# Scientific Supplement

## FA Family Newsletter #17

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## In This Issue:

# Treatment Updates

## G-CSF Treatment of Patients with FA

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This study examines the effect of filgrastim (G-CSF) in 11 patients with Fanconi anemia and an absolute neutrophil count (ANC) less than 1,000/mm$^3$. Two of 13 patients who were evaluated were ineligible due to abnormal bone marrow cytogenetics. G-CSF was started at a commonly used dose. The dose was reduced when the ANC remained at normal levels for a number of weeks. Some patients are now receiving the drug on an every-other-day schedule. All patients have had an increased neutrophil count at week 8. Three patients had an increased platelet count at week 8 without transfusion. Unfortunately, in all 3 of these patients, the platelet count returned to baseline levels when the dose of G-CSF was reduced over time.

Three of 4 patients who did not receive red blood cell transfusion had an increase in the hemoglobin of 1.1 - 2.3 grams at week 8. A fourth patient had a transfusion in week 2 and then had an elevated hemoglobin without subsequent transfusion.

Adverse experiences were as follows: mild fever, which resolved when the G-CSF dose was decreased (1 patient); dental abscess (1 patient). No patient has developed a bone marrow cytogenetic abnormality or myelodysplasia while on G-CSF. These early results suggest that G-CSF is safe and efficacious in patients with Fanconi anemia. G-CSF appears to be a good candidate for use in combination cytokine protocols for patients with Fanconi anemia. Its role in stimulating platelet and red blood cell production needs to be better studied, before drawing any conclusions about its usefulness in patients with low platelets and red blood cells. ••

## Bone Marrow Transplantation for Fanconi Anemia

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Bone marrow transplantation is currently the only proven method for curing FA patients of aplastic anemia and of reducing or eradicating the risk of progression to myelodysplastic syndrome or leukemia. Gene therapy may also be available in the near future.

I have presented data on the outcome of marrow transplants for FA at the Fanconi Anemia Family Meetings for the past several years. This database, the *Fanconi Anemia Transplant Registry (FATR)*, incorporates transplant outcome information obtained from a variety of sources, including the International Bone Marrow Transplant Registry, the National Marrow Donor Program and the International Fanconi Anemia Registry. The FATR also includes comprehensive data from several major transplant programs which have performed five or more transplants in patients with FA. These include the Hopital St. Louis (Paris), the Fred Hutchinson Cancer Research Center (Seattle), the University of Minnesota, Memorial Sloan-Kettering Cancer Center (New York), University Hospital in Curitiba (Brazil), and the Children's Hospital Medical Center in Cincinnati. The information contained in this summary was updated to November, 1994 and presented at the Fanconi Anemia Scientific Meeting at Skamania, Washington.

The FATR includes information on 243 matched sibling donor transplants performed from 1974 to 1994. The overall survival 5 years after transplant in these 243 patients is 61%. However, results have steadily improved with time. Seventy percent of patients transplanted since 1990 are surviving with normal marrow function and freedom from transfusions.

Several important observations have been made among these 243 patients. First, children transplanted before the age of 10 years have a better outcome than those transplanted after age 10 (70% vs 51%, p = 0.004). Patients who have had 10 or more transfusions of platelets or red cells and patients who have been on androgen therapy are also at higher risk of a poor outcome after transplant, predominantly due to graft rejection (transfusion-induced sensitization) or liver dysfunction (androgen therapy, transfusion-transmitted hepatitis, and iron accumulation in the liver).

The preparative therapy utilized for the transplant is also predictive of outcome. Regimens utilizing high dose cyclophosphamide (>30 mg/kg total dose) are associated with a higher incidence of serious side effects (severe mouth sores, diarrhea, skin rashes, liver and kidney dysfunction) and have resulted in an overall survival of 48% at 5 years (N = 80). The regimens with the lowest incidence of serious side effects and with the best survival are those utilizing low dose cyclophosphamide (20 mg/kg total dose) combined with low dose limited field irradiation (400-500 cGy). Survival at 5 years in the 111 patients in the registry receiving this type of preparative therapy is 73%. These include the regimens utilized predominantly at Paris and in Cincinnati. There is also some suggestion that the addition of antithymocyte globulin (ATG) to the preparative regimen results in a lower risk of graft rejection and graft vs host disease (GVHD) and improved survival (84% vs 67%, p = 0.11), but this observation needs further confirmation.

In summary, for patients who have a matched sibling donor, I recommend that a marrow transplant be performed soon after diagnosis, preferably before the age of 10 years, and certainly before the patient develops transfusion dependence or a cytogenetic clone, myelodysplasia, or leukemia. I recommend a regimen utilizing low dose cyclophosphamide and low dose limited field irradiation combined with ATG and cyclosporine prophylaxis for graft rejection and GVHD prophylaxis. Utilizing

*Harris – continued on page 15*

## Antioxidants and Erythropoiesis

*Blanche P. Alter, MD*
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Free radicals which are formed in cells in oxygen may be toxic, and may damage DNA. Antioxidants are agents which detoxify those free radicals, thus reducing the potential damage. The major antioxidants include superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) or other sulfhydryl reducing agents. The medical literature has suggested that FA patients may be low in SOD, although several studies in fact found normal levels. Very limited clinical trials of SOD led to slight decreases in chromosome breakage.

We have examined several antioxidants in tissue culture, using blood mononuclear cells from normal adults, and from normal umbilical cords obtained at birth; we have not yet examined cultured cells from FA patients. Erythroid (red blood cell) colony progenitors were examined in air and in low (5%) oxygen. Cultures included SOD, CAT, 2-mercapto-ethanol (2-ME, the reducing agent used most often in culture), and GSH. We found that erythroid colony growth in air was increased the most with GSH, next with CAT, followed by 2-ME and SOD. In low oxygen, the best were GSH and CAT, then 2-ME and SOD. Combinations were no more effective than GSH alone. GSH at 10 micromolar (which is 1/1000 of the concentration within cells) was the only reagent which was equally effective in air and in low oxygen.

These results suggest that SOD may not be the best antioxidant, and that GSH or its precursor molecules may be potentially effective in FA. The problem in FA may not be primarily lack of antioxidant, but inability to repair damage produced by toxic free radicals. Pharmacological reduction of free radicals may secondarily decrease DNA damage, thereby eliminating the need for repair. ••

# Complementation Studies and Cloning of FA Genes

## FA-C Carrier Screening and Genetic Mapping

*Arleen D. Auerbach, PhD*
*Rockefeller University*
*New York*

Two common mutations (IVS4 +4 A→T and 322delG) and several rare mutations (Q13X, R185X and L554P) have been found in patients affected with FA. We have recently developed a test for the rapid detection of these known mutations in the FA-C gene. We have applied this molecular test, known as an ARMS assay, in a variety of ways: (1) testing for mutations in newly diagnosed FA patients; patients found to carry any of these mutations belong to complementation group C [FA-C]; (2) prenatal testing in FA-C families; (3) carrier testing in FA-C families; (4) carrier testing in healthy individuals in high risk populations. From these studies, we have found that approximately 15% of FA families (34 patients, not including affected siblings) have mutations in the FA-C gene.

Nineteen of the 34 FA-C patients (56%) exhibited the IVS4 +4 A→T mutation and all 19 were homozygous for this mutation, having received identical copies from their mother and father. All were of Jewish ancestry; IVS4 +4 A→T was not found in any FA patient of non-Jewish origin. All IVS4 +4 A→T patients studied had multiple major birth defects associatedwith the classical form of FA. Eleven of the remaining FA-C patients (32%) exhibited the 322delG mutation. Only one individual was homozygous for this mutation, while the other 10 were compound heterozygotes, which means they inherited 322delG from one parent and a different mutation from the other parent. Two patients received 322delG from one parent and Q13X from the other; 2 patients exhibited a combination of 322delG and R185X mutations. The second mutation in the rest of the patients with 322delG is unknown. Patients with 322delG usually do not exhibit any major birth defects. Carriers of 322delG, R185X and L554P have Northern European ancestry; the Q13X mutation is of Southern Italian origin.

In another application of the ARMS assay, we recently confirmed a prenatal diagnosis of an affected fetus which was homozygous for the IVS4 +4 A→T mutation. We have also applied the ARMS assay to carrier testing for relatives of individuals carrying a known mutation in FA-C, thus enabling us to provide improved genetic counseling to FA families. In addition, we have screened DNA samples from more than 2,700 healthy Jewish individuals, primarily of Ashkenazi ancestry, in order to determine the carrier frequency of the IVS4 +4 A→T mutation in this population. These samples, ascertained for carrier screening for Tay Sachs, cystic fibrosis, and other genetic diseases with a high frequency in the Jewish community, were tested for both IVS4 +4 A→T and 322delG mutations; 30 IVS4 +4 A→T carriers were identified, for a carrier frequency of approximately 1%. No 322delG carriers were found in this population. Two of these individuals who are carriers for IVS4 +4 A→T are of Sephardic Jewish ancestry. We hypothesize that IVS4 +4 A→T is a very old mutation, predating the divergence of the Ashkenazi and Sephardic populations.

We have also performed linkage analysis with DNA markers from a region on chromosome 9q that is tightly linked to the FA gene. Results from studies of 49 highly informative families in the International Fanconi Anemia Registry (IFAR), in which there are two or more affected siblings, or in which the parents are related to each other, confirmed our data from the ARMS assay identifying families as FA-C. Thirty-one families could be positively excluded from complementation group C, and these families are being used in new studies to map the genes for the other FA complementation groups. Linkage analysis also enhances our ability to perform molecular based prenatal diagnosis and carrier detection in FA-C families, particularly for those families in which the second mutation is still unknown.

We are happy to provide information to FA families or their physicians regarding mutation screening for FA by our laboratory. ••

## Classification of FA Patients by Complementation Analysis

*Hans Joenje, PhD, Free University, Amsterdam*
*The Netherlands*

The laboratory of Dr. Buchwald has shown that at least 4 complementation groups (separate disease genes) exist in FA. Since these 4 groups were distinguished among only 7 patients, it seems likely that still more FA genes exist. In an attempt to estimate the number of major FA genes we are conducting complementation analysis in a group of 50 unrelated European FA patients.

**Principle of the method:**
Lymphoblastoid cell lines are fused with (genetically marked) derivatives of the 4 reference cell lines HSC-72, HSC-230, HSC-536 and HSC-62, which represent the complementation groups A, B, C and D, respectively. Hybrid cell lines are tested for growth inhibition by mitomycin C (MMC). Resistance of a hybrid indicates complementation (non-identity with the reference line), whereas sensitivity indicates lack of complementation, implying that the cell line is defective in the same gene as the reference line.

**Results:**
By September 1994 23 FA patients had been analyzed and classified as follows: A; 15; C:5; D:2. No FA-B patients were found, while 1 patient's cell line complemented all reference cell lines, suggesting that it represents a new complementation group (E).

In all 5 patients classified as belonging to the C group, mutations were found in both alleles of the FA-C gene, confirming the validity of the complementation test. The pathogenic mutation 322del G was frequently encountered in patients of Dutch ancestry. One cell line having a previously described FA-C sequence variation with uncertain pathogenic status (D195V) was classified as FA-A, indicating that D195V is most likely a non-pathogenic polymorphism.

The complementation studies as carried out thus far have assigned a number of multiplex families to group A, as a result of which positional cloning of FA-A through linkage analysis is now within our reach.

*Supported by the FA Research Fund, The European Cancer Centre, Amsterdam and EUFAR, a concerted action on European Fanconi Anemia Research sponsored by the European Union.*

## Reinvestigation of the Chromosome 20 Linkage in FA-A Families Participating in the EUFAR Program

*Rachael Gibson, Guy's Hospital, London*

Fanconi anaemia is a complex genetic disorder which can be caused by a defect in at least four different genes. Common neutral genetic changes (polymorphisms) occur at random intervals on each chromosome and relatively frequently in the general population. These polymorphisms or markers will eventually allow us to identify the position on a chromosome where the other FA genes are found. This can be achieved by the analysis of different markers from each chromosome in FA families to see whether a specific marker or set of markers is inherited together with the disease (linkage analysis).

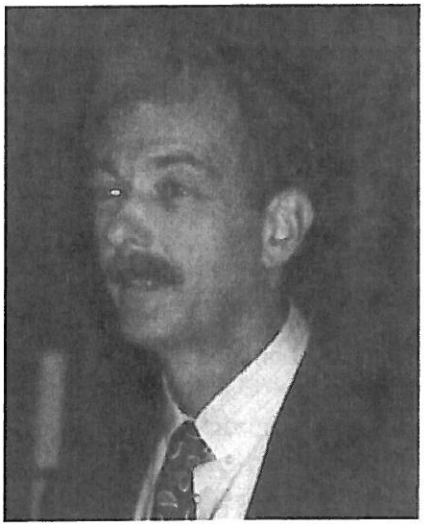
Only the gene defective in FA complementation group C has been identified to date. This gene is located on chromosome 9. The other FA genes are likely to be found on different chromosomes. However, linkage analysis in families from unknown complementation groups is unlikely to be successful. Evidence of linkage to a particular chromosome may be obtained in one family from one complementation group while linkage to a second chromosome may be obtained in a family from another group. If these two results are examined together, no evidence of linkage will be obtained overall.

To overcome this problem, a European collaboration for Fanconi anaemia research (EUFAR) has been established. One of the EUFAR projects includes the complementation analysis of a large set of cell lines, and is being coordinated by Hans Joenje in Amsterdam. Fifteen families have been identified as belonging to complementation group A to date. We initially tested the hypothesis that FA-A was located on the long arm of chromosome 20 because a previous study showed evidence of linkage of FA to this region. However, we found no evidence that the FA-A gene is located at this position. A genome-wide search to locate FA-A on the genetic linkage map, which is being coordinated by Fre Arwert in Amsterdam, is underway.

## Complementation Cloning of Fanconi Anemia Genes

*Robb E. Moses, MD, Oregon Health Sciences University, Portland, Oregon*

Our research group is attempting to identify Fanconi anemia genes by building a library of normal human genes and inserting these into Fanconi anemia cells. The cells are then evaluated for normal resistance to mitomycin C, a drug which creates DNA damage that Fanconi anemia cells cannot repair. We have searched more than one million Fanconi anemia cells treated with such a library and have found five cell lines which appear to be corrected. We are in the process of recovering the genes which we have artificially inserted into these cells, from the cells. Such genes are candidate Fanconi anemia genes. ••



*Robb Moses*

## Epstein-Barr Virus for Cloning and Gene Therapy of Fanconi Anemia

*Jean-Michel Vos, PhD, University of North Carolina at Chapel Hill*

Epstein-Barr virus (EBV) is a B-lymphotrophic human herpes virus, which is carried in a persistent state by over 90% of the worldwide adult population. Our search for an episomal genomic cloning vector (1,2) and an efficient viral gene transfer vector, specific for hematopoietic disorders, has led us to develop a mini-EBV vector, with minimal *cis*-EBV elements for episomal replication, viral amplification and packaging. In an attempt to show the therapeutic potential of the mini-EBV vector, we have constructed a recombinant vector expressing the Fanconi anemia complementation group C cDNA (FA-C), and used it to infect FA-C lymphocytes.

Fanconi anemia is a DNA repair disorder characterized by hypersensitivity to DNA crosslinking agents resulting in chromosomal instability, cell death and increasing propensity to cancer. RT-PCR analysis of RNA's from infected Fanconi anemia lymphoblastoid cells demonstrated stable expression of the gene. Phenotypic correction of the defects to crosslinking agent sensitivity confirmed successful gene transfer into the lymphoblastoid cell line. Molecular and fluorescent *in situ* hybridization (FISH) analysis of the FA-C gene revealed stable maintenance of 140-160 kb mini-EBV episomes in the infected cells. This work clearly demonstrates the generation of a unique, stable episomal gene therapy vector which can transfer potentially large insert into B-lymphocytes by infection.

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## Progress Towards Cloning of the FA-A Gene by Using the Chinese Hamster Cell VH-4 Mutant

*Margaret Z. Zdzienicka, PhD, Leiden University, The Netherlands*

Recently, five complementation groups have been identified among Fanconi anemia patients and at least two more complementation groups were determined among mitomycin C (MMC)-sensitive hamster cell mutants. This indicates a tremendous complexity of the pathway involved in the mammalian cellular response

to crosslinks. The identification and cloning of all the genes involved will help to understand the FA pathway. To identify these genes, we are using MMC-sensitive hamster cell mutants because hamster cells are much better recipients for exogenous DNA allowing the isolation of human genes involved. Also the hamster mutants serve as an excellent tool for the localization of these defective genes on the human genome.

The research on Fanconi anemia conducted by my group has been mainly focused on the following topics: 1) cloning of the FA-A gene, by using the hamster cell MMC-sensitive mutant (V-H4) which is defective in the gene which is homologous to the human FA-A gene (in collaboration with Dr. F. Arwert – Free University of Amsterdam and Dr. A. Westerveld – University of Amsterdam); 2) chromosomal localization of genes involved in the Fanconi anemia pathway by microcell-mediated chromosome transfer into Fanconi anemia cell lines (group A and D), as well as into V-H4 cells (in collaboration with Dr. M. Oshimura - Tottori University, Japan; 3) localization of genes involved in the cellular defense mechanisms against crosslinks (MMC, cis-platin or DEB) in new complementation groups of MMC-sensitive hamster cell mutants isolated in my laboratory.

Presently, human DNA sequences complementing the defect in two independent primary MMC-resistant transformants of V-H4 obtained after transfection of a cosmid library from normal human DNA are being recovered and analyzed. We examined most of the human genome in our search for the chromosomal localization of the FA-A gene. However, so far the complementing chromosome has not yet been identified. The search for the chromosome complementing the defect in V-H4 and FA-A cells is in progress. ••

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## The mus308 gene of Drosophila Encodes a Novel Eukaryotic DNA Polymerase

*Ken Burtis, PhD, University of California, Davis*

We have continued our characterization of the *mus308* gene of Drosophila, which we hope will provide a useful tool for identifying and cloning human genes associated with Fanconi anemia. Mutations in *mus308* result in a specific inability to repair the damage caused by DNA crosslinking agents such as diepoxybutane (DEB), a characteristic shared by cells with mutations in FA genes. By determining the molecular characteristics and DNA sequence of the Drosophila gene, we have been able to search for related genes in other species including humans.

We have determined that the *mus308* gene sequence encodes a new, previously undiscovered DNA polymerase; a type of enzyme that is well known for its role in other types of DNA repair. We are thus optimistic that the *mus308* protein plays a direct role in the repair of crosslinked DNA, and that an analogous human enzyme could likewise be involved in this process. Further, the identification of the specific part of the *mus308* gene that encodes the polymerase activity allows us to narrow our search of the human genome to those parts that contain DNA sequences related to the polymerase sequence, which increased the sensitivity of the search. We used both hybridization and more sensitive polymerase chain reaction (PCR) based approaches in attempts directly to recover related sequences from human genomic DNA. Although these efforts have been thus far unproductive, we were fortunate to discover by computer analyses related sequences in the databases related to the *mus308* polymerase domain. One such sequence, found in unpublished data derived from the *C. elegans* genome project, is clearly the nematode homolog of *mus308*. Of even greater potential interest is an EST (expressed sequence tag) from the human EST database that shows homology to a region of the *mus308* polymerase domain. We have very recently isolated a human cDNA including this EST and are commencing to sequence it to confirm and extend the region of homology. If this cDNA indeed represents a human homolog of *mus308*, we will initiate studies to determine its potential relevance to Fanconi anemia. ••

### Isolation of FA Genes by cDNA Complementation

*Stephen Meyn, PhD*
*Yale University, Connecticut*

Genes responsible for genetic diseases recently have been found by "reverse genetics" approaches that do not require identification of a biochemical defect. We are attempting to isolate the FA-A gene with a reverse genetics method that uses a novel vector that was developed for efficiently transferring genes into and out of human cells. In this approach, copies (cDNAs) of all of the active genes found in a normal human cell are inserted into an episomal expression vector, a special DNA molecule that can replicate in both human cells and bacteria and can be passed from one generation of cells to another. This collection, or "library", of cDNAs then is transferred (transfected) into a population of cells taken from an FA-A patient. Cells which have taken up DNA molecules from the library are forced to grow in the presence of DEB, a DNA-damaging drug that preferentially kills FA cells. Transfected FA cells which survive drug exposure are picked and tested to identify those cells that survived because they had become DEB-resistant. In these experiments, cells usually become drug-resistant because of the cDNAs they carry. Consequently, the cDNAs identified by this approach should include the FA-A gene as well as other genes that are involved in the repair of DNA damage.

Using this approach, we have rescued thirteen cDNAs contained in seven colonies of transfected FA cells that survived DEB treatment and proved to be highly DEB-resistant. Three of these cDNAs, cFA21.3, cFA26.12 and cFA23.4, conferred both DEB and mitomycin C resistance when transferred into fresh FA-A cells, suggesting that they can correct the DNA repair defect of the FA cells. The cFA21.3 cDNA confers DEB resistance to FA-A cells, but does not increase the DEB resistance of normal fibroblasts. This specificity is similar to that of FA-C, which corrects the DEB sensitivity of FA-C fibroblasts only. The cFA23.4 cDNA codes for rpS3, a protein that is able to perform an initial step in the repair of several types of DNA damage. What we have learned so far about the cFA21.3 and cFA26.12 cDNAs suggests that they are not related to any known gene. However, we may find similarities to other genes once we complete the sequencing of these two cDNAs. We now are mapping the cFA21.3 and cFA265.12 genes by a microscopic technique known as fluorescent *in situ* hybridization (FISH). We also are examining the structure and expression of these three complementing genes in cells from both normal individuals and FA patients in order to determine if one of the cDNAs represents the FA-A gene. Our initial results indicate that the cFA21.3 gene has been rearranged in an FA-A cell line. We now are studying the cFA21.3 gene in other cells from this patient, as well as cells from other members of his family, in order to determine whether this alteration in the cFA21.3 gene is an artifact peculiar to this cell line or the cFA21.3 gene rearrangement is present in the DNA of all affected family members. The latter would be strong evidence that the cFA21.3 gene is actually the FA-A gene. ••

## DNA Repair and Protein Function

### Evaluation of the DNA Repair Defect in FA-A

*Muriel Lambert, PhD, University of Medicine and Dentistry of New Jersey, Newark, New Jersey*

Fanconi anemia (FA) cells have been shown to be particularly hypersensitive to the chromosome breaking and cell killing effects of agents which produce interstrand crosslinks in DNA (1,2). Such crosslinks, which are formed between opposite strands of the DNA molecule, prevent the two DNA strands from separating so as to allow DNA replication or RNA transcription to occur, thus leading to loss of function or even cell death (1,2). Cells from individuals with FA have been shown to have a defect in ability to repair DNA damage produced by these agents (reviewed in 2). On the basis of this sensitivity to crosslinking agents and the presence of a DNA repair defect, it has been hypothesized that an underlying mechanism for this disorder may involve a DNA repair defect (1,2).

In human cells, nuclear DNA is found within a complex structure known as chromatin which is composed of DNA and closely associated proteins. We have isolated a chromatin-associated DNA endonuclease complex from normal human cell nuclei which is involved in the repair of DNA interstrand crosslinks (4). This complex is composed of several interacting proteins, one of which is an endonuclease (i.e., an enzyme which produces a cut in a strand of DNA) and another of which is a damage-

recognition protein which recognizes specific sites of damage in DNA. This complex cleaves the DNA strand near the site of the DNA interstrand crosslink. We have isolated this same complex from FA-A cell nuclei and have shown that it is defective in ability to incise DNA containing interstrand crosslinks (2). In addition we have shown that the FA-A complex is defective in a DNA binding protein which recognizes interstrand crosslinks (4).

The goal of the present research was to delineate the exact nature of the DNA repair defect in FA-A cells. For these studies a 136 base pair DNA fragment (i.e., oligonucleotide) was synthesized which contained a single site-directed interstrand crosslink. The agent used to produce this damage was psoralen plus long wavelength UVA light. Psoralens are a group of naturally occurring compounds found in a variety of common plants used in our diet, which in conjunction with long wavelength (UVA) light, are one of the most definitive agents for the production of interstrand crosslinks and monoadducts in DNA. Psoralen monoadducts are formed when just one end of the molecule is joined to only one DNA strand, unlike a crosslink where the other end of the molecule is bound to the opposite DNA strand. The ability of the endonuclease complexes from normal and FA-A cells to incise these damaged oligonucleotides was examined. Sequence analysis showed that the normal complexes could endonucleolytically produce incisions on both sides of the interstrand crosslink and the monoadduct. The FA-A complexes, however, although they could produce incisions on both sides of the monoadduct, were defective in ability to produce dual incisions at the site of the crosslink. These results indicate that there is a definite defect in the initial stages of the DNA repair process in FA-A cells.

Introduction of the normal endonuclease complex into FA-A cells in culture, by a process known as electroporation, enabled these cells to repair damage to their DNA produced by psoralen plus UVA light. The levels of repair were similar to those of the normal cells. These results indicate that this complex contains the protein which is responsible for defective DNA repair in FA-A cells.

We have produced monoclonal antibodies (Mabs) against proteins in the normal human endonuclease complex and using them have identified several of the proteins involved in the initial damage recognition/incision step of the repair of DNA interstrand crosslinks. Studies using these Mabs have shown that FA-A cells are defective in one of these proteins. The Mab directed against this protein will now be used in the isolation of this protein and in determining whether it is the defective damage recognition protein or not.

The question remains as to how this repair defect in FA-A is related to the disorder itself. One hypothesis is that the FA-A gene may be involved in a developmentally important function which could involve a number of diverse cellular systems. The FA-A gene product could play a role not only in DNA repair but also in other cellular processes critical to development and differentiation such as transcription and DNA replication. A defect in such a protein could thus affect hematopoiesis, leukemogenesis, and organ and cytoskeletal development via its pleiotropic effects on these different cellular processes.

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*Muriel and Clark Lambert*

## A Checkpoint Regulates Progress Through S Phase When the DNA Template is Damaged

*Amanda Paulovich, University of Washington*

Chromosomes contain DNA, the genetic material of the cell. Before a cell divides, it must make an exact replica of all of its chromosomes, so that each daughter cells can receive a copy of the genetic information. One of the enzymes involved in DNA replication is DNA polymerase. DNA polymerase replicates DNA with extraordinarily high fidelity.

Often cells suffer damage to their chromosomes. Damage can be induced by chemicals or UV radiation in our environment, as well as by molecules produced within our own cells. When DNA polymerase replicates damaged regions of chromosomes, mistakes are frequently made and may result in mutation. Mutations can cause cells to behave differently. For example, sometimes cells die as a result of mutation. More rarely, mutations can create a cancer cell.

Luckily, cells have additional protein machinery designed to excise and repair damaged regions. These repaired chromosomes can then be replicated with high fidelity, avoiding mutation.

As a result of our research, we believe that replication and repair of damaged chromosomes do not occur independently. Rather, we believe that cells couple repair and replication such that a region of the chromosome is scanned for damage and any damage is repaired before that region is allowed to be replicated. Our current research is aimed at characterizing and understanding how this coupling is achieved.

Cells obtained from patients with Fanconi anemia are unable to repair some types of chromosomal damage. By learning more about how cells with functioning repair systems respond to damage, we might eventually be able to understand the cause of this and other diseases. ••

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## Study of the FA-C Protein

*Alan D. D'Andrea, MD, Dana-Farber Cancer Institute, Boston, Massachusetts*

Fanconi anemia (FA) is a genetic disease resulting from congenital mutations in one of several Fanconi anemia genes. While one of the FA genes has been cloned (for complementation group'C), the other (four or more) FA genes await isolation. In the meantime, our laboratory at the Dana-Farber Center Institute in Boston, has been studying the protein which is made by the FA-C gene.

At this time our laboratory and several other laboratories have made a number of observations regarding the FA-C protein. First, the FA-C protein normally protects cells from mitomycin C or diepoxybutane induced cellular damage. Congenital absence of the FA-C protein results in cellular sensitivity to these drugs. Second, the FA-C protein is normally found in a region of the cell called the cytoplasm. Third, the FA-C protein binds and forms a stable association with other cellular proteins. One of our major goals is to identify proteins that bind to the FA-C protein. We believe that identification of these FA-C-binding proteins will shed light on the cellular function of the FA-C protein. It is possible that the FA-C binding proteins are themselves made by other Fanconi anemia genes.

As a second project in our laboratory, we are interested in studying the Fanconi anemia (FA-C) gene and protein in normal and leukemia cell lines. Normal cells from the body have two copies of each human gene. Carriers (parents) of FA patients have one normal copy and one mutant copy of the Fanconi anemia gene. Patients with FA have two mutant copies of the gene. We have been studying the FA-C gene in adults (non-Fanconi anemia patients) who have developed leukemias. Some normal patients who develop leukemia or cancers as adults appear to have lost FA genes. This result is interesting because it suggests that the FA genes play a more general role in preventing the formation of cancers (leukemias or other cancers), even in individuals who are not born with Fanconi anemia. For these reasons, we are

broadening our research of Fanconi anemia to include normal and leukemic cell lines. In summary, our laboratory is interested in the broad cellular roles that the Fanconi anemia FA-C gene plays in the normal development of the organism, in the prevention of drug toxicity, and in the prevention of cancer. ••

## Molecular Studies of Fanconi Anemia

*Hagop Youssoufian, MD, Brigham and Women's Hospital, Harvard Medical School*

We wish to understand the precise biochemical function of the FA-C protein. Dr. Buchwald's group isolated the FA-C gene by taking advantage of its ability to prolong the survival of FAC cells during treatment with DNA-damaging agents, such as mitomycin C. However, the exact mechanism at the molecular level by which FA-C is able to effect this process is unknown. We have developed a number of new reagents, including an antibody that recognizes the FA-C protein, in order to address the following issues:

Where is the FA-C protein located in the cell? We believe that it is located in the cytoplasm, not the nucleus. This result has implications for the function of FA-C: it suggests that the FA-C protein is not a DNA-repair enzyme.

We are also very interested in characterizing the individual molecular steps that lead from DNA damage to repair. Our best guess at this point is that FA-C works at a step before DNA damage by mitomycin C or other agents. In this sense, we do not think that FA-C is the mechanic that fixes the damage; rather, we prefer to make the analogy with a protective barrier that is able to prevent DNA damage in the first place.

Finally, we have also wondered about the relationship of the FA-C protein to the proteins that are deficient in other FA complementation groups. We have recently identified three proteins that can bind to the FA-C protein specifically. We are attempting to identify them and understand whether or not they are deficient in FA cells of various groups. ••

## Isolation of FA-C-interacting Proteins

*Maureen Hoatlin, PhD, Oregon Health Sciences University, Portland, Oregon*

At least four genes are involved in Fanconi anemia. Three of these genes are unknown, but recently the gene that codes for the type C of Fanconi anemia, called FA-C, was cloned. The predicted FA-C protein is not similar to any other protein yet studied. Recently, several groups have found that the FA-C protein is required for normal growth and development of bone marrow cells, and that the FA-C protein associates with at least one other protein in bone marrow cells.

I am using a new, powerful method called the yeast two-hybrid system to isolate proteins that interact with the FA-C protein. This method was first developed by a group of scientists in 1991 and has already been so successful that many new genes have been cloned. More importantly, the proteins that these new genes encode have been "caught" interacting with other proteins in a cell, so that their function is revealed. I plan to use the newly identified FA-C protein as a "bait" in this system to fish out interacting proteins from an entire library of human proteins. I have already isolated 144 candidates in my first experiment using a portion of the FA-C protein as "bait". I will analyze each of these clones to ensure that they are truly interacting with the FA-C protein. True positives will be sequenced and analyzed. By studying the interacting proteins, I hope that I will be able to determine the function of the FA-C protein. Because FA patients can have at least four separate defects that give rise to the same disorder, the FA-C protein may be part of a protein complex or part of a new pathway. It is possible that I will isolate the genes for the other Fanconi anemia complementation groups by using this method, leading the way for gene therapy for the children that have defective FA genes. ••

**2) Pattern of expression of the mouse *Fac* gene during embryonic development.**

We have used a method called *in situ* hybridization to study how the expression of the mouse FA-C gene is regulated during the development of the embryo. Our results show that early in development high amounts of expression are seen in those tissues that lead to the development of the internal organs and bones. As the embryo becomes more mature, the pattern of expression becomes more specific and localized. During the time that the bones are developed high levels of expression are seen at those places. Our results indicate that the FA-C product is involved in the process that leads to deposition of bone on the cartilage matrix.

**3) Progress towards the production of a mouse model for FA-C.**

Using the techniques of gene targeting in mouse embryonic stem cells, we generated a set of lines in which the mouse FA-C gene was artificially disrupted. These cells were then used to produce mice in which the embryonic stem cells contributed to the germ cells. Breeding of these mice led to mice in which one chromosome had the normal mouse FA-C gene and the other chromosome had a disrupted version. In effect, these mice were heterozygotes for FA-C. Breeding of these mice to each other has generated a set of mice that have both copies of FA-C disrupted and are expected to be homozygous for a deficiency of FA-C. Our hope is that these mice will model some of the features of FA-C patients. To date, the majority of FA-C mice do not appear to have any bone abnormalities, a surprising, though not necessarily unexpected, result. The oldest mice analyzed for their blood picture are 1 month old and appear normal. We are currently checking the mice to make sure that our genetic manipulation did disrupt the function of FA-C. We are also following the mice to older ages to see if they will develop bone marrow failure and/or leukemia. If this occurs, the FA-C mice will be useful for treatment studies. ••

## Correction of FA-C Phenotypic Abnormalities Using a Clinically-Suitable Retroviral Vector Infection Protocol

*D. Wade Clapp, MD, Indiana University Medical Center, Indianapolis, Indiana*

We recently developed a recombinant retrovirus that contains the FA-C gene, originally isolated by Dr. Buchwald and colleagues. We determined that this virus can complement a cell line of patients with the FA-C mutation and enhance the cell growth of blood cells (progenitors) of patients, as well as in experimental tissue cultures.

A major stumbling block in accomplishing gene transfer into human blood cells has been the inability to introduce the retrovirus into a very small population of blood-forming cells called stem cells. We have been testing new protocols to enhance the gene transfer efficiency into these blood-forming cells. The standard way scientists introduce genes into blood-forming cells is to culture recombinant retrovirus supernatant (containing the gene of interest) with stem cells isolated from the patient's blood or bone marrow in plastic dishes. We recently added to this culture system a fragment of a molecule found in the normal bone marrow called fibro-nectin. Addition of this protein to the cultures may more closely mimic the normal bone marrow where the primitive blood cells reside. We found that the gene transfer into normal human blood forming cells is greatly enhanced using this protocol. We are now testing the gene transfer efficiency into stem cells using the fibronectin fragment in experimental animal models. ••

## Particle Bombardment-Mediated Gene Transfer Into CD 34+ Hematopoietic Progenitor Cells from Human Cord Blood

*N.T. Shahidi, MD, School of Medicine, University of Wisconsin*

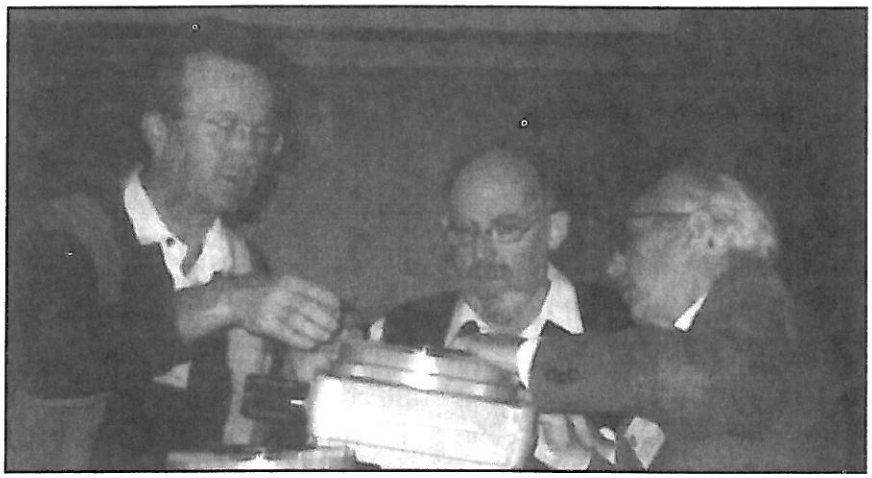
Previous studies of gene transfer into human hematopoietic progenitor cells have mainly used retrovirus vectors. For the past two years, in collaboration with Dr. N.S. Yang, Director of Mammalian Genetics Laboratories (Agracetus Corporation, Middleton) we have used particle bombardment technology to transfer various marker and therapeutic genes into hematopoietic progenitor cells ($CD34^+$ cells). In this technique, microscopic gold beads are coated with DNA and propelled into target cells by a shock wave generated by an electric-discharge. Highly ($\geq 90\%$) purified $CD34^+$ cells, prepared from fresh cord blood, were subjected to this gene transfer method using a number of reporter and cytokine genes, such as β-galactosidase, luciferase, and GM-CSF. Expression of all tested transgenes was readily detected in $CD34^+$ cells, not only from growth factor-stimulated cell cultures, but also from cell samples freshly isolated from human cord blood. To optimize the transgene expression in hematopoietic stem cells, the relative strength of various viral and cellular promoters and special vector designs for gene expression were also examined and yielded reproducible results. High-level transgene expression is reached within 24 hours after a simple physical delivery of a single dose of plasmid DNA, and the expression can last for about a week.

These results combined with our previous studies demonstrate that particle bombardment technology may have several advantages over viral vectors for gene delivery into $CD\ 34^+$ cells: (1) It does not depend on the specific cell surface receptors or membrane features on target cells which are required for virus absorption and entry; (2) It is easy to perform, taking less than 30 seconds for cell culture manipulation and bombardment; (3) it is very flexible for application to non-attached cells in culture, and one can bombard $10^4$ to $10^7$ cells in a 4 to 20 µl suspension; (4) It is highly cost-effective, utilizing only 0.1 to 0.4 µg plasmid DNA in ethanol precipitated dry form as optimal dosage for transfection of each target sample, yet allowing a delivery of more than $5x10^3$ copies of 5 kb plasmid DNA into each target cell; (5) it totally eliminates the expensive mammalian tissue culture system required to produce high-titer virus vectors; (6) It can easily be employed for co-transfection experiments involving delivery of multiple genes, genomic DNA fractions and large size cDNA fragments, and (7) unlike retrovirus vectors, particle bombardment technique does not require cell cycle coordination. ••

## Gene Therapy for FA

*Johnson M. Liu, MD, National Institutes of Health, Bethesda, Maryland*

FA is an autosomal recessive genetic disorder characterized by progressive pancytopenia, congenital abnormalities, and a predisposition of malignancy. Therapy is currently limited to allogeneic bone marrow transplantation, and new therapeutic strategies are needed for patients lacking a suitable donor. At least four different genes complement the FA defect, and one novel gene, FA-C, has been identified. FA-C mutations have been identified in approximately 15% of FA patients. We have been involved in the design and implementation of gene transfer strategies for FA-C patients. We have demonstrated successful in vitro transfer of the normal FA-C gene to mutant hematopoietic cells from FA-C patients. These gene-corrected cells exhibit improved growth when cultured in vitro. Our studies suggest that gene therapy may be feasible, and we are currently designing an experimental trial for FA-C patients. ••



*David Frohnmayer, Manuel Buchwald, and Nasrollah Shahidi*



*Harris - continued from page 3*

such a regimen in 21 patients at my own institution in Cincinnati, all but one currently survive with follow-up from less than one year to over 7 years (median 3 years). To date, all surviving patients remain fully engrafted without significant GVHD, and none has developed leukemia or a secondary cancer.

Some centers such as those in Seattle and Curitiba, Brazil, are investigating regimens without radiation in an effort to reduce the potential risk of late secondary cancers from radiation. There is published evidence in transplants for idiopathic aplastic anemia that regimens which include radiation are associated with an increased risk of developing secondary cancers such as cancers originating in the oropharynx, skin, thyroid gland or reproductive system several years after the transplant. Unfortunately, at least currently, these regimens without radiation are not yet achieving outcome results as satisfactory as those which include radiation.

For the patient without a matched sibling donor, the outcome of marrow transplant is not nearly so encouraging. In the FATR there are data available on 43 unrelated donor transplants and 49 transplants from relatives other than matched siblings (termed "haploidentical" transplants). The overall survival for these 92 "alternative donor" transplants is only 24% at 5 years. Most deaths are due to GVHD, graft rejection, infections, or organ failure – all known significant risks in such

alternative donor transplants. However, these complications seem to be even more common in patients with FA compared to patients undergoing such alternative donor transplants for other diseases such as leukemia. The reasons for this higher incidence of complications in FA patients probably include the inability of such patients to tolerate the high-dose preparative regimens usually necessary to achieve durable engraftment with alternative donor marrow and also the inability of the FA patient to repair damage done to normal tissue cells. Thus, if a patient with FA develops GVHD, then the damage to the organs by the GVHD as well as damage caused by the drugs utilized to treat the GVHD cannot be repaired quickly by the FA patient. The GVHD and its treatment also leads to worsening immune function, infections, then additional damage to organs from the infections as well as the antimicrobial therapy given to treat the infections. Ultimately, this scenario all too often results in the death of the patient. Thus, physicians must carefully select the best matched donor to reduce the risk of GVHD and graft rejection, and they must choose a transplant preparative regimen which will ensure engraftment without leading to too much toxicity. To date, no such regimen has been identified.

Some centers are also investigating alternative approaches to reducing the risk of GVHD, such as partially depleting the donor marrow of some of the cells responsible for GVHD. Such an approach, though, generally increases the risk of graft rejection. Needless to say, much more needs to be done to identify the best choice of donor, the best preparative regimen, and the best method of T-cell depletion to insure the best chance of survival for the patient with FA who does not have a matched sibling donor. Such patients should probably be transplanted only at centers with extensive experience in unrelated or haploidentical transplants and in which the transplant physicians

are fully aware of the risks peculiar to the FA patient.

The choice of a donor for such alternative donor transplants is also a complicated topic. The FATR data suggest that the best alternative donor is a completely matched relative and that the second best donor is a completely matched unrelated donor. A complete match includes matching by DNA technology of the D-locus antigens (often referred to as oligotyping). Transplants utilizing anything other than such a completely matched donor to date have resulted in very poor outcomes. With a completely matched alternative donor, results have been at least tolerable (48% and 34% in patients receiving transplants from completely matched relatives (N = 21) and unrelated donors (N = 32) respectively). A fully matched unrelated donor is preferable to a partially matched related donor (34% vs 12% survival at 5 years, p = 0.05).

I recommend the following for patients who lack a matched sibling donor. First, since outcome with alternative donor transplants is currently poor, alternative therapies should be tried first to delay the transplant to a time when either it must be performed for medical reasons or results of such transplants have improved. Such alternative therapies include androgens and cytokines such as G-CSF and/or erythropoietin. Such patients are encouraged to enroll in open cytokine trials for FA patients so the information obtained from the trials can be of help to future families considering such therapies. Dr. Wayne Rackoff at Riley Children's Hospital in Indianapolis has just completed a trial of G-CSF in such patients and I am currently conducting a trial of the combination of G-CSF and erythropoietin in similar patients. The early results from these two trials are showing that all patients have a rise in their neutrophil counts, and a significant proportion of patients are also exhibiting improvements in their hemoglobin and platelet levels and reductions in their transfusion requirements. These approaches might allow delay of the transplant for several years.

However, the transplant should not be delayed to such a point that the risk of the transplant becomes too great. Thus, patients who are approaching transfusion dependence in spite of trials with androgens and cytokines, patients who cannot tolerate androgens due to excessive liver toxicity or adenoma formation in the liver, and patients who have developed myelodysplasia or leukemia should not delay their transplant but proceed promptly to an alternative donor transplant. Patients who develop a cytogenetic clone in their marrow cells but do not have evidence of myelodysplasia or leukemia should not undergo immediate alternative donor transplant, but should be followed closely with repeat marrow chromosome studies done about every four months. Often these cytogenetic clones disappear without leading to MDS or leukemia. Transplant should probably be delayed until there is evidence of progression in the clone (increasing percentage of cells in the marrow exhibiting the clone) or the patient develops evidence of MDS or leukemia. This is a very controversial area among transplant physicians. Another alternative that might be available on a wider basis in the future is gene therapy. Families should be encouraged to find out the latest results with alternative donor transplants and the current status of gene therapy before making any final decisions for their child lacking a matched sibling donor.

I am willing to speak to any family or physician over the phone about my recommendations in individual situations. Families are encouraged to visit more than one transplant center prior to making a final decision about transplant, especially when an alternative donor transplant is being contemplated. Families should also speak with other FA families in which a family member has undergone a transplant to learn more about transplant and about their specific experience at the center where the transplant was performed. Suggestions for families to contact can be obtained through the Fanconi Anemia Research Fund office or from specific transplant centers.

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