FANCONI ANEMIA RESEARCH FUND, INC.

SCIENTIFIC SUPPLEMENT

FAMILY NEWSLETTER #20

Reports from the FA Family Meeting• Camp Sunshine• Point Sebago, Maine • May 16 - 21, 1996

Blood Cell Growth Factors

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The primary goal of the G-CSF study was to observe the effect of G-CSF on the neutrophil counts of patients with FA. Ten patients have now completed 40 weeks on study. All patients have had excellent neutrophil responses. In most patients, the G-CSF dose has been reduced to every other day, and they are still maintaining good neutrophil counts. Four patients had an increase in their platelet count and five patients had an increase in hemoglobin while on G-CSF.

One patient had a bone marrow clonal cytogenetic abnormality at week 40. By six months after the discontinuation of G-CSF the patient had monosomy 7 without an excess of blasts. The patient was transplanted, died of multiple post-transplant complications.

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The remaining patients have been off study for seven to 24 months. One patient died after a bone marrow transplant from a matched unrelated donor at the conclusion of the study period. Monosomy 7 was found on a routine bone marrow examination 12 months after completion of the study in one patient. G-CSF was stopped and a search for a source of matched unrelated stem cells is in progress. One patient was taken off G-CSF and started on oxymetholone at the conclusion of the study period.

Six patients remain on G-CSF. Four patients are on three-times-per-week or every-other-day doses and a fifth patient receives daily G-CSF. Neutrophil counts for these patients are 1,100 to 5616/ mm^3. One patient remains dependent on platelet transfusions and has had one episode of infection since completing the study. The remaining patients do not require transfusions. Platelet counts for these patients are 2,000/mm^3 to 46,000/mm^3 lower than at study entry. Hemoglobin levels have been maintained close to or above pre-study levels.

This pilot study showed that G-CSF is probably a safe and effective form of treatment in FA. The study was designed to measure hematologic responses, and does not allow conclusions to be

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Blood Cell Growth (Hematopoiesis) and FA

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Patients with FA who have blood problems have deficiencies in one or more cell lines (white cells, red cells, or platelets). Their bone marrow has decreased cellularity (seen most easily in a biopsy sample), with loss of megakaryocytes, erythroblasts, and myeloid cells (precursors of platelets, red cells, and white cells respectively). Blood cell development, or hematopoiesis, is most likely a clonal event, in which one or a few stem cells produce all of the precursors and progeny at any given time. At a different point in time, one or more other stem cells may drive hematopoiesis. A stem cell cannot be distinguished from other blood cells by its morphologic appearance, but rather by its ability to repopulate the bone marrow. Stem cells also have a surface marker, called CD34, which binds to CD34-antibodies. This permits the stem cells to be physically separated from other blood cells. The stem cells can be cultured in the laboratory to form colonies of blood cells.

The cells which can form colonies in culture in the presence of the appropriate cytokines are called progenitor cells. There are several kinds of progenitor cells. CFU-GM develop into colonies of granulocytes and macrophages, BFU-E into bursts of erythroid cells, CFU-E into colonies of erythroid cells (BFU-E and CFU-E require erythropoietin [Ep]), CFU-mega into colonies of megakaryocytes, and CFU-GEMM into colonies of granulocytes, erythroblasts, monocytes, and megakaryocytes.

We cultured progenitor cells from FA patients with varying hematologic severity, where class 1 has the most severe and unresponsive aplastic anemia, on transfusions only, class 2 is receiving but unresponsive to androgens, class 3 is responding to treatment, class 4 is about to start treatment, class 5 is stable but has abnormalities such as anemia, thrombocytopenia, or macrocytosis, and class 6 has no hematologic symptoms. The number of BFU-E was higher in the patients in classes 5 and 6. Adding stem cell factor and using reduced oxygen in the incubator increased the size and number of the erythroid colonies; colony improvement also correlated with the clinical classification. In serial studies, patients who clinically worsened from group 6 to group 5 showed a decrease in colony formation.

Studies done at the National Institutes of Health by J Liu et al showed that colony formation was poor from the progenitor cells of patients with FA-C, and was sensitive to mitomycin C (MMC). Transfection of those cells with a viral vector containing a normal FA-C gene led to restoration of colony numbers and MMC resistance. G Segal and G Bagby were able to treat normal bone marrow

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Studies of the FA-C Protein and its Cell Cycle Regulation

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A major focus of research among laboratories studing FA has been the identification and isolation of FA genes. Once an FA gene has been isolated, several kinds of experimental strategies can be pursued. First, specific mutations can be identified in the FA gene. In this way, FA patients and families can be rapidly assigned to different complementation groups. Second, the gene can be used as a diagnostic test, say, in prenatal screening. Third, the gene can be used, potentially in gene therapy, introduced into blood cells derived from FA patients and thereby used to correct the cellular deficiency.

My laboratory is interested in the actual molecular function of the FA genes. We have initiated several studies focused on the FA-C protein (encoded by the FA-C gene). Studies of this protein have been difficult, since it is a novel protein, unrelated to any other known proteins. To study the FA-C protein we have introduced the normal protein into FA-C cell lines. The normal FA-C protein corrects the sensitivity of these cells to various chemical crosslinking agents such as DEB and mitomycin C.

We have drawn several conclusions from our studies:

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Hematopoietic Stem Cell Transplantation

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Hematopoietic stem cells are the cells that make other blood cells. These blood-forming stem cells can be found in the bone marrow, the blood, and the blood of the placenta and umbilical cord of newborn infants. Stem cells are rare, immature cells which will grow and mature into all types of blood cells, including granulocytes, red blood cells, platelets, and lymphocytes.

Stem cells have special proteins (antigens) on their surface that help the body's immune system recognize them as being a normal part of the body (self) and not foreign, like a bacterium or virus. These molecules are made by genes called the HLA genes. Three of these genes are very important for matching tissue from one person with that of another person. Since every person inherits two copies of each gene, one from each parent, an individual could have six different HLA antigens. A full HLA match is all six antigens matching. Although three different genes are involved, they are very close together (closely linked). Therefore, the chance that a brother or sister will be a full HLA-match is 25%. The chance that a parent will be a full match is less than 2%, and an unrelated donor approximately 1:20,000, depending upon the patient's ethnic background.

Stem cells, whether from bone marrow, blood or umbilical cord blood, can be obtained from matched family members, mismatched family members, matched unrelated donors, or mismatched unrelated donors. The source of stem cells chosen for transplantation is determined by transplant physicians, based upon the stage and aggressiveness of the patient's disease and the availability of the best HLA-matched stem cells. For patients whose FA is slowly progressive, a well-matched donor is preferred since transplantation is not urgent. For those patients with aplastic anemia or leukemic transformation, a less well-matched donor may be chosen due to the aggressiveness of the patient's disease.

Patients with FA are prepared for transplantation with chemotherapy and radiation to the whole body (total body irradiation -TBI) or radiation to the chest and abdomen. The choice of potential preparative regimens depends upon the stage of disease and the stem cell source. The purpose of the preparative regimen is to destroy the abnormal blood and bone marrow cells and to suppress the body's immune system so that the body will not reject the new stem cells. Patients with FA have an increased sensitivity to the effects of radiation and chemotherapy so that lower doses are needed. Even with lower doses, the side effects can be excessive. In addition, as more mismatched stem cell donors are used, there is a greater chance of graft-versus-host disease (GVHD), an immune reaction in which the donor cells attack the patient's body. Techniques to manipulate mismatched family donor stem cells and unrelated marrow donor stem cells to decrease the chance of severe GVHD will be discussed by Dr. John Wagner.

Stem cell transplantation remains the only curative procedure for the blood problems associated with FA. Dr. Richard Harris will present the current results for cord blood transplantation for FA and Dr. Wagner will present the updated experience with unrelated marrow donor transplants. The use of unrelated cord blood stem cells and newer methods to manipulate unrelated bone marrow may further improve the results for the transplantation of children and adults with FA. •••

Drs. John Wagner and Frank Smith confer at Camp Sunshine

Complications in Older FA Patients: Issues of Infertility and Malignancy

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At least 10% of FA patients are not diagnosed until age 16 years or older. In addition, the median survival age of patients with FA has improved with time. Thus, problems unique to the older patients become important.

FA males are often short in stature. They have underdeveloped gonads and abnormal spermatogenesis. Fewer than 5% of those in the literature who had reached age 16 had fathered children.

FA females have delayed onset of menstruation, irregular menses, and early menopause, with the expected risks associated with decreased estrogen, including hot flashes, osteoporosis, cardiac disease, and gynecologic problems. Females are also at risk for gynecologic and other malignancies. Pregnancy can and does occur, and was reported in about 15% of those over age 16. There were 29 pregnancies in 19 patients. FA was diagnosed later than usual, not until during or after pregnancy in 10. Twenty-two babies were delivered, and 21 survive. Eleven FA mothers developed anemia and 8 thrombocytopenia. Six babies were delivered by C-section, usually due to failure of the labor to progress. There were four episodes of pre-eclampsia or eclampsia. Although none of the mothers died during pregnancy or delivery, nine had died (seven from cancer) on average 10 years after delivery, leaving young children. The survival curve for pregnant women with FA is no worse, however, than for pregnant women without FA, who had acquired aplastic anemia prior to or during pregnancy.

Cancer other than leukemia and liver tumors occurred in about 5% of FA cases in the literature. The oropharynx and gastrointestinal tracts were involved in more than half and gynecologic

areas in one-fourth. An unexpected observation is that females acquired cancer at twice the rate of males, even after exclusion of the gynecologic cases. In addition, a few patients with solid tumors also had liver tumors or leukemia, or multiple primaries of solid tumors. Patients with cancer were diagnosed with FA at a later than average age (mean 12); cancer developed at a mean of 23 years, with a range from one month to 38 years.

Liver tumors also were noted in about 5% of cases. All but one had received androgen therapy. Adenomas and hepatocellular carcinomas were reported in equal numbers. Truly malignant (i.e. metastatic) liver tumors were rare, and deaths were from aplastic anemia, sepsis, leukemia, or other tumors. Liver tumors may regress when androgens are stopped. Just as bone marrow cells may appear dysplastic, we may speculate that liver cells do so as well, and the distinction between adenoma and hepatocellular carcinoma may not be totally clear.

At least 4 cancers (all tongue) occurred in patients who had received bone marrow transplants. All had received Cytoxan and irradiation as preparation. It is not clear whether the cancers were direct results of immunosuppression and transplant, or because the patients lived long enough to develop this complication, unrelated to the transplant procedure.

The median ages at which various complications occur in FA are 8 for aplastic anemia, 14 for leukemia, 13 for liver tumors, 17 for myelodysplastic syndrome, and 26 for solid tumors. Thus, the older patients in particular warrant close monitoring for the development of malignancies. •••

Complementation Studies

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Blood lymphocytes normally have a short life-span when cultured in the laboratory. However, by using Epstein-Barr virus (EBV) they can be "immortalized" into a cell line that has the potential to divide indefinitely. Such cell lines are extremely useful for experiments that may lead to a better understanding of blood disease. Also, they are an unlimited source of DNA, which can be used for gene-finding studies (linkage analysis), gene cloning and mutation detection. FA can be studied at the cell level, because, in principle, all cells of an FA patient exhibit the characteristic hypersensitivity to crosslinking agents (DEB or MMC). EBV-immortalized cell lines, also called "lymphoblastoid cell lines" or "lymphoblasts", are usually also hypersensitive to MMC, though in a proportion of patients (patients with "mosaicism", see below), surprisingly enough they are MMC resistant. Lymphoblasts, if MMC sensitive, can also serve to determine a patient's complementation group, by cell fusion techniques.

A full complementation analysis involves an extensive investigation, which altogether may take 3 to 12 months to complete. The patient's lymphoblasts are mixed with lymphoblasts of a known complementation group, for example FA-A. The mixed culture is then treated with polyethylene glycol, a sticky substance that causes the cells to "fuse", producing hybrid cells that contain the two sets of 46 chromosomes from both fusion partners. In such hybrids one can determine whether the disease phenotype (MMC sensitivity) is complemented (corrected or "cured"). If both cell lines that make up the hybrid have mutations in the same FA gene, the hybrid will have four mutated (defective) copies of the gene and will therefore still be sensitive to MMC. In this example (fusion with reference FA-A lymphoblasts) failure to complement means that the patient belongs to group A. However, if the hybrid were MMC resistant, the patient would be classified as "non-A". In that case more fusions would have to be carried out with cell lines belonging to each of the other known complementation groups. If the cells fail to complement with the reference C-group cell line, the patient would be classified as FA-C. If the patient's cell line appears to complement all other known groups, we conclude that he/she belongs to a new complementation group. By doing such an analysis on patients from a great diversity of ancestral backgrounds, we hope to be able to tell how many complementation groups there are. Since it is thought that each complementation group represents a distinct FA gene, such experiments provide information about the number of existing FA genes. Our current data suggest that there are as many as 8 different complementation groups. However, it remains to be seen whether each group indeed represents a separate gene. Ultimate proof that each complementation group represents a distinct gene requires isolation of the gene and finding mutations in patients who belong to that group. FA-C is currently the only group for which such ultimate proof is available. For groups A and D, positions on different chromosomes have been established (chromosomes 16 and 3, respectively). These groups must therefore represent distinct FA genes, which are also different from FA-C, since the group C gene is known to be on chromosome 9. Whether the other groups (B, E-H) also represent distinct FA genes awaits the establishment of their chromosomal localization and/or cloning of the genes.

How can mutations in different genes cause the same disease? A plausible explanation is that the proteins encoded by all FA genes act in concert to serve some function in cells and that all of these proteins are equally important for this function. Consequently, a defect in any one of the FA genes will have the same effect: FA. A great challenge of FA research is to elucidate the precise function of the proteins encoded by the FA genes.

Genetic reversion complicates complementation analysis. Assignment to complementation groups is complicated by the phenomenon of genetic reversion, which occasionally happens in fusion hybrids. From the observation of mosaicism in patients (see below) we know

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Gene Therapy Trial Protocol Update

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Currently, a trial of hematopoietic stem cell transduction is being conducted at the Clinical Center of the National Institutes of Health for FA-C patients lacking a compatible bone marrow transplant donor (please see *Scientific Supplement 19* for details). The protocol is a pilot study to determine the feasibility of FA-C gene transduction of hematopoietic progenitor and stem cells from patients with FA-C mutations. The mutations of the three patients on study (so far) include the three major categories of FA-C mutations—exon 1, exon 14, and intron 4. The study has confirmed that transfer of the FA-C gene to multipotential progenitor cells (those able to develop into multiple blood lineages) is possible. Furthermore, function of the normal FA-C gene is suggested by the marked increase in progenitor numbers with each cycle of gene transduction in the one patient who has completed the protocol. Mitomycin C (MMC)-resistant colonies have also increased with each cycle of gene transduction in this patient. He also has had stable blood counts following the four cycles of transduction, despite decreasing his dose of oxymetholone. These data are encouraging and warrant further analysis.

Summary of Protocol Design
Studies in our laboratory have suggested that FA-C may be a good candidate disease for gene therapy. We have placed the normal FA-C gene into a retroviral vector and introduced the gene into cells derived from FA-C patients. In the laboratory, when we have compared cells lines and bone marrow cells from FA patients before and after this procedure, we see a return towards normal of cell growth, resistance to the chemical agents that harm FA cells, and a more normal appearance of the cells' chromosomes. Because the cells containing the normal FA-C gene grow better, these cells should have a competitive advantage compared to unaltered FA-C cells.

The purpose of this research protocol is to test whether we can safely introduce the normal FA-C gene into stem cells of patients with this disease. Stem cells are the cells in the bone marrow and blood that give rise to the white cells, platelets, and red cells. We will treat blood cells from FA-C patients in a test tube, using the vector containing the normal FA-C gene, and then return these cells to patients. If the cells are genetically altered, we expect to be able to detect the normal gene in blood and bone marrow cells afterwards. We hope that cells that contain the normal FA-C gene will grow well in the bone marrow, and that we will be able to detect normal stem cells in special tissue culture studies. If we are successful, we should also be able to correct the chromosome abnormality. It may even be possible to increase blood counts in patients with FA using this procedure. However, it should be clear that the major purpose of this protocol is to test the safety of this technique and to determine whether we can transfer the FA-C gene successfully.

In order to obtain sufficient stem cells from patients with Fanconi anemia, we will treat each patient with a hematopoietic growth factor called G-CSF or granulocyte-colony stimulating factor. G-CSF will be given for one week, at the end of which time blood cells will be removed by a process called apheresis for treatment with the FA-C gene vector. •••

Clonal Abnormalities: Their Implications for Treatment

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Approximately 10% of FA patients are reported to develop leukemia. Of those, the sex ratio and age at diagnosis of FA was similar to all FA patients, although almost half had leukemia as the presenting feature, without prior aplastic anemia. Most of the leukemias were of the acute myeloid type (AML), although the usual leukemia in non-FA children is lymphoid.

An important question in FA is the meaning of "preleukemia". This term is perhaps not appropriate, because it implies that leukemia can actually be predicted, and we do not know this for sure. The term "myelodysplastic syndrome (MDS)" is preferable. MDS may be suggested by the characteristic appearance of

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Phenotypic Consequences of Mutations in the FA-C Gene: an International FA Registry (IFAR) Study

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FA patients comprise at least 5 complementation groups (FA-A to FA-E). The gene for group C (FA-C) has been cloned. Mutations in the FA-C gene have been identified. We have developed a special assay (ARMS) to look for the six most common FA-C mutations and have used this assay to screen patients in the IFAR Registry. Fifty-nine FA-C patients have been identified and their FA-C mutations have been studied. We have separated the FA-C patients into three genotype groups, defined by where the mutation is in the FA-C gene: exon 1 (at the beginning), IVS4 (in the middle), and exon 14 (at the end). We then studied and compared the clinical outcome for each group.

Two of the subgroups, IVS4 and exon 14, are associated with a poor prognosis ("poor risk") manifest by onset of hematologic disease before three years of age and median survival of 10–15 years. These two subgroups have a significantly worse prognosis than the non-C IFAR population, who experience a median onset of hematologic disease at 6.6 years and a median survival of 23 years. Exon 1 patients have a better prognosis than the other two FA-C subgroups with a later onset of hematologic disease and a survival comparable to the non-C IFAR group.

Bone marrow transplantation remains the only curative therapy for FA-related hematologic disease. Recent analysis of 151 HLA-matched sibling transplants from Cincinnati show that increased survival is associated with younger age, less severe hematologic disease, and absence of malignant transformation. Therefore, optimal BMT results require careful monitoring of hematologic progression and advance donor selection. Although most clinicians perform HLA-typing of family members of all newly diagnosed FA patients, it is imperative that typing be performed as soon as possible in the patients in the poor risk groups defined in this study. Most of these patients will require transplant before the age of five years.

In these poor risk patients, the knowledge that there is no HLA-matched sibling is also important. Many families in this situation will pursue future pregnancies in hopes of having a non-affected HLA-matched sibling. Alternatively, searches for unrelated bone marrow or cord blood donors are often conducted. Although the results of unrelated bone marrow transplants are inferior to those with HLA-matched siblings, and the experience is limited with unrelated cord blood transplants, they may be the only option for the patient with severe hematologic disease or evidence of early malignant transformation. Unrelated searches can take months, and sometimes years, to locate a suitable donor. In poor risk patients the search should begin at the time of diagnosis.

Sixteen of the 59 FA-C patients (27%) have developed AML. The incidence of leukemia in each of the FA-C subgroups ranges from 19-37%. Twenty-eight of the 59 FA-C patients have died. Leukemia was the cause of death in 13 of the 28 (46%).

In addition to the identification of two poor risk groups in the FA-C population, this study clearly demonstrates that leukemia is a significant complication and cause of morbidity in the FA-C patients. IVS4 and exon 14 patients develop leukemia at a younger age than exon 1 patients. The careful surveillance of bone marrow for evidence of clonal disease is extremely important. For all FA patients we recommend yearly bone marrow aspirations for morphologic examination and cytogenetic analysis. Patients with hematologic disease, who are at higher risk for leukemia, should have bone marrow examinations every six months. These frequent observations will allow future study of the evolution of AML in FA, and are important in making decisions regarding the timing of transplant, especially in those patients without a matched sibling donor.

Since only 15% of FA patients in the IFAR are FA-C, these results affect only a small

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Unrelated Donor Stem Cell Transplant for the Treatment of FA: University of Minnesota

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The only definitive therapy for the treatment of the hematologic manifestations of FA (i.e. bone marrow failure, myelodysplastic syndrome or leukemia) is stem cell transplantation. While the probability of cure is more than 85% for children less than 10 years of age with FA who have an HLA-identical sibling donor (data presented by Dr. Harris), others not fitting this description (i.e., older patients or patients with stem cells from other related or unrelated donors) have a substantially lower cure rate. In an attempt to increase the chance of success for patients with FA without HLA-matched donors, we have investigated both the efficacy of marrow T cell depletion using elutriation, and umbilical cord blood as sources of stem cells for transplantation.

To date, more than 400 transplants (from sibling and unrelated donors) have been performed using elutriation as the method of T cell depletion, with the majority of unrelated transplants having been performed at the University of Minnesota. While most patients have had diseases other than FA, we have used this technique as part of an FDA monitored phase I-II trial since February 1995 to treat patients specifically with FA who do not have HLA-matched sibling donors. Certain requirements must be satisfied to be eligible for an unrelated donor or non-matched related donor transplant:

- Patient must have confirmatory DEB testing and complementation group analysis. In those instances where DEB testing has been performed elsewhere, confirmatory DEB testing will be performed by Dr. Arleen Auerbach.
- Patient must have bone marrow failure and be either unresponsive to androgen/steroid therapy or unable to receive such therapy due to significant side effects (e.g., liver adenoma)—or myelodysplastic syndrome with cytogenetic evaluation consistent with clonal evolution—or presence of leukemia.
- Patient must have a suitable donor.
- Patient must have adequate kidney, heart, lung and liver function.

Optimally, patients referred to the University of Minnesota will have initiated a donor search before they are heavily transfused (>20 transfusions) or have leukemia. An unrelated donor search may take years or may be completed within months; the average search time is 3.5 months. It is clear from the results with HLA-matched sibling donor transplants that patients who have been heavily transfused or who have developed leukemia do not have as good an outcome as those not heavily transfused and without leukemia.

Once it has been determined that a patient meets the eligibility criteria and the patient and family desire transplant therapy, the patient is treated with cyclophosphamide (Cytoxan, CY) 10 mg/kg/day intravenously over one hour for four days followed by total body irradiation (TBI) 450 cGy given as a single dose. During this time, the patient also receives methylprednisolone (MP) 1 mg/kg and anti-thymocyte globulin (ATG) 15 mg/kg intravenously twice daily over the five day period of CY and TBI in an attempt to reduce the risk of graft rejection. On the day of transplant, the patient receives either bone marrow that has been T cell depleted by elutriation, or unprocessed cord blood. Regardless of the source of stem cells, the patient will receive cyclosporin (CSA) and a short course of methylprednisolone to further prevent the risk of severe GVHD.

The most significant risks of the transplant procedure are: 1) multi-organ failure that results from the excessive sensitivity of FA patients to CY and TBI; 2) graft failure that results from the partial survival of the patient's own immune system that in turn results from the inability to give higher doses of CY and TBI to patients with FA; 3) severe GVHD with overwhelming infection that results from the activation of donor T cells against the patient's vital organs (primarily skin, gut and liver). In an attempt to minimize the risk of graft failure, we have increased the dose of CY and TBI, as

compared to those doses given to patients with matched sibling donors, and have added methylprednisolone (MP) and ATG. In an attempt to minimize the risk of GVHD and infection, we eliminate 97% of the donor T cells in the bone marrow by elutriation, or alternatively, use cord blood. While multiorgan failure can occur, the doses of CY and TBI prescribed can be used successfully in the majority of patients.

The decision to use bone marrow or cord blood depends upon: 1) the degree of HLA match or mismatch; 2) numbers of cells in the cord blood unit; 3) the patient's disease status. *Most patients will not have a matched or 1-antigen mismatched cord blood donor after testing for HLA-A, B and DR (with DR further tested using high resolution DNA based assays).* For those with a matched or 1-antigen mismatched cord blood donor, cord blood will be offered as the first choice if there are adequate numbers of cells. For those without a closely matched cord blood donor, a matched or 1-antigen mismatched bone marrow donor will be offered. Most patients will have such a donor registered with the National Marrow Donor Program. For those few patients for whom a matched or 1-antigen mismatched cord blood or bone marrow donor cannot be found, a 2 or 3 antigen mismatched cord blood will be considered. Although there are some encouraging preliminary data with 2 and 3-antigen mismatched cord blood transplants in patients with FA, follow-up has been short in general. As more is learned about mismatched unrelated donor cord blood transplantation, our recommendations may change.

Seventeen patients have been treated using the University of Minnesota protocol for patients with FA who do not have an HLA-identical sibling. While seven patients transplanted before February 1995 received unprocessed bone marrow (marrow that was not T cell depleted), patients transplanted after February 1995 received either T cell depleted bone marrow (eight patients) or umbilical cord blood (two patients) as described above. Ten of 17 patients are alive three months to five years after transplantation. Of the eight patients who received T cell depleted marrow, five are alive; and, of the two patients who received umbilical cord blood, one is alive. Notably, no patient receiving T cell depleted bone marrow or umbilical cord blood has developed severe GVHD.

Multi-Institutional Trail Supported by the Fanconi Anemia Research Fund, Inc.

Since patient accrual is slow at any one institution, it is hoped that a multi-institutional cooperative trial will help us more rapidly determine the optimal transplant approach for patients with FA. Thus far, 12 transplant centers have agreed in principle to the development of a single multi-institutional treatment protocol. While there has been general agreement on the use of CY and TBI, as outlined above for the University of Minnesota, the exact method of T cell depletion has not been determined. Because elutriation is not readily available at all sites, further discussions on the method or methods of T cell depletion to be used in this study are required.

This work is being done in collaboration with Dr. Stella Davies at the University of Minnesota, and with the National Marrow Donor Program and many other individuals who are instrumental to the success of this endeavor: most notably, Arleen Auerbach (Rockefeller University, New York, NY), Eliane Gluckman (Hôpital St. Louis, Paris, France), Alfred Gillio (Tomorrow's Children's Institute, Hackensack Medical Center, Hackensack, NJ), Richard Harris (Children's Hospital of Cincinnati, Cincinnati, OH), Gaye Crooks (Children's Hospital of Los Angeles, Los Angeles, CA), Joachim Deeg (Fred Hutchinson Cancer Research Center, Seattle, WA), Eva Guinan (Children's Hospital of Boston, Boston, MA) and Jim Casper (Medical College of Wisconsin, Milwaukee, WI). Support provided by the Fanconi Anemia Research Fund, Inc. will be used to facilitate the successful development of this cooperative group study. Progress will be communicated to families and physicians.

General Comments about Transplantation in the Treatment of FA

Donor search can be a lengthy process. A search should begin as early as possible, once the eligibility requirements for transplant have been satisfied. Also, remember that every insurance carrier is different and

that policies within a company vary significantly between individuals. While an insurance policy may state that there is coverage for bone marrow transplantation, there is no guarantee that the amount of coverage will be acceptable to any transplant center. Therefore, it is important to designate a transplant center early in the process so that the details can be worked out between the institution and insurer. In cases where the insurer will cover transplant expenses but not the donor search, contact the Office of Patient Advocacy at the National Marrow Donor Program: 1-612-627-5800. In cases where the insurer will cover transplant expenses, but only at a center without experience with unrelated donor transplantation for FA, it is important to know that occasionally exceptions have been granted due to the unique aspects of this patient population. When choosing a transplant center, you and your physician are encouraged to consult with several centers. The choice of transplant center should be based on: 1) experience in unrelated marrow donor transplantation; 2) experience with patients with FA; 3) location of transplant center. In some cases, however, the insurer may dictate the center because of existing contracts.

In general, the patient will be required to stay near the transplant center for 100 days after transplantation. At most centers, housing will be made available to you. Centers vary widely in their rules and restrictions, and these should be discussed in detail.

Prenatal Diagnosis and Genetic Counseling

It is extremely important that couples at risk for having children with FA seek genetic counseling prior to pregnancy.

For those couples electing to have children, prenatal diagnosis can be performed on tissue obtained from a chorionic villus sample (CVS) or from amniocytes collected by amniocentesis. Each procedure has a low risk of causing fetal death, and the test results will provide information to the couple as to whether the unborn child has FA or is HLA-identical with another affected child. In instances where the unborn child is not affected by FA and is HLA-identical or 1-antigen mismatched with an affected child in the family, the umbilical cord blood should be collected at the time of birth and stored for future transplantation. The collection of this blood takes nothing from baby or mother. In cases where the umbilical cord blood donor is HLA 2 or 3-antigen mismatched with the patient, collection may still be considered since it may still serve as the stem cell source if no "better" donor can be identified.

Preimplantation Genetic Diagnosis

In addition to conventional prenatal testing such as CVS or amniocentesis, *a new technology is now available which should allow some couples to begin their pregnancy knowing that their fetus does not have FA. The technology is called Preimplantation Genetic Diagnosis, or PGD.* Using this new technique, over 65 healthy babies have been born to couples at high genetic risk of having a child with a serious inherited disorder such as cystic fibrosis, muscular dystrophy, hemophilia, Huntington disease, thalassemia, Tay- Sachs, etc. This technology can be used in families in which the specific gene mutation(s) responsible for FA is known: for instance, when the defect is in the FA-C gene. *Soon this will be an option for FA-A families as well.*

The procedure of PGD is as follows: The couple uses "*in vitro* fertilization," a medical procedure which has been employed for 18 years to assist infertile couples to have a baby. The woman is treated with follicle stimulating hormone, administered intramuscularly for 12–14 days starting two days after the last menstrual period, so that her ovary produces more than the usual one egg that month (perhaps 10–12 or so). Using a very small needle under ultrasound guidance, the eggs are collected from the surface of the ovary and fertilized with the husband's sperm. The eggs are allowed to grow *in vitro*, and divide to the 8-cell stage, which takes about two days. One cell (called a blastomere) is removed from each egg and is tested for the presence or absence of the FA mutations known to exist in the mother and father. The

"pre-embryos" which do not have the FA mutation(s) are then transferred to the woman's womb, allowing her to start her pregnancy knowing the baby will not have FA.

PGD has been used successfully for numerous genetic disorders, though not yet for FA, but the technology is available now, and some families are considering this as one of their reproductive options. The procedure can be expensive (depending on whether a research protocol assists with the costs) and will require travel. Furthermore, while the results with PGD are promising, there are no guarantees that the couple will become pregnant. Of course, this is a new test and despite successes with other genetic disorders, it is still too early to know its reliability in avoiding FA. For this reason, standard procedures such as CVS or amniocentesis can be performed later, to verify that the fetus is unaffected.

Researchers have added a twist to PGD for disorders such as FA, where a transplant is needed. From the same cell (blastomere) upon which genetic testing is performed to detect the disease mutation, *HLA testing can also be performed. This allows the couple to initiate a pregnancy without FA in which cord blood from the baby would be an identical match to a sibling with FA.* Comprehensive counseling will be made available that may include a discussion of PGD for eligible couples. At this time, however, not all families can benefit from this procedure. PGD is limited to families in which 1) the genetic mutation(s) have been determined for both mother and father, and 2) the HLA-DR or DO typing can distinguish the genes (i.e. HLA-A, B, DR), inherited from mother and father (if testing is requested). It is not likely to be covered by third party payers.

Summary

Most patients with Fanconi anemia will develop bone marrow failure, myelodysplasia or leukemia before the age of 20 years. As supportive medical care with androgens, growth factors, etc., improve, it is likely that patients will live longer. There remains a lot that is still not understood about the natural history of this disease—for example: How frequently do patients develop leukemia? How frequently do clonal cytogenetic abnormalities come and go? Are there risk factors that will allow us to predict early death or higher risk of developing leukemia? The only way to answer these questions is to examine the bone marrow of all patients routinely. It is not in the patient's best interest to study the bone marrow only after the bone marrow "catastrophe" has occurred, such as with the onset of transfusion-dependence or the development of leukemia. *All patients should have bone marrow examinations performed yearly even if all the blood counts are normal, and more frequently once abnormalities are discovered. Patients with myelodysplasia should plan on bone marrow examinations every 4 months.* If the myelodysplasia and/or cytogenetic abnormality disappears, then bone marrow examinations might be spread out; if the myelodysplasia and/or cytogenetic abnormality worsens, then bone marrow transplantation should be considered. While bone marrow transplantation is associated with significant risk, it offers the best chance for cure. *As the patient's disease advances from myelodysplasia to leukemia, the chance of cure after transplantation decreases, because of the added risk of leukemia relapse months to years later.* However, while early results have been promising, unrelated donor stem cell transplantation remains a high-risk procedure. •••

Joenje - continued from page 5

that a mutated FA gene sometimes can return to the normal (healthy) state. Suppose we had a MMC-sensitive hybrid (for example FA-A x FA-A) in which one of the 4 defective FA-A gene copies had reverted to normal. This hybrid would be MMC resistant instead of sensitive, since one healthy copy of the FA gene (even with three defective ones) is sufficient to correct the disease phenotype completely. The appearance of complementation, due to the reversion of one defective gene, would lead us to define this cell line as "non-A". Since in this example the patient is in fact A, all the non-A FA genes in this patient are likely to be normal (reverted). Therefore, fusions with all the other groups will result in complemented hybrids, and the patient will be erroneously assigned to a new complementation group. Thus, some of the patients classified as belonging to the E-H groups might represent cases that are particularly prone to genetic reversion under the conditions of cell fusion. Again, ultimate proof for the validity of these groups will be finding the corresponding genes and demonstrating mutations in patients.

Other methods to determine an FA patient's subtype. In Dr. Grompe's laboratory complementation analysis is carried out by a different method, in which fibroblasts grown from a patient's skin biopsy are used to perform fusions with an immortalized group A fibroblast cell line. This allows the assignment of a patient to group A or not. To assess if a patient is group C or non-C, in many laboratories blood cells or fibroblasts are used to search for mutations in the FA-C gene. If definite mutations are found, the patient is diagnosed as group C. However, not all DNA sequence alterations found so far in the FA-C gene are actually pathogenic (responsible for the disease). Today a total of 10 pathogenic mutations have been identified in this gene. In addition to these, an equal number of non-pathogenic variations have been found. Such variations (called "polymorphisms") still allow the gene to function normally. Finding pathogenic mutations in both copies of the FA-C gene in a patient is conclusive evidence for the patient being type C. However, current screening methods detect only approximately 90% of all possible mutations. Thus, not finding mutations in the FA-C gene does not completely exclude the C group. In such cases complementation analysis can help, since assignment to group C on the basis of a cell fusion experiment would indicate that the FA-C gene must carry pathogenic mutations. A more extensive investigation should then be carried out to identify these mutations. Knowledge of the ancestry of the patient can make a mutation search more efficient, since certain mutations may be particularly frequent in certain ethnic groups. For example, most of the FA patients of Ashkenazy Jewish ancestry have the FA-C mutation IVS4+4 A->T, whereas Dutch FA-C patients are most likely to have the 322delG mutation.

From the patients analyzed to date by cell fusion studies, the great majority (over 65%) appear to belong to the A group. As soon as the gene for group A has been isolated, rapid mutation screening methods will become available. These screens will allow most patients to be recognized as "A" on the basis of finding pathogenic mutations in the FA-A gene.

Cloning the FA-A gene.
Essentially two methods are being followed to clone the FA-A gene:

1. Positional cloning. This method is based on the gene's position on the genetic map, as determined by genetic linkage analysis. It is known that the FA-A gene must be located at the tip of the long arm of chromosome 16, in a region technically described as 16q24.3. The job is to identify genes in this area and determine which one is mutated in FA-A patients. This method can take a long time, since many genes may have to be screened, but sooner or later the correct gene will be found. Research teams in Europe, the US and Australia have joined forces to search for the FA-A gene in the 16q24.3 area.

2. Expression cloning. This method is more direct than positional cloning but it is also riskier, because for technical reasons it may not work for the

gene one is searching for. This is the method that was used successfully in Dr. Buchwald's laboratory to clone the FA-C gene. The method makes use of a so-called cDNA expression library and the capacity of the correct gene to complement the MMC sensitivity of FA-A lymphoblasts. The library is essentially a collection of all genes that are expressed in a given cell type, built into a so-called expression vector. This vector, when delivered into FA-A lymphoblasts, causes the gene that it contains to be expressed, which means that the corresponding protein is made. If one of the vectors in the library contains the FA-A gene, the cell that takes up this vector will be complemented, meaning that its MMC hypersensitivity will be corrected. This particular corrected cell can then be propagated in the presence of MMC at a level that is sufficient to kill all the other cells that do not contain this vector. Finally one can recover the vector from the complemented cells and see if it contains the gene. We are now at the stage of having identified a cDNA that may be viewed as a strong candidate for being the FA-A gene, because it complements FA-A cells and localizes to the correct region on chromosome 16. Final proof, however, will come from finding mutations in this gene in FA-A patients. Until then, the status of this cDNA remains that of a "strong candidate."

Mosaicism

Mosaicism refers to the situation in which an individual contains cells that are genetically non-identical. A proportion of FA patients appear to be mosaics, according to observations on their peripheral blood lymphocytes. Two kinds of observation have suggested mosaicism. First, in a chromosomal breakage test two cell types are sometimes detected, one type behaving like FA cells and the other behaving like normal cells. Second, sometimes a lymphoblastoid cell line derived from an FA patient may be MMC resistant. Apparently in a proportion of blood cells from mosaic patients the disease phenotype has disappeared or "reverted" to normal. We have recently been able to determine the origin of such a "reversion" in a female group C patient. This patient had inherited a different FA-C mutation from each parent, one located at the beginning of the gene and the other at the end. When looking at the MMC-resistant (normal) cells from this patient it appeared that both mutations were found in the FA-C gene on one chromosome, whereas the FA-C gene on the homologous chromosome was normal. The explanation comes from a process called genetic recombination. During this process, parts of the homologous maternal and paternal chromosomes pair and undergo a mutual exchange: a breakage- and-reunion type of event that causes the paternal chromosome to be linked up with the maternal chromosome and vice versa. If recombination takes place between the two mutations in the FA-C gene, this process leads to one chromosome with an FA-C gene containing both mutations and one chromosome with a normal gene. **See diagram.**

Since only one copy of a normal gene is sufficient for normal cell function (as in the parents of FA children), the recombination has actually *cured* this particular cell. If this occurs in a hematopoietic stem cell, this cell should be capable of normal hematopoiesis and may ultimately improve the patient's hematological condition. We have seen several examples of patients with mosaicism who had unusually mild or no hematological symptoms. Thus, mosaicism in FA may be clinically relevant and should be studied in more detail. Since patients with a high proportion of reverted cells are sometimes not correctly diagnosed in the standard chromosomal breakage test, we are currently trying to optimize the test to improve its efficacy for detection of mosaic FA cases. Clearly, more elaborate studies will be necessary to elucidate the diagnostic and clinical implications of mosaicism in FA. •••

Clonal - continued from page 6

the bone marrow cells. Some subcategories of MDS include refractory anemia (RA), refractory anemia with excess blasts (RAEB), and refractory anemia with excess blasts in transformation (RAEBIT). Although the incidence of leukemia in non-FA patients with these types of MDS is increased, it is not possible on an individual basis to determine which patients will actually develop leukemia. Another component of "preleukemia" is the presence of a clonal cytogenetic abnormality, defined as 2 or more cells with the same abnormality. However, since only 25 cells are usually examined, there is a large statistical probability of missing a clone which is present in small numbers.

Although FA patients develop leukemia, and patients with leukemia may have abnormal clones, there is no evidence so far that the presence of a clone means that leukemia is inevitable. This must be remembered when the topic of high risk unrelated donor transplant comes up. When we reviewed the literature, we found 31 patients with "MDS", of whom 30 had abnormal clones. Only six actually developed leukemia, while 15 died from other causes at up to 12 years later, and 10 were alive without leukemia at up to three years. The chromosome most commonly abnormal in FA with leukemia is 7, while in those with MDS an abnormal chromosome 7 was no more frequent than many others. In addition, clonal abnormalities fluctuated, sometimes disappearing, sometimes reappearing, and sometimes replaced by a different and unrelated clone.

At the University of Texas Medical Branch and Mount Sinai Medical Center we performed bone marrow biopsies on a series of 22 patients. Six were "MDS" by morphology. Fifteen yielded sufficient cells for cytogenetics, and six had clonal abnormalities. Fluctuation was seen in three. One patient did develop leukemia three years after the first clone was seen (which was the first marrow examined). The explanation for the clonal fluctuation might be that all hematopoiesis is clonal, and that the number of clones populating the marrow at any time is small. At different times, different clones are replicating. DNA damage and repair are continual processes; in FA, repair is defective, and some cells may be identifiable by cytogenetics. The presence of a clone may merely mark the common ancestry of the progeny, without necessarily meaning that leukemia will develop. The age of onset of MDS in the literature cases is later than the age of onset of leukemia, and the survival is longer for MDS. These data do not support the contention that MDS must precede leukemia.

The current recommendation is that bone marrow examinations be done at the time of diagnosis and then annually in asymptomatic patients, as well as whenever a clinical change occurs. The marrow aspirate should be examined by a hematologist or hematopathologist who is experienced in aplastic anemia, myelodysplasia, and leukemia. A biopsy is important to assess cellularity. For research purposes, hematopoietic cultures may be useful. Most important, cytogenetics should be done by a laboratory which focuses on cancer cytogenetics, and has available the ability to develop FISH (fluorescence *in situ* hybridization) analyses, both for common leukemic clones (e.g. monosomy 7), as well as tailored to the specific abnormality of a given patient. A long term, prospective study of large numbers of patients is needed to determine whether there may be specific cytogenetic and/or morphologic markers which do predict leukemia. Until then, one must weigh the risks before choosing an unrelated bone marrow transplant to prevent leukemia. •••

Gillio - continued from page 7

portion of the FA population. However, the FA-A gene, which is defective in approximately 65% of FA patients, is now being characterized. FA-A mutation analysis is likely to yield genotype-phenotype differences in this heterogeneous population, again allowing subgroup segregation and prediction.

Further identification of mutant FA-C alleles may allow more precise genotype segregation and perhaps more clinical homogeneity between subgroups. Nevertheless, this study enables us to define this clinically heterogeneous disorder genotypically in order to predict clinical outcome and aid decision-making regarding therapeutic options for a subset of FA patients. •••

Rackoff - continued from page 1

drawn concerning the prevention of infections or other complications in patients with FA. Despite the multilineage responses seen in some patients, it is clear that this drug often does not provide adequate treatment of thrombocytopenia (low platelet count) and anemia. The patients who had multilineage responses were, for the most part, those who had the least severe bone marrow failure. In some patients, the best responses were seen while the patients were taking androgen and G-CSF, evidence that androgen continues to play an important role in the supportive treatment of this disorder. The question of whether the apparent multilineage effects of G-CSF will prove to work together with other cytokines, such as IL-11 and thrombopoietin, awaits study. •••

D'Andrea - continued from page 2

First, the FA-C protein is a cytoplasmic protein and therefore does not play a direct role in repairing DNA. **Second,** the FA-C protein binds to three other normal cellular proteins. The identification of these other proteins may help us identify the cellular function of the FA-C protein. **Third,** specific alterations in the FA-C protein alter its activity. Some of these altered FA-C proteins fail to correct sensitivity of FA cells to crosslinking agents. These same alterations block the ability of FA-C to bind the three normal host cell proteins.

We have recently made the provocative observation that the FA-C protein binds to some proteins involved in normal cell division and normal cell cycle progression. This observation suggests that the normal function of the FA-C protein is critical to cell division. Perhaps this is the reason that FA patients have so many different types of developmental abnormalities including skeletal and hematological abnormalities. FA patients also demonstrate considerable phenotypic variability. Approximately twenty-five percent of FA patients have no obvious developmental abnormalities. Interestingly, the clinical severity of FA patients correlates, at least in part, with the specific FA-C mutations that different FA families express. See the report by Al Gillio.

Our long term goal is to understand, in great detail, the molecular function of the FA-C protein. In the meantime, we will also study the FA-C binding proteins and the proteins encoded by other FA genes, as they are cloned and identified. Understanding the molecular function of the FA protein will lead to more informed treatment strategies for FA patients. For instance, if FA proteins normally play a role in correcting cellular damage or in controlling cell division, drug treatments or prevention strategies that address these processes may be rationally applied. •••

Hematopoiesis - continued from page 2

types of studies indicate that the FA-C protein is intimately involved in hematopoiesis, either directly or indirectly.

Many investigators have examined the role of cytokines in FA. IL-6 levels are low in FA,while TNF-alpha is high. IL-6 decreases production of TNF-alpha, while TNF-alpha fails to stimulate production of IL-6 in FA. IL-6 inhibits apoptosis (programmed cell death). If IL-6 is low in FA, and TNF-alpha is high but does not increase IL-6 in a regulatory loop, perhaps FA patients have increased apoptosis, which may contribute to hematopoietic failure.

The accumulation of FA cells in sub-G1 (early) parts of the cell cycle might reflect apoptosis. In normal cells, MMC increases the expression of an oncogene called p53, which in turn increases apoptosis. In FA, MMC increases p53, but increases apoptosis independent of p53, because cells mutant in p53 still undergo apoptosis. The FA-C gene product is in the cytoplasm, and might prevent a DNA crosslinker from increasing apoptosis. One speculation which has not been tested is that the FA-C protein increases IL-6. •••

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Editing, Layout, and Design of Scientific Supplement
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WE THANK THE FOLLOWING DONORS:
Editing, Layout and Design:
Joyce L. Owen
Support for distribution:
The Edwin & June Cone Fund of the Oregon Community Foundation
Amgen, Incorporated

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MADISON, WISCONSIN

OCTOBER 10 – 12, 1996