Congenital Neutropenia: An Intrinsic Cell Defect Demonstrated by Electron Microscopy of Soft Agar Colonies

By D. Zucker-Franklin, P. L’Esperance, and R. A. Good

Congenital neutropenia (CN), a disease characterized by recurrent infections leading to death in infancy, shows a maturation arrest of the myeloid series at the promyelocyte-myeocyte level. The potential value of marrow transplantation in this disease would be determined by the nature of the underlying defect. However, studies to date have failed to define whether the defect is intrinsic in the cells or attributable to “environmental” factors. Therefore, marrow of four patients with CN was cultured on soft agar, and the colonies were analyzed by a newly developed ultrastructural method. In parallel, patients’ cells were used in feeder layers for normal marrow. Although the patients’ colonies appeared grossly normal in size and number, electron microscopy showed only rare neutrophil colonies. These colonies contained markedly aberrant cells exhibiting asynchronous nuclear-cytoplasmic maturation, convoluted nuclei, excessive cytoplasm, and dearth of granules. Monocyte and eosinophil colonies differentiated normally. Patients’ cells and sera supported growth of normal colonies. The studies have demonstrated unequivocally that the neutrophil cell line of patients with CN is intrinsically defective and suggest that attempts at marrow grafting are warranted.

CONGENITAL NEUTROPENIA (CN), a rare disease which was not described as a separate entity until 1956,1 has aroused an unusual degree of interest during the last few years.2 12 The widespread concern appears to be intimately related to the question of whether patients afflicted with this fatal disease would benefit from bone marrow transplantation. In part, the answer to this question depends on whether the defect is intrinsic to the cell series affected—in which case, the patient’s “environment” might permit normal differentiation of grafted stem cells—or if factors extrinsic to the cells are at fault. Unfortunately, as poignantly demonstrated by two articles which have appeared in this journal within a period of 6 mo,11 12 pertinent studies conducted, so far, have yielded controversial results.

Initially, when bone marrow cells of patients with CN were grown in soft agar enriched with spleen conditioned medium, their cloning efficiency appeared normal. This observation led to the conclusion that the patients lacked an inducer necessary for normal differentiation of leukocytes.8 On the other hand, it had also been generally recognized that transfusion of normal blood or plasma did not supply a missing factor, since not even a transitory increase in mature neutrophils ensued. Moreover, when colonies obtained in soft agar

From the New York University Medical Center, and the Sloan-Kettering Institute, New York, N.Y.

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Address for reprint requests: Dr. Dorothea Zucker-Franklin, New York University Medical Center School of Medicine, 550 First Ave., New York, N.Y. 10016.

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culture were isolated and subjected to routine staining procedures, they proved to consist primarily of monocytes and eosinophils. The rare neutrophil colonies found were arrested at the myeloblast-promyelocyte stage of differentiation, as was the case in vivo. In subsequent studies utilizing histochemical techniques, it was noted that some neutrophils stained for alkaline phosphatase, an enzyme which normally is elaborated after the promyelocyte stage of maturation. It thus seemed possible, once again, that the cells were able to attain a more mature stage in vitro than in vivo. In order to assess more definitively to what extent bone marrow cells of patients with congenital neutropenia could differentiate and mature normally, the present study utilized a newly developed ultrastructural technique to analyze intact soft agar colonies of four patients with the disease. The results of this study, presented in preliminary form elsewhere, have shown unequivocally that the neutrophil series of patients with CN are abnormal.

MATERIALS AND METHODS

Bone marrow specimens obtained from one female and three male patients whose disease was recognized 5 days to 4 wk after birth were cultured in soft agar as described previously. At the time of culture, the boys were aged 5 mo, 10 mo, and 2 yr, respectively, and the girl was 10 mo. The severity of the infections did not appear to influence the absolute granulocyte counts, which remained below 100 x 10³/liter throughout the time of study. All patients had a marked decrease or complete absence of mature neutrophils and a maturation arrest at the myeloblast-promyelocyte level. As in most other cases reported, there was a "compensatory" monocytosis and eosinophilia, while erythroid and platelet maturation remained normal. As mentioned in our previous report, although all patients had somewhat elevated levels of polyclonal immunoglobulins, none had dysgammaglobulinemia or anti-neutrophil antibodies.

The bone marrow aspirates were washed three times in McCoy’s 5A medium and plated at a concentration of 2 x 10⁵ cells/ml in 0.3% agar containing 15% fetal calf serum. Feeder layers consisted of 1 x 10⁶ of the patients’ own peripheral blood buffy coat cells or the same concentration of normal buffy coat cells suspended in 0.5% agar. In parallel experiments, the patients’ buffy coat cells were used to support the growth of colonies obtained from the bone marrow of normal subjects. Ethical considerations precluded marrow aspirations from healthy infants. Therefore, control cultures were set up with bone marrow specimens obtained from four patients with leukemia in remission. All of the controls were less than 6 yr of age, but no specimen obtained from an infant less than 1 yr old was available.

The cultures were maintained at 37°C for 7, 15, 20, and 25 days. At these time intervals, colonies were removed one by one, smeared, and stained for light microscopy and enzyme studies, or the entire content of the petri dish was fixed in situ with 3% glutaraldehyde. Dehydration and embedding for electron microscopy were carried out by a special method developed for this purpose and described in detail elsewhere. In essence, this method yielded a “block” equivalent in size to the original petri dish in which individual colonies had been left undisturbed, and no mixture of cells between colonies had occurred. Selected colonies were then cut from this block with a jeweler’s saw. They were serially sectioned with an LKB ultratome, contrasted with uranyl acetate and lead citrate, and examined with an Elmiskop I electron microscope at instrument magnifications ranging from 600 to 30,000.

RESULTS

As had been observed by others, inspection of the cultures limited to routine examination with an inverted microscope supported the impression that the colonies grown from the bone marrow of patients with CN were normal. However, as early as day 7 of culture, electron microscopy disclosed that a few colonies (± 2%) consisted of markedly aberrant “blasts.” These
abnormal cells had a convoluted nucleus, excessive cytoplasm, and a villous surface. By day 15, such colonies comprised up to 1000 cells closely apposed to each other (Fig. 1). Their nuclei were deeply indented and contained one or more large nucleoli. Although granulation was not conspicuous, there were many atypical inclusions and vacuoles. Long villous processes, which characterized the surface membrane, interdigitated with those of adjacent cells (arrows in Fig. 1). It should be stressed, however, that, even on electron
Fig. 2. Detail of a myeloblast-promyelocyte colony at 15 days in culture. Since the majority of colonies had a similar appearance, it is likely that the cells represent differentiating monocytes. \( \times 3500 \).

Fig. 3. Detail of a colony in the same culture as the one illustrated in Fig. 2. The nuclei are primitive and display abnormally large nucleoli. Mitoses are frequent, the cell on the right being in metaphase. The large number of small granules in the cell indicated by the arrow, suggest that this colony probably consists of aberrant neutrophil precursors. L, lipid inclusion. \( \times 4000 \).
microscopy, by day 15 the vast majority of colonies did not appear unusual. As is the case in normal specimens at this time of culture, many cells had attained the promyelocyte stage of differentiation, which made it sometimes difficult to distinguish monocyte precursors from cells destined to become neutrophils (Figs. 2 and 3). However, by day 20, colonies in normal cultures always permitted identification by virtue of the fact that they contained at least some cells whose ultrastructure resembled that of mature leukocytes in circulating blood. In the patients’ cultures, this was more difficult. Neutrophil colonies were not only sparse, but the cells were morphologically aberrant, making it sometimes necessary to section the entire colony before definitive identification could be made. The most striking pathologic feature was the discrepancy of nuclear vis-a-vis cytoplasmic differentiation (Figs. 4–7). While the nuclei often seemed mature as judged by their degree of segmentation, the amount and distribution of heterochromatin, and the absence of nucleoli (Figs. 4, 5, and 7), cytoplasmic maturation had lagged behind. Many nuclei were hypersegmented, and some of them had undergone convolutions never seen in normal cells (Figs. 5 and 6). Granulation of the cytoplasm was poor, and the majority of granules resembled primary lysosomes in size and electron density (Figs. 4 and 7). Only very few cells possessed inclusions characteristic of the specific granules of mature neutrophils (Figs. 5 and 6). These may have been responsible for the observation that some cells stained positively for alkaline phosphatase when the colonies were studied by light microscopy. The excessive number of mitochondria, profiles of rough endoplasmic reticulum, and free ribosomes also attested to the degree of cytoplasmic immaturity. It may be of interest to mention the incidental finding of some cells with granules typical of basophils. Since the ultrastructure of basophils grown

Fig. 4. Example of neutrophils in 20-day-old cultures. The nuclei appear mature and segmented (N), but the granules have the size and density of primary (azurophil) granules (A), which are primarily lysosomes. Glycogen (GI) is abundant. × 7500.
Fig. 5. Cell from a colony in a 20-day-old culture believed to represent an aberrant neutrophil. The nucleus is mature and hypersegmented. Very few specific granules (S) are seen in the cytoplasm, which contains many mitochondria (M) and profiles of endoplasmic reticulum and glycogen (GI). x 8000.

Fig. 6. Cell with highly abnormal circinate nucleus (N) encountered in rare neutrophil colony. The cytoplasm is replete with small granules and mitochondria (M). x 8500.
Fig. 7. Neutrophil showing a normal mature segmented nucleus but asynchronous cytoplasmic maturation. Only few primary granules (A) are seen and no specific granules have developed. × 8000.

Fig. 8. Cell presumed to be a basophil found in a neutrophil colony. Granulation is sparse but typical. The area delimited by the rectangle is shown at higher magnification in Fig. 9. × 8000.

in soft agar has not been described before, an illustration is included (Figs. 8 and 9). The granules had the characteristic appearance described elsewhere, but also seemed to be less numerous than those of basophils in normal circulating blood.

Fig. 9. Area seen delimited by the rectangle in Fig. 8 at higher magnification shows characteristic basophil granules (BG) consisting of particles bound by a membrane. Polysomes and rough endoplasmic reticulum (RER) are indicative of synthetic ability. × 45,000.
Monocytes and Eosinophils

Electron microscopy has confirmed the impression gained earlier\(^9\) that the vast majority of colonies consist of monocytes or eosinophils. Under conditions prevalent in soft agar cultures, monocytes appear more differentiated than those seen in freshly obtained human blood (Fig. 10). They have an irregular contour and an abundant amount of cytoplasm which is replete with lysosomes, vesicles, vacuoles, mitochondria, small profiles of rough ER, and other inclusions characteristic of the "activated" state.

Eosinophil colonies differentiated normally, most cells reaching the promyelocyte stage by day 15 (Fig. 11). In cultures fixed after 20 days of incubation, eosinophil colonies showed metamyelocytes, as well as mature eosinophils with characteristic granules.

Plasma Cells

A most unexpected observation in the cultures of each of the four patients studied was the appearance of one to three plasma cell colonies. As illustrated in Fig. 12, the cells comprising such colonies had mature nuclei and fully developed cisternae of rough ER, which were in no way distinguishable from plasma cells in fresh bone marrow or lymphoid tissue obtained from normal individuals. Although neoplastic plasma cell lines have been established in liquid media for almost a decade, this was the first time that plasma cell colonies were observed in soft agar tissue culture of primary human isolates.

Finally, it should be reiterated that bone marrow and serum of all patients with CN, when incorporated in feeder layers supporting specimens obtained from healthy subjects, were able to stimulate colonies which were completely normal in size and number.

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*Fig. 10. Cell representative of those seen in monocyte colonies 20–25 days in culture. Granules are usually more abundant than those in circulating blood. M, mitochondria; G, Golgi; L, lipid inclusions. × 6000.*
DISCUSSION

The observations recorded here leave little doubt that the neutrophilic cell line in patients with congenital neutropenia is intrinsically defective. Soft agar cultures containing media and feeder cells able to support the growth of neutrophil colonies derived from normal marrow were unable to induce orderly differentiation and maturation of the patients’ progenitor cells. A similar conclusion was reached by Parmley et al., who studied the marrow of one patient with CN by a comparable technique. It should be emphasized that the number and size of colonies appeared normal when the cultures were scanned with a dissecting microscope, as is done routinely when the soft agar technique is applied to clinical problems. Electron microscopy of the colonies was necessary to establish that the vast majority of colonies consisted of monocytes, and that the neutrophil colonies, which could be identified with certainty, contained aberrant cells. This observation may explain the discrepant conclusions in previous reports.

The most striking feature displayed by the abnormal cells was “maturation anarchy,” a term coined by Bessis to describe the asynchrony in nuclear cytoplasmic development seen in leukemic leukocytes. Although many nuclei appeared to have developed to a mature, segmented stage, granulation was poor and arrested at the promyelocyte level. Rare cells, containing inclusions which resembled neutrophil specific granules, had pathologic appearing nuclei. Thus, when the progenitor cells were permitted to differentiate in culture, their
Fig. 12. Detail of a plasma cell colony in which the cells appear fully mature with well-developed cisternae of rough endoplasmic reticulum and large Golgi zones. × 8000.

Ultrastructure came to look identical to that of the cells in the patient's freshly obtained marrow. In this context, it should be recalled that the ultrastructural pathology just described was very reminiscent of the type of abnormality found in many patients with myelogenous leukemia. Moreover, colonies containing the type of cells depicted in Fig. 1 have not been seen in bone marrow or soft agar cultures heretofore. Though similar colonies have been observed by
one of us in the marrow culture of one patient with AML, ultrastructural analyses on soft agar cultures have been too limited so far to assess the significance of this observation. Nevertheless, it is conceivable that CN represents a form of myelogenous leukemia, a possibility supported by the fact that at least one patient developed this disease terminally.7

Apart from the clinical implications which these studies entail, some theoretical aspects may also be noteworthy. Thus, it is of interest that the monocytosis and eosinophilia shown by patients with CN is reflected by the increased number of monocyte and eosinophil colonies in their cultures. Although on the basis of spleen colony-forming unit (CFU-s) assay in rodents it is generally believed that granulocytes and monocytes have common stem cells,2 it is not at all clear at what time and under what influences differentiation of the various cell lines diverge. Since the present studies have shown that some “blast” colonies and all neutrophilic promyelocytes are aberrant, whereas monocyte colonies develop normally, one may assume that in order to escape a similar pathologic destiny, the monocyte progenitor must already have been distinct from the neutrophil precursor at a stage which, so far, has eluded morphological identification. The same considerations apply to the eosinophil series, the differentiation of which has been discussed in more detail elsewhere.22 Indeed, in contrast to what is known about hematopoiesis in rodents,21 it is not known until what stage of development man possesses truly pluripotential stem cells. The possibility exists that all stem cells in postnatal human bone marrow are already committed to differentiate along recognized cell lines. In any case, the observations presented here mitigate strongly against earlier theories that neutrophils and monocytes are derived from the same myeloblast or promyelocyte.24

Some comment as regards the plasma cell colonies in the cultures of patients with CN is also warranted. It has been generally recognized that the semisolid culture system is selective for the proliferation of granulocytes and monocytes, since plasma cell or lymphocyte colonies have not been seen before under conditions employed in this study. Only recently, following modifications of technique involving addition of mercaptoethanol and sheep red blood cells to the medium, has it become possible to grow immunoglobulin-producing B-lymphocyte colonies from marrow and lymphoid tissue of normal mice.25 As illustrated in Fig. 12, some colonies of the patients with CN consisted of fully developed mature plasma cells. The possibility has not been excluded that bone marrow cells of healthy infants would normally yield plasma cell colonies, since studies on such specimens have of necessity been extremely limited. However, four control marrows obtained from young children with leukemia in remission cultured and analyzed in our laboratory by the same method have not given rise to such colonies.

Finally, it should be pointed out that although the objective of this study has been accomplished in that it has been demonstrated that the patients’ cells or sera have no detrimental effect on the development of normal cells and that the defect is intrinsic to the patients’ leukocyte precursors, one reservation must moderate our optimism as regards the success of marrow transplantation. It is still conceivable that an extrinsic agent detrimental specifically for the precursor of the neutrophil series could be operative. Such an agent would be as damaging
to the stem cells of a potential donor as for those of the host. An example of such a transplant sequel has been reported in myelogenous leukemia. However, such failure is amply balanced by the encouraging results of marrow transplantation obtained in other conditions and warrants an attempt with this mode of therapy in congenital neutropenia.

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