Congenital Dysgranulopoietic Neutropenia: Clinical, Serologic, Ultrastructural, and In Vitro Proliferative Characteristics

By Richard T. Parmley, William M. Crist, Abdelsalam H. Ragab, Laurence A. Boxer, Ahmad Malluh, Victor K. Lui, and Charles P. Darby

Six children with severe congenital neutropenia and repeated life-threatening infections were investigated by examining clinical features and myeloid cell ultrastructure, cytochemistry, and in vitro proliferation. Despite the presence of neutropenia, normal numbers of colony-forming cells (CFC) were present in blood and marrow specimens, and colony-stimulating activities (CSA) from blood cells and serum were normal or slightly increased in all patients. In vitro maturation of the progenitors to neutrophils was also uniformly present in the colonies. No patients had demonstrable antineutrophil antibodies or serum inhibitors of myelopoiesis. Serum lysozyme levels were normal. Ultrastructural and cytochemical studies of directly sampled marrow cells revealed several abnormalities in most neutrophilic myeloid cells from each of the patients consistent with an intrinsic myeloid precursor cell defect. These included (1) the defective synthesis or degeneration of primary granules, (2) an absence or marked decrease of secondary granules in the few late neutrophils observed in the bone marrow, and (3) the presence of autophagy. Phagocytosis of intact myeloid cells with subsequent degeneration was not observed; however, neutrophil debris was evident in phagocytic vacuoles of marrow macrophages. Our demonstration of ultrastructurally dysmorphic neutrophilic granulocytes, intramedullary cell lysis, normal stem cell numbers, and negative serology is comparable to similar observations of erythroid cells from patients with congenital dyserythropoietic anemia. We therefore hypothesize that the dysgranulopoiesis in these children results in neutropenia and propose the descriptive name congenital dysgranulopoietic neutropenia.

CHILDREN with congenital neutropenia comprise a heterogeneous group of patients for which present classification methods are inadequate. Some patients have a relatively benign clinical course characterized by occasional skin infections, whereas others have repeated bouts of severe life-threatening infections. The diseases occur as an acquired phenomenon or as a genetic disturbance that can be sporadic, autosomal recessive, autosomal dominant, or sex-linked.

Evaluation of laboratory data from these patients is often difficult. Marrow findings have varied widely between samples from different patients and samples obtained on different occasions from the same patient. Marrow examination may demonstrate an apparent “maturation arrest” at the promyelocyte, myelocyte, or later stages of neutrophil development, as well as variable increases in monocytes, eosinophils, and plasma cells. Culture of blood and marrow cells from these patients has been performed in different laboratories using varied techniques with substantially heterogeneous findings, making interpretation unclear. Some patients have been described with abnormal maturation of bone marrow colonies, which “mimic” the disease, and other patients have had “paradoxical” normal neutrophil colonies or defective colony-stimulating activity (CSA). Occasionally, laboratory studies have identified extrinsic factors causing neutropenia, such as antineutrophil antibodies and/or splenic sequestration, or have provided evidence for intrinsic neutrophil defects, such as abnormal chromosomes, defective mobility, or abnormal ultrastructure.

The recent availability of therapies, such as immnosuppression, leukocyte transfusions, and marrow transplantation, with their inherent risks, mandates further studies of patients with severe congenital neutropenia so that laboratory classification and natural history information can be correlated and provide a basis for recommendations concerning management. The present study has utilized ultrastructural, cytochemical, serologic, and granulocyte culture techniques to characterize and subclassify six severely symptomatic patients from a larger group of similarly studied children with congenital neutropenia. A preliminary report of this study has been previously published.

MATERIALS AND METHODS

Patient Data and Routine Laboratory Studies

Fourteen patients with congenital neutropenia were studied similarly in our laboratory, 6 of these were severely symptomatic and form the basis of this report. Patient 1 was a white male diagnosed...
at 4 mo of age with pneumonia; patient 2, a black male diagnosed at 1 day of age with omphalitis and sepsis; patient 3, a white male diagnosed at 1 mo of age with fever of unknown origin; patient 4, a white female diagnosed at 8 mo of age with cellularis; patient 5, a white male diagnosed at the age of 11 mo with cellularis; and patient 6, a white female diagnosed at the age of 14 mo with sepsis. Although blood smears obtained from the patients were not available for review prior to diagnosis, they were presumed to be neutropenic since birth. Medical records of 2 patients from other hospitals did report the presence of up to 1000 neutrophils/cumm in blood specimens, however, these slides were not available for review by the investigators. All patients have been followed by the authors for 9 mo–9 yr without resolution of the neutropenia.

All patients have had serious life-threatening bacterial infections. Four of the children (patients 1, 4, 5, and 6) have had pseudomonas cellularis with significant morbidity involving the perirectal area, groin, and face. Four patients have had sepsis with the following organisms: Pseudomonas aeruginosa (patient 6), Klebsiella pneumoniae (patient 6), Staphylococcus aureus (patient 2), Escherichia coli (patient 3), Clostridium clostridiforme (patient 1), and Bacteroides fragilis (patient 1). Patients 2 and 6 have had recurrent lung abscesses containing Pseudomonas aeruginosa that were unresponsive to antibiotic therapy and required surgical intervention in patient 6. Patient 2 died at 10 mo of age with progressive pulmonary infection. Patient 1 died at 4.25 yr of age with gangrenous colitis, peritonitis, and septicemia with multiple organisms. Patients 1, 2, 3, and 4 required multiple leukocyte transfusions (in addition to antibiotics) for treatment of expanding cellularis, pneumonia, and sepsis. Patients 1, 3, and 6 have had persistent perioral infections requiring special dental management as reported previously for patient 6. After 2 yr of age, infection-related-hospital admissions decreased considerably (patients 1, 4, 5, and 6) despite continued severe blood and marrow neutropenia. However, despite this apparent improvement, susceptibility to life-threatening infections still persisted, as evidenced by the sudden septic death of patient 1 at 4.25 yr of age.

Examination of Wright's stained marrow aspirates demonstrated that the percent of late marrow neutrophils was consistently low, with normal to increased numbers of promyelocytes and myeloblasts in all patients. Many of these cells contained a variable number of cytoplasmic vacuoles (Figs. 1 and 2). Except in patient 4, M:E ratios were less than 1:0. The percentage of myeloid cells (200 nucleated cells scored) varied as follows: 0–4% myeloblasts, 1–12% promyelocytes, 0–4% myelocytes, 0–3% metamyelocytes, 0–4% band neutrophils, and 0–1% segmented neutrophils. Marrow samples contained normal to increased eosinophils (4%–18%), monocytes (4%–15%), and plasma cells. Marrow aspirates were sampled on multiple occasions from several patients and demonstrated variability within the range outlined above.

Leukocyte alkaline phosphatase (LAP) failed to stain any neutrophils in marrow aspirates from patients 2, 3, and 6. Occasional cells appeared stained in specimens from patients 4 and 5. Although bone marrow samples from patient 1 failed to demonstrate LAP-positive neutrophils in samples obtained at 24 mo of age, a sample obtained at 37 mo of age demonstrated several LAP-positive neutrophils (although still <2% of nucleated cells).

In all patients, blood neutrophil counts rarely exceeded 300/cumm. Approximately 4 days after hospital admission of patient 1 for treatment of a terminal episode of sepsis, a total leukocyte count of 7000/cumm was observed that contained a large number of vacuolated, hypogranular neutrophils with bizarre nuclear configuration apparent in Wright's stained blood smears. Differential counts revealed 30% segmented neutrophils, 34% band neutrophils, 1% metamyelocytes, 4% myelocytes, 6% progranulocytes, 2% myeloblasts, 10% monocytes, and 11% lymphocytes. Similar counts were observed until the patient's death on hospital day 7, despite cessation of leukocyte transfusions on hospital day 2. The majority of segmented neutrophils demonstrated moderate to strong LAP positivity.

Rebuck skin windows in patients 1 and 6 showed no evidence of neutrophil infiltration from 3 to 24 hr (the other patients were not tested). No blood neutrophils were observed in patient 1 after typhoid or epinephrine injections (the other patients were not tested). Serum lysozyme levels were 8.95 µg/ml, 11.10 µg/ml, and 8.20 µg/ml for patients 3, 4, and 5, respectively, with a normal range of 7.66 ± 2.59 µg/ml. Chromosome abnormalities were not identified in marrow specimens from patients 1, 2, and 6 (the other patients were not tested). Hemograms of the parents and siblings of each of these patients were normal. There was no family history of neutropenia. Although patient 1 had 4 siblings, 2 of which shared major HLA loci, none of these individuals had neutropenia. All blood and marrow specimens for additional tests (outlined below) were obtained after receiving informed consent.

**Serology**

Test serum samples from each patient were stored at -20° C for up to 3 mo prior to testing. Normal blood neutrophils (designated test cells) were obtained as previously described. The cells were incubated with 133 µl of heat-inactivated test serum (30 min at 56° C) per 10⁷ neutrophils at 25° C for 30 min in the presence of 10 mM 2-deoxyglucose to inhibit anaerobic metabolism of the test cells; 15 µl of 0.15 M sodium chloride was then added to the tubes, and the tubes were centrifuged at 400 g and the pellets suspended in 0.2 ml modified Krebs Ringer phosphate (KRP) buffer, pH 7.4, at a final concentration of 9 x 10⁶ cells/ml and added to 0.8 ml of 2 x 10⁵ peripheral blood leukocytes in KRP buffer (indicator cells) containing a final concentration of 1.0 µCi of [¹⁴C]-1-glucose and 1 mM of carrier glucose. The initial rate of glucose oxidation by the indicator phagocytic leukocytes was determined as previously described. The test was considered positive if the glucose oxidation rates exceeded twice those of the control (n = 14).

**Culture of Blood and Marrow Cells**

The in vitro agar culture technique was utilized for the investigation of granulopoiesis in patients 1–5. Specimens from patients 1 and 2 were studied at the University of Alabama in Birmingham and those from patients 3–5 were studied at Emory University in Atlanta, Ga., with similar techniques. Specimens from patient 6 were evaluated with the methyl cellulose culture technique, and the results were reported previously.

Underlayers were prepared from normal blood leukocytes from single donors. McCoy's 5A medium enriched with 15% fetal calf serum was mixed in a 9:1 ratio with boiled 5% agar (purified bacteriologic agar, Difco) and held at 40°C. The blood cells, diluted to a concentration of 10⁶ nucleated cells/ml, were then added to the mixture, and 1-ml aliquots were pipetted into 35 mm Petri dishes. The agar medium was allowed to gel at room temperature.

McCoy's medium (containing 15% fetal calf serum) was mixed in a 9:1 ratio with 3% agar. Then, in patients 1 and 2, either the blood cells at 4 x 10⁵ or 5 x 10⁵ nucleated cells/ml or the bone marrow cells at 10⁵/ml were added, and this mixture was placed over the previously prepared underlayers and allowed to gel at room temperature. The cultures were incubated at 37°C with a 7.5% flow of carbon dioxide for 14 days with 100% humidity. All cultures were performed in quadruplicate. After 2 wk, colonies containing 50 cells or more were counted, using an inverted microscope. Patients 3–5 were studied identically, except Ficoll-Hypaque separation of cells was performed at Emory University prior to culture. This resulted in a concentrating effect of CFC for both patients and controls (Table
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Fig. 1. Directly sampled marrow cells obtained when the patient was asymptomatic. Numerous cytoplasmic vacuoles are evident in this promyelocyte (Patient 1; Wright's stained light micrograph, x 1050.)

Fig. 2. Patient 2 marrow cells obtained when the patient was asymptomatic. Similar cytoplasmic vacuoles are present in this mitotic promyelocyte. Also present in the field is a hypogranular cell with a bilobed nucleus (arrow). (Wright's stained light photomicrograph, x 1050.)

Fig. 3. Marrow cells obtained when patient 5 was asymptomatic. Portions of promyelocytes (P), lymphocytes (L), and monocytes (M) are illustrated in this low-power electron micrograph. The promyelocytes demonstrate variable numbers of cytoplasmic vacuoles (V) and a few lipid droplets (LD). The extreme vacuolation of the middle promyelocyte is suggestive of impending cell death. These changes are not apparent in nearby monocytes and lymphocytes. (Thin section from morphological preparations counterstained with lead citrate and uranyl acetate; x 5800.)

Nevertheless, conclusions regarding CFC number were similar using either technique.

The CSA of peripheral leukocytes from patients 1 and 2 was assayed by substituting them for normal blood cells (at 10⁶ cells/ml) in the underlayers and plating control marrow cells over them (cells of patients with idiopathic thrombocytopenic purpura or solid tumors without marrow involvement). The resultant number of colonies formed was compared to that obtained from the same control marrow cells when normal donor peripheral leukocytes were used in the underlayers. The specimens from patients 3–5 were processed identically at Emory University except adherent cells were removed from the overlayer marrows by placing them sequentially in two glass Petri dishes at 37°C for 1 hr each and then culturing nonadherent cells (at 10⁶ cells/ml) over nucleated peripheral blood cells from patients 3–5 and over normal underlayer peripheral blood cells. Removal of adherent cells from marrow specimens eliminated a possible extraneous source of CSA. Nevertheless, conclusions regarding data were similar using either technique.

Serum CSA from the patients was also investigated. Marrow cells from controls (similar to those mentioned above) were cultured in agar on an underlayer prepared from the peripheral blood of a normal individual. Additionally, 0.2 ml of the patients' sera or normal sera was added to control marrow cells and also cultured in the same manner. All cultures were performed in quadruplicate. Marrow cells from patients 3–5 were cultured identically except that the cells were Ficoll-Hypaque separated before culture. Control marrow cells plated at 2 x 10⁵/ml were used. This resulted in CFC concentration but in no difference in conclusions regarding serum inhibitor status. The colony counts with and without the serum were then compared (Table 2).

Electron Microscopy

Heparinized venous blood and marrow aspirate samples were obtained from each of the patients and processed immediately (by the same investigator) for ultrastructural, morphological, and cytochemical studies. The patients were not receiving antibiotics and lacked evidence of clinical infection at the time of sampling. One blood sample from patient 1 was obtained during an episode of
sepsis during which he demonstrated a significant elevation in neutrophil count (see patient data). The specimens were centrifuged in 1-ml glass tubes at 1200-1500 g for 3 min to obtain a buffy coat, the plasma was removed, and cold 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.35, was gently layered over the buffy coat at 4°C for 10 min. The buffy coats were then removed, finely minced, and fixed for another 50 min in the glutaraldehyde solution at 4°C. The specimens were then rinsed 3 times in 7 g/dl sucrose, 0.1 M cacodylate buffer, pH 7.35.

Aliquots of marrow specimens were processed for the following cytochemical studies: peroxidase was demonstrated according to a modification23 of the method of Graham and Karnovsky24 using diaminobenzidine as a substrate; acid phosphatase was localized according to the method of Barka and Anderson, using β-glycerol phosphate as the substrate; sulfated mucusubstance (glycosaminoglycan) was localized according to Spicer's high iron diamine method.25

After 8 or 14 days of soft agar culture (patients 1 and 2), a 3% glutaraldehyde 0.1 M cacodylate solution was layered over the agar containing the cells, and the Petri dishes were held for 7-10 days at 4°C. The colonies were then removed with a Pasteur pipette and processed for routine morphology. Specimens from patient 6 (methyl cellulose culture) were processed as described previously.1,11,23

The morphological and cytochemical preparations were then post-fixed in 2% osmium tetroxide, dehydrated in alcohol and propylene oxide, and embedded in Spurr low viscosity medium. Thin sections were examined with a Philips 300 electron microscope at an accelerating voltage of 60 kV.

As a comparative control, marrow samples from 8 patients with a relatively benign clinical course and congenital neutropenia (blood neutrophil count was less than 500/cumm in 6 patients and less than 100/cumm in 2 other patients) and 1 child with severe acquired neutropenia (blood neutrophils less than 100/cumm) were processed similarly. Parallel marrow samples from non-neutropenic or chronic benign neutropenic subjects were processed simultaneously with the specimens from 4 of the 6 patients with dysgranulopoietic neutropenia.

RESULTS

Bone Marrow Culture

Normal numbers of CFCs were demonstrated from blood and marrow of all patients and CSA was also noted to be normal (Table 1). The size of the colonies and maturation to granulocytes and/or macrophages within individual colonies were exactly as seen in normal controls. The patients’ sera did not inhibit colony formation from control marrow CFCs plated over normal underlayers (Table 2).

Serology

None of the sera contained opsonic antineutrophil activity that exceeded the control mean by twofold.

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**Table 1. Assay of Colony-Forming Cells in Blood and Marrow**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age of Study (mo)</th>
<th>Method of Assay (A or B)</th>
<th>Blood CFC/5 × 10⁶ Nucleated Cells Plated (Mean ± SEM)</th>
<th>BM CFC/10⁶ Nucleated Cells Plated (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37.0</td>
<td>A</td>
<td>5.0 ± 0.9</td>
<td>36.0 ± 3.8</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>A</td>
<td>9.8 ± 1.0</td>
<td>26.0 ± 2.7</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>B</td>
<td>36.1 ± 1.3</td>
<td>55.0 ± 8.3</td>
</tr>
<tr>
<td>4</td>
<td>8.5</td>
<td>B</td>
<td>21.5 ± 2.1</td>
<td>100.3 ± 6.2</td>
</tr>
<tr>
<td>5</td>
<td>17.0</td>
<td>B</td>
<td>—</td>
<td>78.8 ± 8.4</td>
</tr>
</tbody>
</table>

*Patients 1 and 2 studied without Ficoll-Hypaque separation of bone or marrow cells prior to culture (University of Alabama at Birmingham).
†Ficoll-Hypaque separation concentrated CFC in patients 3-5 and resulted in higher colony counts from these specimens (Emory University).
‡For method A, these compare with a normal range of 0-15 colonies/5 × 10⁶ unseparated nucleated blood cells plated (mean ± 5.2) for 19 normal children ages 3 wk-16 yr; for method B, 0-5 colonies/5 × 10⁶ Ficoll-Hypaque separated nucleated blood cells from 7 normal adults.
§For method A, these compare with a normal range of 12-94 colonies/10⁶ nucleated marrow cells plated (mean 33.7 ± 6.7) for 8 control children 3 wk-2 yr of age (patients with ITP or benign tumor without marrow involvement); or for method B, 39-222/10⁶ Ficoll-Hypaque separated marrow cells (mean 128.7 ± 22.5) for 9 control children 1 mo-2 yr of age.

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**Table 2. Colony-Stimulating Activity* of Blood Cells and Serum**

<table>
<thead>
<tr>
<th>Patient</th>
<th>CSA From Mononuclear Cells†</th>
<th>Serum CSA or inhibition†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Marrow CFC Over Normal Underlayer (Mean ± SEM)</td>
<td>Control Marrow CFC Over Patient Underlayer (Mean ± SEM)</td>
</tr>
<tr>
<td>1</td>
<td>16.3 ± 1.5</td>
<td>19.3 ± 1.3</td>
</tr>
<tr>
<td>2</td>
<td>40.3 ± 7.4</td>
<td>34.0 ± 0.9</td>
</tr>
<tr>
<td>3</td>
<td>164.0 ± 5.4</td>
<td>222.0 ± 7.8</td>
</tr>
<tr>
<td>4</td>
<td>164.0 ± 5.4</td>
<td>167.3 ± 9.7</td>
</tr>
<tr>
<td>5</td>
<td>110.5 ± 5.9</td>
<td>129.0 ± 6.7</td>
</tr>
</tbody>
</table>

*Control marrow overlayers (from patients with ITP or solid tumors without marrow involvement) cultured in quadruplicate at 10⁶ nucleated cells/plate. Control marrow cells were obtained either by extraction from the marrow buffy layer after sedimentation or by a Ficoll-Hypaque separation and were plated over underlayers consisting of 10⁶ nucleated blood cells (from normal single donors)/plate.
†For patient monocyte counts see Table 2; average monocyte count in the normal underlayers was 450 ± 80 cu mm as determined from 10 widely separated samples.
‡Ficoll-Hypaque separated bone marrow cells plated at 2 × 10⁶ cells/plate. (Higher plating concentration and Ficoll-Hypaque separation with concentration of CFC from control marrow account for higher CFC counts.)
Fig. 4. Patient 5 marrow cells obtained when patient was asymptomatic. Numerous abnormally lucent primary granules are present in this promyelocyte. Some of these granules contain heterogeneous or membrane-like material (arrows). A few primary granules appear normally condensed. The Golgi cisternae (G), rough endoplasmic reticulum (ER), centriole (C), and nucleus (N) appear intact. (Electron micrograph prepared as in Fig. 3; x 20,500.)

Fig. 5. Marrow cells from asymptomatic patient 1. A large vacuole containing cytoplasmic debris is located adjacent to the Golgi complex (G) of this neutrophilic granulocyte. (Electron micrograph prepared as Fig. 3; x 12,500.)

Fig. 6. Marrow cells obtained when patient 5 was asymptomatic. Abnormal primary granules contain heterogeneous material (arrows) in this mitotic promyelocyte with a metaphase chromosome distribution. (C, partially illustrated). (Electron micrograph prepared as in Fig. 3; x 25,000.)
Serum lysozyme levels were generally within the normal range.

Electron Microscopy

Morphology

Several morphological abnormalities were observed in directly sampled neutrophilic granulocytes from each patient. At least 20 electron micrographs of these dysmorphic cells were examined for each patient sample. Most marrow neutrophilic myeloid cells were at an early stage (promyelocyte) of development (Fig. 3). Abnormal granule genesis appeared confined to the neutrophil series (Figs. 4–6). Cytoplasmic maturation, as evidenced by decreased amounts of polyribosomes, rough endoplasmic reticulum, and mitochondria, exceeded nuclear maturation in many of these cells. Late neutrophils frequently contained abundant diffusely distributed glycogen particles (Fig. 7). Degenerating cells containing lipid droplets and cytoplasmic vacuoles were frequently observed and were confined to the neutrophilic series. Autophagic and/or heterophagic vacuoles, which enclosed cytoplasmic granules, mitochondria and other membranous structures, were evident in several neutrophilic granulocytes at all stages of development (Figs. 5 and 6). Early promyelocytes (containing few primary granules) generally lacked discernable abnormalities, whereas most late promyelocytes (containing several primary granules, Fig. 4) and myelocytes, and all band and segmented neutrophils (Fig. 7) demonstrated severe ultrastructural abnormalities. In patients 4 and 6, the majority of promyelocytes appeared to have normal morphology; however, distinct abnormalities in granule genesis were observed in most myelocytes and all late neutrophils from these patients. A minority (5%–10%) of neutrophilic cells demonstrated abnormal nuclear morphology as evidenced by excessive nuclear lobulation (Fig. 7) or folding and/or nuclear pockets (Fig. 8). These findings were more frequently observed in late neutrophils. Abnormal nuclear lobulation was rarely observed at the light microscope level and could not be significantly quantitated because of the sparse number of late neutrophils present.

Neutrophilic granulocytes often contained fewer primary granules than expected for the developmental stage (Fig. 3). These granules varied from normal to grossly abnormal in appearance. Generally, all maturation stages of primary granule genesis could be identified: the dense material in immature granules appeared flocculent and was widely separated from granule membranes, whereas the dense material in mature granules extended to the membrane and occasionally contained a central lucent crystalloid as described in normal cells. In contrast, abnormal primary granules contained myelin figures or were unusually lucent (Figs. 3 and 4). Some granules were abnormally fused or were identified in autophagic vacuoles (Figs. 4–6). The latter morphological findings were similar to the granule lysis observed during disposal of defective or unused secretory granules in endocrine cells.

Secondary granules were not identified in late myeloid cells from patients 2, 3, and 6, whereas rare secondary granules were present in specimens from patient 5, and moderate but significantly decreased numbers of secondary granules were present in late myeloid cells from patient 4. Coincidentally, this latter patient has had a milder clinical course (see patient data). Although no secondary granules were initially identified in specimens obtained at 4 and 24 mo of age in patient 1, a few secondary granules were observed in a specimen obtained at 37 mo of age. The rare secondary granules that were observed averaged 0.3 μm in diameter and contained an electron-dense rim with an electron-lucent core (Fig. 9) as described previously in normal cells. It could not be determined if these sparse granules participated in the autophagic process observed in myeloid cells.

Monocytes and macrophages in marrow specimens

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Fig. 7. Asymptomatic patient 1 marrow cells. This segmented neutrophil cell profile contains 4 nuclear lobes with moderate, but incompletely condensed chromatin. The cytoplasm appears mature with a single mitochondrion (M), sparse endoplasmic reticulum (ER), and diffusely distributed glycogen particles (typical of mature neutrophils and enlarged in the inset). Only a few primary granules and some small dense granules are present and no secondary granules can be identified. Two abnormal cytoplasmic vacuoles (V) contain variably dense material. Both immature (noncrystalloid containing) and mature (crystalloid containing) granules of a nearby eosinophil (lower right) appear normal (Electron micrograph prepared as in Fig. 3; × 14,000.)

Fig. 8. Blood cells obtained during a fatal episode of sepsis in patient 1. The nucleus of this band neutrophil demonstrates nuclear envelope reduplication (arrows) and the presence of cytoplasmic-nuclear pockets. Although not a frequent finding in other patients, this abnormality was present in 28% of late neutrophils (n = 50) from patient 1. Also note the hypogranular appearance of the cytoplasm, which lacks identifiable secondary granules and contains abundant particulate glycogen (G) typical of late neutrophils. (Electron micrograph prepared as in Fig. 3; × 14,000.)

Fig. 9. Blood cells obtained during a fatal episode of sepsis in patient 1. This late neutrophil with 3 nuclear lobes contains several primary granules that appear homogeneously dense with an occasional electron-lucent rim. Some of these granules appear abnormally enlarged (upper granule of inset). Rare secondary granules are evident and contain an electron-lucent central portion with an electron-dense rim (lower granule of inset). (Electron micrograph prepared as in Fig. 3; × 13,000, inset × 26,000.)
were occasionally observed engulfing disrupted neutrophils and cell debris (Fig. 10). The maturation of nuclei, cytoplasmic granules, and organelles appeared normal in monocytes (Fig. 3), macrophages (Fig. 10), lymphocytes (Fig. 3), eosinophils (Fig. 7), and basophils. Phagocytic vacuoles (autophagic and/or heterophagic) were observed in a few monocytes and eosinophils.

Myeloid cells were rarely identified in ultrastructural studies of blood samples from these patients. A blood specimen taken from patient 1 during a terminal episode of sepsis contained a large number of late neutrophils (see patient data). As in marrow specimens obtained when the patient was asymptomatic, these cells contained autophagic vacuoles, degenerating primary granules, and rare secondary granules, despite complete nuclear segmentation (Figs. 8 and 9).

Cytochemistry

Peroxidase reactivity was evident in most neutrophil primary granules and confirmed the morphological observation of absent or markedly decreased secondary granules in metamyelocyte, band, and segmented neutrophils (Fig. 11). Cytoplasmic vacuoles often lacked peroxidase reactivity.

Acid phosphatase variably stained neutrophil primary granules, and confirmed the morphological observation of absent or markedly decreased secondary granules in metamyelocyte, band, and segmented neutrophils. Only rare staining was observed in autophagic vacuoles of these cells.

Comparative and Parallel Controls

The abnormalities in neutrophilic granulocytes described above for marrow specimens were not observed in similarly (and in several instances simultaneously) processed specimens from normal individuals or from eight patients with congenital neutropenia and a relatively benign clinical course or one patient with severe acquired neutropenia.

Cultured Marrow Cells

A variety of morphological abnormalities was apparent in cultured neutrophilic granulocytes from patient and control specimens. The majority of cells contained several cytoplasmic vacuoles, which often made differentiation of monocytic and neutrophilic cells difficult, as described previously. Although primary granules were observed in several stages of maturation, distinct secondary granules could not be identified. Similar morphological abnormalities were observed in patient and control cells cultured in methyl cellulose as described previously.

DISCUSSION

The findings of dysmorphic neutrophilic granulocytes, evidence of neutrophil disruption, normal or increased numbers of colony-forming cells with normal proliferative capacity, and lack of antineutrophil antibodies or inhibitors noted in all of these patients are similar to the findings described in the erythroid cells of the congenital dyserythropoietic anemias. Consequently, we have used the descriptive name, congenital dysgranulopoietic neutropenia, to describe these severely symptomatic patients. We have previously reported a single patient (patient 6 of this study) with severe congenital neutropenia, cytoplasmic and nuclear abnormalities in neutrophilic granulocytes, and normal neutrophil proliferation in vitro. Other investigators have described ultrastructural lesions in cultured neutrophils from abnormal neutrophil colonies, "mimicking the disease." These previous studies only assessed single patients or failed to investigate directly sampled marrow cells and did not correlate clinical and laboratory findings of the patient population. Thus, the collective data obtained in this study have separated six patients from a larger group of similarly studied neutropenic patients from our clinics and have shown that these children have severe clinical symptomatology associated with dysgranulopoiesis.

Many of the findings (cytoplasmic vacuoles, autophagy, and lipid inclusions) in neutrophilic granulocytes from our patients can be interpreted as nonspecific alterations related to impending cell death and intramedullary cell lysis. However, the presence of abnormalities in granule genesis, cytoplasmic maturation, and to a lesser extent nuclear maturation are more consistent with an intrinsic cell defect. These extensive ultrastructural abnormalities in neutrophilic granulocytes suggest an underlying lesion that affects many cell organelles, comparable to that observed in dyserythropoietic anemia.

The most frequent morphological expressions of the neutrophil dysfunction appear in the granules or lysosomes. The presence of quantitative and qualitative granule abnormalities suggests both impaired synthesis as well as degeneration of granule content or granule lysis. The latter interpretation is supported by the presence of intragranular inclusions, decreased density of some granules, and the presence of granules in phagocytic vacuoles. The present studies have not determined whether the abnormalities in granule genesis result from failure of other cellular organelles.
or whether lysosomal instability adversely affects adjacent organelles. Also, more than one type of congenital dysgranulopoietic neutropenia may exist, as seen in congenital dyserythropoietic anemia.34

The absence or decrease in secondary granules of late myeloid cells from our patients could result from either a primary defect in synthesis or from cell injury. The latter possibility may occur as a result of primary granule disruption and damage to adjacent organelles. The observation of LAP activity in some neutrophils, despite the absence or marked decrease in secondary granules, supports biochemical and cytochemical stud-
ies localizing LAP in cytoplasmic vesicles rather than secondary granules of human neutrophils. A decrease in late neutrophils and secondary granules would suggest a concomitant decrease in lactoferrin thought to be present in these sites. Lactoferrin has recently been demonstrated to provide feedback inhibition of myelopoiesis by inhibiting CSA production from monocytes. However, the apparent normal CSA and normal number of CFCs in our patients would suggest that either lactoferrin is present in organelles other than secondary granules or that additional feedback mechanisms may exist.

Abnormalities in neutrophil granule genesis have been described in other neutrophil disorders but have not resulted in excessive destruction or neutropenia. However, autophagy, granule lysis, and lysosomal disruption were not frequent findings in non-neutrophic patients, and these may be distinguishing factors heralding destruction of abnormal granulocytic cells in this group of neutrophic patients. Previous studies have similarly associated excessive autophagy with neutropenia, and increased marrow destruction of neutrophils in vitamin B12 deficiency, and the Chediak-Higashi syndrome. However, the presence of hypergranular neutrophils in the former disease and giant granules in the latter disease are clearly distinct from the hypogranular neutrophils observed in the present study. Excessive autophagy also occurs in dyserythropoietic anemia but appears limited by the small number of lysosomes in erythroid cells.

Abnormal nuclear, cytoplasmic, and/or granule maturation has been observed in vitamin B12-deficient neutrophils, pharmacologically altered neutrophils, and normal granulocytes grown in soft agar or methyl cellulose culture in some laboratories. Direct sampling of marrow cells in this study eliminates the possibility of culture-induced abnormality, and the failure to demonstrate serum inhibitors, antineutrophil antibodies, abnormal CSA, or nutritional disease would further argue against a primary extrinsic etiology for the neutropenia observed in these patients. Nevertheless, the normal in vitro proliferation of the patient’s CFCs, despite their persistent abnormal ultrastructure, indicates that the abnormal cells survive longer in vitro than in vivo and that factors in vivo may augment the destruction of the mature dysmorphic myeloid cells.

The observation in marrow specimens of neutrophil disruption and neutrophil debris in macrophages provides direct evidence for the intramedullary destruction of defective neutrophils. The decrease in lysozyme-containing primary and secondary granules in neutrophils from the patients in this study precludes the use of elevated serum lysozyme as a method for detecting increased granulocyte destruction and provides an explanation for the near-normal values observed.

Some investigators have recommended marrow transplantation for severely symptomatic patients with intrinsically abnormal neutrophils. However, recent improvement in supportive care and the observation here of decreased infections with increasing age (possibly related to specific antibody production) are noteworthy. Nevertheless, despite these improvements, the death of two of our patients and the morbidity of infectious complications in our other patients attest to the inadequacy of present therapeutic modalities. Longer survival of these patients may allow emergence of other manifestations of a stem cell defect, such as the development of leukemia, which has been reported in three patients and a sibling of a patient with some type of congenital neutropenia. If this proves to be a common occurrence, support for marrow transplantation would be further strengthened.

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DYSGRANULOPOIETIC NEUTROPENIA


Congenital dysgranulopoietic neutropenia: clinical, serologic, ultrastructural, and in vitro proliferative characteristics

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