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# Granulopoiesis in Severe Congenital Neutropenia

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**The pathogenesis of the granulopoietic failure in three children with severe congenital neutropenia was studied. Mature neutrophils were absent from both peripheral blood and bone marrow. Assay of bone marrow granulocyte colony-forming cells (CFU-C) in a methylcellulose tissue culture system using colony-stimulating activity (CSA) from peripheral blood leukocytes demonstrated normal or increased concentrations of CFU-C compared to those from marrows of 60 age-matched controls. Colonies were of normal size and by light microscopy appeared to contain granulocytes in all stages of maturation including the mature polymorphonuclear neutrophil. CFU-C from peripheral blood of two patients were normal. Production and activity of CSA from the patients' peripheral blood leukocytes and urinary CSA excretion were normal. No serum inhibitors against CFU-C or CSA could be demonstrated using both control and autologous marrow. The defect did not appear to be due to a lack of granulocytic stem cells, a reduction of humoral stimulators of granulopoiesis, nor the presence of an inhibitor as measured by these techniques.**

THE AVAILABILITY of a tissue culture assay for committed granulocyte progenitor cells (colony-forming units in culture, CFU-C) in man[1,2] and the discovery of factors (colony-stimulating activity, CSA) capable of stimulating colony growth[3-6] have made possible the study of early events in human granulopoiesis. Congenital neutropenias constitute a heterogeneous group of disorders, and in the absence of knowledge of the pathophysiology of these disorders, classification is tentative.[7] Based on present concepts of granulopoiesis several possible mechanisms could cause neutropenia due to a failure of granulocyte production. These include (1) an absence of CFU-C, (2) an intrinsic cellular defect of CFU-C prohibiting differentiation to mature granulocytes, (3) a deficiency of humoral substances acting on CFU-C,[8] (4) an inhibitor directed against CFU-C (similar to that directed against erythroid precursors in acquired pure red cell aplasia[9]), (5) an inhibitor directed against a "granulopoietin" (CSA), and (6) an abnormality of the marrow microenviron-

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**Table 1. Patients With Neutropenia at Time of Study**

| | | Blood | | | | | | | Marrow Granulocytic Series (% of Total Differential) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient | Age | Hb (g/100 ml) | WBC (X $10^3$/cu mm) | Poly (%) | Lymph (%) | Mono (%) | Eo (%) | Platelets (X $10^3$/cu mm) | Blast | Promyelo | Myelo | Meta | Band | Poly | Eo |
| 1a* | 5 yr | 11.6 | 4.2 | 2 | 92 | 5 | 1 | 258.0 | 0.2 | 2.6 | 3.6 | — | — | — | 7.6 |
| b | | 12.2 | 5.1 | 1 | 55 | 29 | 15 | 265.0 | 0.2 | 1.8 | 26 | 1.4 | — | — | 9.2 |
| 2 | 3 yr | 10.7 | 4.1 | 1 | 85 | 13 | 1 | 306.0 | 0.2 | 0.6 | 21 | 0.2 | — | — | 15 |
| 3 | 3 mo | 8.8 | 9.6 | 0 | 72 | 27 | 1 | 336.0 | 1.4 | 2.4 | 6.0 | — | — | — | 10 |

*Patient 1 had two studies.

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ment (similar to the abnormal microenvironment for erythroid precursors in Sl/Sl$^d$ mice[10]). We have studied three children with severe congenital neutropenia to explore these possible mechanisms. Our results suggest that the defect in these patients may not be intrinsic to the stem cell and could reside in an abnormal marrow microenvironment.

## MATERIALS AND METHODS

### *Subjects*

These three male children all presented with infection during the first month of life, and continued to have frequent severe life-threatening infections, usually due to pyogenic staphylococci or *Escherichia coli*. Sites of involvement included the umbilicus, skin, middle ear, lung, lymph nodes, and blood. Soft tissue infections frequently progressed to abscess formation containing pus that consisted primarily of monocytes with some lymphocytes. Patient 1 has an associated dysgammaglobulinemia with very low IgG, absent IgA, and elevated IgM. We considered patients 2 and 3 to have congenital agranulocytosis similar to that described by Kostmann.[11] Kostmann described an autosomal recessive neutropenia with onset in infancy resulting in death, associated with a characteristic maturation arrest of the granulocytic series of the bone marrow. At present, patient 1 is reasonably well on regular injections of commercial gamma globulin. Patient 2 died with chronic slowly progressive lung abscesses which were present for 24 mo. Patient 3 died of septicemia before our investigations could be completed. Total white blood counts were usually low normal. The differentials consisted primarily of small lymphocytes with a variable monocytosis. Neutrophils were usually entirely absent and never made up more than 1% or 2% of the differential count in patients 2 and 3. Patient 1 was somewhat different in that he has responded to infections with a mild increase in neutrophils. When he was well his neutrophils were less than 100/cu mm, the same as the other patients. Bone marrow aspirates had normal cellularity with a striking "maturation arrest" at the myelocyte stage in all three patients. On multiple examinations, no bands or polymorphonuclear neutrophils were present, and only rarely was a metamyelocyte seen. We have grouped these patients together because of the identical marrow morphology. Results of blood and marrow examination at the time of study are summarized in Table 1. Patient 1 was studied on two occasions 3 mo apart. Routine blood counts on all parents were normal.

Attempted mobilization of the patients' neutrophils with epinephrine and TAB vaccine (endotoxin) was ineffective. Rebuck "skin windows"[12] demonstrated a delayed inflammatory response with an absence of neutrophils. Monocytes, eosinophils, and lymphocytes migrated normally. Monocytes could effectively phagocytose and reduce nitroblue tetrazolium (NBT) dye. Leukoagglutinins could not be demonstrated.[13] All studies were done at a time when there was no evident infection. Control bone marrow was obtained from 60 children ranging in age from 3 days to 15 yr who needed bone marrow examination for medical reasons and in whom no marrow pathology was found. Control patients were being investigated for metastatic tumor, hepatosplenomegaly, storage diseases, or idiopathic thrombocytopenic purpura (ITP). All had normal white blood counts and differentials. Marrow was not used from children with infection or any hematologic disease other than ITP. This procedure was approved by the Human Experimentation Committee of The Hospital for Sick Children.

### *CFU-C Assays*

Two to five milliliters of bone marrow were aspirated into preservative-free heparin and allowed to sediment. The buffy coat was removed and the cells were washed, resuspended in alpha medium,[14] and counted. Plating was carried out according to a modification of the method of Iscove et al.,[3] with final concentrations of 0.8% methylcellulose and 20% fetal calf serum (FCS) in alpha medium. The cultures were set up in 1-ml volumes in 35 × 10-mm plastic culture dishes (Lux) with cell concentrations of 0.5, 1.0 and 2.0 × $10^5$ nucleated cells/ml. The plates were incubated in 100% humidity and 5% $CO_2$ in air at 37°C for 14 days. All cultures were done in duplicate. In each study, cultures were set up with and without the addition of a standard CSA (conditioned medium).

For assay of CFU-C from peripheral blood, the method of Rubin and Cowan[15] was used.

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Heparinized venous blood was diluted 1:3 with phosphate-buffered saline, and 25-ml aliquots of this suspension layered over 10 ml of Ficoll Hypaque solution (12 parts double deionized 5% Ficoll, 5 parts 34% Hypaque). The gradients were centrifuged at 300 *g* for 20 min at 4°C, and the leukocyte layer at the interface between plasma and Ficoll-Hypaque was aspirated. The cells were washed three times in alpha medium and counted. Plating was similar to that for marrow-cell suspensions, except that nucleated cell concentrations ranged between 2.5 and $10 \times 10^5$/ml.

### *Colony Scoring and Identification*

Colonies were defined as discrete clumps of 20 or more cells. Using an inverted microscope, the total number of colonies in each dish was counted, and the duplicate results were averaged and expressed as colonies per $10^5$ nucleated cells. Representative colonies were picked from the culture, spread on glass slides, and stained with Giemsa to confirm colony identification using light microscopy.

### *Preparation and Assay of CSA*

A standard CSA was produced from the peripheral blood leukocytes[3] of one normal adult donor. Leukocytes obtained from heparinized blood were immobilized in 10 ml of 0.5% agar at a concentration of $10^6$ cells/ml in 100-mm culture dishes and overlaid with 15 ml of 20% fetal calf serum in alpha medium. The dishes were incubated for 7 days in 100% humidity and 5% $CO_2$ at 37°C. The cell-free supernatant was harvested, washed free of agar, and stored in aliquots at −20°C. This medium "conditioned" with CSA was used in the CFU-C assay. A new batch of conditioned medium was used only if it demonstrated activity which varied less than 10% from previous batches when tested with control marrow in the CFU-C assay system.

To study normal variations in CSA activity, 25 studies were done using CSA produced from peripheral blood leukocytes of normal adult volunteers, and the activity on control marrows was compared to that of the standard CSA. The number of colonies produced with standard CSA was defined as 100% activity, and an individual result was expressed as a percentage of the standard activity on the same marrow. The patients' ability to produce CSA was assayed in a similar fashion using autologous marrow and at least five control marrows. The results were averaged. Twenty-four hour urinary CSA excretion was measured on patients 1 and 2 by Dr. E. R. Stanley of the Ontario Cancer Institutes as previously described.[16]

### *Inhibitor Assays*

To test the effect of human serum on the CFU-C assay system, pooled normal AB serum was substituted in part for FCS in 26 studies. The concentration of human serum in the cultures ranged from 1.7% to 20%, but the total serum concentration was always made up to 20% with FCS. To detect inhibitors against CFU-C or CSA, patients' sera were used in the same manner with autologous marrow and at least six control marrows, both with and without added CSA. The number of colonies produced with standard FCS was defined as 100%, and the result with human serum was expressed as a percentage of the standard. The results were averaged.

## RESULTS

### *CFU-C Assays*

Results of CFU-C assays in control marrows with added CSA are shown in Fig. 1. When colony counts were plotted according to age, it became evident that greater numbers were obtained in children under age 3 yr. Therefore, the data were separated into two groups by age. For 23 subjects less than 3 yr, the mean value was 68 colonies/$10^5$ nucleated marrow cells, and for 37 subjects 3 yr and over, the mean value was 36 colonies/$10^5$ nucleated marrow cells. Although the ranges were wide, the difference between the two groups was highly significant: $p < 0.001$, Wilcoxon rank sum.[17] Colonies reached a maximum size of 2000–3000 cells. All stages of granulocyte maturation to the mature poly-

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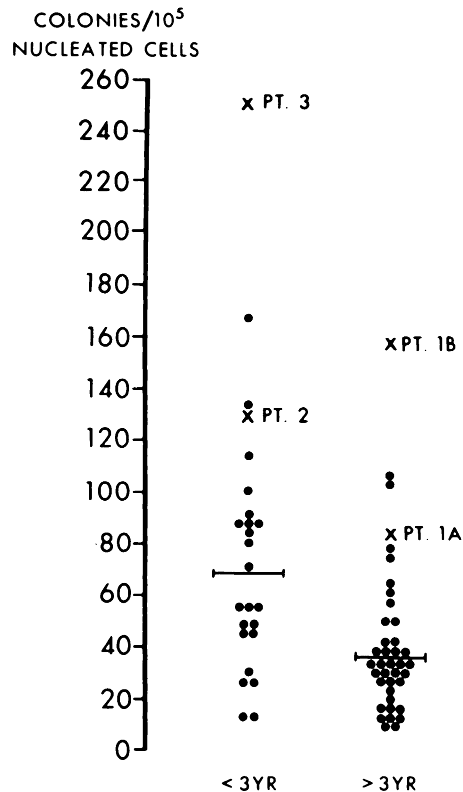


**Fig. 1. Marrow CFU-C from control children and three patients with neutropenia. Patient 1 was studied twice. Cultures contain standard CSA.**

morph appeared to be present. The predominant cells were myelocytes and metamyelocytes.

In cultures without added CSA, granulocyte colonies were decreased in number and size. The mean control value for patients under 3 yr was $43/10^5$ nucleated marrow cells (range 7–133), and, for patients 3 yr and over, $22/10^5$ cells (range 1–76). Colonies reached a maximum size of 500–1000 cells. All stages of granulocyte maturation appeared to be present.

Results of CFU-C assays with added CSA on the neutropenic patients are also shown in Fig. 1. It is evident that all three patients had high concentrations of CFU-C for their age groups. The colonies were similar in size and morphology to controls. Although mature polymorphs and bands were absent from the plated marrows, these cells appeared to be present in picked colonies. Similar results were obtained from cultures without added CSA.

Peripheral blood CFU-C determined on six normal adults ranged from 3.6 to 19.2 (mean 8.7) colonies per $10^5$ nucleated blood cells. Values for patients 1 and 2 were 9.6 and 2.7, respectively.

*CSA Assays*

Results of CSA assays are shown in Table 2. The activity of CSA from normal individuals ranged widely in comparison to the standard CSA. CSA from the patients was just as effective in stimulating colony growth as normal CSA.

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**Table 2. Colony-stimulating Activity From Peripheral Blood Leukocytes of Neutropenic Patients Expressed as Percentage of Activity of Standard CSA**

| CSA Source | Activity (%) | |
|---|---|---|
| | Mean | Range |
| Standard | 100 | |
| Normals | 94 | 56–178 |
| Patient 1a | 88 | 51–106 |
| Patient 1b | 107 | 94–128 |
| Patient 2 | 106 | 39–320 |

Urinary CSA excretions were slightly increased in the two patients tested: 226,000 and 240,000 units/24 hr (urinary protein concentration of 0.32 and 0.39 mg/ml, respectively; normal, 10,000–220,000 units/24 hr).

*Inhibitor Assays*

A mixture of equal parts pooled human serum (NHS) and FCS gave the most reliable results, and the results of inhibitor studies using this mixture are shown in Table 3. The patients' serum did not inhibit colony formation; in fact as seen with NHS there was a moderate stimulatory effect on both control marrow and autologous marrow. Colony size and morphology did not change. The results of studies on cultures without added CSA were similar. Sera from the parents of patient 3 were tested against the patient's marrow with no inhibition detected.

## DISCUSSION

There are few published studies of in vitro granulocyte colony growth in severe congenital neutropenia.[18–20] All the reported patients had normal numbers of CFU-C, although studies of controls were limited. The patient of Barak and co-workers produced colonies with normal neutrophil maturation,[18] whereas L'Esperance and co-workers were able to detect very few mature neutrophils in colonies from their patients.[19,20] Our patients had high normal or increased concentrations of marrow CFU-C and normal peripheral blood CFU-C. Because of differences in marrow cell populations between patients and controls, it is impossible to conclude that the total absolute number of CFU-C was increased. The morphologic study of picked colonies is technically difficult and, unfortunately, not quantitative. Frequently, it is impossible to

**Table 3. Effect of Patients' Serum and Normal Human Serum on CFU-C Expressed as a Percentage of Colony Number With FCS**

| Serum Source | Control Marrow (%) | | Neutropenic Marrow (%) | |
|---|---|---|---|---|
| | Mean | Range | Mean | Range |
| 20% FCS | 100 | | 100 | |
| 10% NHS + 10% FCS | 137 | 46–310 | 124 | 89–150 |
| 10% Patient 1 + 10% FCS | 113 | 65–203 | 107 | |
| 10% Patient 2 + 10% FCS | 183 | 100–267 | 166 | |
| 10% Patient 3 + 10% FCS | — | | 103 | |

FCS, fetal calf serum; NHS, normal human AB serum.

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identify accurately individual cells by light microscopy. Electron microscopy may prove to be a better way of examining single colonies.[20] Nevertheless we feel that the colonies from our patients were probably able to develop mature polymorphonuclear neutrophils in vitro. From these data, we conclude that the granulocyte stem cell may not be intrinsically abnormal. Therefore, these patients may not be good candidates for bone marrow transplantation.

CSA production by peripheral blood leukocytes was within normal limits in the two patients tested. Urinary CSA excretion was slightly increased. L'Esperance and co-workers reported similar findings.[20] The assay for CSA using whole human marrow is crude because of endogenous CSA production by marrow cells. Therefore, small changes in CSA production by patients' leukocytes would not be detected. Using separated marrow fractions, Senn and co-workers have been able to demonstrate decreased CSA production in two adults with chronic neutropenia.[21] Granulocyte colonies from our patients' marrows responded normally to added CSA by increasing in number and size. It is unlikely that a deficiency of CSA-producing cells, a defect of CSA production by these cells, or failure to respond to CSA, plays a role in the neutropenia of these patients.

No serum inhibitors to CSA or CFU-C have been found by other investigators[18,19] or by us in patients with congenital neutropenia. There is the remaining possibility of an inhibitor at a local level which is not detectable in serum.

CFU-C have not been extensively studied in children without hematologic disease. Numbers of CFU-C from older children appear similar to adults. It is of interest that control marrows from infants less than 3 yr of age have about double the concentration of CFU-C, with or without added CSA, as older children and adults. It is well known that marrows of infants normally contain an increased proportion of immature mononuclear cells. Some of these cells may be the colony-forming cells. Cord blood has been reported to contain markedly increased numbers of CFU-C.[22]

The pathogenesis of severe congenital neutropenia remains unclear. We believe the defect could lie in the microenvironment of the bone marrow. There is evidence for the existence of a hemopoietic-inductive microenvironment in vivo in mice.[10] This mechanism is postulated to involve interaction of stromal cells with hemopoietic stem cells, resulting in the commitment of the latter to various lines of differentiation. While it is possible that this microenvironment is defective in neutropenia, it is difficult to explain the successful differentiation that occurs solely in the presence of culture medium and a semisolid base, unless the medium provides a nutrient not present in the patients, or a class of cells that is inhibitory in vivo is selectively diminished or eliminated in the course of the assay. Related to this latter possibility is the speculation that normal or abnormal granulocyte precursors could be destroyed in the marrow by phagocytic cells before full differentiation has occurred (ineffective granulopoiesis). However, examination of bone marrow slides does not reveal phagocytosis.

It is also possible that the lesion resides in an as yet unknown granulopoietic function, not assessed by CFU-C or CSA assays. Although the demonstration

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of such a lesion must await further knowledge about granulopoiesis, CFU-C and CSA assays should continue to be used in studies of neutropenias, since defects in these functions could be present in some cases.

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## REFERENCES

1. Senn JS, McCulloch EA, Till JE: Comparison of colony-forming ability of normal and leukemic human marrow in cell culture. Lancet 2:597-598, 1967

2. Robinson WA, Pike BL: Colony growth of human bone marrow cells in vitro, in Stohlman F Jr (ed): Hemopoietic Cellular Proliferation. New York, Grune & Stratton, 1970, pp 249-259

3. Iscove NN, Senn JS, Till JE, McCulloch EA: Colony formation by normal and leukemic human marrow cells in culture: Effect of conditioned medium from human leukocytes. Blood 37:1-5, 1971

4. Chervenick PA, Boggs DR: Bone marrow colonies: Stimulation in vitro by supernatant from incubated human blood cells. Science 169:691-692, 1970

5. Pike BL, Robinson WA: Human bone marrow colony growth in agar-gel. J Cell Physiol 76:77-84, 1970

6. Stanley ER, Metcalf D: Purification and properties of human urinary colony stimulating factor (CSF), in Harris R, Allin P, Viza D (eds): Cell Differentiation. Copenhagen, Munksgaard, 1972, pp 272-276

7. Kauder E, Mauer AM: Neutropenias of childhood. J Pediatr 69:147-157, 1966

8. Bjure J, Nilsson LR, Plum CM: Familial neutropenia possibly caused by deficiency of a plasma factor. Acta Paediatr 51:497-508, 1962

9. Krantz SB, Moore WH, Zaentz SD: Studies on red cell aplasia. V. Presence of erythroblast cytotoxicity in $\gamma$G-globulin fraction of plasma. J Clin Invest 52:324-336, 1973

10. Trentin JJ: Determination of bone marrow stem cell differentiation by stromal hemopoietic inductive microenvironments (HIM). Am J Pathol 65:621-628, 1971

11. Kostmann R: Infantile genetic agranulocytosis. Acta Paediatr 45 (suppl 105): 1-78, 1956

12. Rebuck JW, Crowley JH: A method of studying leukocytic functions in vivo. Ann NY Acad Sci 59:757-805, 1955

13. Payne R: Leukocyte agglutinins in human sera; correlation between blood transfusions and their development. Arch Intern Med 99:587-606, 1957

14. Stanners CP, Eliceiri GL, Green H: Two types of ribosome in mouse-hamster hybrid cells. Nature (New Biol) 230:52-54, 1971

15. Rubin SH, Cowan DH: Assay of granulocytic progenitor cells in human peripheral blood. Exp Hematol 1:127-131, 1973

16. Stanley ER, Metcalf D, Maritz JS, Yeo GF: Standardized bioassay for bone marrow colony stimulating factor in human urine: Levels in normal man. J Lab Clin Med 79:657-668, 1972

17. Wilcoxon F: Individual comparisons by ranking methods. Biometrics Bull 1:80-83, 1945

18. Barak Y, Paran M, Levin S, Sachs L: In vitro induction of myeloid proliferation and maturation in infantile genetic agranulocytosis. Blood 38:74-80, 1971

19. L'Esperance P, Brunning R, Good RA: Congenital neutropenia: In vitro growth of colonies mimicking the disease. Proc Natl Acad Sci USA 70:669-672, 1973

20. L'Esperance P, Zucker-Franklin D, Hansen J, Brunning R, Good RA: Abnormal in vitro differentiation of four patients with congenital neutropenia (CN). Blood 44:953, 1974

21. Senn JS, Messner HA, Stanley ER: Analysis of interacting cell populations in cultures of marrow from patients with neutropenia. Blood 44:33-39, 1974

22. Knudtzon S: In vitro growth of granulocytic colonies from circulating cells in human cord blood. Blood 43:357-361, 1974

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