HUMAN GENE THERAPY 10:2337–2346 (September 20, 1999)
Mary Ann Liebert, Inc.

Engraftment of Hematopoietic Progenitor Cells Transduced with the Fanconi Anemia Group C Gene (*FANCC*)

JOHNSON M. LIU,[1] SONNIE KIM,[1] ELIZABETH J. READ,[2] MAKOTO FUTAKI,[1] INDERJEET DOKAL,[3] CHARLES S. CARTER,[2] SUSAN F. LEITMAN,[2] MICHAEL PENSIERO,[4] NEAL S. YOUNG,[1] and CHRISTOPHER E. WALSH[5]

ABSTRACT

Fanconi anemia (FA) is an autosomal recessive disorder that leads to aplastic anemia. Mutations in the *FANCC* gene account for 10–15% of cases. FA cells are abnormally sensitive to DNA-damaging agents such as mitomycin C (MMC). Transfection of normal *FANCC* into mutant cells corrects this hypersensitivity and improves their viability *in vitro*. Four FA patients, representing the three major *FANCC* mutation subgroups, were entered into a clinical trial of gene transduction aimed at correction of the hematopoietic defect. Three patients received three or four cycles of gene transfer, each consisting of one or two infusions of autologous hematopoietic progenitor cells that had been transduced *ex vivo* with a retroviral vector carrying the normal *FANCC* gene. Prior to infusion, the *FANCC* transgene was demonstrated in transduced CD34-enriched progenitor cells. After infusion, *FANCC* was also present transiently in peripheral blood (PB) and bone marrow (BM) cells. Function of the normal *FANCC* transgene was suggested by a marked increase in hematopoietic colonies measured by *in vitro* cultures, including colonies grown in the presence of MMC, after successive gene therapy cycles in all patients. Transient improvement in BM cellularity coincided with this expansion of hematopoietic progenitors. A fourth patient, who received a single infusion of transduced CD34-enriched BM cells, was given radiation therapy for a concurrent gynecologic malignancy. The *FANCC* transgene was detected in her PB and BM cells only after recovery from radiation-induced aplasia, suggesting that *FANCC* gene transduction confers a selective engraftment advantage. These experiments highlight both the potential and difficulties in applying gene therapy to FA.

OVERVIEW SUMMARY

Fanconi anemia (FA) is a genetic disorder that leads to hematopoietic failure and hypersensitivity to DNA-damaging agents such as mitomycin C (MMC). Mutations in *FANCC* account for 10–15% of cases. Hematopoietic progenitor cells from four FA-C patients were transduced *ex vivo* with an *FANCC*–retroviral vector and then reinfused without prior myeloablation. In each patient, engraftment of short- or long-lived progenitor cells occurred, as documented by the presence of *FANCC*–vector sequences in blood and marrow cells. Normal *FANCC* transgene function was suggested by a marked increase in the number of hematopoietic colonies grown *in vitro,* including colonies resistant to MMC, after successive transduction cycles. Transient improvement in marrow cellularity coincided with this expansion of hematopoietic progenitors. In a fourth patient, who received radiation therapy for a concurrent gynecologic malignancy, the *FANCC* transgene was detectable only after recovery from radiation-induced aplasia. *FANCC*-transduced cells may have a relative growth or engraftment advantage.

[1]Hematology Branch, National Heart, Lung, and Blood Institute (NHLBI), Bethesda, MD 20892.
[2]Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD 20892.
[3]Department of Haematology, Hammersmith Hospital, London, UK.
[4]Genetic Therapy, Inc./Novartis, Gaithersburg, MD 20878.
[5]University of North Carolina School of Medicine, Center for Gene Therapy, Chapel Hill, NC 27599.

INTRODUCTION

FANCONI ANEMIA (FA) is a rare genetic syndrome manifested by aplastic anemia, physical malformations, and cancer susceptibility (Fanconi, 1967). The hallmark of the FA cell is hypersensitivity to DNA-damaging agents such as mitomycin C (MMC), and the disease is now defined by this assay (Liu *et al.,* 1994). FA can be divided into at least eight groups, FA-A through FA-H (Joenje *et al.,* 1997). The gene defective in the FA-C group was identified after phenotypic correction, or complementation, of mutant cells was observed after transfection with the normal *FANCC* gene (Strathdee *et al.,* 1992). Although its exact biochemical function is unclear, FANCC has been reported to interact with a number of cytoplasmic proteins including cytochrome *P*-450 reductase (Kruyt *et al.,* 1998) and may form a complex in the nucleus with other FA gene products (Yamashita *et al.,* 1998) to participate in some aspect of DNA repair (reviewed by Carreau and Buchwald, 1998). FANCC may also normally function to retard programmed cell death (Cumming *et al.,* 1996). Transfer of the normal *FANCC* gene into mutant hematopoietic cells markedly enhances colony formation *in vitro* (Walsh *et al.,* 1994a,b). These studies suggest that bone marrow failure in FA-C patients may be related to an exaggerated susceptibility to hematopoietic cell death and that gene transfer could ameliorate the pathophysiologic process.

Gene transfer is unproven as a therapeutic modality (Liu *et al.,* 1995b). Although some encouraging results have been reported for adenosine deaminase deficiency (Bordignon *et al.,* 1995; Kohn *et al.,* 1995), the concept of gene augmentation has been difficult to realize, in part because of low transduction efficiency. The hematopoietic stem cell has been targeted for genetic manipulation because of its potential for continuous production of blood elements (Brenner *et al.,* 1993; Brenner, 1996). However, despite intensive efforts to alter the conditions of transduction, human stem cell marking with currently available vectors has been disappointingly inefficient (Dunbar *et al.,* 1995). We reasoned that gene transfer might be useful in disorders in which the corrected cell has a selective advantage *in vivo.*

Two features of FA suggest it as a logical candidate for gene augmentation. First, FA affects the hematopoietic stem cell, and the hematologic manifestations of FA can be treated by allogeneic stem cell transplantation from a histocompatible donor (Gluckman *et al.,* 1989, 1995). (Stem cell transplantation would not be expected to alter predisposition to malignancy in nonhematopoietic tissues). Second, observations have suggested that the mutant FA stem cell clone may be at a selective disadvantage relative to a normal clone (Lo Ten Foe *et al.,* 1997). Spontaneous reversion of the FA phenotype has been described in a few patients, resulting in reconstitution of a normal allele at the disease locus. In one patient, the degree of reversion was sufficient to allow replacement of nearly all of the mutant cells, implying that a single revertant stem cell had gained dominance over mutant cells.

Hematopoietic stem cells (Lu *et al.,* 1996) can be isolated from bone marrow (BM), growth factor-mobilized peripheral blood (PB), or umbilical cord blood. The cell fraction enriched for the CD34 antigen is thought to include both lineage-committed progenitor cells (capable of hematopoietic colony formation *in vitro*) and a small number of stem cells (capable of repopulating a myeloablated host). Initially, we designed a protocol for *FANCC* gene transduction of PB CD34-enriched hematopoietic cells. Four cycles (Liu *et al.,* 1995a) of mobilization, collection, transduction, and infusion were used in an attempt to increase the number of gene-corrected cells. *Ex vivo* transduction conditions were based on pilot studies indicating a requirement of cytokine prestimulation (Walsh *et al.,* 1995). Because of the hypoplastic state of the FA host bone marrow, ablative chemotherapy was not administered prior to cell infusion. Two of the patients described in this study also received transduced CD34-enriched cells derived from BM rather than mobilized PB.

MATERIALS AND METHODS

Study subjects

Three children and one adult with FA-C were studied in a protocol approved by the National Heart, Lung, and Blood Institutional Review Board, NIH Office of Recombinant DNA Activities, NIH Biosafety Committee, and the Food and Drug Administration. Clinical features of these subjects are presented in Table 1.

Collection of G-CSF-mobilized autologous hematopoietic progenitor cells

For each cycle of gene therapy, patients received a 5-day course of granulocyte colony-stimulating factor (G-CSF; Amgen, Thousand Oaks, CA), 10 μg/kg per day subcutaneously, followed by one to three daily consecutive leukapheresis procedures to collect circulating hematopoietic progenitor cells. Procedures were performed using the Fenwal CS3000 Plus (Baxter, Deerfield, IL) or the Spectra cell separator (Cobe, Lakewood, CO). For each procedure, a whole blood volume of 5 to 15 liters was processed, using central venous access and acid–citrate–dextrose (ACD-A; Baxter) anticoagulation. In patient 3, who weighed only 12 kg, packed red blood cells were used to prime the apheresis device, and heparin was substituted for ACD-A to avoid citrate toxicity. The interval between gene therapy cycles was 3 to 4 months.

Bone marrow harvest

Bone marrow was collected by standard methods from two patients under general anesthesia. A volume of approximately 1 liter of bone marrow was aspirated into heparinized syringes from posterior iliac crests, pooled, and filtered prior to further processing.

Preparation and infusion of gene-transduced autologous hematopoietic progenitor cells

Each leukapheresis or bone marrow collection was either processed separately or pooled with a second leukapheresis collection prior to processing on a Ceprate SC system (CellPro, Bothell, WA). CD34-enriched cells obtained by this method were transduced in equal volumes of culture medium (Dulbecco's modified Eagle's medium [DMEM]; BioWhittaker, Walkersville, MD) and clinical-grade retroviral vector supernatant in D10 medium (G1FASvNa.52; Genetic Therapy, Gaithersburg, MD), at cell concentrations of 1.0–5.0 $\times$ 10^5

TABLE 1. CLINICAL FEATURES OF PATIENTS IN STUDY

Patient	*Hematologic presentation*	*Bone marrow cytogenetics*	FANCC *mutation*
1: 11-year-old male	Aplastic anemia	Normal XY	Exon 1[a]
2: 14-year-old male	Refractory anemia with excess blasts	47 XY+ derivative of chromosome 3	Exon 1/exon 14 (compound heterozygote)[b]
3: 11-year-old female	Aplastic anemia	Translocation of X and 3	Intron 4[c]
4: 29-year-old female	Aplastic anemia	Normal XX	Exon 1[a]

[a]delG322 *FANCC* mutation on one allele; second undetermined *FANCC* mutation on the other allele.
[b]delG322 *FANCC* mutation on one allele; leucine-to-proline change at position 554 (L554P mutation) on the other allele.
[c]Mutation of the fourth intron (IVS4+4 A → T mutation) on one allele; second undetermined *FANCC* mutation on the other allele.

cells/ml, in polystyrene flasks at 37°C in 5% CO_2. The G1FASvNa.52 retroviral vector, containing the *FANCC* and neomycin resistance (*neo*-) coding sequences, has a titer of 5×10^6 infectious particles/ml (Walsh *et al.,* 1995). The culture transduction medium also contained 10% fetal bovine serum (FBS; BioWhittaker), protamine sulfate (4 μg/ml; Elkins-Sinn, Cherry Hill, NJ), interleukin 3 (IL-3, 20 ng/ml; Sandoz, East Hanover, NJ), stem cell factor (SCF, 100 ng/ml; Amgen), interleukin 6 (IL-6, 50 ng/ml; Sandoz), and gentamicin sulfate (50 ng/ml; GIBCO, Gaithersburg MD). Medium was changed 24 and 48 hr after cultures were initiated. At 72 hr, cells were harvested, washed in DMEM supplemented with preservative-free porcine heparin sodium (10 units/ml; Fujisawa USA, Deerfield, IL), and resuspended in 30 ml of Plasma-Lyte A (Baxter) supplemented with 2% human serum albumin (Albutein; Alpha Therapeutics, Los Angles, CA) for infusion. Samples of blood, leukapheresis or bone marrow collections, positively selected hematopoietic progenitor cells, and transduced cells were obtained for automated cell counting and flow cytometric quantitation of $CD34^+$ cells (Mavroudis *et al.,* 1996). Gene-transduced hematopoietic progenitor cells were infused into a peripheral vein over 5 min after premedication with acetaminophen and diphenhydramine.

Colony culture and molecular analysis of CD34-enriched cells prior to infusion cycle

Samples (2×10^6 cells) of the untransduced and transduced CD34-enriched cell fractions were resuspended in Iscove's modified Dulbecco's medium (IMDM; GIBCO) containing 20% FBS with SCF (100 ng/ml), IL-6 (50 ng/ml) IL-3 (25 ng/ml), and erythropoietin (5 U/ml). Cells were mixed with methylcellulose (Stem Cell Technologies, Vancouver, BC, Canada) and plated into duplicate wells (1.5 ml each) with either 0, 1, or 5 n*M* concentrations of mitomycin C (MMC). The cultures were incubated for 14 days at 37°C with 5% CO_2. At the end of the incubation period, colonies at 0, 1, and 5 n*M* MMC were counted; some were isolated and transferred to 0.5-ml tubes containing 50 μl of H_2O. Each culture from a single dish was then dissolved in phosphate-buffered saline (PBS) and transferred to a 15-ml conical tube to pool the remaining colonies. The cells were centrifuged and resuspended in 50 μl of digestion buffer containing 5 m*M* Tris-HCI (pH 8.0), 0.45% Nonidet P-40, and 0.45% Tween 20. DNA from each colony and the pooled cultures pre and post-transduction was extracted with proteinase K (10 mg/ml). DNA polymerase chain reaction (PCR) amplification (Liu *et al.,* 1995a) was performed using β-actin (5′-CAT TGT GAT GGA CTC CGG AGA CGG-3′ and 5′-CAT CTC CTG CTC GAA GTC TAG AGC-3′), G1 backbone (5′-CCT GCG TCT GTA CTA GTT AGC TAA CTA G-3′ and 5′-GAT GCT GCA GCG CTG CAG CAG ACA AGA C-3′), and *neo*^{r} (5′TCC ATC ATG GCT GAT GCA ATG CGG C-3′ and 5′-GAT AGA AGG CGA TGC GCT GCG AAT CG-3′) primers at 95°C for 2 min for 1 cycle, 95°C for 1 min, 55°C for 1 min, 72°C for 2 min for 35 cycles, with a final extension of 72°C for 8 min.

Molecular analysis of postinfusion peripheral blood and bone marrow samples

PB and BM samples were processed after Ficoll-Hypaque separation to isolate mononuclear cells. Cells were incubated at 37°C overnight with proteinase K (10 mg/ml) and 2× lysis buffer containing 20 m*M* Tris-HCI (pH 7.5), 0.6 *M* NaCl, 10 m*M* EDTA, and 1% sodium dodecyl sulfate (SDS). DNA was extracted from these cells with phenol–chloroform and precipitated with ethanol. A standard curve was generated from DNA isolated from cells known to have a single copy of the *neo*^{r} gene per cell. Patient PB and BM DNA samples were PCR amplified (as for the colony analysis) and the signal intensity compared with those from the dilutions of the single-copy standard. RNA from PB mononuclear cells was isolated using RNA STAT-60 (Tel-Test, Friendswood, TX), and RNA PCR was performed with reverse transcriptase (Perkin-Elmer RNA PCR kit; Roche Molecular Systems, Branchburg, NJ) and the same primers and conditions as described for the DNA PCR.

In some instances, DNA was isolated from PB cell samples sorted for B lymphocytes (anti-CD19 antibody) and T lymphocytes (anti-CD2 antibody), using a Coulter (Hialeah, FL) Epics Elite fluorescence-activated cell sorter. Granulocytes were isolated on the basis of light scatter properties.

RESULTS

Collection, transduction, and infusion of autologous hematopoietic progenitor cells

Results of G-CSF mobilization and infusion of hematopoietic progenitor cells in patients 1–3 are presented in Table 2.

Table 2. Autologous Cellular Products and Colony Numbers

Patient	Cycle No.	Circulating PB $CD34^+$ cells after G-CSF (per μl)	Infused $CD34^+$ cells		Colony numbers prior to cycle of transduction[a]		
			Total ($\times 10^6$)	Dose (10^6/kg)	Mitomycin C		
					0 nM	1 nM	5 nM
1	1	0–1.1	0.6	0.011	272	ND	ND
	2	0.7–3.4	0.8	0.02	472	26	0
	3	0	1.0	0.02	804	272	0
	4	0	0.12	0.003	TNTC	TNTC	134
2	1	43	28.1	0.46	162	32	0
	2	0.9–4.1	0.18	0.003	52	16	0
	3	33.6–116	56	0.85	TNTC	TNTC	134
	4	12–16	4.8	0.07	12	2	0
3	1	26–30	13.5	1.1	148	4	2
	2	92	20.4	1.9	66	36	0
	3	19–34	16	1.3	TNTC	288	10

Abbreviations: TNTC, Too numerous to count; ND, not done.

[a]Hematopoietic colonies were enumerated manually from replicate samples, and the numbers averaged. Numbers of colonies were determined prior to vector exposure for each cycle and thus reflect *in vivo* changes during the preceding months. For patient 1, 2×10^5 CD34-enriched cells/ml were plated. For patients 2 and 3, 1×10^5 cells/ml were plated.

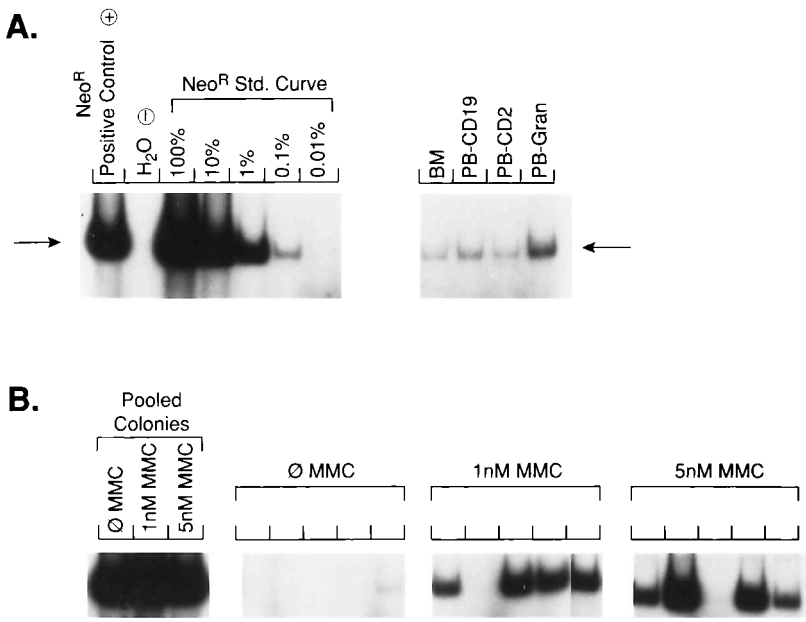

FIG. 1. **(A)** DNA was isolated from bone marrow (BM) mononuclear cells and from peripheral blood (PB) cell samples, sorted for B lymphocytes (CD19 positive), T lymphocytes (CD2 positive), and granulocytes, in patient 1. DNA samples were PCR amplified and the signal intensities compared with those from dilutions of the *neo*^{r} gene single-copy standard. "100%" signifies that 100% of cells have 1 copy of the *neo*^{r} gene, with serial 10-fold dilutions as shown. In comparison, the BM and PB samples range from 0.1 to 1% (1 in 1000 to 1 in 100 cells), with the strongest signal from the granulocyte fraction. The positive control was DNA from a cell line transfected with the G1FASvNa.52 retroviral vector. H_2O was the negative control. **(B)** After *FANCC* retroviral transduction of CD34-enriched progenitor cells in patient 1, vector sequences could be detected in DNA samples from either pools of colonies (left) or randomly selected individual colonies (right) grown in the presence of 0, 1, or 5 n*M* concentrations of MMC.

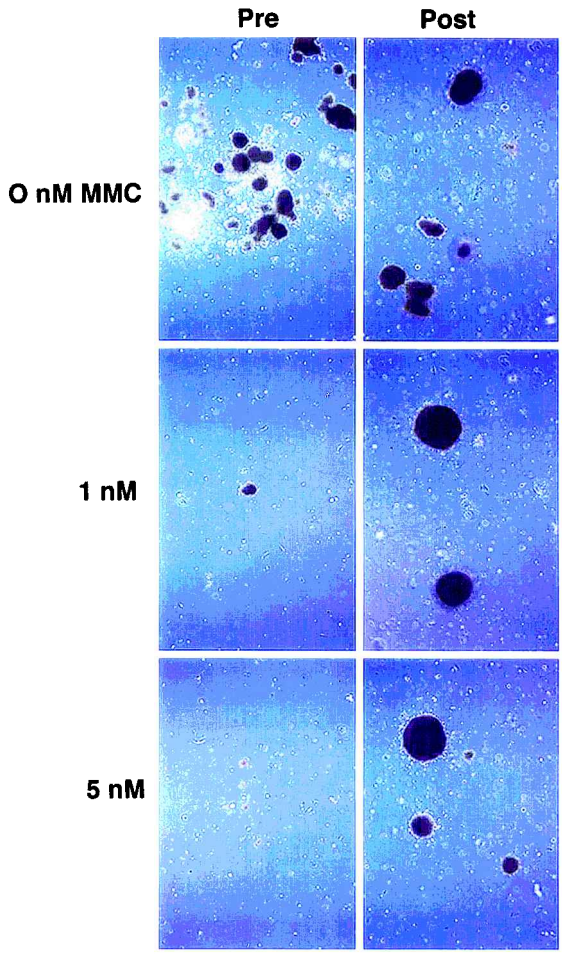

FIG. 2. Appearance of hematopoietic progenitor colonies before (labeled "Pre") and after (labeled "Post") cycle 3 for patient 1. Progenitors were grown in the presence of 0, 1, or 5 n*M* concentrations of MMC. After *FANCC* transduction, increased numbers of colonies were observed in the presence of 1 and 5 n*M* MMC. With successive transduction cycles, the morphologic appearance of the colonies changed, with larger numbers of cells in each colony or cluster. In addition, erythroid colonies were seen after cycle 2 and persisting thereafter, whereas prior to transduction, the colonies from this patient were almost entirely granulocytic or granulocyte/macrophage-like.

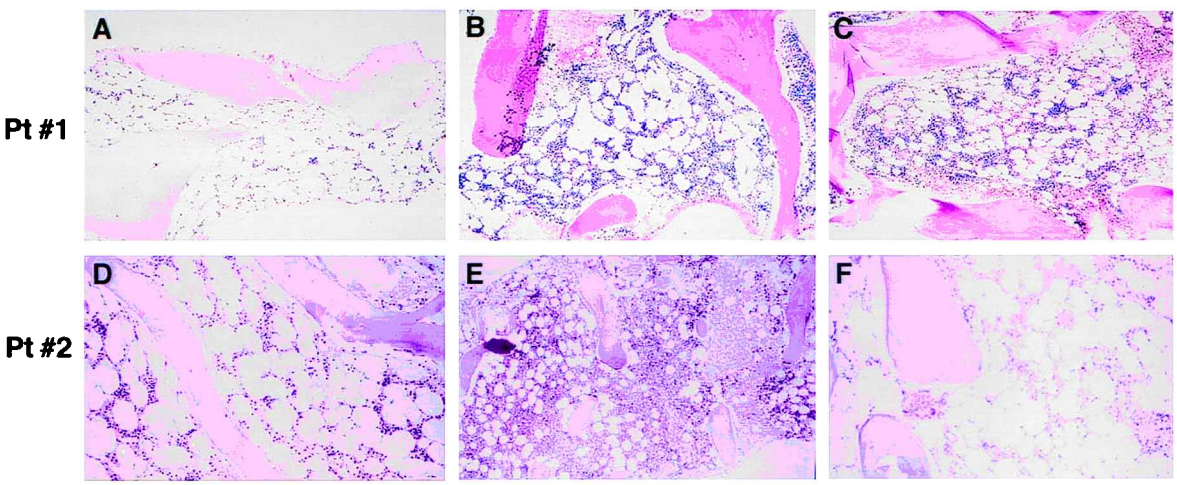

FIG. 3. Trephine bone marrow biopsies were obtained from the iliac crest of patients 1 and 2. For patient 1, specimens were obtained immediately prior to cycle 1 **(A),** cycle 2 **(B),** and cycle 4 **(C).** For patient 2, specimens were obtained immediately prior to cycle 2 **(D),** cycle 3 **(E),** and cycle 4 **(F).** The biopsies were stained with hematoxylin and eosin. Original magnification: ×25.

Each patient had a distinct pattern of $CD34^+$ cell mobilization, and the number of $CD34^+$ cells collected (and therefore available for transduction) was proportional to the number circulating. For all four cycles in patient 1, $CD34^+$ cell yields were extremely low, whereas patient 3 demonstrated the most consistent rise in circulating $CD34^+$ cells. Final infused $CD34^+$ cell numbers ranged from 6×10^5 in patient 1, who failed to mobilize, to 20.4×10^6 in patient 3 and 56×10^6 in patient 2, both following successful mobilization. For patient 3, the $CD34^+$ cell doses (per body weight) in the three separate infusions were 1.1, 1.9, and 1.3×10^6 cells/kg, values within the range used for autologous hematopoietic rescue after high-dose chemotherapy. After infusion of the transduced cells, none of the patients experienced fever, chills, or other immediate adverse effects.

Clinical course and FANCC *transduction for patient 1*

The clinical course of patient 1, whose PB and BM mononuclear cell samples were repeatedly positive for *FANCC* vector sequences for nearly 16 months after the first infusion, was notable for transient improvements in blood counts temporally related to gene therapy cycles. Immediately prior to the first gene therapy, cycle, he had a leukocyte count of $2800/mm^3$ (absolute neutrophil count of $532/mm^3$), hemoglobin of 8.2 g/dl, and a platelet count of $25{,}000/mm^3$. Prior to cycle 1, he was treated with oxymetholone at a dose of 100 mg/day and G-CSF at a dose of 180 μg three times weekly. After each cycle of gene therapy, hemoglobin values rose without intervening transfusions, reaching a high of 12.6 g/dl immediately prior to cycle 2 and again peaking at 12.4 g/dl 3 months after cycle 4. Platelet counts also rose without intervening transfusions, peaking at $46{,}000/mm^3$ immediately prior to cycle 3 and again at $45{,}000/mm^3$ 1 month after cycle 4. White blood cell (WBC) counts rose, without intervening infections, to a peak of $21{,}400/mm^3$ 1 month before cycle 3. Because of the improvement in hematologic indices, both oxymetholone and G-CSF were tapered. In contrast, patients 2 and 3 did not show consistent changes in blood counts in relationship to gene therapy cycles. The clinical course for patient 4 is described below.

Detection of transduced mononuclear cells in peripheral blood and bone marrow

For patient 1, the *FANCC* transgene was detected in fewer than 0.01% of PB mononuclear cells 1 month after cycle 1. However, by 4 months after cycle 1, the transgene was present in approximately 0.1% (1 in 1000) of PB mononuclear cells (Fig. 1A). Furthermore, analysis of DNA from cells separated by fluorescence-activated sorting indicated that granulocytes, B cells, and T cells were all marked 4 months after cycle 1 (Fig. 1A).

Patient 2, who received four infusions of transduced cells, had *FANCC* vector sequences detected in approximately 1–3% of PB mononuclear cells, 2 months after cycle 1 (data not shown). However, all subsequent samples from him were negative. Patient 3, who received three infusions of transduced cells, had *FANCC* vector sequences initially detected in the peripheral blood and bone marrow at a low level ($<0.01\%$ of PB mononuclear cells) but no detectable sequences after cycle 3 (data not shown).

Colony growth and functional analysis of FANCC*-transduced CD34-enriched cells*

Function of the *FANCC* transgene was confirmed for each autologous cellular product by assessing colony growth and MMC resistance of the CD34-enriched cell fractions (untransduced and transduced). As shown for patient 1 in Fig. 1B, *FANCC* vector sequences could easily be detected in DNA from either individual colonies or pools of colonies in the transduced CD34-enriched cell fraction. During each cycle, transfer of *FANCC* to the CD34-enriched cell fraction increased the total number of colonies grown in the absence and presence of low concentrations of MMC. Figure 2 shows the appearance of these colonies before and after cycle 3 for patient 1. In patient 2, we were able to obtain additional confirmation of transgene expression by noting the growth of primitive erythroid progenitors (BFU-E) resistant to 1 mg of geneticin (G-418), per milliliter, whereas none was present prior to the gene therapy cycles (data not shown).

In vivo *expansion of hematopoietic progenitors after gene therapy cycles*

Between gene therapy cycles, all three patients developed marked increases in total numbers of progenitor-derived colonies and colonies resistant to low concentrations of MMC added in culture (Table 2). Numbers of colonies were determined prior to vector exposure with each cycle and presumably reflected *in vivo* changes during the preceding months; this increase in hematopoietic colony formation did not occur immediately but only after at least two gene therapy cycles.

Bone marrow morphology

Increases in bone marrow cellularity for patients 1 and 2, which coincided with the expansion of hematopoietic progenitor cells, are shown in Fig. 3. For patient 1, cellularity increased from 10–15 to 30%. Patient 2 initially had a hypoplastic bone marrow with approximately 5% myelomonocytic blasts. After cycle 2, bone marrow cellularity increased transiently to 60%; fewer blasts were detected by microscopic examination because of the overall increased cellularity. However, shortly before cycle 4, marrow cellularity decreased to 10–15%, similar to prestudy values.

Transduced BM-derived $CD34^+$ progenitor cells: Course of patients 1 and 4

Because of the variability in mobilization of PB $CD34^+$ cells, we obtained permission to collect and transduce autologous bone marrow hematopoietic progenitor cells. Marrow cells were CD34 selected, transduced by methods described above, and cryopreserved. Patients 1 and 4 received transduced BM $CD34^+$ cells. As described above, patient 1 had evidence of *FANCC* gene transfer for nearly 16 months after the first infusion of transduced PB $CD34^+$ cells, corresponding to increases in progenitor numbers, bone marrow cellularity, and blood counts. After loss of the *FANCC* transgene signal in this patient, there was a concurrent and progressive deterioration in hematologic indices. To determine whether the apparent improvements related to *FANCC* gene transfer, we performed a bone marrow harvest 16 months after the last infusion (cycle 4) and returned trans-

duced BM $CD34^+$ cells 7 months later. Despite the infusion of a number of transduced $CD34^+$ cells similar to what had been obtained with each cycle of PB mobilization and processing, we could not detect evidence of *FANCC*-marked cells after infusion.

Patient 4, a 29-year-old woman with the same exon 1 mutation as patient 1, also received transduced BM $CD34^+$ cells. After collecting 7.5×10^9 nucleated BM cells with a low content of $CD34^+$ cells, only 1.13×10^6 transduced $CD34^+$ cells could be infused. This patient suffered from mild pancytopenia and had a 6-year history of recurrent genital warts. Her hematologic indices included a leukocyte count of 2000/mm^3 (absolute neutrophil count of 1000/mm^3), hemoglobin of 11.8 g/dl, and a platelet count of 114,000/mm^3. Fifty days after the infusion of transduced BM cells, she was diagnosed with rapidly progressive squamous cell carcinoma of the vulva, without evidence of lymph node involvement. She underwent radiation therapy to the pelvic field with a target dose of 4500 cGy in 25 fractions. Within 3 weeks of these daily radiation treatments, she developed worsening pancytopenia with a nadir in the absolute neutrophil count to 200/mm^3 (approximately 80 days after the infusion of *FANCC*-transduced cells), prompting a 6-week delay before her radiation treatments were resumed (on day 120 after infusion). Radiation therapy was completed by day 180. Whereas PB samples collected prior to and immediately after the radiation treatments did not show evidence of *FANCC* gene-marked cells, both PB and BM mononuclear cell samples collected 220 days after infusion (and after recovery from radiation-induced myelosuppression) were weakly positive for marked cells (data not shown). DNA PCR assays confirmed the presence of marked BM and PB cells on day 332 (Fig. 4A, left). Analysis of DNA from cells separated by fluorescence-activated sorting indicated that granulocytes, B cells, and T cells were marked by day 360 (Fig. 4A, right). Finally, RNA PCR assays confirmed transgene RNA expression in PB mononuclear cells isolated on day 414 (Fig. 4B).

DISCUSSION

Ideally, hematopoietic cell gene augmentation in FA would lead to correction of the phenotypic defect in some stem cells, leading to proliferation of corrected clones capable of permanently replacing abnormal cells. This outcome would be demonstrated by the presence of the *FANCC* transgene in all marrow and peripheral blood hematopoietic cells, with normal function of all of these cells, including resistance to MMC. In our trial, we have not observed stem cell correction to date. However, infusion of *FANCC* gene-transduced autologous hematopoietic cells resulted in a marked increase in bone marrow cellularity and numbers of hematopoietic progenitor cells, as measured in clonogenic assays. These results were uniformly observed in spite of the genotypic and phenotypic variability among the three patients who received transduced PB CD34-enriched cells. Because we were able to demonstrate, after *FANCC* transduction, increased numbers of progenitors capable of colony formation in the presence of MMC, it is likely that the *in vivo* expansion of hematopoietic cells was related to *FANCC* transgene function and not merely to the infusion of mobilized, cultured hematopoietic progenitor cells. This interpretation is further supported by our negative results after a fifth infusion in patient 1 of transduced BM-derived cells, which did not lead to engraftment of *FANCC*-transduced cells and was not associated with any change in his hematologic indices. In addition to the highly significant increases in progenitor numbers, including those resistant to MMC (or to G418), we also observed transgene RNA expression in peripheral blood mononuclear cells from patient 4 (Fig. 4B), clearly demonstrating persistent transduction with our retroviral vector and corroborating our finding of *FANCC* transgene function.

As shown in Fig. 1A, in which comparison is made between gene transfer levels into bone marrow mononuclear cells, B

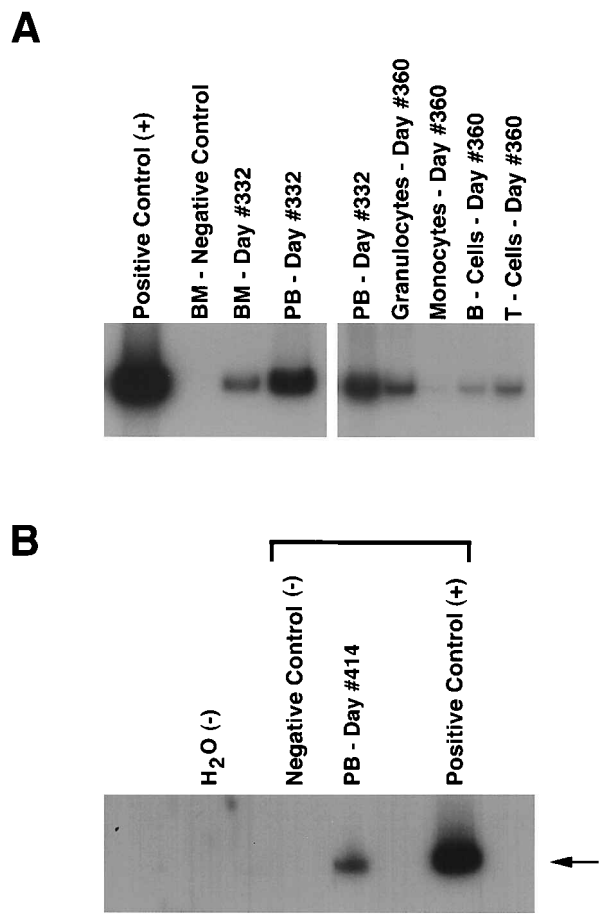

FIG. 4. **(A)** DNA was isolated from bone marrow (BM) mononuclear cells and from peripheral blood (PB) cell samples from patient 4. DNA samples were amplified by PCR, using primers specific for the *FANCC* retroviral vector. Shown are DNA PCR analyses of DNA from cells collected 332 days after infusion. The positive control was DNA from a cell line transfected with the G1FASvNa.52 retroviral vector. DNA from a mock-transduced BM mononuclear cell sample served as a negative control. As shown on the right, cells were also separated by fluorescence-activated sorting: granulocytes, B cells, and T cells were marked at day 360 (a weak signal was also seen from the monocyte fraction). **(B)** RNA PCR assays confirmed transgene RNA expression in PB mononuclear cells isolated on day 414.

cells, T cells, and granulocytes in patient 1, the percentage of positive cells in the marrow (below 0.1%) is not higher than in peripheral blood (compare with granulocyte fraction, for example). This contrasts with some other published studies of hematopoietic progenitor cell gene transfer that reported discrepancies between gene transfer levels into cells from bone marrow versus peripheral blood. Cell lineage analyses for both patients 1 and 4 (the two having had the longest duration of gene transfer from the time of infusion) documented transduction of T cells and B cells (Figs. 1 and 4). This finding argues that patient 2, in whom gene transfer was detected only transiently in peripheral blood mononuclear cells, may have had a short-lived progenitor cell transduced, giving rise to myeloid cells but not including or giving rise to lymphocytes in peripheral blood. Patient 2 also had little evidence of hematopoietic correction, correlating with our expectation that persistent *FANCC* gene transfer to a multipotent progenitor cell would be required for a clinically significant result. In patient 4, we believe that a long-lived multipotential progenitor cell was transduced, since these findings were obtained more than 1 year after a single infusion of transduced CD34-enriched BM cells (only further analysis can determine if a true stem cell was transduced). Our results also suggest that *FANCC* gene transfer may have conferred a selective growth and/or engraftment advantage after recovery from a known toxic insult to blood cell production in patient 4 (see discussion below).

Successful transduction of hematopoietic stem cells, which may exist in a quiescent state, has been hampered because retroviral vectors require active replication of their target cells for integration (Liu *et al.,* 1995b). Prestimulation with cytokines has resulted in efficient transduction of more mature progenitor cells but not of stem cells. Our retroviral vector was able to transduce 10–20% of progenitor cells, as assessed by DNA analysis of individual colonies after each cycle of transduction (our unpublished data, 1998). An additional challenge for this trial was the uncertainty as to the most appropriate methods for mobilization, collection, and hematopoietic transduction for FA, an intrinsic stem cell disorder. Our patients were representative of the three major FA-C subgroups identified on the basis of genotype–phenotype analyses (Gillio *et al.,* 1997): intron 4 (patient 3), exon 14 (patient 2), and exon 1 (patients 1 and 4). As predicted by such analyses, patients 2 and 3 suffered from early-onset hematologic abnormalities. With the use of G-CSF mobilization prior to harvest of autologous cells from the peripheral blood and transduction conditions that included cytokine stimulation and a high-titer retroviral vector, a range of outcomes was observed in these individuals. Of particular interest is the variability in mobilization, which likely had an effect not just on the quantity but also on the nature of the cell populations available for transduction. One patient (patient 1) failed to mobilize CD34-positive cells into the peripheral blood and had the lowest number of CD34-positive cells in infused cell suspensions, but *FANCC*-transduced cells circulated for 4 months after cycle 1, and for nearly 16 months total. Conversely, patient 3 received cell doses within the range used for conventional autologous hematopoietic rescue after myeloablation. Although colonies were marked at the beginning of cycle 3, PB samples were only transiently positive. These findings demonstrate a lack of correlation between the number of CD34-positive cells in the infusion and the persistence of detectable circulating cells.

The safety of administering gene-transduced cells, especially in repeated cycles, continues to be a concern for clinical trials of gene therapy. In this study, patients generally tolerated the procedures well, but the occurrence of bleeding associated with placement of apheresis catheters was notable, while not unexpected owing to preexisting thrombocytopenia. Even after multiple infusions, there were no allergic, anaphylactic, or serum sickness-like reactions, which have been reported to occur in association with repeated exposure to cells cultured with fetal bovine serum and retroviral vector supernatant (Selvaggi *et al.,* 1997). Extensive testing after each transduction cycle failed to reveal the presence of replication-competent retrovirus. Finally, we did not observe amplification of *FANCC*-transduced myeloblasts in the patient with preexistent myelodysplasia.

Despite the growth advantage of transduced cells *in vitro,* to date there has been no evidence of amplification of a hematopoietic stem cell *in vivo,* in the three patients who received either PB or BM $CD34^+$ cells alone. Possibly, CD34-enriched cells are not the optimal targets for complementation, as there continues to be debate regarding the phenotypic identification of true stem cells (Osawa *et al.,* 1996). Alternatively, amplification of the transduced cell clone may require additional selective pressure, as appeared to be the case with patient 4, in whom we could detect *FANCC* gene-marked cells only after recovery from radiation-induced aplasia 220 days after the cell infusion. This result mirrors our finding in *Fancc* knockout mice that received syngeneic BM cells transduced by our *FANCC* retroviral vector (C.E. Walsh, unpublished data, 1998). In these animals, we observed a marked increase in the number of gene-corrected cells 1 to 2 months after induction of pancytopenia with injections of MMC. The human and mouse experiments imply that the transduced fraction of progenitor and stem cells may be quiescent and that proliferation of these cells may be triggered by radiation- or drug-induced aplasia. The delay in engraftment with gene-corrected cells suggests that our results may not be explained entirely by direct cytotoxicity against mutant FA cells. It may be necessary to use either DNA cross-linking agents, radiation, or biological agents in order to create new marrow space or to trigger the proliferation of gene-corrected cells. With the identification of the *FANCA* (Fanconi Anaemia/Breast Cancer Consortium, 1996; Lo Ten Foe *et al.,* 1996) and *FANCG* (De Winter *et al.,* 1998) genes, transduction strategies (Fu *et al.,* 1997) eventually may become applicable in other FA patients. First, however, research will be needed on methods of isolating the optimal hematopoietic cell targets for transduction and on application of selective pressure to their advantage (Liu, 1998).

ACKNOWLEDGMENTS

We thank Drs. A. Nienhuis, M. Cottler-Fox, and H. Safferstein for intellectual contributions. We also thank the 2 West Hematology/BMT nursing staff and the patients and families involved in our protocols. The clinical-grade G1FASvNa.52 retroviral vector supernatant was manufactured by Genetic Therapy, Inc./Novartis under the auspices of a collaborative research agreement with the NHLBI.

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Address reprint requests to:
Dr. Johnson M. Liu
Hematology Branch
National Heart, Lung, and Blood Institute
Bldg. 10, Rm. 7C103, ACRF
Bethesda, MD 20892

E-mail: LiuJ@gwgate.nhlbi.nih.gov

Received for publication March 17, 1999; accepted after revision July 2, 1999.