training fellowship from the Frank A. Campini Foundation.

Alfrd Au, Charles Fezzie, Zabi Wardak, Myla Sanchez Vikas Arora, and Abigail Peterson are technical personnel in the investigator's laboratories who have received partial salary support from this award.

(9) CONCLUSIONS

Our progress on each Technical Objective with plans for the duration of this award are presented in detail in the Body. The nature of translational research is that it involves considerable effort in generating reagents, performing the experiments, and obtaining long-term follow-up. These studies are proceeded well to date and are on or ahead of schedule. We tentatively conclude that MM is unlikely to provide benefit in treating tumors that arise in individuals with NF1.

(10) REFERENCES

Bollag, G., Clapp, D. W., Shih, S., Adler, F., Zhang, Y., Thompson, P., Lange, B. J., Freedman, M. H., McCormick, F., Jacks, T., and Shannon, K. (1996). Loss of *NF1* results in activation of the Ras signaling pathway and leads to aberrant growth in murine and human hematopoietic cells. Nature Genet *12*, 144-148.

Heim, R., Kam-Morgan, L., Binnie, C., Corns, D., Cayouette, M., Farber, R., Aylsworth, A., Silverman, L., and Luce, M. (1995). Distribution of 13 truncating mutations in the neurofibromatosis 1 gene. Human Mol Genet *4*, 975-981.

Heim, R., Silverman, L., Farber, R., Kam-Morgan, L., and Luce, M. (1994). Screening for truncated NF1 proteins. Nature Genet *8*, 218-219.

Jacks, T., Shih, S., Schmitt, E. M., Bronson, R. T., Bernards, A., and Weinberg, R. A. (1994). Tumorigenic and developmental consequences of a targeted *Nf1* mutation in the mouse. Nature Genet *7*, 353-361.

Kalra, R., Paderanga, D., Olson, K., and Shannon, K. M. (1994). Genetic analysis is consistent with the hypothesis that *NF1* limits myeloid cell growth through $p21^{ras}$. Blood *84*, 3435-3439.

Kataoka, Y., Perrin, J., Hunter, N., Milas, L., and Grdina, D. J. (1996). Antimutagenic effects of amifostine: clinical implications. Semin Oncol *23 (suppl 8)*, 53-57.

Largaespada, D. A., Brannan, C. I., Jenkins, N. A., and Copeland, N. G. (1996). *Nf1* deficiency causes Ras-mediated granulocyte-macrophage colony stimulating factor hypersensitivity and chronic myeloid leukemia. Nature Genet *12*, 137-143.

Mahgoub, N., Taylor, B., Le Beau, M., Gratiot, M., Carlson, K., Jacks, T., and Shannon, K. M. (1999). Myeloid malignancies induced by alkylating agents in Nf1 mice. *Blood 93*, 3617–3623

Mahgoub, N., Taylor, B. R., Gratiot, M., Kohl, N. E., Gibbs, J. B., Jacks, T., and Shannon, K. M. (1999). In vitro and In vivo effects of a farnesyltransferase inhibitor on Nf1- deficient hematopoietic cells. Blood *94*, 2469-76.

Major, I. R., and Mole, R. H. (1978). Myeloid leukaemia in x-ray irradiated CBA mice. Nature *272*, 455-6.

Maris, J. M., Wiersma, S. R., Mahgoub, N., Thompson, P., Geyer, R. J., Lange, B. J., and Shannon, K. M. (1997). Monosomy 7 myelodysplastic syndrome and other second malignant neoplasms in children with neurofibromatosis type 1. Cancer *79*, 1438-46.

Meng, Q., Skopek, T. R., Walker, D. M., Hurley-Leslie, S., Chen, T., Zimmer, D. M., and Walker, V. E. (1998). Culture and propagation of Hprt mutant T-lymphocytes isolated from mouse spleen. Environ Mol Mutagen *32*, 236-43.

Miles, D. K., Freedman, M. H., Stephens, K., Pallavicini, M., Sievers, E., Weaver, M., Grunberger, T., Thompson, P., and Shannon, K. M. (1996). Patterns of hematopoietic lineage involvement in children with neurofibromatosis, type 1, and malignant myeloid disorders. Blood *88*, 4314-4320.

Nishinakamura, R., Nakayama, N., Hirabayashi, Y., Inoue, T., Aud, D., McNeil, T., Azuma, S., Yoshida, S., Toyoda, Y., Arai, K., Miyajima, A., and Murray, R. (1995). Mice deficienct for the IL-3/GM-CSF/IL-5 ßc receptor exhibit lung pathology and impaired immune response, while $ß_{IL3}$ receptor-deficienct mice are normal. Immunity *2*, 211-222.

Shannon, K. M., O'Connell, P., Martin, G. A., Paderanga, D., Olson, K., Dinndorf, P., and McCormick, F. (1994). Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. N Engl J Med *330*, 597-601.

Side, L., Emanuel, P., Taylor, B., Franklin, J., Thompson, P., Castleberry, R., and Shannon, K. (1998). Mutations of the *NF1* gene in leukemias from children without evidence of neurofibromatosis, type 1. Blood *92*, 267-273.

Side, L., Taylor, B., Cayouette, M., Connor, E., Thompson, P., Luce, M., and Shannon, K. (1997). Homozygous inactivation of the NF1 gene in bone marrow cells from children with neurofibromatosis type 1 and malignant myeloid disorders. N Engl J Med *336*, 1713-1720.

Upadhyaya, M., Shaw, D., and Harper, P. (1994). Molecular basis of neurofibromatosis type 1 (NF1): mutation analysis and polymorphisms in the NF1 gene. Human Mutation *4*, 83-101.

Zhang, Y., Vik, TA, Ryder, JW, Srour, EF, Jacks, T, Shannon, K, Clapp, DW (1998). Nf1 regulates hematopoietic progenitor cell growth and Ras signaling in response to multiple cytokines. J Exp Med *187*, 1893-902.

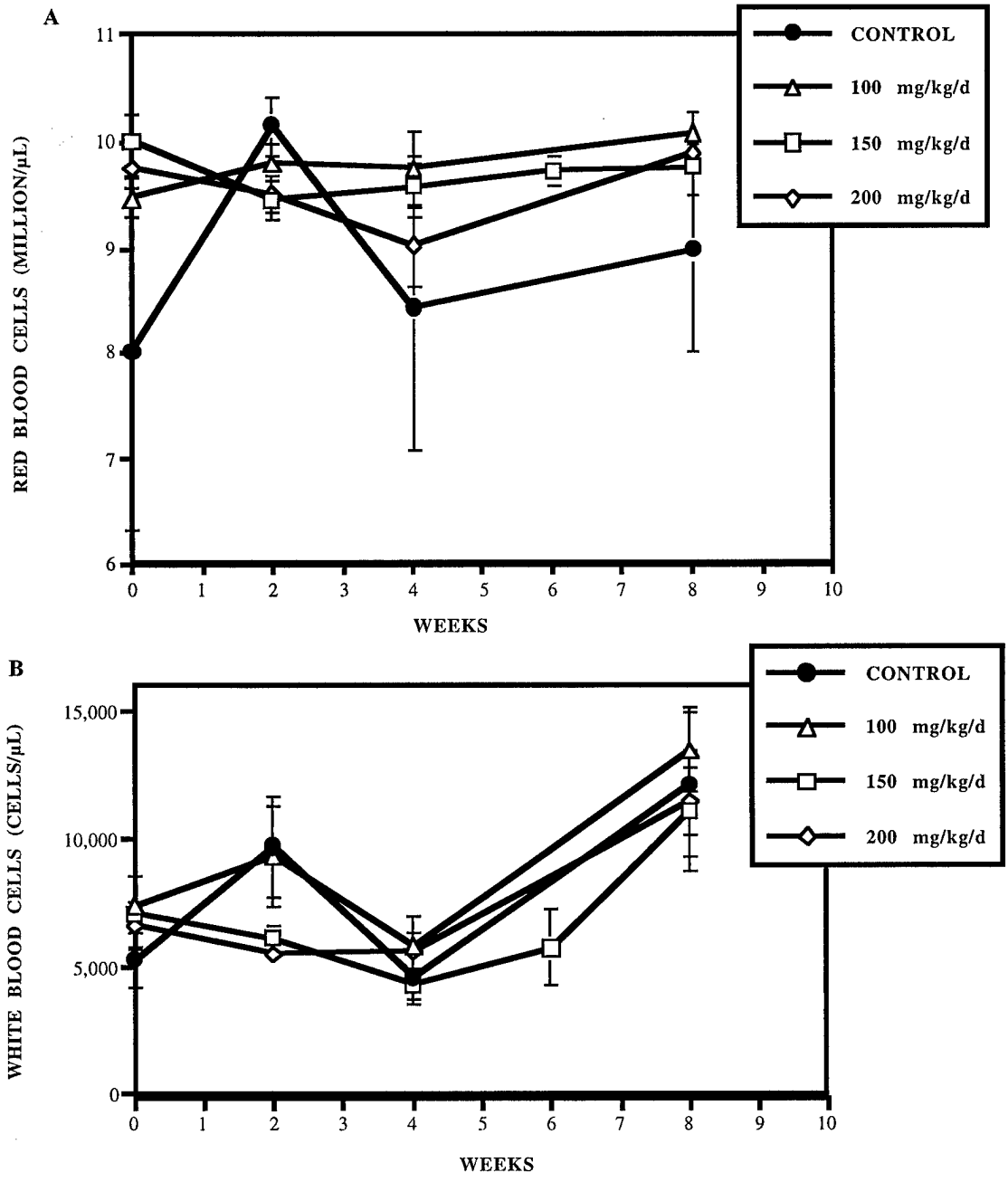


Figure 1. Red blood cell (panel A) and white blood cell counts (panel B) in wild type mice treated with 100-200 mg/kg /day of MM (n = 5 in each group).

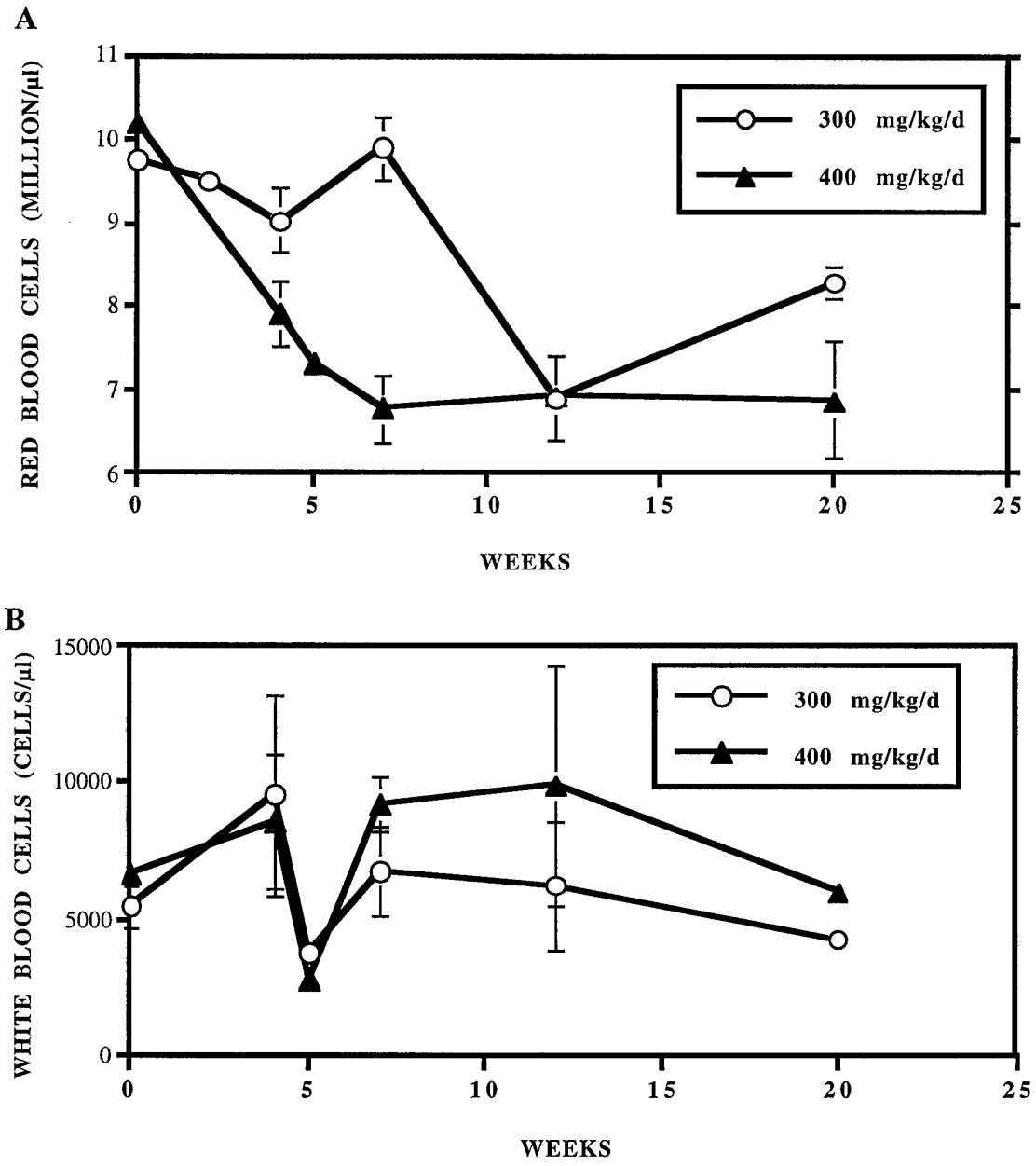


Figure 2 Red blood cell (panel A) and white blood cell counts (panel B) in wild type mice treated with 300 or 400 mg/kg /day of MM for 20 weeks (n = 5 in each group). Hemoglobin and hematocrit values declined in parallel with RBC counts.

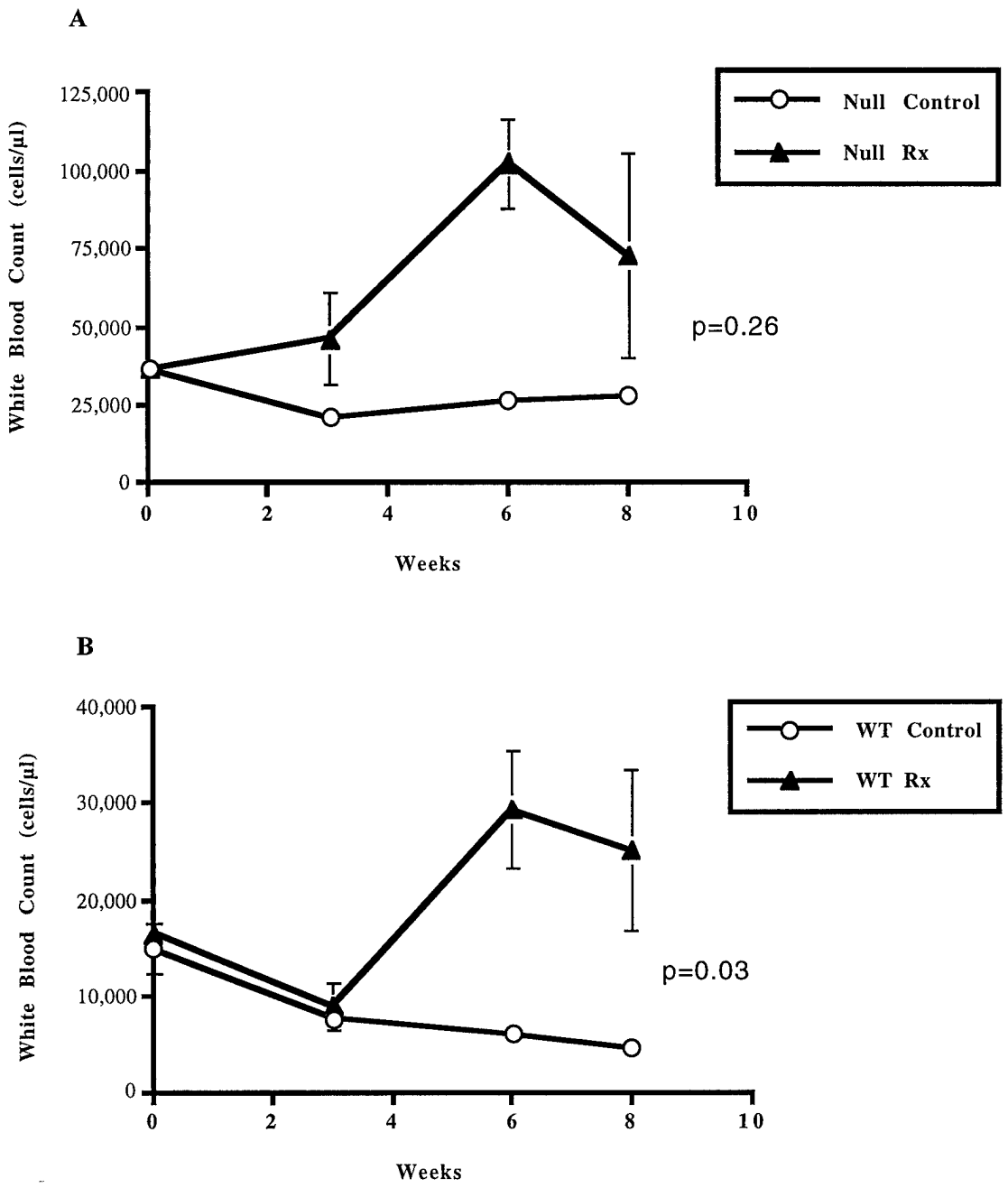


Figure 3. White blood cell counts in recipient mice engrafted with *Nf1-/-* fetal liver cells (panel A) or with wild type fetal liver cells (panel B). Control mice were not treated and "Rx" mice received MM at a dose of 400 mg/kg/day . Note that the baseline white blood cell counts were much higher in recipients of *Nf1-/-* cells. The increase in white blood cell counts was due to a rise in myeloid cells in both groups of Rx mice (n = 7-10 in each group).

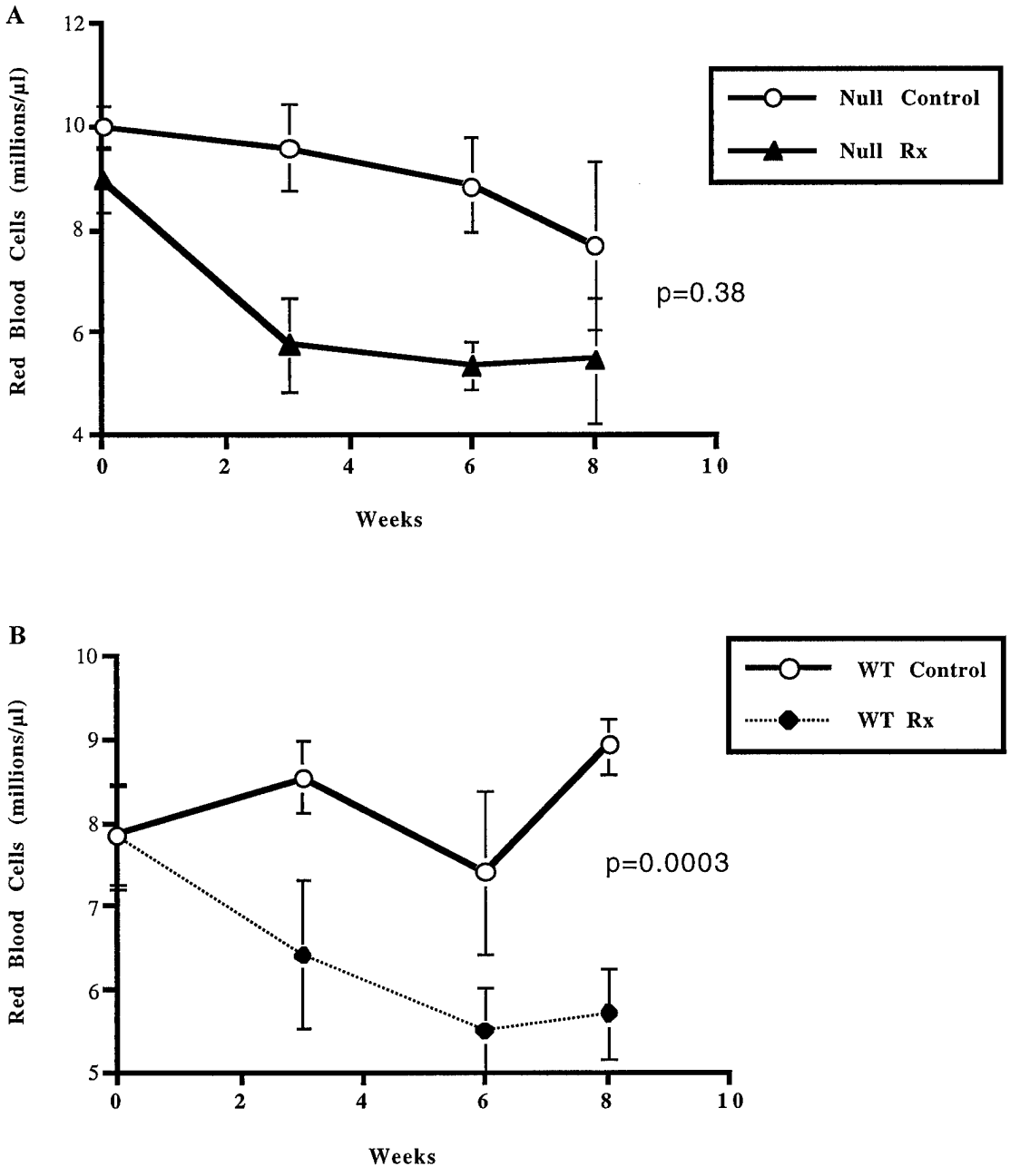


Figure 4. Red blood cell counts in recipient mice engrafted with *Nf1-/-* fetal liver cells (panel A) or with wild type fetal liver cells (panel B). Control mice were not treated and "Rx" mice received MM at a dose of 400 mg/kg/day (n = 7-10 in each group).

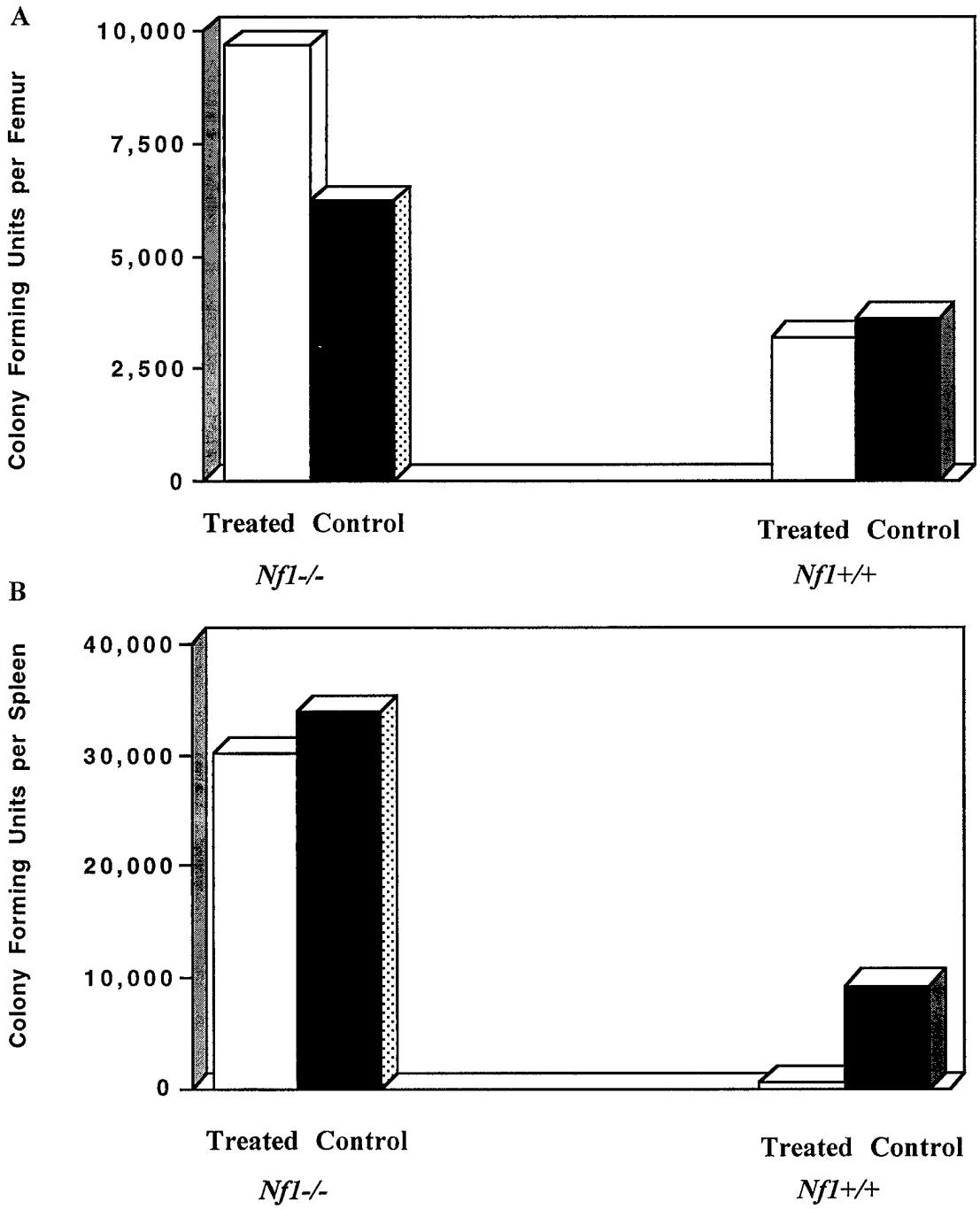
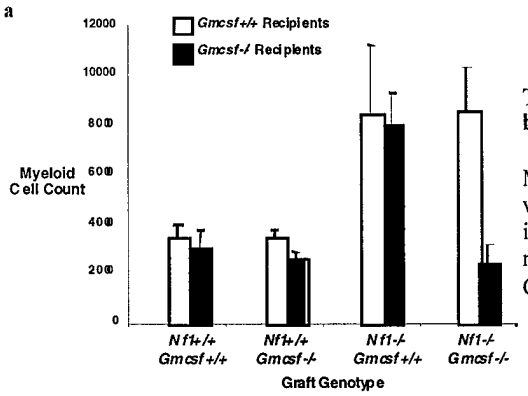


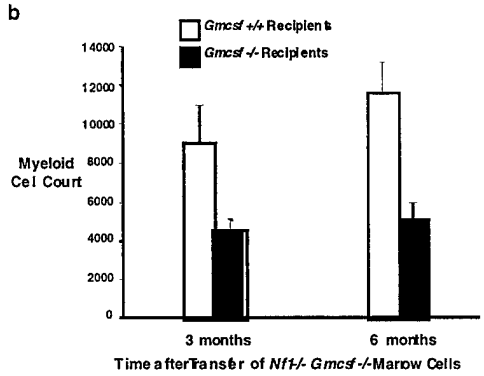
Figure 5. Total numbers of CFU-GM progenitor colonies enumerated in the femurs (panel A) and spleens (panel B) in recipients of *Nf1-/-* or wild type fetal liver cells. Note that the baseline numbers of CFU-GM is markedly expanded in *Nf1-/-* recipients at baseline. (n = 4 in *Nf1-/-* recipients, and n = 6 in wild type recipients).

Figure 6. Influence of *Nf1* and *Gmcsf* Genotype on Myeloid Cell Counts in Primary and Secondary Recipients



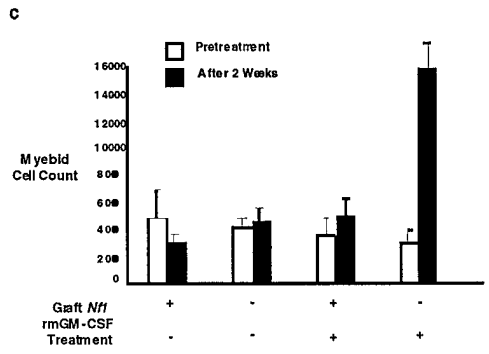
The MPD induced by adoptive transfer of *Nf1* null cells is attenuated by the lack of GM-CSF in primary recipients.

Myeloid cell counts (± SEM) three months after adoptive transfer in surviving mice engrafted with donor fetal liver cells (n = 75). *Gmcsf-/-* recipients of *Nf1-/- Gmcsf-/-* grafts showed lower myeloid cell counts than recipients in which either host ($p<0.01$) or graft ($p<0.005$) could produce GM-CSF.



The MPD induced by the adoptive transfer of *Nf1 -/-* cells is attenuated by the lack of GM-CSF in secondary recipients.

Ten pairs of irradiated secondary wild type (open bars) and *Gmcsf-/-* (closed bars) recipients received the same bone marrow cells. Myeloid cell counts (± SEM) were elevated in wild type versus *Gmcsf-/-*hosts 3 and 6 months later ($p<0.05$ and $p<0.005$, respectively). The myeloid cell counts of the *Gmcsf-/-* recipients are in the normal range for wild type mice.



The MPD can be re-established by treatment with sub-therapeutic doses of GM-CSF in recipients of *Nf1-/- Gmcsf-/-* grafts.

Myeloid cell counts (± SEM) in rmGM-CSF-treated and control recipients immediately prior to and after 2 weeks of treatment with rmGM-CSF. Recipients of *Nf1-/- Gmcsf-/-* grafts that received rmGM-CSF had elevated myeloid counts when compared to all controls ($p<0.01$).

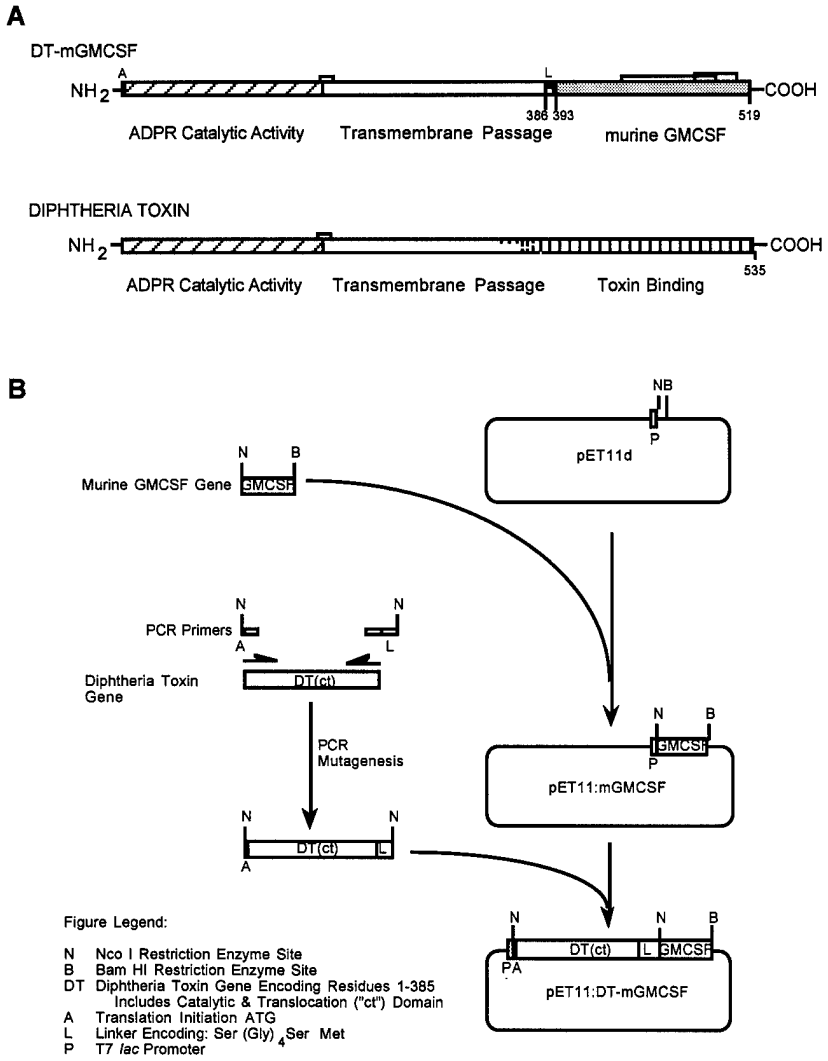


Figure 7. (A) Structure of the DT_{ct}GM-CSF fusion toxin and comparison to diphtheria toxin. The native receptor-binding domain of diphtheria toxin was genetically deleted and replaced with murine GM-CSF, separated by a short linking sequence ("L": Ser-(Gly)4-Ser-Met). The genetic addition of an ATG codon was used to introduce a methionine residue to the amino terminus of the fusion toxin ("A" = Met). (B) Construction of the recombinant growth factor - toxin fusion expression vector pET11d-DT-mGMCSF. A synthetic cDNA encoding mature murine GMCSF was cloned between the NcoI and BamHI sites of plasmid pET11d downstream of the T7 promoter to produce pET11d-GMCSF. PCR mutagenesis of the diphtheria toxin gene was employed to obtain a NcoI DT gene cassette that included: (i) the addition of an ATG methionine translation initiation codon immediately 5' of the initial GGC glycine codon of mature native diphtheria toxin, (ii) a short 3' linker sequence encoding seven amino acid [Ser-(Gly)4-Ser-Met] residues downstream of diphtheria toxin lysine residue 385, and (iii) flanking NcoI restriction endonuclease sites. Expression plasmid pET11dDT-mGMCSF was constructed by the cloning of the intact DT NcoI gene cassette into the NcoI site of pET11d-mGMCSF.

DT_{ct}GM-CSF EXPRESSION AND PURIFICATION

DT_{ct}GM-CSF Transformed HMS174(de3)plysS
↓ OD = 0.6
IPTG - INDUCED CULTURE (1 HR)
↓→ SUPERNATANT
PELLET
↓
LYSIS
↓→ SUPERNATANT
PELLET / TRITON X100
↓
HOMOGENIZATION
↓
24,000 g
↓→ SUPERNATANT
PELLET – INCLUSION BODIES
↓
SOLUBILIZATION (GUANIDINE) 16 HRS 22°C
↓
40,000 g
↓→ PELLET
SUPERNATANT
↓
REFOLDING BUFFER
100 X DILUTION 10°C x 48 HR
↓
DIAFILTRATE / ULTRAFILTRATE
↓
SEPHAROSE 0.3 M NaCl
↓
SEPHAROSE Q GRADIENT 0.06-0.4 M NaCl
↓
TSK-gel G2000
↓
ENDOTOXIN REMOVAL

Figure 8. Expression and purification of the recombinant DT_{ct}GM-CSF fusion toxin (see text for details).

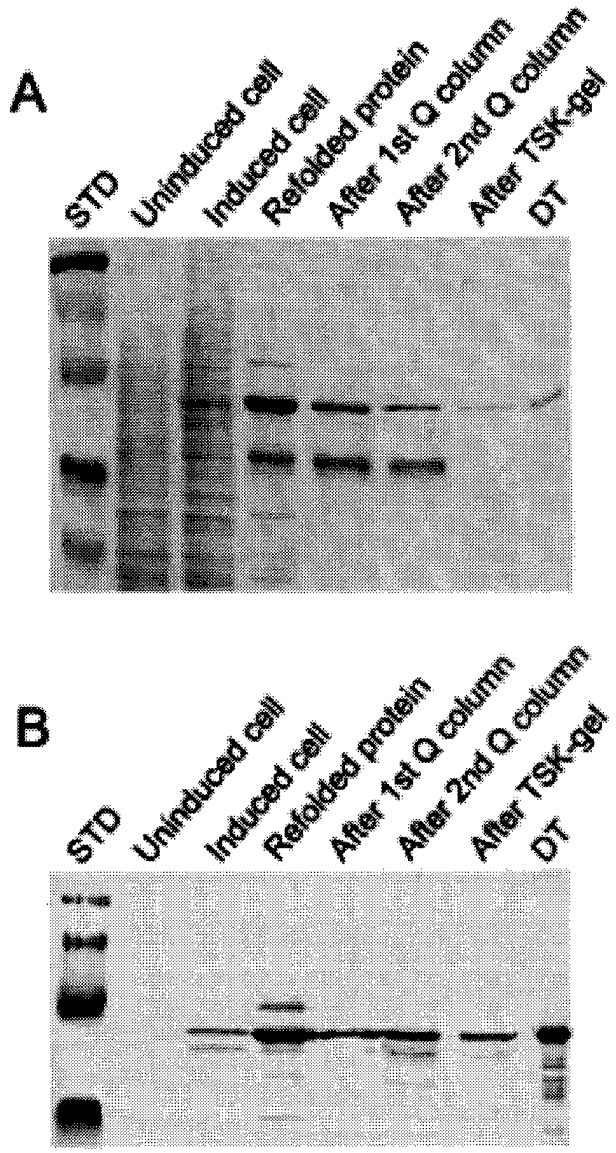


Figure 9. Purification of recombinant DT_{ct}GM-CSF . The expression and sequential Q sepharose columns, TSK-gel G2000 column purification of DT_{ct}GM-CSF from IPTG-induced 500ml cultures of E. coli were analyzed by SDS-PAGE (A), and anti-DT immunoblots (B), detecting the 58 kDa DT-mGMCSF protein. The migration of prestained molecular weight size standards is indicated, and for immunoblot analysis, native DT was used as controls.

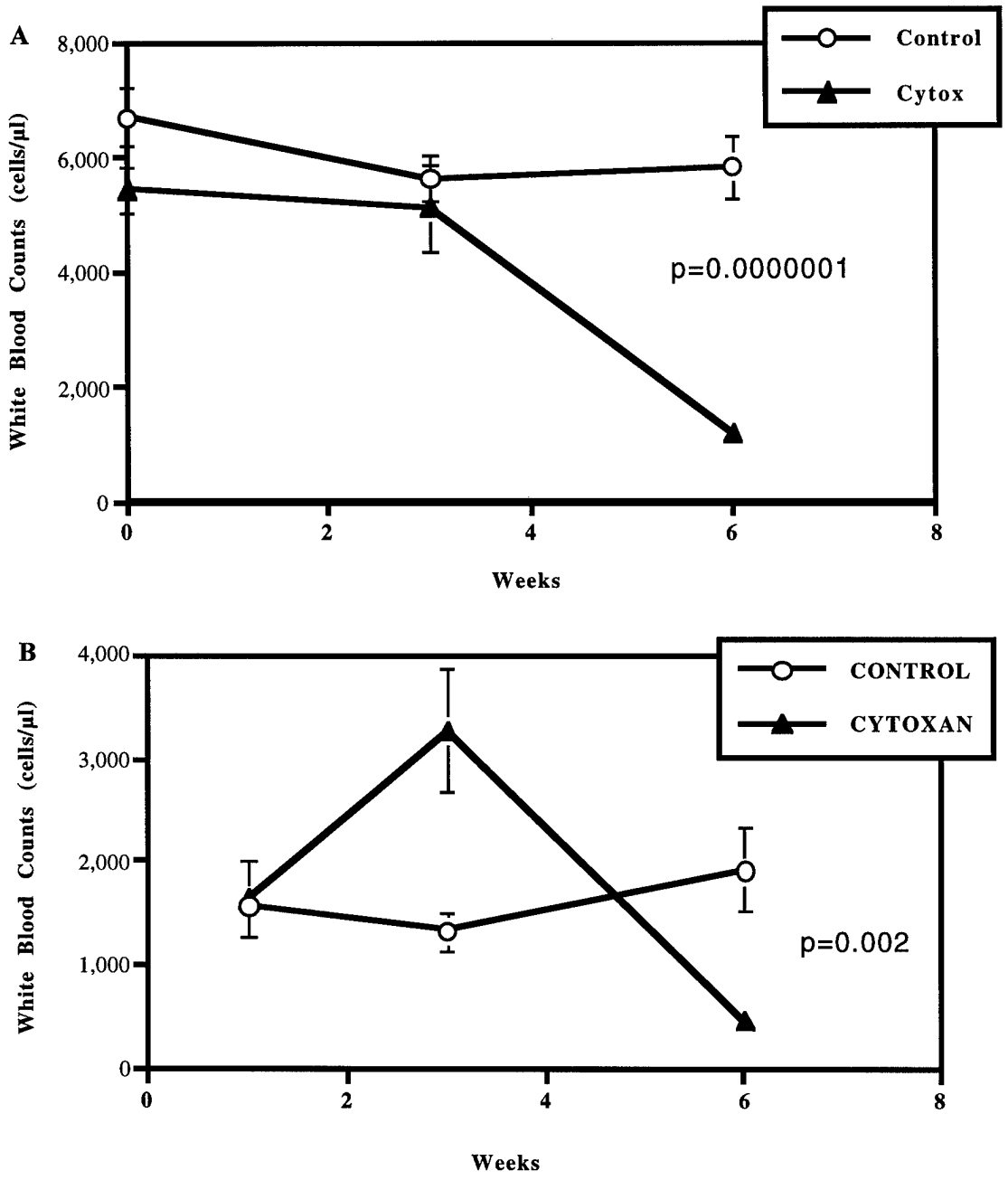


Figure 10. Total white blood cell counts (panel A) and blood neutrophil counts (panel B) in untreated control mice and in mice that received 200 mg/kg/week of cyclophosphamide for 6 weeks (n = 20 in each group).

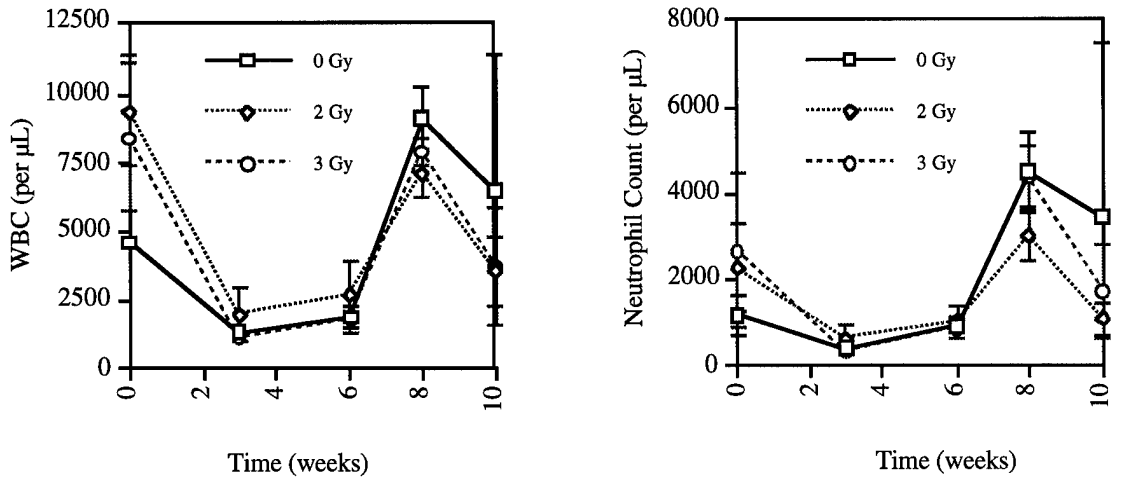


Figure 11. Total white blood cell counts (left panel) and blood neutrophil counts (right panel) in mice that received 200 mg/kg/week of cyclophosphamide for 6 weeks followed by either no radiation, 2 Gy, or 3 Gy (n =15).

AMERICAN JOURNAL OF MEDICAL GENETICS (SEMIN. MED. GENET.) 89:14–22 (1999)

ARTICLES

Hyperactive Ras as a Therapeutic Target in Neurofibromatosis Type 1

BRIAN WEISS, GIDEON BOLLAG, AND KEVIN SHANNON*

The *NF1* gene encodes neurofibromin, a GTPase-activating protein (GAP) for members of the $p21^{ras}$ (Ras) family, which negatively regulates Ras output by accelerating the conversion of active Ras·GTP to inactive Ras·GDP. Analysis of tumors from patients with neurofibromatosis type 1 (NF1) has shown biochemical evidence of hyperactive Ras as well as frequent loss of the normal *NF1* allele, consistent with its role as a tumor suppressor gene. Taken together, these data suggest that novel therapeutics directed against components of the Ras signaling cascade might provide effective treatments for certain pathological complications of NF1. Here we summarize data that support a role for hyperactive Ras in NF1 disease, including Ras processing, activation, and down-regulation. We review targets for rational drug design, provide preliminary results, and discuss implications for future studies. Am. J. Med. Genet. (Semin. Med. Genet.) 89:14–22, 1999. © **1999 Wiley-Liss, Inc.**

KEY WORDS: neurofibromatosis type 1; experimental therapeutics; Ras

INTRODUCTION

Before the gene for neurofibromatosis type 1 (NF1) was discovered, the cancer predisposition and propensity to develop multiple cutaneous neurofibromas in affected individuals suggested that the disease gene encoded a protein that negatively regulated cell growth. Furthermore, the autosomal dominant inheritance pattern of NF1 was consistent with the theory that *NF1* functions genetically as a tumor suppressor gene (TSG) in certain tissues. Both of these hypotheses could be tested only after the gene was identified in 1990 [Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990]. At that time, a segment of *NF1* cDNA unexpectedly showed strong homology with yeast and mammalian GTPase-activating proteins (GAPs) for members of the $p21^{ras}$ (Ras) family [Buchberg et al., 1990; Xu et al., 1990].

Ras proteins play a central role in cellular growth and differentiation. Ras output is tightly regulated by cycling between an active GTP-bound conformation (Ras·GTP) and an inactive GDP-bound state (Ras·GDP) [reviewed in Bourne et al., 1990; Wittinghofer, 1998]. Ras has a slow intrinsic GTPase activity that is enhanced by GAPs. These proteins greatly increase the rate of GTP hydrolysis and thereby act as negative regulators of Ras output [reviewed in Boguski and McCormick, 1993; Bernards, 1995; Wittinghofer, 1998]. Oncogenic *RAS* alleles carry single point mutations at the Gly12, Gly13, or Gln61 positions that greatly reduce the intrinsic GTPase activity and render these proteins resistant to GAPs [reviewed in Wittinghofer, 1998].

Ras proteins regulate cell fates by transducing signals from the plasma membrane to the nucleus via a series of downstream effectors [reviewed in Wittinghofer, 1998]. Ras·GTP recruits Raf kinase to the membrane, where its kinase activity is effective. Raf, in turn, activates a kinase cascade involving MEK kinase and the Erk1 and Erk2 isoforms of mitogen-activated protein (MAP) kinase (Fig. 1). The activation states of the phosphoinositol-3′-kinase (PI3K) and Rac/Rho pathways are also regulated by Ras·GTP in many cell types. The consequences of Ras activation are influenced by the cellular context and by cross talk between its downstream effectors.

Neurofibromin, the product of *NF1*, has a putative GAP-related domain (GRD); biochemical analysis of recombinant peptides corresponding to its GRD have demonstrated that neurofibromin is an authentic GAP for Ras [reviewed in Boguski and McCormick, 1993; Bernards, 1995]. Yeast strains with mutations in the *NF1* homologues *ira1* and *ira2* are susceptible to heat shock owing to an inability to down-regulate *ras1*. The observation that expressing the neurofibromin GRD in these yeast strains could restore a normal heat-shock response provided direct in vivo evidence supporting its role as a GAP for Ras [Ballester et al., 1990]. Furthermore, structural analysis of crystals containing a fragment of neurofibromin have shown a homologous "arginine finger" within the GAP domain

Brian Weiss is a fellow in pediatric hematology/oncology at the University of California, San Francisco (UCSF). His research focuses on translational studies of myeloid leukemia in *NF1* mice. Gideon Bollag is research director for small molecule therapeutics at Onyx Pharmaceuticals. His research involves exploiting signal transduction pathways for the development of cancer therapeutics. Kevin Shannon is in the Department of Pediatrics and is leader of the program in hematopoietic malignancies at UCSF. His laboratory studies inherited predispositions to myeloid leukemia.

Contract grant sponsor: NIH; Contract grant number: CA72614; Contract grant sponsor: U.S. Army Neurofibromatosis Research Program; Contract grant number: NF960048; Contract grant sponsor: American Cancer Society; Contract grant number: DB 80030; Contract grant sponsor: Frank A. Campini Foundation.

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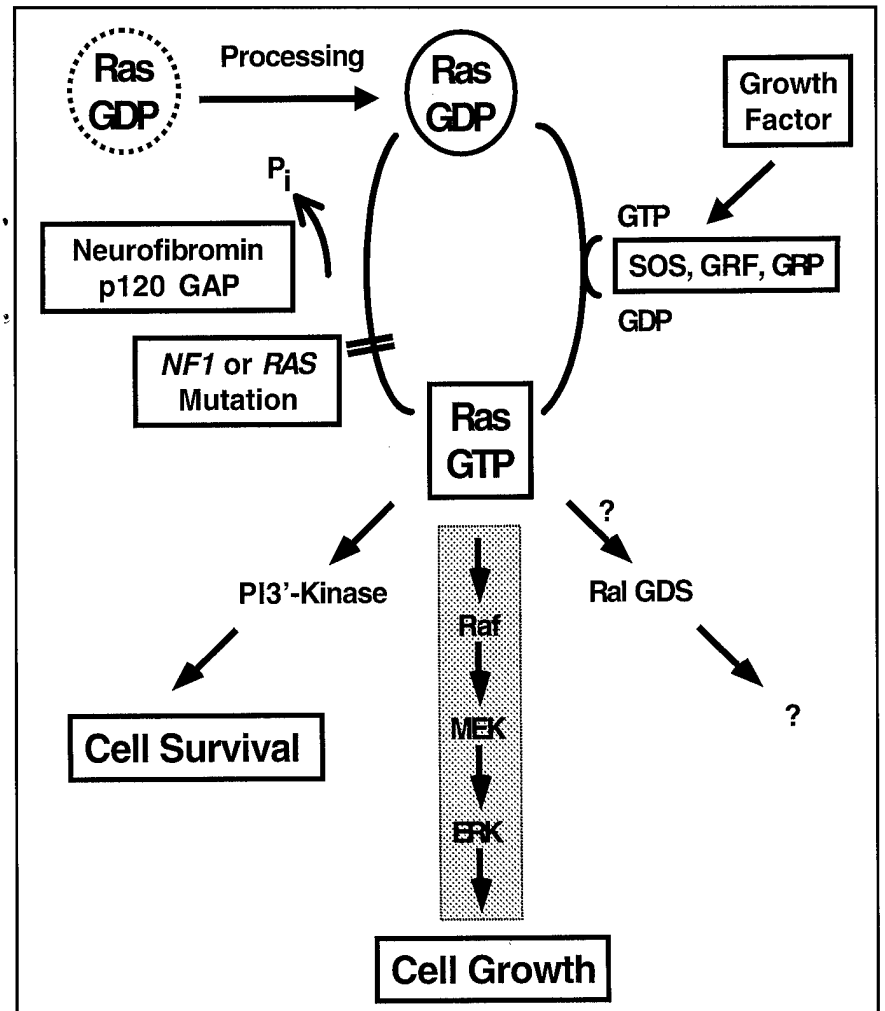

Figure 1. Overview of Ras activation by GEFs and signaling through downstream effectors. Ras cycles between an active GTP-bound conformation (Ras·GTP) and an inactive GDP-bound state (Ras·GDP). Growth factors induce cell growth, in part by activating the GEFs Sos, GRF, and GRP that bind Ras and stimulate nucleotide dissociation. Nucleotide exchange increases the percentage of Ras·GTP because the intracellular concentration of free GTP vastly exceeds that of GDP. Signaling terminates when Ras·GTP is hydrolyzed to Ras·GDP. Neurofibromin and p120 GAP regulate this process by accelerating the conversion of Ras·GTP to Ras·GDP. Oncogenic *RAS* mutations or inactivation of *NF1* perturb Ras signaling by favoring the GTP-bound state, as described in the text. Ras·GTP activation of the Raf/MEK/ERK kinase cascade stimulates proliferation in many cell types, and activation of the PI3K pathway has been shown to promote cellular survival. Ras interacts with other downstream effectors, such as Ral-GDS; all of the components of this pathway remain to be defined.

[Scheffzek et al., 1998]. Recently, structural analysis of crystals containing a fragment of p120 GAP in complex with Ras identified an arginine finger within the GAP domain that comes into close proximity to Gly12 and Gln61 and stabilizes a transition state between Ras·GTP and Ras·GDP [Scheffzek et al., 1997].

The GAP activity of neurofibromin has profound implications both for understanding the pathological complications of NF1 and for designing rational therapies. Studies of anti-Ras therapeutics are broadly applicable because *RAS* is the most common oncogene mutated in human malignancies. This review summarizes data that support a role for hyperactive Ras in NF1 disease, including Ras processing, activation, and down-regulation. It explores targets for rational drug design and preliminary results and presents implications for future studies.

HYPERACTIVE RAS IN NF1 PATHOLOGICAL MANIFESTIONS

Cells derived from patients with NF1 and *Nf1* knockout mice have been used to test the hypothesis that *NF1* functions as a TSG in mammalian cells and to determine if loss of function is associated with hyperactive Ras. Individuals with NF1 are predisposed to specific cancers, including malignant peripheral nerve sheath tumor (MPNST), astrocytoma, pheochromocytoma, and juvenile myelomonocytic leukemia (JMML). Somatic loss of constitutional heterozygosity (LOH), a hallmark of DNA segments that harbor TSGs, has been detected at *NF1* in MPNST, pheochromocytoma, and JMML [Skuse et al., 1989; Glover et al., 1991; Xu et al., 1992; Shannon et al., 1994]. In patients with familial NF1, LOH has invariably been associated with loss of the allele inherited from the unaffected parent. A subset of cutaneous neurofibromas also shows LOH at *NF1* [Colman et al., 1995]. Homozygous inactivation of *NF1* has been demonstrated directly in a case of MPNST, in a neurofibroma, and in a number of leukemias from children with NF1 [Legius et al., 1993; Sawada et al., 1996; Side et al., 1997]. Similarly, heterozygous *Nf1* knockout mice are predisposed to pheochromocytoma and myeloid leukemia, and these neoplasms show loss of the wild-type *Nf1* allele. Together, these human and murine data demonstrate that *NF1* functions as a TSG in at least a subset of tumors.

A number of observations support the idea that inactivation of *NF1* is associated with hyperactive Ras in tumor cells. MPNST cell lines derived from

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patients with NF1 show decreased in vitro GAP activity and markedly elevated levels of Ras·GTP [Basu et al., 1992; DeClue et al., 1992]. Increased Ras·GTP levels have also been reported in primary MPNSTs removed from patients with NF1 [Guha et al., 1996] and in Schwann cells isolated from homozygous *Nf1*-deficient embryos [Kim et al., 1995].

The role of neurofibromin in myeloid growth control has been studied extensively in humans and in mice. In a large series of children with JMML and related disorders, activating *RAS* mutations were detected only in bone marrow samples from patients without NF1, suggesting that inactivation of *NF1* and somatic *RAS* point mutations are functionally equivalent [Kalra et al., 1994]. In addition, leukemic cells from children with NF1 show a reduction in neurofibromin-specific GAP activity, elevated levels of Ras·GTP, and activation of MAP kinase [Bollag et al., 1996]. JMML cells form excessive numbers of hematopoietic progenitor colonies in cultures containing low concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF) [Emanuel et al., 1996].

Although homozygous mutant *Nf1* murine embryos die in utero of complex cardiac defects, fetal hematopoiesis is normal [Brannan et al., 1994; Jacks et al., 1994]. Like JMML cells, *Nf1*−/− fetal liver cells are hypersensitive to GM-CSF in culture [Bollag et al., 1996; Largaespada et al., 1996], and adoptive transfer into irradiated recipient mice induces a JMML-like disorder [Largaespada et al., 1996]. Unstimulated *Nf1*−/− hematopoietic cells demonstrate constitutive MAP kinase activity and hyperactivation in response to GM-CSF and other hematopoietic growth factors [Zhang, 1998]. Finally, *Myb*-transformed hematopoietic cell lines prepared from *Nf1*−/− fetal livers show an exaggerated and prolonged increase in Ras·GTP levels in response to GM-CSF [Largaespada et al., 1996]. Taken together, these human and murine studies strongly support a model whereby the tumor suppressor function of *NF1* is mediated through the ability of neurofibromin to down-regulate Ras output in immature myeloid and neural crest cells. Furthermore, the hypersensitivity of *Nf1*-deficient hematopoietic cells to GM-CSF and of *Nf1*−/− fetal neurons to neurotrophins (Vogel et al., 1995) implicates abnormal responses to growth factors that activate Ras in the excessive deregulated proliferation of these cells.

Although most *NF1* mutations truncate neurofibromin and are thought to function as null alleles, a few families with missense mutations have been reported. In one kindred, a missense mutation at a highly conserved residue in the GAP domain (Lys-1423) was not associated with an unusual phenotype [Li et al., 1992]. Recently, a mother and two sons with NF1 were identified in whom the disease was inherited with a novel missense mutation of Arg-1276 (the arginine finger) [Klose et al., 1998]. This mutation does not impair secondary or tertiary protein structure, does not reduce neurofibromin levels, and does not significantly influence neurofibromin binding to Ras; however, as predicted from the structural analysis, this mutation completely disables GAP activity. Most affected family members have café au lait spots, freckling, and scoliosis, and both children have a learning disability. The proband developed mutliple schwannomas, one of which underwent malignant degeneration [Klose et al. 1998]. The example of this family suggests that many of the clinical phenotypes associated with NF1 disease can be accounted for by a selective inability of neurofibromin to interact with and down-regulate Ras output and illustrates how molecular analysis of human patients can enhance our understanding of disease pathobiology.

A number of observations support the idea that inactivation of NF1 is associated with hyperactive Ras in tumor cells.

While all of these observations are consistent with the idea that *NF1* negatively regulates cell growth by accelerating GTP hydrolysis on Ras, this simple model does not explain other data [Boguski and McCormick, 1993; Bernards, 1995]. Acquired *RAS* mutations are uncommon in neural crest tumors, and overexpressing oncogenic *RAS* induces differentiation, rather than transformation, in some neural crest cell lines [Bar-Sagi and Feramisco, 1985; Noda et al., 1985]. Furthermore, neuroblastoma and melanoma cell lines that have inactivated both *NF1* alleles still show normal levels of Ras·GTP [Andersen et al., 1993; Johnson et al., 1993; The et al., 1993]. A conserved *Drosophila* homologue, with 60% homology to human *NF1*, is widely expressed at low amounts during all stages of pupal development [The et al., 1997]. Homozygous disruption of the *Drosophila NF1* gene produces viable and fertile mutants with a growth defect that is fully rescued by expression of a heat shock–inducible *NF1* transgene [The et al., 1997]. However, *Drosophila NF1* mutants do not demonstrate phenotypic abnormalities associated with Ras activation, and neither reducing nor increasing signaling through the Ras-Raf1 pathway affects the impaired growth phenotype. In contrast, stimulating the adenosine 3′5′-monophosphate (cAMP)-PKA pathway rescues the size defect in *NF1*-deficient pupae and restores a neuromuscular junction defect that is insensitive to Ras manipulations [Guo et al., 1997; The et al., 1997]. These data raise the possibility that *NF1* may negatively regulate growth by mechanism(s) that are independent of the level of Ras·GTP and that may involve the cAMP pathway in some cell types [Andersen et al., 1993; Johnson et al., 1993; The et al., 1993]. Involvement of the cAMP-PKA pathway in the neuromuscular junction phenotype in *Drosophilia* raises the possibility that some of the learning disabilities in individuals with NF1 and in *Nf1*+/− mice are not entirely due to hyperactive Ras.

A relevant question in considering therapeutic strategies is whether inacti-

vation of a single *NF1* allele (i.e., haplo-insufficiency) contributes to any of the complications of the disease. While few data speak to this question, it is possible that haplo-insufficiency at *NF1* induces subtle biochemical alterations that underlie some of the learning disabilities, pigmentary lesions, and tumors in affected individuals. Heterozygous *Nf1* mice have specific learning defects that are compatible with this idea [Silva et al., 1997]. In addition, recent data showing that some of the tumors that arise in heterozygous *p53* knockout mice retain a functional *p53* allele illustrate that TSGs may promote tumorigenesis by either homozygous inactivation (Knudson model) or dosage [Venkatachalam et al., 1998]. If some of the complications of NF1 develop in cells that retain a normal allele, therapies directed at enhancing gene expression represent a rational approach. This strategy is predicated on the observation that none of the NF1 mutations identified to date have been shown to encode proteins that function in a dominant negative manner.

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RAS ACTIVATION BY GUANINE NUCLEOTIDE EXCHANGE FACTORS AND SIGNALING THROUGH DOWNSTREAM EFFECTORS

Because Ras is one of the most intensively studied proteins in modern biology, a great deal has been learned about Ras activation in response to extracellular stimuli. A number of the downstream targets of Ras·GTP are also known (Fig. 1). We summarize these processes briefly, since they provide a context for discussing approaches to rational drug design in NF1. Guanine nucleotide exchange factors (GEFs) bind to either Ras·GDP or Ras·GTP; this interaction results in guanine nucleotide dissociation from Ras followed by passive rebinding. Because the intracellular concentration of free GTP vastly exceeds that of GDP in cells, nucleotide exchange on Ras increases the percentage of Ras·GTP and enhances output. Signaling terminates when Ras·GTP is hydrolyzed to Ras·GDP. GAPs play an integral role in this process by stabilizing a transition state between Ras·GTP and Ras·GDP; this accelerates the t1/2 of the Ras GTPase from minutes to seconds. Structural analysis of Ras co-crystalized with the GRD of p120 GAP strongly implicates an arginine finger formed by Arg-1276 of neurofibromin as critical for this interaction [Klose et al., 1998]. As discussed earlier, a missense mutation at this codon was recently characterized in a family with NF1.

Three types of GEFs for Ras are currently known: Sos, GRF, and GRP. The mammalian Sos1 and Sos2 [Bowtell et al., 1992; Wei et al., 1992] were identified on the basis of homology to the *Drosophila* "son of sevenless" gene [Simon et al., 1991]. The mammalian GRF1 and GRF2 proteins [Cen et al., 1992; Martegani et al., 1992; Shou et al., 1992; Fam et al. ,1997] were identified as homologues of the budding yeast Cdc25 gene product [Broek et al., 1987; Robinson et al., 1987]. Ras GRP was found in a functional screen searching for proteins capable of transforming fibroblasts [Ebinu et al., 1998; Tognon et al., 1998].

Each of these GEFs is regulated in a different fashion. The Sos proteins are ubiquitously expressed and function downstream of tyrosine kinase growth factor receptors [Buday and Downward, 1993; Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993]. Activated tyrosine kinase growth factor receptors recruit adaptor proteins, such as Shc and Grb2, which interact through SH2 and SH3 domains and recruit Sos to the complex. This membrane localization of Sos results in activation of GEF function and, hence, GTP loading of Ras. The GRF proteins are tissue specific (primarily brain) and can be activated by both calcium [Farnsworth et al., 1995] and heterotrimeric G-protein-mediated phosphorylation [Mattingly and Macara, 1996]. Ras GRP expression is also limited, with the highest levels in lymphoid tissues and brain [Ebinu et al., 1998; Tognon et al., 1998]. This GEF contains calcium- and diacylglycerol-binding domains, suggesting that its activity may be regulated by phospholipase C.

Effector pathways downstream of Ras appear to be numerous and complementary (Fig. 1). Since the discovery that Raf kinase binds directly to the GTP-bound form of Ras [Moodie et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993], many experiments have established the Raf/MEK/ERK kinase cascade [Crews and Erikson, 1993] as a key growth-stimulating pathway. Experimental evidence suggests that recruitment of Raf kinase to the membrane is the main function of Ras binding [Leevers et al., 1994; Stokoe et al., 1994].

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Phosphoinositol 3′-kinase (PI3K) represents another effector for Ras [Rodriguez-Viciana et al., 1994]. The observation that products of PI3K promote cellular survival [Yao and Cooper, 1995] suggests that dysregulation of this pathway may also be an important determinant of malignancy. Further investigations suggest that lipid products of PI3K inhibit apoptosis by activating a kinase cascade that includes Atk (also known as protein kinase B [PKB]) [Franke et al., 1995] and its upstream

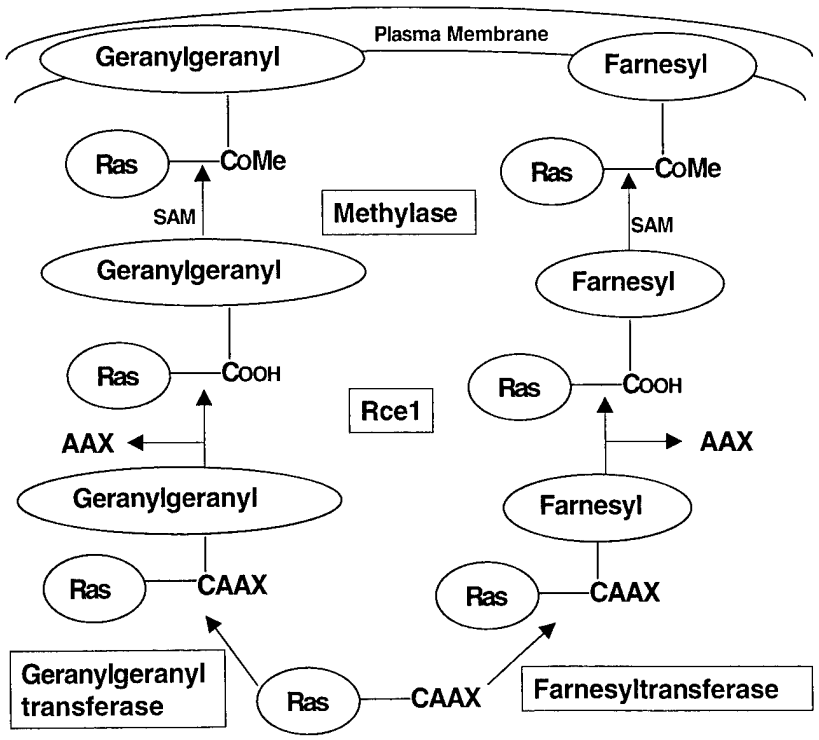

Figure 2. Post-translational processing of Ras. Post-translational modification of Ras is initiated by attachment of either a farnesyl or geranylgeranyl isoprenoid lipid to the cysteine residue of the carboxy terminal CAAX box. These reactions are catalyzed by farnesyl- and geranylgeranyltransferase, respectively. The last three amino acids (i.e., the -A-A-X) are then removed by a specific endoprotease, Rce1. The final step in Ras processing involves methylation of the carboxyl group by an endoplasmic reticulum–associated methyltransferase.

activating kinase phosphoinosotide dependent kinase (PDK) [Alessi et al., 1997; Stokoe et al., 1997].

Roles for additional effectors of Ras have been inferred from studies using Ras effector domain mutants [White et al., 1995; Rodriguez-Viciana et al., 1997]. A set of mutants with differential affinities for a variety of effector proteins suggests that binding to a single effector is insufficient for complete transformation of cells. These studies implicate an effector besides Raf kinase and PI3K. Among the candidates for these additional effectors are Ral GDS, a GEF for the small GTPase Ral [reviewed in Feig et al., 1996]. In addition, there is a growing list of candidates containing a Ral GDS-like Ras binding domain that may also serve as effectors [reviewed in Ponting and Benjamin, 1996]. In spite of the tremendous resources that have been expended to elucidate the Ras signal transduction pathway, it appears likely that additional targets for therapeutic intervention are yet to be discovered.

POST-TRANSLATIONAL PROCESSING OF RAS

Ras proteins undergo post-translational processing at a common C-terminal CAAX sequence where C is cysteine, A is an aliphatic amino acid, and X is any amino acid [Gibbs et al., 1994; Kohl et al., 1995a; Cox and Der, 1997; Gibbs and Oliff, 1997] (Fig. 2). Ras processing is intiated by cytosolic protein prenyltransferases that attach either a farnesyl or a geranylgeranyl isoprenoid lipid to the thiol group of the cysteine residue [Casey and Seabra, 1996]. In general, the cysteine is geranylgeranylated when the X residue is a leucine or a phenylalanine; otherwise, the protein is farnesylated [Finegold et al., 1991; Moores et al., 1991]. The isoprenoid groups that are transferred in these reactions are donated by geranylgeranyl pyrophosphate and by farnesyl pyrophosphate, respectively. This initial lipid modification targets Ras to membranes and is required for the biological activity of both normal and oncogenic Ras. After the prenyl group is attached, the last three amino acids of Ras proteins (i.e., the -A-A-X) are removed by a specific endoprotease [Schmidt et al., 1998]. Recent data from a line of knockout mice suggest that a single gene called *Rce1* encodes the -A-A-X endoprotease activity in mammalian cells (Fig. 2). *Rce1*−/− embryos die late in gestation, and about 50% of the Ras is mislocalized in *Rce1*− /− cells [Kim et al., 1999]. The final step in Ras processing involves methylation of the carboxyl group of the prenylcysteine by an endoplasmic reticulum–associated methyltransferase [Clarke et al., 1988; Clarke, 1992, 1993]. While prenylation of Ras is essential for plasma membrane targeting, biological function, and transformation, the importance of the proteolysis and methylation steps is less certain.

ANTI-RAS THERAPEUTICS

Inhibitors of Ras Processing

A number of farnesyltransferase inhibitors (FTIs) are presently being administered to patients with refractory cancer in Phase 1 and 2 clinical trials. Studies evaluating different FTIs in a number of in vitro and in vivo systems have provided preclinical data supporting selective antitumor effects of these compounds [Cox and Der, 1997; Gibbs and Oliff, 1997]. FTIs have been shown to block Ras-induced transformation in tissue culture cells, to inhibit the growth of many cancer cell lines, and to halt proliferation of Ras-activated xenografts in nude mice [Cox and Der, 1997; Gibbs and Oliff, 1997]. One FTI, L-744,832, also showed efficacy in two transgenic mouse models of breast cancer in which *RAS* expression is driven from a mammary tumor virus (MMTV) promoter [Kohl et al., 1995b; Mangues, 1998]. Barrington and co-

workers [1998] recently reported that L-744,832-induced breast tumor regression was associated with apoptotic cell death that was partially independent of *p53* function in MMTV-*HRAS* mice.

The idea that hyperactive Ras underlies the abnormal cellular proliferation in NF1 provides a rationale for the potential therapeutic value of FTIs and other inhibitors of Ras processing in treating NF1-associated complications. Indeed, Yan and co-workers [1996] found that FTI treatment induced growth inhibition and morphological reversion in an *NF1*-deficient MPNST cell line. We recently investigated the effects of FTI L-744,832 in *Nf1*-deficient hematopoietic cells in vitro and in vivo. L-744,832 inhibited H-Ras prenylation in cell lines and in primary hematopoietic cells and abrogated the growth of myeloid progenitor colonies in response to GM-CSF [Mahgoub et al., 1999]. This FTI also partially blocked GM-CSF-induced MAP kinase activation but did not reduce constitutively elevated levels of MAP kinase activity in primary *Nf1*−/− cells. Injection of a single dose of L-744,832 increased the amount of unprocessed H-Ras in bone marrow cells but had no detectable effect on N-Ras.

Treatment of irradiated recipient mice that had developed the JMML-like myeloid disorder following adoptive transfer of fetal liver cells was not associated with clinical improvement [Mahgoub et al., 1999]. It is likely that the lack of efficacy was due to the resistance of N-Ras and K-Ras processing to inhibition by this FTI. It is not clear if the differences seen when FTIs were tested on MPNST cell lines and in mice engrafted with *Nf1*-deficient hematopoietic cells are accounted for by cell lineage–specific factors, by genetic variations between immortal tumor-derived cell lines and primary cells, by the ability to achieve higher FTI concentrations in tissue culture than in whole animals, or by the use of different compounds in the two studies.

Potential therapeutics targeting Ras effector pathways are not yet in clinical trials. Published inhibitors include two different MEK inhibitors—PD98059 [Dudley et al., 1995] and U0126 [Favata et al., 1998]—as well as an inhibitor of PI3K, LY294002 [Vlahos et al., 1994]. The specific effects these inhibitors have on cells are indeed impressive. The MEK inhibitors specifically block Ras-induced ERK phosphorylation and revert many aspects of cellular transformation. The PI3K inhibitor was critical in establishing a role for 3′-phosphorylated lipids in cellular survival pathways; thus, LY294002 is a potent inducer of apoptosis [Yao and Cooper, 1995]. These downstream components of the Ras signaling pathway are thus promising targets for NF1 therapeutics.

Potential therapeutics targeting Ras effector pathways are not yet in clinical trials.

Another potential therapeutic strategy for some of the pathogenic complications of NF1 is suggested by data showing that some *NF1*-deficient cells are hypersensitive to specific growth factors. For example, a peptide antagonist of the GM-CSF receptor has been shown to inhibit the growth of human JMML cells both in vitro and in immunodeficient mice [Iversen et al., 1996; Iversen et al., 1997]. As the growth factors that contribute to other hyperproliferative complications of NF1 are elucidated, the development of specific antagonists would represent a rational therapeutic approach for NF1-related tumors.

While gene therapy strategies to correct *NF1* mutations in affected tissues are theoretically appealing, there are significant obstacles to this approach. First, the large size of *NF1* cDNA will make it difficult to construct vectors that can be transduced and expressed efficiently. Using a smaller fragment encoding the *NF1* GAP domain offers a potential solution to this problem; however, it is uncertain if it would be regulated or functional in the same way as full-length neurofibromin. Another problem involves the need to correct a high percentage of cells in multiple lineages. Finally, because Ras plays an integral role in many normal cellular functions, it is likely that exogenous *NF1* will need to be expressed at physiological levels in target cells. In summary, while gene therapy clearly represents an intriguing therapeutic strategy, it is likely that it would be applied to NF1 only after it is validated in such diseases as inherited immunodeficiencies or thalassemia, where the technical issues are less complex. *Nf1* knockout mice may prove useful for modeling specific aspects of gene therapy as applied to NF1.

SUMMARY AND FUTURE PERSPECTIVES

In many ways, NF1 provides an excellent model for assessing strategies that utilize basic information about abnormal cell growth to devise and test rational therapeutics. A large body of genetic and biochemical data implicate hyperactive Ras in many of the pathological complications seen in NF1 patients. Because of its involvement in 30% of human cancers, hyperactive Ras is being intensively investigated as a target for novel drugs. While some of the compounds developed through this effort may prove effective for treating cancers that carry oncogenic *RAS* mutations as well as certain pathological complications of NF1, it is essential that investigators become aware of potential genetic and biochemical differences between these disease states. In particular, oncogenic Ras proteins show defective intrinsic GTP hydrolysis and are resistant to GAPs. In contrast, the Ras proteins present in *NF1*-deficient cells have normal intrinsic biochemical ac-

In many ways, NF1 provides an excellent model for assessing strategies that utilize basic information about abnormal cell growth to devise and test rational therapeutics.

tivities, including responsiveness to p120 GAP. The degree and duration of Ras·GTP activation may differ markedly in cells with different genetic lesions, and this may have profound effects on cellular survival and proliferation [Marshall, 1995; Lloyd et al., 1997; Sewing et al., 1997]. From a therapeutic perspective, it should not be assumed that an agent that is effective or ineffective in cells with oncogenic *RAS* mutations will show efficacy in treating the complications of NF1, and vice versa. If Ras does not function as a simple binary switch in regulating cell growth, reducing Ras·GTP toward (but not all the way back to) normal levels may have unanticipated adverse effects in some cell types.

Despite these potential concerns, anti-Ras therapeutic strategies represent an exciting avenue for NF1 research. The *Nf1* knockout mouse models a number of the important complications of human NF1 and therefore provides a logical system for preclinical studies of novel therapies. These trials should include correlative biochemical studies to ascertain any effects on Ras signaling in primary cells whenever possible. Human clinical trials of new treatments should include a careful assessment of the ratio of risks versus benefits for each patient; this is especially important in NF1, given the great clinical variability between affected individuals. Finally, the development of therapeutic strategies will benefit greatly from continued investments in basic research aimed at defining critical effectors of hyperactive Ras in cell lineages affected in NF1 patients, the role of haplo-insufficiency in various pathological complications, and the contributions of growth factors and of other extracellular stimuli to excessive cellular growth in this disorder.

ACKNOWLEDGMENTS

B.W. is supported by a research fellowship from the Frank A. Campini Foundation.

REFERENCES

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B α. Curr Biol 7:261–269.

Andersen LB, Fountain JW, Gutmann DH, Tarle SA, Glover TW, Dracopoli NC, Housman DE, Collins FS. 1993. Mutations in the neurofibromatosis 1 gene in sporadic malignant melanoma cell lines. Nature Genet 3: 118–121.

Ballester R, Marchuk D, Boguski M, Saulino A, Letcher R, Wigler M, Collins F. 1990. The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. Cell 63:851–859.

Barrington RE, Subler MA, Rands E, Omer CA, Miller PJ, Hundley JE, Koester SK, Troyer DA, Bearss DJ, Conner MW, Gibbs JB, Hamilton K, Koblan KS, Mosser SD, O'Neill TJ, Schaber MD, Senderak ET, Windle JJ, Oliff A, Kohl NE. 1998. A farnesyltransferase inhibitor induces tumor regression in transgenic mice harboring multiple oncogenic mutations by mediating alterations in both cell cycle control and apoptosis. Mol Cell Biol 18:85–92.

Bar-Sagi D, Feramisco JR. 1985. Microinjection of the *ras* oncogene protein into PC12 cells induces morphological differentiation. Cell 42:841–848.

Basu TN, Gutmann DH, Fletcher JA, Glover TW, Collins FS, Downward J. 1992. Aberrant regulation of *ras* proteins in malignant tumour cells from type 1 neurofibromatosis patients. Nature 356:713–715.

Bernards A. 1995. Neurofibromatosis type 1 and Ras-mediated signaling: filling in the GAPs. Biochim Biophys Acta 1242:43–59.

Boguski M, McCormick F. 1993. Proteins regulating Ras and its relatives. Nature 366:643–653.

Bollag G, Clapp DW, Shih S, Adler F, Zhang Y, Thompson P, Lange BJ, Freedman MH, McCormick F, Jacks T, Shannon K. 1996. Loss of *NF1* results in activation of the Ras signaling pathway and leads to aberrant growth in murine and human hematopoietic cells. Nature Genet 12:144–148.

Bourne HR, Sanders DA, McCormick F. 1990. The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348:125–132.

Bowtell D, Fu P, Simon M, Senior P. 1992. Identification of murine homologues of the Drosophila son of sevenless gene: potential activators of ras. Proc Natl Acad Sci U S A 89:6511–6515.

Brannan CI, Perkins AS, Vogel KS, Ratner N, Nordlund ML, Reid SW, Buchberg AM, Jenkins N, Parada L, Copeland N. 1994. Targeted disruption of the neurofibromatosis type 1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. Genes Dev 8:1019–1029.

Broek D, Toda T, Michaeli T, Levin L, Birchmeier C, Zoller M, Powers S, Wigler M. 1987. The *S. cerevisiae CDC25* gene product regulates the *RAS*/adenylate cyclase pathway. Cell 48:789–799.

Buchberg A, Cleveland L, Jenkins N, et al. 1990. Sequence homology shared by neurofibromatosis type-1 gene and IRA-1 and IRA-2 negative regulators of the RAS cyclic AMP pathway. Nature 347:291–294.

Buday L, Downward J. 1993. Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell 73:611–620.

Casey PJ, Seabra MC. 1996. Protein prenyltransferases. J Biol Chem 271: 5289–5292.

Cawthon RM, Weiss R, Xu GF, Viskochil D, Culver M, Stevens J, Robertson M, Dunn D, Gesteland R, O'Connell P, White R. 1990. A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. Cell 62:193–201.

Cen H, Papageorge AG, Zippel R, Lowy DR, Zhang K. 1992. Isolation of multiple mouse cDNAs with coding homology to Saccharomyces cerevisiae CDC25: identification of a region related to Ber, Vav, Dbl and CDC24. EMBO J 11:4007–4015.

Clarke S. 1992. Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. Annu Rev Biochem 61:355–386.

Clarke S. 1993. Protein methylation. Curr Opin Cell Biol 5:977–983.

Clarke S, Vogel JP, Deschenes RJ, Stock J. 1988. Posttranslational modification of the Ha-ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. Proc Natl Acad Sci U S A 85:4643–4647. (Published erratum appears in Proc Natl Acad Sci U S A 1988;85(20):7556.)

Colman SD, Williams CA, Wallace MR. 1995. Benign neurofibromas in type 1 neurofibromatosis (NF1) show somatic deletions of the *NF1* gene. Nature Genet 11:90–92.

Cox AD, Der CJ. 1997. Farnesyltransferase inhibitors and cancer treatment: targeting simply ras? Biochim Biophys Acta 1333:F51–F71.

Crews CM, Erikson RL. 1993. Extracellular signals and reversible protein phosphorylation: what to Mek of it all. Cell 74:215–217.

DeClue JE, Papageorge AG, Fletcher JA, Diehl SR, Ratner N, Vass WC, Lowy DR. 1992. Abnormal regulation of mammalian $p21^{ras}$ contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell 69:265–273.

Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci U S A 92:7686–7689.

Ebinu JO, Bottorff DA, Chan EY, Stang SL, Dunn RJ, Stone JC. 1998. RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. Science 280:1082–1086.

Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA. 1993. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature 363:45–51.

Emanuel PD, Shannon KM, Castleberry RP. 1996. Juvenile myelomonocytic leukemia: molecular understanding and prospects for therapy. Mol Med Today 2:468–475.

Fam NP, Fan WT, Wang Z, Zhang LJ, Chen H, Moran MF. 1997. Cloning and characterization of Ras-GRF2, a novel guanine nucleotide exchange factor for Ras. Mol Cell Biol 17:1396–1406.

Farnsworth CL, Freshney NW, Rosen LB, Ghosh A, Greenberg ME, Feig LA. 1995.

Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. Nature 376:524–527.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273:18623–18632.

Feig LA, Urano T, Cantor S. 1996. Evidence for a Ras/Ral signaling cascade. Trends Biochem Sci 21:438–441.

Finegold AA, Johnson DI, Farnsworth CC, Gelb MH, Judd SR, Glomset JA, Tamanoi F. 1991. Protein geranylgeranyltransferase of *Saccharomyces cerevisiae* is specific for Cys-Xaa-Xaa-Leu motif proteins and requires the *CDC43* gene product but not the *DPR1* gene product. Proc Natl Acad Sci U S A 88:4448–4452.

Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tsichlis PN. 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 81:727–736.

Gibbs JB, Oliff A. 1997. The potential of farnesyltransferase inhibitors as cancer chemotherapeutics. Annu Rev Pharmacol Toxicol 37:143–166.

Gibbs JB, Oliff A, Kohl NE. 1994. Farnesyltransferase inhibitors: Ras research yields a potential cancer therapeutic. Cell 77:175–178.

Glover TW, Stein CK, Legius E, Andersen LB, Brereton A, Johnson S. 1991. Molecular and cytogenetic analysis of tumors in von Recklinghausen neurofibromatosis. Genes Chromosom Cancer 3:62–70.

Guha A, Lau N, Huvar I, Gutmann D, Provias J, Pawson T, Boss G. 1996. Ras-GTP levels are elevated in human NF1 peripheral nerve tumors. Oncogene 12:507–513.

Guo HF, The I, Hannan F, Bernards A, Zhong Y. 1997. Requirement of Drosophila NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. Science 276: 795–798.

Iversen P, Lewis I, Turczynowicz S, Hasle H, Niemeyer C, Schmiegelow K, Bastiras S, Biondi A, Hughes TP, Lopez AF. 1997. Inhibition of granulocyte-macrophage colony-stimulating factor prevents dissemination and induces remission of juvenile myelomonocytic leukemia in engrafted immunodeficient mice. Blood 90:4910–4917.

Iversen P, Rodwell RL, Pitcher L, Taylor KM, Lopez AF. 1996. Inhibition of proliferation and induction of apoptosis in JMML cells by the granulocyte-macrophage colony-stimulating factor analogue E21R. Blood 88:2634–2639.

Jacks T, Shih S, Schmitt EM, Bronson RT, Bernards A, Weinberg RA. 1994. Tumorigenic and developmental consequences of a targeted *Nf1* mutation in the mouse. Nature Genet 7:353–361.

Johnson MR, Look AT, DeClue JE, Valentine MB, Lowy DR. 1993. Inactivation of the NF1 gene in human melanoma and neuroblastoma cell lines without impaired regulation of GTP-Ras. Proc Natl Acad Sci U S A 90:5539–5545.

Kalra R, Paderanga D, Olson K, Shannon KM. 1994. Genetic analysis is consistent with the hypothesis that *NF1* limits myeloid cell growth through $p21^{ras}$. Blood 84:3435–3439.

Kim E, Ambroziak P, Otto JC, Taylor B, Ashby M, Shannon K, Casey PJ, Young SG. 1999. Disruption of the mouse Rce1 gene results in defective Ras processing and mislocalization of Ras within cells. J Biol Chem 274: 8383–8390.

Kim HA, Rosenbaum T, Marchionni MA, Ratner N, DeClue JE. 1995. Schwann cells from neurofibromin deficient mice exhibit activation of $p21^{ras}$, inhibition of cell proliferation and morphological changes. Oncogene 11:325–335.

Klose A, Ahmadian MR, Schuelke M, Scheffzek K, Hoffmeyer S, Gewies A, Schmitz F, Kaufmann D, Peters H, Wittinghofer A, Nurnberg P. 1998. Selective disactivation of neurofibromin GAP activity in neurofibromatosis type 1. Hum Mol Genet 7: 1261–1268.

Kohl NE, Conner MW, Gibbs JB, Graham SL, Hartman GD, Oliff A. 1995a. Development of inhibitors of protein farnesylation as potential chemotherapeutic agents. J Cell Biochem 22:145–150.

Kohl NE, Omer CA, Conner MW, Anthony NJ, Davide JP, DeSolms J, Giuliani EA, Gomez RP, Graham SL, Hamilton K, Handt LK, H. G.D., Koblan KS, Kral AM, Miller PJ, Mosser SD, O'Neill TJ, Rands E, Schaber MD, Gibbs JB, Oliff A. 1995b. Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in *ras* transgenic mice. Nat Med 1:792–797.

Largaespada DA, Brannan CI, Jenkins NA, Copeland NG. 1996. *Nf1* deficiency causes Ras-mediated granulocyte-macrophage colony stimulating factor hypersensitivity and chronic myeloid leukemia. Nature Genet 12:137–143.

Leevers S, Peterson H, Marshall C. 1994. Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature 369:411–414.

Legius E, Marchuk DA, Collins FS, Glover TW. 1993. Somatic deletion of the neurofibromatosis type 1 gene in a neurofibrosarcoma supports a tumour suppressor gene hypothesis. Nature Genet 3:122–126.

Li N, Batzer A, Daly R, Yajnik V, Skolnik E, Chardin P, Bar-Sagi D, Margolis B, Schlessinger J. 1993. Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature 363:85–88.

Li Y, Bollag G, Clark R, Stevens J, Conroy L, Fults D, Ward K, Friedman E, Samowitz W, Robertson M, Bradley P, McCormick F, White R, Cawthon R. 1992. Somatic mutations in the neurofibromatosis 1 gene in human tumors. Cell 69:275–281.

Lloyd AC, Obermuller F, Staddon S, Barth CF, McMahon M, Land H. 1997. Cooperating oncogenes converge to regulate cyclin/cdk complexes. Genes Dev 11:663–677.

Mahgoub N, Taylor B, Le Beau M, Gratiot M, Carlson K, Jackts T, Shannon KM. 1997. Effects of farneysl transferase inhibitor (FT1) L744,832 in a *RAS*-activated murine myeloid leukemia model. Blood 90(suppl 1): 497a.

Mangues R, Corral T, Kohl NE, Symmans, WF, Lu S, Malumbresm M, Gibbs JB, Oliff A, Pellicer A. 1998. Antitumor effect of a farnesyl protein transferase inhibitor in mammary and lymphoid tumors overexpressing N-ras in transgenic mice. Cancer Res 58: 1253–1259.

Marchuk DA, Saulino AM, Tavakkol R, Swaroop M, Wallace MR, Andersen LB, Mitchell AL, Gutmann DH, Boguski M, Collins FS. 1991. cDNA cloning of the type 1 neurofibromatosis gene: complete sequence of the *NF1* gene product. Genomics 11:931–940.

Marshall C. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179–185.

Martegani E, Vanoni M, Zippel R, Coccetti P, Brambilla R, Ferrari C, Sturani E, Alberghina L. 1992. Cloning by functional complementation of a mouse cDNA encoding a homologue of *CDC25*, a *Saccharomyces cerevisiae* RAS activator. EMBO J 11:2151–2157.

Mattingly RR, Macara IG. 1996. Phosphorylation-dependent activation of the Ras-GRF/CDC25Mm exchange factor by muscarinic receptors and G-protein $\beta\gamma$ subunits. Nature 382:268–272.

Moodie SA, Willumsen BM, Weber MJ, Wolfman A. 1993. Complexes of Ras·GTP with Raf-1 and mitogen-activated protein kinase kinase. Science 260:1658–1661.

Moores SL, Schaber MD, Mosser SD, Rands E, O'Hara MB, Garsky VN, Marshall MS, Pompliano DL, Gibbs JB. 1991. Sequence dependence of protein isoprenylation. J Biol Chem 266:14603–14610.

Noda M, Ko M, Ogura A. 1985. Sarcoma viruses carrying the ras oncogene activate differentiation-associated properties of a neuronal cell line. Nature 318:73–75.

Ponting CP, Benjamin DR. 1996. A novel family of Ras-binding domains. Trends Biochem Sci 21:422–425.

Robinson LC, Gibbs JB, Marshall MS, Sigal IS, Tatchell K. 1987. *CDC25:* a component of the *RAS*-adenylate cyclase pathway in *Saccharomyces cerevisiae*. Science 235:1218–1221.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J. 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370:527–532.

Rodriguez-Viciana P, Warne PH, Khwaja A, Marte BM, Pappin D, Das P, Waterfield MD, Ridley A, Downward J. 1997. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell 89:457–467.

Rozakis-Adcock M, Fernley R, Wade J, Pawson T, Bowtell D. 1993. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature 363:83–85.

Sawada S, Florell S, Purandare S, Ota M, Stephens K, Viskochil D. 1996. Identification of NF1 mutations in both alleles of a dermal neurofibroma. Nature Genet 14:110–112.

Scheffzek K, Ahmadian MR, Kabsch W, Wiesmuller L, Lautwein A, Schmitz F, Wittinghofer A. 1997. The Ras-RasGAP complex: structural basis for GTPase activation and its

loss in oncogenic Ras mutants [see comments]. Science 277:333–338.

Scheffzek K, Ahmadian MR, Wiesmuller L, Kabsch W, Stege P, Schmitz F, Wittinghofer A. 1998. Structural analysis of the GAP-related domain from neurofibroma and its implications. EMBO J 17:4313–4327.

Schmidt WK, Tam A, Fujimura-Kamada K, Michaelis S. 1998. Endoplasmic reticulum membrane localization of Rce1p and Ste24p, yeast proteases involved in carboxyl-terminal CAAX protein processing and amino-terminal a-factor cleavage. Proc Natl Acad Sci U S A 95:11175–11180.

Sewing A, Wiseman B, Lloyd AC, Land H. 1997. High-intensity Raf signal causes cell cycle arrest mediated by p21Cip1. Mol Cell Biol 17:5588–5597.

Shannon KM, O'Connell P, Martin GA, Paderanga D, Olson K, Dinndorf P, McCormick F. 1994. Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. N Engl J Med 330:597–601.

Shou C, Farnsworth CL, Neel BG, Feig LA. 1992. Molecular cloning of cDNAs encoding a guanine-nucleotide-releasing factor for Ras p21. Nature 358:351–354.

Side L, Taylor B, Cayouette M, Connor E, Thompson P, Luce M, Shannon K. 1997. Homozygous inactivation of the NF1 gene in bone marrow cells from children with neurofibromatosis type 1 and malignant myeloid disorders. N Engl J Med 336: 1713–1720.

Silva AJ, Frankland PW, Marowitz Z, Friedman E, Lazlo G, Cioffi D, Jacks T, Bourtchuladze R. 1997. A mouse model for the learning and memory deficits associated with neurofibromatosis type I. Nature Genet 15:281–284.

Simon MA, Bowtell DDL, Dodson GS, Laverty TR, Rubin GM. 1991. Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. Cell 67:701–716.

Skuse GR, Kosciolek BA, Rowley PT. 1989. Molecular genetic analysis of tumors in von Recklinghausen neurofibromatosis: loss of heterozygosity for chromosome 17. Genes Chromosom Cancer 1:36–41.

Stokoe D, MacDonald S, Cadwallader K, Symons M, Hancock J. 1994. Activation of Raf as a result of recruitment to the plasma membrane. Science 264:1463–1466.

Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, Hawkins PT. 1997. Dual role of phosphatidylinositol 3,4,5 trisphosphate in the activation of protein kinase B. Science 277:567–570.

The I, Hannigan GE, Cowley GS, Reginald S, Zhong Y, Gusella JF, Hariharan IK, Bernards A. 1997. Rescue of a Drosophila NF1 mutant phenotype by protein kinase A. Science 276:791–794.

The I, Murthy AE, Hannigan GE, Jacoby LB, Menon AG, Gusella JF, Bernanrds A. 1993. Neurofibromatosis type 1 gene mutations in neuroblastoma. Nature Genet 3:62–66.

Tognon CE, Kirk HE, Passmore LA, Whitehead IP, Der CJ, Kay RJ. 1998. Regulation of RasGRP via a phorbol ester–responsive C1 domain. Mol Cell Biol 18:6995–7008.

Van Aelst L, Barr M, Marcus S, Polverino A, Wigler M. 1993. Complex formation between RAS and RAF and other protein kinases. Proc Natl Acad Sci U S A 90:6213–6217.

Venkatachalam S, Shi YP, Jones SN, Vogel H, Bradley A, Pinkel D, Donehower LA. 1998. Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. EMBO J 17:4657–4667.

Viskochil D, Buchberg AM, Xu G, Cawthon RM, Stevens J, Wolff RK, Culver M, Carey JC, Copeland NG, Jenkins NA, White R, O'Connell P. 1990. Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. Cell 62:187–192.

Vlahos CJ, Matter WF, Hui KY, Brown RF. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem 269:5241–5248.

Vogel KS, Brannan CI, Jenkins NA, Copeland NG, Parada L. 1995. Loss of neurofibromin results in neurotropin-independent survival of embryonic sensory and sympathetic neurons. Cell 82:733–742.

Vojtek AB, Hollenberg SM, Cooper JA. 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell 74:205–214.

Wallace M, Marchuk D, Andersen L, et al. 1990. Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients. Science 249:181–186.

Warne PH, Viciana PR, Downward J. 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature 364:352–355.

Wei W, Mosteller RD, Sanyal P, Gonzales E, McKinney D, Dasgupta D, Li P, Liu BX, Broek D. 1992. Identification of a mammalian gene structurally and functionally related to the CDC25 gene of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 89: 7100–7104.

White MA, Nicolette C, Minden A, Polverino A, Van Aelst L, Karin M, Wigler MH. 1995. Multiple Ras functions can contribute to mammalian cell transformation. Cell 80:533–541.

Wittinghofer A. 1998. Signal transduction via Ras. Biol Chem 379:933–937.

Xu G, O'Connell P, Viskochil D, Cawthon R, Robertson M, Culver M, Dunn D, Stevens J, Gesteland R, White R, Weiss R. 1990. The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell 62:599–608.

Xu W, Mulligan L, Ponder MA, Liu L, Smith BA, Mathew CG, Ponder BA. 1992. Loss of alleles in pheochromocytomas from patients with type 1 neurofibromatosis. Genes Chromosom Cancer 4:337–341.

Yan N, Ricca C, Fletcher J, Glover T, Seizinger BR, Manne V. 1996. Farnesyltransferase inhibitors block the neurofibromatosis type 1 (NF1) malignant phenotype. Cancer Res 55:3569–3575.

Yao R, Cooper GM. 1995. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 267:2003–2006.

Zhang XF, Settleman J, Kyriakis JM, Takeuchi-Suzuki E, Elledge SJ, Marshall MS, Bruder JT, Rapp UR, Avruch J. 1993. Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 364:308–313.

Zhang Y, Vik TA, Ryder JW, Srour, EF, Jacks T, Shannon K, Clapp DW. 1998. Nf1 regulates hematopoietic progenitor cell growth and Ras signaling in response to multiple cytokines. J Exp Med 187:1893–1902.

RAPID COMMUNICATION

Myeloid Malignancies Induced by Alkylating Agents in *Nf1* Mice

By Nidal Mahgoub, Brigit R. Taylor, Michelle M. Le Beau, Mary Gratiot, Katrin M. Carlson, Susan K. Atwater, Tyler Jacks, and Kevin M. Shannon

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.
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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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Submitted December 22, 1998; accepted March 25, 1999.

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Supported by American Cancer Society Research Grant No. DB-80030 and by National Institutes of Health (NIH) Grants No. R01 CA72614 and P01 CA40046. N.M. was supported by NIH Training Grant No. DK07636. T.J. is an Investigator of the Howard Hughes Medical Institute.

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0006-4971/99/9311-0054$3.00/0

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%)

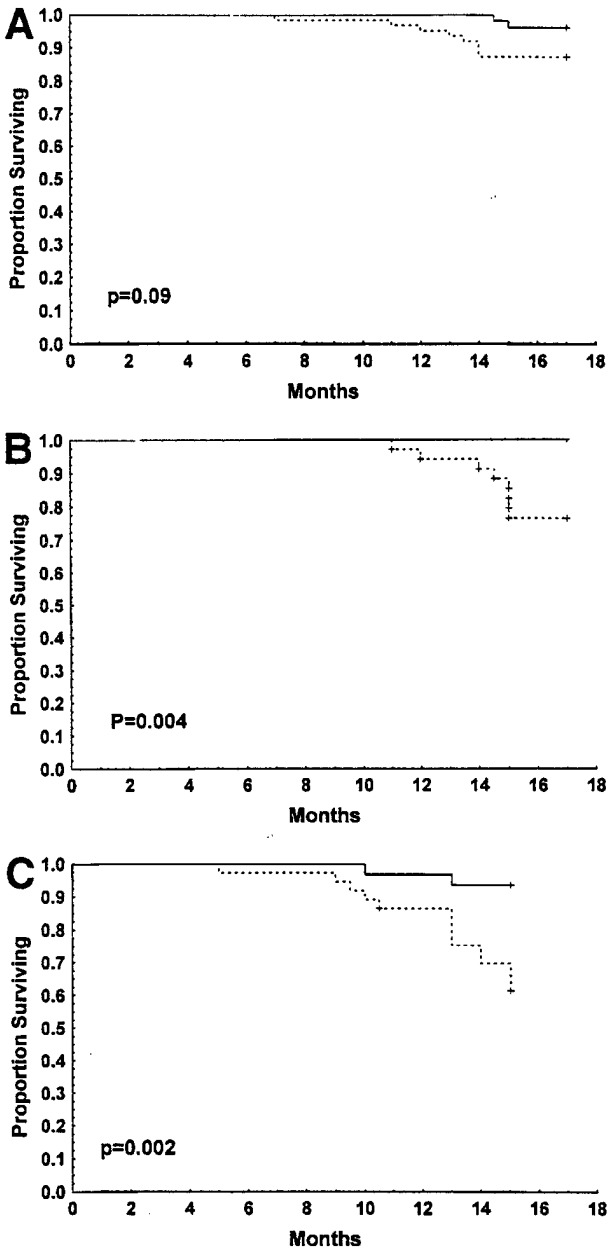

Fig 1. Kaplan-Meier plots showing the proportion of *Nf1*+/+ and *Nf1*+/− mice surviving without leukemia. *Nf1*+/+ mice are shown as an unbroken line and *Nf1*+/− mice as a dotted line. (A) Data from untreated mice; (B) data from the etoposide group; (C) data from the CY group.

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

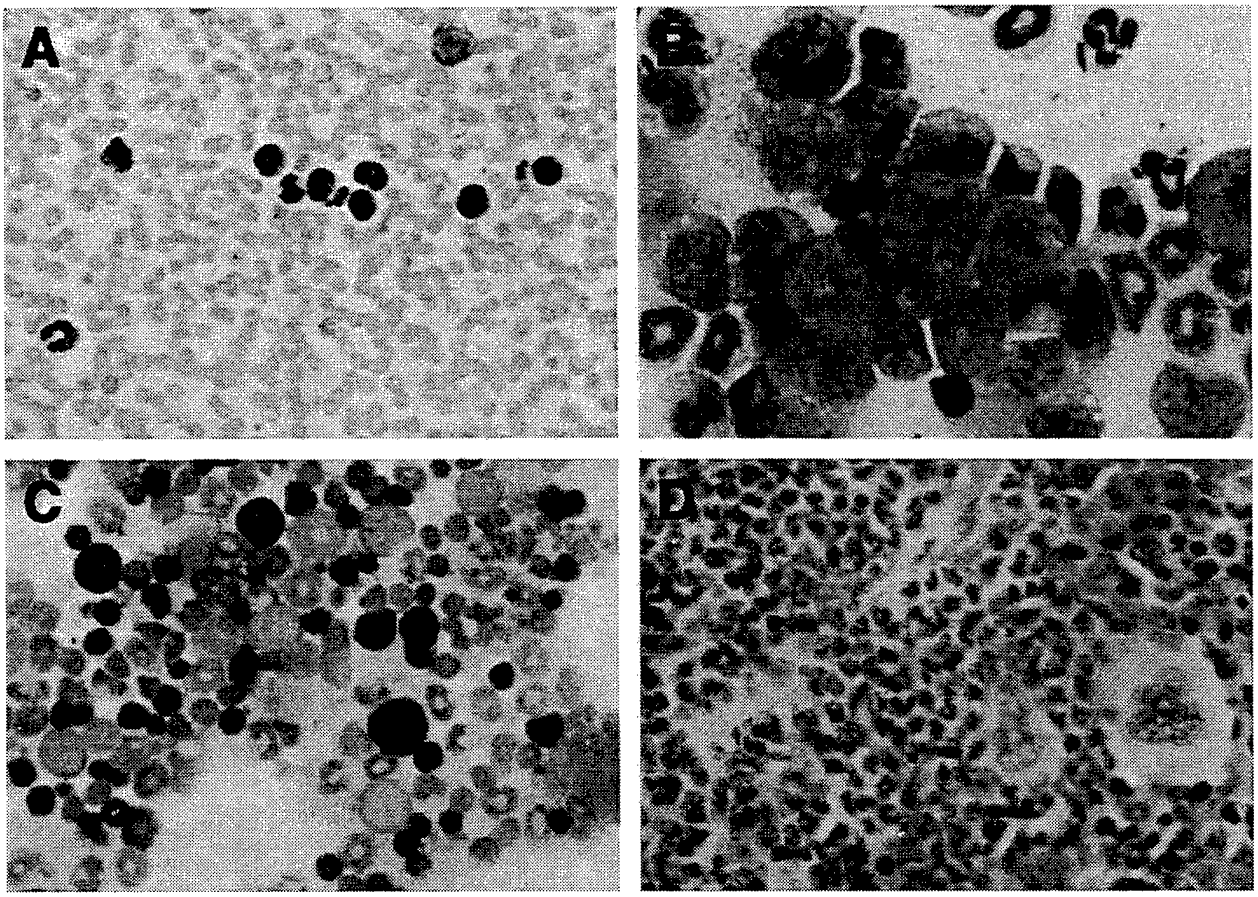

Fig 2. Tissue sections from CY-treated *Nf1*+/− mice with MPD. (A and B) Blood and bone marrow smears showing immature and well differentiated myeloid cells. (C) A cytocentrifuge preparation of spleen cells stained with the myeloid lineage marker nonspecific esterase demonstrates many positive cells (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%)

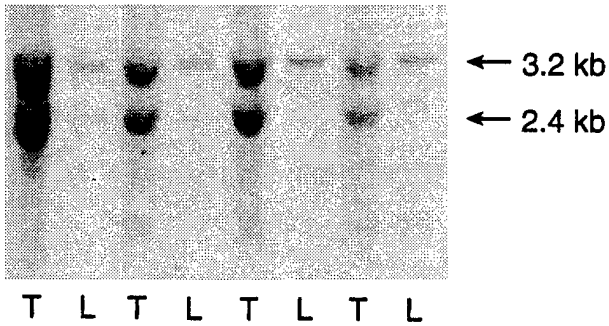

Fig 3. Southern blot analysis of tissues from *Nf1*+/− mice with MPD. The 3.2-kb restriction fragment corresponds to the targeted *Nf1* allele, and the 2.4-kb band is derived from the wild-type allele. DNA extracted from the bone marrows or spleens of five mice with leukemia (L) show absence or a marked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

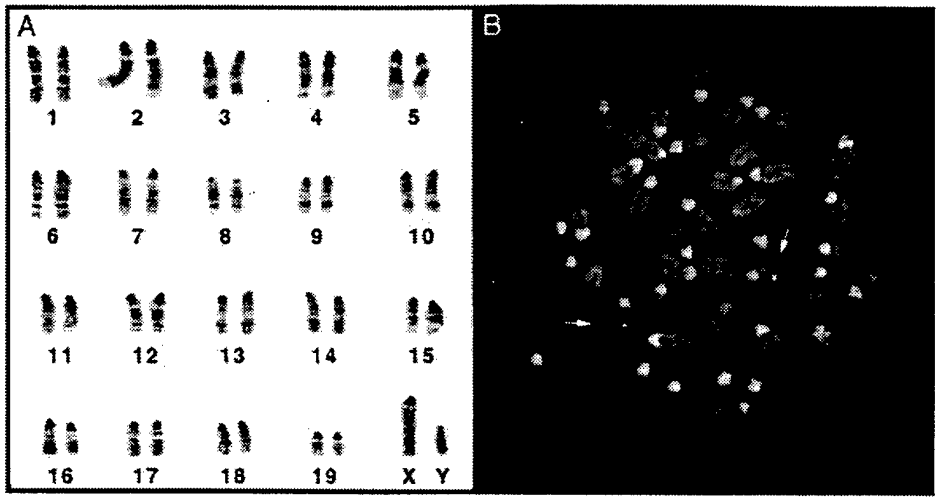

Fig 4. (A) Cytogenetic analysis of spleen cells from a CY-treated mouse with LOH at *Nf1* shows a normal diploid karyotype. (B) FISH of the same specimen shown in (A) with a genomic *Nf1* probe shows 1 copy of the gene on each chromosome 11 homolog.

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.

ACKNOWLEDGMENT

We thank Peter Houghton for suggesting the chemotherapy drug schedules used in this study, Rafael Espinosa III for assistance with FISH analysis, Anthony Fernald for assistance with cytogenetic studies, Nancy Zeleznik-Le and Janet Rowley for the *MLL* probe, Alexander Muller and Arnold Levine for information about polymorphic loci in 129/Sv × C57BL/6 hybrid mice, Sandra-Luna Fineman and Nancy Swigonski for assistance with statistical analyses, and Ben Yen and Ivy Hseih for preparing sections of murine tissues.

REFERENCES

1. Rowley JD, Golomb HM, Vardiman J: Nonrandom chromosomal abnormalities in acute nonlymphocytic leukemia in patients treated for Hodgkin disease and non-Hodgkin lymphoma. Blood 50:759, 1977

2. Le Beau M, Albain KS, Larson RA, Vardiman J, Davis E, Blough R, Golomb H, Rowley J: Clinical and cytogenetic correlations in 63 patients with therapy-related myelodysplastic syndromes and acute nonlymphocytic leukemia: Further evidence for characteristic abnormalties of chromosomes 5 and 7. J Clin Oncol 3:325, 1986

3. Pedersen-Bjergaard J, Phillip P: Cytogenetic characteristics of therapy-related acute nonlymphocytic leukemia, preleukemia, and acute myeloproliferative syndrome: Correlation with clinical data in 61 consecutive cases. Br J Hematol 66:199, 1987

4. Pedersen-Bjergaard J, Rowley JD: The balanced and unbalanced chromosomal aberrations of acute myeloid leukemia may develop in different ways and may contribute differently to malignant transformation. Blood 83:2780, 1994

5. Ratain MJ, Kaminer LS, Bitran JD, Larson RA, Le Beau MM, Skosey C, Rowley JD: Acute nonlymphoblastic leukemia following etoposide and cisplatin combination chemotherapy for advanced small cell carcinoma of the lung. Blood 70:1212, 1987

6. Levine E, Bloomfeld C: Leukemias and myelodysplastic syndromes secondary to drug, radiation, and environmental exposure. Semin Oncol 19:47, 1992

7. Pui C-H, Behm FG, Raimondi SC, Dodge RK, George SL, Rivera GK, Mirro JJ, Kalwinsky DK, Dahl GV, Murphy SB, Crist WM, Williams DL: Secondary acute myeloid leukemia in children treated for acute lymphoid leukemia. N Engl J Med 321:136, 1989

8. Smith MA, Rubinstein L, Ungerleider RS: Therapy-related acute myeloid leukemia following treatment with epipodophyllotoxins: Estimating the risks. Med Pediatr Oncol 23:86, 1994

9. Riccardi VM, Eichner JE: Neurofibromatosis. Baltimore, MD, Johns Hopkins University Press, 1986

10. Bader JL, Miller RW: Neurofibromatosis and childhood leukemia. J Pediatr 92:925, 1978

11. Stiller CA, Chessells JM, Fitchett M: Neurofibromatosis and

childhood leukemia/lymphoma: A population-based UKCCSG study. Br J Cancer 70:969, 1994

12. Arico M, Biondi A, Pui C-H: Juvenile myelomonocytic leukemia. Blood 90:479, 1997

13. Boguski M, McCormick F: Proteins regulating Ras and its relatives. Nature 366:643, 1993

14. Shannon KM, O'Connell P, Martin GA, Paderanga D, Olson K, Dinndorf P, McCormick F: Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. N Engl J Med 330:597, 1994

15. Kalra R, Paderanga D, Olson K, Shannon KM: Genetic analysis is consistent with the hypothesis that *NF1* limits myeloid cell growth through $p21^{ras}$. Blood 84:3435, 1994

16. Jacks T, Shih S, Schmitt EM, Bronson RT, Bernards A, Weinberg RA: Tumorigenic and developmental consequences of a targeted *Nf1* mutation in the mouse. Nature Genet 7:353, 1994

17. Largaespada DA, Brannan CI, Jenkins NA, Copeland NG: *Nf1* deficiency causes Ras-mediated granulocyte-macrophage colony stimulating factor hypersensitivity and chronic myeloid leukemia. Nat Genet 12:137, 1996

18. Bollag G, Clapp DW, Shih S, Adler F, Zhang Y, Thompson P, Lange BJ, Freedman MH, McCormick F, Jacks T, Shannon K: Loss of *NF1* results in activation of the Ras signaling pathway and leads to aberrant growth in murine and human hematopoietic cells. Nat Genet 12:144, 1996

19. Miles DK, Freedman MH, Stephens K, Pallavicini M, Sievers E, Weaver M, Grunberger T, Thompson P, Shannon KM: Patterns of hematopoietic lineage involvement in children with neurofibromatosis, type 1, and malignant myeloid disorders. Blood 88:4314, 1996

20. Side L, Taylor B, Cayouette M, Connor E, Thompson P, Luce M, Shannon K: Homozygous inactivation of the NF1 gene in bone marrow cells from children with neurofibromatosis type 1 and malignant myeloid disorders. N Engl J Med 336:1713, 1997

21. Maris JM, Wiersma SR, Mahgoub N, Thompson P, Geyer RJ, Lange BJ, Shannon KM: Monosomy 7 myelodysplastic syndrome and other second malignant neoplasms in children with neurofibromatosis type 1. Cancer 79:1438, 1997

22. Shannon KM, Turhan AG, Chang SSY, Bowcock AM, Rogers PCJ, Carroll WL, Cowan MJ, Glader BE, Eaves CJ, Eaves AC, Kan YW: Familial bone marrow monosomy 7: Evidence that the predisposing locus is not on the long arm of chromosome 7. J Clin Invest 84:984, 1989

23. Cowell JK: A photographic representation of the variability in the G-banded structures of the chromosome in the mouse karyotype. Chromosoma 89:294, 1984

24. Rowley JD, Diaz MO, Espinosa R, Patel YD, van Melle E, Ziemin S, Le Beau MM: Mapping chromosome band 11q23 in acute leukemia with biotinylated probes: Identification of 11q23 breakpoints within a yeast artifical chromosome. Proc Natl Acad Sci USA 87:9358, 1990

25. Anderson D, Bishop JB, Garner RC, Ostrosky-Wegman P, Selby PB: Cyclophosphamide: Review of its mutagenicity for an assessment of potential germ cell risks. Mutation Res 330:115, 1995

26. Venkatachalam S, Shi YP, Jones SN, Vogel H, Bradley A, Pinkel D, Donehower LA: Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. EMBO J 17:4657, 1998

27. Heim R, Silverman L, Farber R, Kam-Morgan L, Luce M: Screening for truncated NF1 proteins. Nat Genet 8:218, 1994

28. Heim R, Kam-Morgan L, Binnie C, Corns D, Cayouette M, Farber R, Aylsworth A, Silverman L, Luce M: Distribution of 13 truncating mutations in the neurofibromatosis 1 gene. Human Mol Genet 4:975, 1995

29. Shannon KM, Watterson J, Johnson P, O'Connell P, Lange B, Shah N, Kan YW, Priest JR: Monosomy 7 myeloproliferative disease in children with neurofibromatosis, type 1: Epidemiology and molecular analysis. Blood 79:1311, 1992

30. Smith GS, Walford RL, Mickey MR: Lifespan and incidence of cancer in selected long-lived inbred mice and their F1 hybrids. J Natl Cancer Inst 50:1195, 1973

31. Shoemaker AR, Luongo C, Moser AR, Marton LJ, Dove WF: Somatic mutational mechanisms involved in intestinal tumor formation in Min mice. Cancer Res 57:1999, 1997

32. Luongo C, Moser AR, Gledhill S, Dove WF: Loss of Apc+ in intestinal adenomas from Min mice. Cancer Res 54:5847, 1994

33. Takaku T, Oshima M, Miyoshi M, Matsui M, Seldin MF, Taketo M: Intestinal tumorigenesis in compound mutant mice of both Dpc4 and Apc genes. Cell 92:645, 1998

34. Stephens K, Weaver M, Leppig K, Side LE, Shannon KM, Maruyama K: Somatic confined uniparental disomy of the NF1 gene region in myeloid leuekmic cells of an NF1 patient mimics a loss of hetrozygosity due to deletion (abstract). Am J Human Genet 59:A5, 1996 (suppl)

35. Haran-Ghera N, Peled A, Krautghamer R, Resnitzkt P: Initiation and promotion of radiation-induced myeloid leukemia. Leukemia 6:689, 1992

36. Sawyers CL, Denny CT: Chronic myelomonocytic leukemia: tel-a-kinase what ets all about. Cell 77:171, 1994

37. Shannon KM: The Ras signaling pathway and the molecular basis of myeloid leukemogenesis. Curr Opin Hematol 3:305, 1995

38. Pedersen-Bjergaard J, Janssen JWG, Lyons J: Point mutations of the *ras* protooncogenes and chromosome aberrations in acute nonlymphocytic leukemia and preleukemia related to therapy with alkylating agents. Cancer Res 48:1812, 1988

39. Side LE, Teel K, Wang P, Mahgoub N, Larson R, Le Beau MM, Shannon KM: Activating *RAS* mutations in therapy-related myeloid disorders associated with deletions of chromosomes 5 and 7 (abstract). Blood 88:566a, 1996 (abstr, suppl 1)