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Granulopoiesis in Fanconi's Aplastic Anemia (40607)[1]

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Fanconi's aplastic anemia is a rare syndrome of pancytopenia associated with one or more of the following congenital malformations: thumb and skeletal abnormalities, microcephaly, strabismus, renal malformations, mental retardation, and skin pigmentation. The onset of pancytopenia usually occurs between the ages of 4 to 7 years. The pathophysiology of this pancytopenia is especially interesting. Pancytopenia may result from a defect in pluripotent hematopoietic stem cells, a defect in the microenvironment of the bone marrow and/or the presence of a humoral inhibitor or cell-mediated suppression of hematopoiesis (1).

The *in vitro* culture of hematopoietic cells has been used recently to study various hematologic diseases. Discrete colonies in culture are derived from the proliferation of different committed progenitor cells which in turn are the progeny of more primitive pluripotent stem cells. With colony-stimulation factor (CSF) from peripheral leukocytes, granulocytic progenitor cells will proliferate and form colonies in agar culture. By using a double-layer agar culture technique, one can quantitate these colony-forming units in culture (CFU-C) and CSF or evaluate the possibility of humoral inhibitor and/or cell-mediated suppression of granulopoiesis.

Recently, we encountered four patients with Fanconi's aplastic anemia. Factors related to granulopoiesis including granulocytic progenitor cell, colony-stimulation factor, cell-mediated suppression, and humoral inhibition of granulopoiesis were studied. We found very similar abnormalities in all four patients.

Materials and Methods. Four patients with clinical and laboratory findings of Fanconi's anemia had complete peripheral blood counts, fetal hemoglobin determination, bone marrow aspiration, chromosomal analysis, intravenous pyelogram, and skeletal survey. They all have typical pancytopenia, increased fetal hemoglobin, macrocytic erythrocyte, decreased cellularity and megakaryocytes in marrow, and increased chromosome breakage (by standard Giemsa banding karyotype). There were varied degrees of congenital abnormalities. Cases three and four were fraternal twins. Details of the hematologic data, chromosome study, and congenital abnormalities were reported elsewhere (2).

All procedures were performed at a time when patients were free of infection. Cases 1, 3, and 4 received no prior blood transfusion or medication. In case 2, oxymetholone treatment (100 mg/kg/day) was begun 4 months before the study. The hemoglobin in this patient was 12 g/dl at the time of the study. The patient had required frequent transfusions before oxymetholone therapy.

Assay of colony-forming units (CFU-C) and colony-stimulation factor (CSF). The double-layer agar culture technique of Kurnick and Robinson (3, 4) was used with slight modification. Alpha-medium (K-C Biological Lenexa, Kansas) was used instead of McCoy's 5A medium and 10% fetal calf serum plus 5% horse serum was substituted for the 15% fetal calf serum supplementation. Peripheral white cells from two healthy adults who had repeated CSF values within ±15% of each other were used for preparation of the normal feeder layer.

Bone marrow aspirates were obtained from either posterior or anterior iliac crest. Informed consent was obtained in all cases. Nucleated cells of bone marrow aspirate and peripheral white cells were washed and plated as a 1 ml of suspension of 2×10^5 (bone marrow) or 10^6 cells/ml (peripheral blood) upon the feeder layer. Four replicate plates were used for each specimen. Colony numbers were scored with an inverted microscope

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at 40× magnification after 12–14 days incubation in a humidified incubator at 37.5°C with constant flow of 7.5% carbon dioxide in air.

Control bone marrows were obtained from patients with solid tumors whose bone marrow was free of involvement. The marrow aspirate from patients with idiopathic thrombocytopenic purpura was also used as the source of colony-forming cells for quantitation of CSF, evaluation of serum inhibitor, and mixing experiments.

To evaluate CFU-C, the patients' peripheral white cells or nucleated marrow cells were plated upon normal feeder layers. The capacity of the patients' washed peripheral white cells to provide CSF for colony formation of marrow cells was compared with CSF of normal peripheral leukocyte feeder layer studied under identical conditions. CSF of normal feeder layers was arbitrarily considered as 100%.

Serum inhibitor study. In order to study the possibility of the existence of a humoral inhibitor in these patients, 0.15 ml serum of control or patients' serum was added to the culture. The bone marrow cells were plated upon normal feeder layers for this study.

Mixing experiments. In order to determine the possible role of cell-mediated suppression of granulopoiesis, various mixing experiments were performed. Bone marrow cells (2×10^5 cells) mixed with either patient's peripheral white blood cells (2×10^5 cells) in case 2 or mononuclear cells (2×10^5) in cases 2, 3, and 4 were plated upon normal feeder layers. Mononuclear cells were prepared by Ficoll–Hypague separation. In two patients (case 3 and 4) peripheral white blood cells (1×10^6 cells) were mixed with normal peripheral WBC (1×10^6 cells) to prepare the feeder layer. Bone marrow cells were then plated on these mixed feeder layers.

Results. Colony-forming units. All four patients showed a marked decrease in marrow and peripheral CFU-C (Table I). In case 2, marrow CFU-C was slightly more than the rest of the patients but was still much lower than that of the control even though she had been treated with oxymethalone and had no anemia at the time of the study.

Colony-stimulation factor. All four patients showed either a moderate or marked decrease in CSF as compared to normal adults (Table I). The colonies grown over the feeder layer prepared from the patients' peripheral leukocytes were also smaller than those on the normal feeder layer.

Serum inhibitor. There was no significant difference in colony number with addition of normal or patients' serum (Table I). This observation implies that patients' serum (at this concentration) did not contain an inhibitor to *in vitro* colony formation.

Mixing experiment. There was no suppressive effect from the patients' peripheral white cells or mononuclear cells on bone marrow granulocyte progenitor cells (Tables II, III). There were also no suppressive effects when the patients' peripheral leukocytes were mixed with normal leukocytes as feeder layer (Table IV).

Discussion. All four patients have clinical and laboratory characteristics of Fanconi's aplastic anemia, although the congenital malformations vary from minimal abnormalities in case 2 to involvement of almost all organs known to be affected in this disease in case 1. We observed very similar changes in colony-forming units (CFU-C), colony-stimulation factor (CSF), serum inhibitor study, and mixing experiments in all four patients, regardless of the variety of congenital abnormalities, degree of anemia, or androgen therapy.

A decrease in CFU-C in both bone marrow and peripheral blood suggests the possibility

TABLE I. COLONY-FORMING UNITS, COLONY-STIMULATION FACTOR, AND SERUM INHIBITOR STUDIES

	Control	Case 1	Case 2[a]	Case 3	Case 4
Marrow CFU-C (per 2×10^5 nucleated cells)	97 (40–318)[b]	4 ± 1[c]	8 ± 2	3 ± 1	2 ± 1
Peripheral CFU-C (per 10^6 WBC)	20 (5–30)	2 ± 1	1 ± 1	2 ± 1	2 ± 1
Peripheral blood CSF (% of control)	92 (80–102)	21–51[d]	23–25	0–28	0–16
Serum inhibitor study (% of control without serum)	96,112	116	88	124	128

[a] Study done after treatment with oxymetholone at age of 7.
[b] Median and range.
[c] Mean ± SD.
[d] Range of two or three experiments using different marrow cells as source of colony-forming cells.

TABLE II. EFFECTS OF PERIPHERAL MONONUCLEAR CELLS ON COLONY FORMATION OF BONE MARROW CELLS[a]

Without	36 ± 2
With control 1	60 ± 6 (14)[b]
With control 2	71 ± 6 (31)
With case 2	46 ± 2 (4)
With case 3	33 ± 3 (1)
With case 4	39 ± 2 (2)

[a] 2×10^5 marrow cells mixed with or without 2×10^5 peripheral mononuclear cell of patients or control subjects plated on normal feeder layer.

[b] Figures in parentheses indicate colony formation from 2×10^5 peripheral mononuclear cells on normal feeder layer.

TABLE III. EFFECT OF PERIPHERAL LEUKOCYTES ON COLONY FORMATION OF BONE MARROW CELLS[a]

Without	17 ± 4
With case 2	14 ± 2
With control 1	19 ± 3
With control 2	13 ± 2

[a] 2×10^5 marrow cells mixed with or without 2×10^5 peripheral WBC from patients or control subjects plated on normal feeder layers.

TABLE IV. EFFECT OF PERIPHERAL LEUKOCYTES IN THE FEEDER LAYER ON COLONY FORMATION OF BONE MARROW CELLS[a]

Without	29 ± 2
With case 3	26 ± 3
With case 4	27 ± 3
With control	28 ± 4

[a] 10^6 normal peripheral leukocytes with or without 10^6 peripheral leukocytes from patients or control subjects as feeder layer.

of a reduction in number of the committed granulocyte progenitor cells, or an intrinsic defect of these progenitor cells. There was also a moderate to severe decrease in CSF. Reduction of CSF does not occur in all children with aplastic anemia. Ragab *et al.* (5) showed that 5 of 10 children with aplastic anemia of various etiologies had more than 80% activity, when compared to the control group. They also demonstrated a severe decrease in CSF in two patients with Fanconi's aplastic anemia and a moderate decrease in another such patient. The decrease in CSF in these patients further emphasizes the difference between Fanconi's aplastic anemia and aplastic anemia of other etiologies. There may be different causative mechanisms leading to neutropenia in various types of aplastic anemia. CSF was produced by monocytes in the peripheral leukocyte feeder layer in this culture system. Because the total number of white cells (1×10^6/ml) and the percentage of monocytes (4–7%) are similar to the control, an intrinsic defect in monocyte production of CSF may exist.

We did not detect a circulating humoral factor capable of inhibiting *in vitro* colony formation. There was also no cell-mediated suppression of *in vitro* granulopoiesis by peripheral white cells or mononuclear cells. Cell-mediated suppression of granulopoiesis by lymphocytes has been reported in a patient with acquired aplastic anemia (6). Cell-mediated suppression of *in vitro* erythropoiesis has been noted in some patients with aplastic anemia and congenital hypoplastic anemia (7, 8). However, more recent studies fail to demonstrate such cell-mediated suppressions in congenital hypoplastic anemia (9, 10). Our data also showed no evidence of cell-mediated suppression of granulocyte progenitor cells in Fanconi's aplastic anemia.

The pattern of CFU-C and CSF in Fanconi's aplastic anemia is essentially identical to that of "preleukemia" as reported by Greenberg *et al.* (4). According to their criteria, Fanconi's aplastic anemia can be classified as preleukemia. Two of their seven patients with preleukemia subsequently developed acute myelogenous leukemia (4). Similarly, a high rate of conversion to acute leukemia has been observed in Fanconi's aplastic anemia (11).

Summary. An *in vitro* agar culture system was used to evaluate the granulocyte colony-forming cells and other factors possibly affecting granulopoiesis in four patients with Fanconi's aplastic anemia. There was a marked decrease in the number of granulocytic colony-forming cells in both bone marrow and peripheral blood in these patients. Colony-stimulation factor from peripheral leukocytes was also decreased in all four patients. No apparent humoral inhibitor of granulocytic colony formation was detected. In three patients studied, peripheral leukocytes or mononuclear cells did not suppress the colony formation of "normal" bone marrow cells.

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