In Vitro Induction of Myeloid Proliferation and Maturation in Infantile Genetic Agranulocytosis

By Yigal Barak, Michael Paran, Stanley Levin, and Leo Sachs

A 3½-yr-old boy with infantile genetic agranulocytosis (IGA) has had recurrent infections since the age of 3 mo. Characteristically he shows permanent, almost complete neutropenia with relative monocytosis in the peripheral blood, together with a bone marrow rich in proliferative myeloid cells, but lacking in mature ones. Results of in vitro cloning of the patient's bone marrow cells in soft agar indicate that human-spleen-conditioned medium can induce the formation of large granulocyte colonies with normal maturation. The cloning efficiency, colony size, and degree of maturation were no less than in cultures seeded with normal marrow cells. Neither the addition of patient's serum to normal marrow culture, nor the addition of normal serum to patient's marrow culture, caused any change in cloning efficiency or degree of maturation. These observations suggest that precursors of granulocytes in cases of IGA are potentially capable of normal proliferation and maturation provided that they are supplied with an inducer such as that derived from human spleen.

The term infantile genetic agranulocytosis (IGA) was applied by Kostman1 in 1956 to a syndrome characterized by markedly decreased or absent peripheral blood neutrophils beginning in infancy, multiple infections, and frequently ending in early death. The striking feature of the marrow is its normal appearance or even hypercellularity, the myeloid series being dominated by promyelocytes and myelocytes, with very few more mature cells. Erythropoiesis and thrombopoiesis are normal, with eosinophilia and monocytosis being associated findings. Subsequent reports have recently been summarized by Miller2 and Gilman et al.3

The pathogenesis of the disorder remains obscure. Neither the theory of a block in differentiation of myeloid cells from proliferative to mature cells4 leading to ineffective granulopoiesis5 nor that of marrow exhaustion resulting from excessive demand due to increased peripheral destruction of granulocytes6 has satisfactorily explained the association of peripheral neutropenia with normocellular or even hypercellular marrow with adequate number of granulocyte precursors.

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Table 1.—Bone Marrow Differential Count (1000 Cells)

<table>
<thead>
<tr>
<th></th>
<th>Patient (3 mo old)</th>
<th>Normal (3 mo old)</th>
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<tbody>
<tr>
<td></td>
<td>(Per Cent of Cells)</td>
<td>(Per Cent of Cells)</td>
</tr>
<tr>
<td>Myeloblasts</td>
<td>0.4</td>
<td>0–4</td>
</tr>
<tr>
<td>Promyelocytes</td>
<td>32</td>
<td>1.5–5.0</td>
</tr>
<tr>
<td>Myelocytes</td>
<td>16</td>
<td>0.5–16</td>
</tr>
<tr>
<td>Metamyelocytes</td>
<td>2</td>
<td>3–33</td>
</tr>
<tr>
<td>Bands</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>Polymorphonuclear leukocytes</td>
<td>0.6</td>
<td>2–24</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>3.5</td>
<td>0.5–5</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.6</td>
<td>0–1</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>34.6</td>
<td>31–81</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>1.0</td>
<td>0–2</td>
</tr>
<tr>
<td>Normoblasts</td>
<td>7.8</td>
<td>3.5–33.5</td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td>0.4</td>
<td>0</td>
</tr>
</tbody>
</table>

The development of an in vitro system for growing and cloning rodent and human hematopoietic cells in soft agar has provided an opportunity to determine the potentialities for growth and differentiation of marrow cells from IGA patients. The results of such a study are the basis of this paper.

CASE REPORT

M.M. has been admitted to hospital several times since the age of 3 mo because of recurrent skin, middle ear, and pulmonary infections. The family history was noncontributory and complete blood counts of the parents and siblings were normal. During the course of his multiple infections, repeated blood counts revealed consistent neutropenia, monocytosis, lymphocytosis, occasional eosinophilia, mild hypochromic anemia, and adequate thrombocytosis. Total leukocytes ranged from 3700 to 11,100 per cu mm with bands 0–540, neutrophils 0–280, lymphocytes 3590–9000, monocytes 320–2680, eosinophils 0–960, and basophils 0–150. The bone marrow was hypercellular with normal erythropoiesis and thrombopoiesis; the myeloid-erythroid ratio was 7.1:1. Myelopoiesis was arrested at the myelocyte stage, and only 1.4% band forms and mature neutrophils were found (Table 1). Many large promyelomonocytes with kidney-shaped nucleus, basophilic cytoplasm, and azurophilic fine granulation were noted. No leukoagglutinins could be demonstrated in the patient’s serum. Protein electrophoresis and assay of immunoglobulins were within normal limits. Attempts to stimulate granulopoiesis with corticosteroids, androgens, and repeated fresh plasma infusions failed to induce any hematological or clinical change. At age 3½ yr, the patient is thriving poorly, is physically retarded, and is suffering from chronic pulmonary infection.

MATERIALS AND METHODS

Cytochemical Staining and Leukocyte Function Tests

Smears from peripheral blood,uffy coat, and bone marrow were stained to show alkaline phosphatase, peroxidase, and esterase (with naphtol AS-D chloracetate as a substrate) activities. Culture of peripheral blood for lymphocyte transformation and karyotype analysis was made employing Mellman’s modification of the Moorhead technique. Histochcmical nitroblue-tetrazolium (NBT) reduction by phagocytizing neutrophils was performed according to the method described by Park. An epinephrine stimulation test was done as described by Miller. Local inflammatory response was determined by the method of Rebuck.
In Vitro Cloning of Bone Marrow Cells

Bone marrow cells were obtained by aspiration from the posterior superior iliac crest. The cells were transferred to a glass tube containing heparin solution, and allowed to sediment during 1 hr. After centrifugation, the cells were resuspended in serum-free Eagle's medium with fourfold concentration of amino acids and vitamins (EM). Then $2 \times 10^5$ nucleated cells were seeded for cloning on 50-mm plates. The cells in EM with 20% calf serum were cloned in soft agar (0.33%) on a harder agar base (0.5%) as described previously. Human-spleen-conditioned medium was prepared from mass culture of human spleen cells after splenectomy from patients having thalassemia major, hereditary spherocytosis, or traumatic rupture of spleen. This conditioned medium was added to the lower agar layer. Colonies were counted with an inverted microscope at 10 days following seeding and each calculation of the number of cells per colony was based on a count of at least 30 pooled colonies. For staining, colonies were picked from the agar with capillary tube at 14 days after seeding. The cells were examined directly as wet preparations by phase-contrast microscopy and after staining with May-Grunwald-Giemsa and cytochemical stains for alkaline phosphatase, peroxidase, and periodic-acid-Schiff (PAS).

RESULTS

Cytochemical staining of the patient's blood and marrow cells showed that both granulocytes and monocytes stained positively for peroxidase activity. Alkaline phosphatase activity was detected in neutrophilic granulocytes. The promyelomonocytoid cells in the marrow smears showed marked peroxidase and esterase activities, the latter indicating that these cells belonged to the granulocytic series. Peripheral lymphocyte short-term culture with phytohemagglutinin (PHA) stimulation showed 85% transformation, while reduction of NBT by neutrophils taken from the patient during an acute infection showed 70% NBT-positive cells. The epinephrine stimulation test showed no response in the number of total leukocytes, neutrophils, and mononuclear cells during 60 min, whereas in a normal control total leukocytes rose from 7000 to 13,100 after 10 min with almost a twofold rise in the number of neutrophils. The results of the local inflammatory response (Rebuck window), showed
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Table 2.—Colony Formation by Three Samples of Bone Marrow Cells From the IGA Patient

<table>
<thead>
<tr>
<th></th>
<th>No. of Colonies per 2 x 10⁶ Cells Seeded With Added 20% Calf Serum</th>
<th>No. of Cells per Colony* After 14 days</th>
<th>No. of Colonies per 2 x 10⁶ Cells Seeded With Added 20% Patient Serum</th>
<th>No. of Cells per Colony* After 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>279</td>
<td>370</td>
<td>282</td>
<td>300</td>
</tr>
<tr>
<td>Normal</td>
<td>429</td>
<td>1160</td>
<td>450</td>
<td>1000</td>
</tr>
<tr>
<td>Patient</td>
<td>410</td>
<td>2300</td>
<td>385</td>
<td>2500</td>
</tr>
<tr>
<td>Normal</td>
<td>259</td>
<td>510</td>
<td>269</td>
<td>500</td>
</tr>
<tr>
<td>Patient</td>
<td>740</td>
<td>2500</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT, not tested.
* Mean of 30 pooled colonies ± 5%.

that in the patient's "window" there was no response to stimulation until 6 hr, when an exudate with 50% monocytes and 26% lymphocytes appeared. Mononuclear cells predominated during the subsequent period under study with a slight increase (up to 40%) in the neutrophil count. In the normal control a cellular exudate composed of 94% neutrophils appeared after 4 hr and continued until about 18 hr when an increased number of mononuclear cells was noted (Fig. 1).

Studies using bone marrow cells aspirated from normal individuals performed in the present and a previous study have shown that with the addition of human-spleen-conditioned medium, granulocytic colonies developed, whereas no colonies were obtained without this conditioned medium. The average cloning efficiency was about one colony per 10⁶ cells seeded, and an average colony size was of about 800 cells at 14 days after seeding. The colonies contained cells in various stages of differentiation from myeloblasts to mature granulocytes. The cytochemical tests with alkaline phosphatase, peroxidase, and PAS showed positive reaction in the mature cells.

The results of three experiments with bone-marrow cells from the patient with IGA are shown in Table 2. The cloning efficiency and the number of cells per colony were not significantly different from those obtained with marrow cells from normal individuals. The addition of the patient's serum in 20% concentration neither inhibited nor enhanced the cloning efficiency of the patient's or normal marrow cells. Examination of the colonies picked from the agar after 14 days of culture showed neutrophilic granulocytes in various stages of maturation (Fig. 2), similar to those seen with normal marrows. Mature granulocytes in colonies from the patient's marrow stained positively for alkaline photophatase, peroxidase, and PAS. The addition of patient's and normal serum at 20% concentration to the patient's and normal marrow cells cultures in various combinations failed to give any significant change in the ratio of mature cells (bands and segmented neutrophils) to immature cells (blasts, promyelocytes, myelocytes, and metamyelocytes).

DISCUSSION

The clinical and hematological findings, including cytochemical staining and function studies of leukocytes observed in this patient closely resemble those
Fig. 2.—Cell maturation in a granulocyte colony from bone marrow cell culture from a case of infantile genetic agranulocytosis 14 days after seeding, showing (A) promyelocyte, (B) myelocyte, (C) metamyelocyte, and (D, E) band forms; (F, G) segmented granulocytes; (H) myeloid cell in mitosis (cells stained with May-Grunwald Giemsa, × 2730); (I) low-powered view (× 335) of unstained colony 10 days after seeding.

in other cases of IGA, and clearly distinguish the present patient from the various forms of chronic granulocytopenia which rarely occur in infancy.

The normal behavior of the patient's marrow myeloid cells in culture indicates that these cells, when grown in the presence of an inducer derived from human spleen, are potentially capable of normal development. It is interesting
to note that despite the fact that the patient’s marrow was rich in promyelocytes (32%) the cloning efficiency (2 colonies per $10^3$ cells seeded) was not significantly greater than that from normal marrows. However, since the identification of the colony-forming cells has not yet been definitely established, one cannot predict the cloning efficiency from the number of promyelocytes observed. The observation that the addition of 20% patient’s serum to the marrow culture from the patient or from normal persons failed to influence the rate of colony formation, colony size, and the degree of cellular maturation suggests that the block in maturation of granulocytes in these patients is probably not related to a humoral factor causing inhibition.

In experiments on mass cultures of bone marrow cells from adult patients with benign idiopathic neutropenia characterized morphologically by a paucity of mature neutrophils, Lau, Brody, and Beizer$^{18}$ reported normal mature/immature cell ratios in the cultures. In an IGA patient, Wriedt et al.$^{19}$ demonstrated that the proliferative activity of the bone marrow in vitro was normal, whereas marrow cells grown in mass tissue cultures for 28 days failed to produce mature neutrophils. Short-term culture of marrow cells obtained from Miller’s IGA patient$^{2}$ with and without added normal serum also failed to produce myeloid maturation. Wriedt et al.$^{20}$ have recently reported in a further two IGA patients that when marrow fragments were cultured in vitro, neutrophils failed to develop from one patient and developed only after a prolonged interval in the other. It would be interesting to determine by our cloning procedure whether these results were due to an induction of differentiation or to a selection of mature cells in the mass cultures.

The results obtained in the present study do not indicate whether in IGA patients the absence of mature cells is due to maturation arrest caused by deficiency of an inducer or to an increased mature cell removal. It is suggested that since there is no evidence that the latter occurs, it is more likely that in IGA patients there is an inherent deficiency of a substance that induces myeloid maturation. It will be of interest to test this by attempting in vivo stimulation of myeloid maturation in IGA patients with this inducer.

The inducer used in the present study was obtained from cultures of human spleen cells. However, since a defect in myeloid maturation does not develop following splenectomy or in cases of congenital absence of spleen, it is presumably also produced by other body tissues. It has been shown with rodents that the inducer can be produced by a variety of rodent cells, such as cultures of mixed embryo cells, young and adult kidney cells, and some leukemic and other tumor cells, as well as adult spleen cells.$^{21}$ Human hematopoietic cells can also be induced to form colonies by feeder layers of normal human peripheral white blood cells.$^{22}$ Further studies$^{23}$ have shown the human cell types that can produce the inducer.

**References**

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