Congenital Neutropenia: Neutrophil Proliferation With Abnormal Maturation

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A child with congenital neutropenia was studied using bone marrow culture and ultrastructural and cytochemical techniques. The patient's marrow cells formed a large number of granulocytic colonies of normal size in culture, and her peripheral blood leukocytes produced adequate colony-stimulating factor. No serum inhibitors were identified. The patient's promyelocytes from direct marrow and culture appeared normal in ultrastructure, and primary granules, contained peroxi-
dase and acid phosphatase activity. Myelocytes and rare segmented neutrophils from direct marrow specimens demonstrated atypical notched nuclei, myelin figures in Golgi lamellae and primary (azurophilic) granules, and no identifiable secondary (specific) granules. These data indicate an intrinsic neutrophil defect which allows normal proliferation of precursors, but results in abnormal granulogenesis and apparent inability to form secondary granules.

IN RECENT YEARS, a number of children have been reported with severe persistent neutropenia which has been described with eponyms such as infantile genetic agranulocytosis,1-4 Kostmann's disease,5 infantile agranulocytosis,6 congenital agranulocytosis,7,8 congenital neutropenia,9-14 and infantile congenital aneutrocytosis.15 Although all of these children present early in life with severe, chronic, and eventually fatal infections, surveys of the reported cases show certain differences that may indicate more than one basic abnormality. The patients initially described by Kostmann1 and later by others5,11,12 were found to have a probable autosomal recessive genetic deficiency, whereas in most other cases no such familial pattern was demonstrable. Chromosome anomalies have also been described in a patient with the disease.5 In many patients, severe neutropenia had apparently been present in the first few months after birth, whereas in others neutrophils had been present, on occasion, in moderate numbers (> 1000/cu mm) during the first several months to 1 yr of life.1,3,7,11 The majority of patients had a normal or increased population of bone marrow promyelocytes and generally a decrease in or absence of myelocytes.1,4,8,11,13 Others, however, had normal or increased numbers of myelocytes.1-3,11,14 In addition, some patients with this disease had eosinophilia, monocytosis, thrombocytosis, hypergammaglobulinemia, and increased numbers of eosinophil precursors and plasma cells in bone marrow specimens.1,11,12

The recent use of bone marrow culture techniques has shown in some cases neutrophil colonies which demonstrate abnormal proliferation.7,9,10,14 However,
PARMLEY El AL.267 others have had normal development and maturation of bone marrow colonies. In order to define further the classification and pathogenesis of this disorder, we studied the bone marrow of a patient with congenital neutropenia using cell culture, cytochemical and ultrastructural techniques.

CASE REPORT

The patient is a 5 1/2-yr-old white girl with a history of numerous bouts of ear and throat infections, diarrhea, and oral lesions during the first year of life. She was first hospitalized at another medical center at 14 mo of age with mental obtundation and necrotic lesions of the nostril, fingers, and perineal area. Pseudomonas and Klebsiella were cultured from blood, urine, mouth, throat, rectum, and tracheal aspirate, and pathogenic Escherichia coli, group C, was cultured from stool specimens. The initial hemogram showed a hematocrit of 27%, a total leukocyte count of 10,800/cu mm with 31% neutrophils, 26% band forms, 30% lymphocytes, 12% monocytes, and 65,000 platelets per cu mm. Examination of the cerebrospinal fluid was negative except for the presence of eight lymphocytes per cu mm. She suffered a cardiac and respiratory arrest on hospital day 4 but was successfully resuscitated. Her neutrophil count remained elevated until hospital day 20, when it dropped to 1% of a total leukocyte count of 6000/cu mm. Six days later, her neutrophil count rose to 19% and remained in the range of 2%-10% of 6000-7000/cu mm total leukocytes for the next 30 days. The patient’s neutrophil counts continued to drop intermittently in a cyclic pattern over the next 90 days. Cultures of blood and necrotic lesions remained positive for Pseudomonas for approximately the first 30 days of hospitalization. During the course of hospitalization, the patient was treated with parenteral gentamicin, methicillin, hydrocortisone, Pseudomonas hyperimmune serums, oral nafcillin and cephalaxin, as well as digitalis and furosemide.

The child was then followed by her family physician for the next 15 mo and maintained continuously on cephalaxin and erythromycin. At 3 yr of age, she presented to the Medical University of South Carolina with pneumonia and lung abscess which responded to parenteral methicillin and gentamicin. At this time, the patient, who had been taking oral iron supplements, had a hemoglobin of 9.2 g/100 ml, total leukocyte count of 8300/cu mm with 4% neutrophils, 53% lymphocytes, 41% monocytes, and 1% eosinophils. The reticulocyte count was 3.6%, and the platelet count was 200,000/cu mm. Since this time, the patient has had approximately 12 hospital admissions for treatment of pneumonia which has responded to treatment with methicillin and/or gentamicin. During the admissions, the bacterial flora of the upper airway have consistently contained Pseudomonas aeruginosa, Proteus species, Klebsiella, and occasional Streptococcus pneumoniae. The patient has also had persistent mastoiditis, otitis, and stomatitis. At age 5, the patient was in the 60th percentile for height and 50th percentile for weight. She has reached mental milestones expected for her age. Pertinent physical findings included a perforated left tympanic membrane, severe dental caries, fine bilateral rales, nasal and perineal fistulas, and clubbing of the fingers. There was no hepatosplenomegaly, significant lymphadenopathy, or bone deformities. There was no family history of neutropenia or persistent infections, and peripheral blood counts of the mother, father, and three siblings were normal.

Additional Laboratory and Light-microscopy Studies

The patient has had over 50 hemograms performed in the past 3 yr which consistently revealed an absence of peripheral blood neutrophils and very occasionally 1% neutrophils in total leukocyte counts from 5000 to 13,000/cu mm. Peripheral blood smears also demonstrated persistent monocytosis and occasional eosinophilia (up to 8%). Repeated bone marrow aspirations since age 3 have shown normal numbers of immature blasts (1.9%-4.1%) and promyelocytes (2.3%-8.2%), decreased myelocytes (0.4%-1.6%), a marked decrease in polymorphonuclear cells (0%-0.3%), and an increase in eosinophil precursors (8%-13%), plasma cells (6%-7%), and monocytes (7.2%-15.5%). Alkaline phosphatase activity appeared markedly decreased in bone marrow preparations. At age 5, a Rebuck skin window placed on the arm using first strength PPD showed marked decrease in neutrophil migration throughout the 24-hr period. Examination of ear exudate revealed only monocytes with occasional fragmented nuclei confirmed by electron microscopy. Nitroblue tetrazolium reaction on peripheral smears showed a marked increase in dye uptake
and reduction by the monocytes. Quantitative immunodiffusion showed IgG, 3000 mg/100 ml; IgA, 560 mg/100 ml; and IgM, 220 mg/100 ml. The patient's serum was strongly positive for cold agglutinins. Complement fixation test for mycoplasma was negative. Serum C3 level was normal. The patient's karyotype was that of a normal female. The mitogenic response of the patient's lymphocytes was normal when stimulated with phytohemagglutinin, concanavalin A, and pokeweed mitogen. No evidence of opsonic antineutrophil antibodies could be demonstrated (courtesy of Dr. L. A. Boxer, The Children's Hospital Medical Center, Boston, Mass.). These studies were performed following appropriate informed consent.

MATERIALS AND METHODS

Bone Marrow Culture

The proliferative capacity of the marrow granulocytic precursor cells was assessed using a modification of the clonal cell culture technique described by Iscove et al. Modifications included substitution of CMRL 1066 with α medium (Flow Laboratory, Rockville, Md.) and omission of bovine serum albumin. A healthy volunteer and two patients with anemia and normal granulopoiesis were the sources for control marrow specimens. Marrow aspirates from the patient and control subjects were received in 6-ml tissue culture tubes containing heparin without preservatives (Connaught Medical Research Laboratory, Willowdale, Ontario, Canada). For studies of the proliferative capacity of the marrow cells, nucleated cells from the marrow buffy coat after washing were directly plated into 35-mm Lux standard non-tissue culture dishes (Flow Laboratory) containing α medium, 0.8% methylcellulose, 20% fetal bovine serum (Flow Laboratory), and conditioned medium (CM) derived from peripheral leukocytes.

Conditioned medium was prepared from cultures of the patient's and the normal peripheral leukocytes using the technique described by Iscove et al. The patient's peripheral leukocytes and those from normal individuals prepared with Aminco cell centrifuge were washed twice with α medium, and nucleated cells in concentrations of 10⁶ cells per ml were immobilized in 0.45% agar in the presence of α medium and 20% fetal bovine serum. Alpha medium containing 20% fetal bovine serum was layered on top of the agar, and the culture was continued for 1 wk. At the conclusion of the incubation, the liquid top layer was harvested, stored at 4°C, and used as CM.

Colony-stimulating activity (CSA) of the patient's CM was tested against control marrow cells and compared to that of normal CM. To eliminate endogenous sources of CSA, test marrow cells were made free of adherent cells using the technique described by Messner et al. Marrow cells devoid of adherent cells after such manipulation are incapable of forming colonies in culture unless stimulated with active CM.

Ultrastructure and Cytochemistry

The patient's bone marrow was obtained on two occasions by needle aspiration from the anterior iliac crest. The cells were fixed immediately at 4°C in 3% glutaraldehyde buffered with 0.1 M, pH 7.4, cacodylate. Three per cent cacodylate-buffered glutaraldehyde was added to an equal amount of culture medium containing bone marrow colonies (following 14 and 20 days incubation) from the patient and normal controls. The cells were fixed in suspension for 1 and 18 hr at 4°C, centrifuged into a pellet at 1000 rpm for 3 min, and then rinsed in 7.5% sucrose-buffered cacodylate.

To demonstrate peroxidase activity, cell pellets from direct and cultured bone marrow were incubated for 30 min in diaminobenzidine (DAB) substrate medium. Also, an aliquot of cells was incubated overnight at 4°C in DAB medium lacking H₂O₂, followed by a 30-min reaction in complete substrate medium. Cryostat sections of bone marrow and bone marrow culture specimens fixed 1 hr in glutaraldehyde were incubated at 37°C for 1 hr in a modified Gomori medium to demonstrate acid phosphatase using glycerol phosphate as the substrate.

All specimens were then rinsed in the buffered sucrose and postfixed in 2% osmium tetroxide. This process was followed by routine dehydration and embedding of Epon. Thin sections of the morphologic preparations were stained with a uranyl acetate-lead citrate sequence. Thin sections of specimens processed for cytochemistry were not stained or were stained with lead citrate only. The sections were examined in Hitachi HS-8 and AEI-EM 6B electron microscopes at an accelerating voltage of 50 KV.
Table 1. Colony Formation by Patient’s and Control Marrow Cells (per 10^5 Cells)

<table>
<thead>
<tr>
<th></th>
<th>Normal CM</th>
<th>Patient’s CM</th>
<th>Normal Serum*</th>
<th>Patient’s Serum*</th>
<th>Without CM</th>
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<tbody>
<tr>
<td>Marrow Buffy Coat Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Patient</td>
<td>43 ± 8</td>
<td>44 ± 6</td>
<td>37 ± 10</td>
<td>45 ± 10</td>
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<tr>
<td>Control 1</td>
<td>20 ± 2</td>
<td>20 ± 5</td>
<td>18 ± 2</td>
<td>19 ± 2</td>
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<tr>
<td>Marrow cells devoid of adherent cells</td>
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<tr>
<td>Control 2</td>
<td>58 ± 3</td>
<td>60 ± 8</td>
<td>—</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Control 3</td>
<td>44 ± 8</td>
<td>40 ± 5</td>
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*Sera in the final concentration of 10% were added to the standard culture condition containing normal CM.

RESULTS

Cell Culture Studies

The proliferative ability of the patient’s marrow cells was assessed in culture in the presence of both normal conditioned medium (CM) and the CM made from the patient’s peripheral leukocytes (see Materials and Methods). The patient’s marrow cells produced approximately 43 neutrophilic and eosinophilic colonies per 10^5 cells in both CM, as depicted in Table 1. The large neutrophilic colonies were estimated to contain between 1000 and 2000 cells. A representative neutrophilic colony is presented in Fig. 1. Both the number and size of the colonies were considered appropriate according to the standard in our tissue culture laboratory and that reported elsewhere. These results clearly demonstrated that the proliferative ability of the patient’s granulocytic precursors was normal.

Fig. 1. This is a typical neutrophilic colony of the patient grown in the presence of normal conditioned medium.
The effect of the patient’s serum on the proliferative capacity of the patient’s and control marrow cells was tested by addition of 10% of the patient’s serum to the above described cell culture conditions. A control using normal serum was also performed. Neither the patient’s nor the control serum inhibited colony formation, as described in Table 1.

Next, the ability of the patient’s CM to sustain colony formation by marrow granulocytic precursor cells in culture was examined. Messner et al. reported that marrow cells deprived of adherent cells were incapable of colony formation unless stimulated with active CM. Two control marrows devoid of an adherent cell population were cultured in concentrations of 10⁵ cells per ml, with or without the presence of CM, and the results are presented in the lower half of Table 1. Both marrows were stimulated in culture by patient’s and normal CM, while no colony formation was observed in their absence.

Ultrastructure and Cytochemistry of Neutrophils in Direct Marrow

Many myeloblasts and promyelocytes were identified in normal numbers and generally displayed morphologic features previously described for these cells in normal specimens. However, many promyelocytes contained up to six myelin figures at a given sectional level.

Myelocytes were less prevalent than normal and usually revealed an indented nucleus with marginated heterochromatin. Occasional neutrophils containing atypical multiple small nuclear lobes or extremely notched nuclei were observed. Myelocytes (Fig. 3) and multinucleated cells (Fig. 4) exhibited characteristic neutrophil primary granules which ranged from 0.2 to 0.4 μm in diameter and contained dense material occasionally enclosing a lucent crystalloid (Fig. 4). Secondary granules could not be identified in myelocytes or segmented neutrophils, which thus differed strikingly from those in normal myelocytes. The endoplasmic reticulum appeared sparse and collapsed. The cytoplasmic sap seemed much denser than that in myeloblasts, promyelocytes, or monocytes. Numerous myelin figures were present in the primary granules and Golgi apparatus of these cells (Figs. 3 and 4).

Acid phosphatase activity was demonstrated in primary granules and the Golgi apparatus of myeloid cells as has been shown in those from normal bone marrow specimens. Often all cytoplasmic granules of myelocytes and late neutrophils contained diaminobenzidine reaction product (Fig. 5).

Ultrastructure and Cytochemistry of Neutrophils in Culture

Myeloblasts, promyelocytes (Fig. 6), and cells containing segmented nuclei were identified in bone marrow culture specimens of the patient. The ultrastructure and cytochemistry of these cells were essentially the same as cultured marrow cells from three normal donors as reported previously from our laboratory. Numerous acid phosphatase- and diaminobenzidine-reactive primary granules in various stages of maturation were identified in the cytoplasm, but secondary granules were not evident in cultured neutrophils from the patient and normal controls.
See legends on facing page.

Figures 2, 3, 4, and 6 were taken from thin sections counterstained with uranyl acetate and lead citrate and that from Fig. 5 was obtained from a thin section stained with lead citrate only.
Fig. 4. This neutrophil from the patient's marrow aspirate contains an atypically notched nucleus with condensed chromatin. The dense cytoplasm encloses collapsed endoplasmic reticulum, some glycogen particles, and a number of primary granules. Some primary granules display myelin figures, and one granule enlarged in the lower inset contains the lucent crystallloid characteristic of the neutrophil primary granules. Numerous myelin figures are also seen in Golgi lamellae (arrows, upper inset). No secondary granules are apparent. × 19,000; upper inset × 57,000; lower inset × 60,000.

Fig. 2. This promyelocyte found in a direct marrow aspirate of the patient contains an eccentric nucleus with dispersed chromatin, abundant dilated endoplasmic reticulum, and numerous primary granules at various stages of maturation. Some primary granules appear extracted peripherally and one granule, enlarged in the inset, contains a typical lucent crystallloid. × 11,200; inset × 35,000.

Fig. 3. This myelocyte from the patient's marrow aspirate contains a nucleus with marginated chromatin and displays collapsed endoplasmic reticulum and numerous primary granules. No structures resembling secondary granules are present. Myelin figures are seen in Golgi lamellae (arrow) and in primary granules (inset). × 11,200; inset × 35,000.
Other Cell Types

A large number of promonocytes and monocytes were observed in the patient’s direct marrow specimens and appeared normal ultrastructurally and cytochemically. They contained fewer cytoplasmic granules than myeloid cells, and their granules differed from primary granules in that they were smaller, more pleomorphic, and lacked peripheral extraction or central lucent crystalloids.

Eosinophils and basophils and their precursors appeared normal ultrastructurally and cytochemically in the patient’s bone marrow. Late eosinophil leukocytes characteristically displayed crystalloid granules and a few of the small granules. Many normal-appearing plasma cells and histiocytes and occasional megakaryocytes were also identified.

Eosinophils, macrophages, and mononuclear cells were also demonstrated in the patient’s bone marrow culture. These cells generally displayed the ultrastructural and cytochemical characteristics previously described for macrophages and eosinophils in tissue culture.
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Fig. 6. The patient's cultured promyelocyte demonstrates many of the morphologic characteristics observed in promyelocytes of the direct marrow specimens. The numerous primary granules in the cytoplasm (enlarged in the upper inset) are similar to those in Fig. 3. Myelin figures are seen, and many of the granules appear extracted peripherally. Some granules appear to be lying free in large vacuoles (lower inset). x9800; upper inset x34,000; lower inset x22,400.

DISCUSSION

The child described here exhibited clinical and laboratory findings consistent with previously reported cases of congenital neutropenia. The patient has been susceptible to serious and nearly fatal infections as a result of a profound and persistent neutropenia. Bone marrow specimens contained promyelocytes in normal numbers, decreased myelocytes, and essentially no band and polymorphonuclear neutrophils. Intermittently for a 3-mo period, the patient demonstrated a moderate neutrophil response similar to that occasionally observed in other patients with congenital neutropenia. However, the failure of this response to affect her overwhelming sepsis suggested its ineffective quality. In addition, the patient had a monocytosis, occasional eosinophilia, and hypergammaglobulinemia described in previous cases of congenital neutropenia. The severity of this patient's infections, the profound and persistent nature of her neutropenia, and the bone marrow maturation arrest at the promyelocytomyelocyte stage differentiated this patient from those described as having chronic granulocytopenia of childhood. The possibility of a drug-induced neutropenia could not be absolutely ruled out, since the patient was treated...
with antibiotics previously reported to cause neutropenia, such as methicillin and cephalothin. However, the onset of severe infections prior to treatment with antibiotics, the absence of leukoagglutinins or inhibitors, and most importantly, the prolonged nature of the neutropenia and failure to respond upon withdrawal of the medication make this diagnosis unlikely.

The proliferative capacity of our patient's marrow cells was seen to be normal in culture. Previous studies are not in agreement concerning growth in vitro of neutrophils from these patients. Barak et al. reported that colony formation in agar was normal when the patient's marrow was stimulated by human spleen-conditioned medium. L'Esperance et al. and others, however, reported defective colony formation using the agar culture technique of Pike and Robinson. More recently, however, L'Esperance et al. and Amato et al. have reported normal colony formation with the soft agar and methylcellulose media, respectively. This discrepancy could be explained on the basis of different subclasses of congenital neutropenia, one of which is associated with the defective proliferation of the granulocytic precursor cells. Alternatively, the colony assay system using agar as support may not be appropriate for the study of granulocytic proliferation. Shohan et al. reported that marrow cells cultured in this assay system contained only eosinophils and monocyte-macrophages. Zucker-Franklin, however, has reported the presence of neutrophils in cultures of peripheral blood grown in soft agar. Clonal cell culture assay using methylcellulose as supporter as described in this report assesses proliferation of neutrophils and eosinophils.

Conditioned medium derived from peripheral leukocytes was necessary for colony formation by normal marrow cells. The present results demonstrated the capacity of the patient's adherent cells to produce a colony-stimulating substance. However, adherent cells from some patients with acquired neutropenia have been shown to produce abnormal levels of CSA. Since the patient had normal levels of CSA and her serum did not contain an inhibitor to granulocytic precursors in culture or leukoagglutinins, the defective maturation appeared to be an intrinsic property of the patient's granulocytic precursors.

The apparent lack of secondary granules in myelocytes and in the occasionally observed segmented neutrophils confirms the presence of a specific lesion in the patient's neutrophils. An inability to synthesize secondary granules probably results from a spontaneous or inherited genetic defect in the cell. The myelin figures seen in primary granules and Golgi complex of mature cells suggest an additional abnormality in primary granulogenesis. Despite the lack of complete cytoplasmic maturation in these cells, the nucleus is capable of becoming segmented. However, the apparent extreme notching of presegmented nuclei and atypical small segmented lobes attest to an abnormality in the segmentation process. Abnormal neutrophil granulopoiesis has been demonstrated ultrastructurally in grey collie dogs with cyclic neutropenia and has been shown to result from a stem cell defect. However, the defect in these cells appears mainly confined to primary granules.

The ultrastructure and cytochemical studies of the patient's cultured marrow cells should be interpreted with caution. Although defective maturation of the neutrophil series was seen in these cells, the maturation of granulocytes from
three control subjects also appeared defective in culture. The morphologic similarity between the neutrophils in the patient’s bone marrow in vivo and neutrophils from normal marrow grown in culture suggests that the defect in the patient’s granulocytes possibly results from nutritional or other factors subject to experimental control.

In conclusion, the present studies have shown normal proliferative activity of the granulocytic precursors in the bone marrow of a patient with congenital neutropenia. In addition, normal colony-stimulating factor and absence of a serum inhibitor to granulocytic precursors have also been demonstrated. These observations, coupled with the ultrastructural and cytochemical demonstration of abnormal cytoplasmic maturation in the patient’s neutrophils, strongly suggest that the basic defect is intrinsic to the granulocytic precursors.

ACKNOWLEDGMENT

We are grateful to Miss Joanne Wright and Mrs. Sara S. Keirn for their technical assistance and to Mrs. Linda S. Todd for her assistance in the preparation of this manuscript.

REFERENCES

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