Chronic Granulomatous Disease

EXPRESSION OF THE METABOLIC DEFECT BY IN VITRO CULTURE OF BONE MARROW PROGENITORS

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ABSTRACT Chronic granulomatous disease (CGD), an often fatal syndrome of recurrent infections, results from the inability of patients' peripheral blood phagocytic leukocytes to generate superoxide despite otherwise normal phagocytic functions such as ingestion and degranulation. Circulating granulocytes and monocytes are the progeny of bone marrow progenitor cells, colony-forming units in culture. We compared the function of cells grown in two different in vitro culture systems from the bone marrow of a CGD patient with those from normal subjects. The cells of normal colony-forming unit in culture colonies grown in semisolid medium reduced nitroblue tetrazolium dye when stimulated by phorbol myristate acetate; none of the cells from colonies derived from CGD marrow did so. Cells grown in liquid suspension culture from normal marrow generated superoxide nearly as well as normal peripheral blood granulocytes; those from CGD marrow produced no superoxide. Similarly cultured cells from both normal and CGD marrow ingested opsonized bacteria at rates equal to peripheral blood granulocytes. CGD marrow-derived cells showed increased exocytic degranulation relative to both normal marrow-derived cells and normal peripheral blood granulocytes. These studies demonstrate that the basic functional characteristics of CGD are embedded in the genetic program of granulocyte progenitors.

INTRODUCTION

Chronic granulomatous disease (CGD) is a syndrome of recurrent purulent infections beginning in infancy and resulting in death by age seven in nearly a quarter of reported cases (1, 2). Phagocytic cells from these patients ingest particles and degranulate normally but fail to kill certain microorganisms because of a failure to generate superoxide (O2-) and related toxic oxygen species (H2O2, hydroxyl radical, singlet oxygen) (1). The disease exhibits heterogeneity of inheritance, with \( \sim 5:3 \) ratio of X-linked recessive to autosomal recessive kindreds (1, 2). It may represent the expression of a variety of defects in the activation, function, and substrate supply of the pyridine nucleotide oxidase that produces \( \text{O}_2^- \) (1, 3, 4).

Delineation of the metabolic basis of CGD has depended on the study of mature peripheral blood granulocytes and monocytes. The present study examines the function of cells derived from in vitro cultures of bone marrow progenitor cells. The func-

1 Abbreviations used in this paper: CGD, chronic granulomatous disease; CFU-C, colony-forming unit in culture; NBT, nitroblue tetrazolium; \( \text{O}_2^- \), superoxide; PMA, phorbol myristate acetate.
tional capacity of progenitor cells cannot be examined directly, for they have yet to be identified or purified. Furthermore, they are highly unlikely to directly express respiratory burst activity; human bone marrow myelocytes, promyelocytes, and myeloblasts do not reduce nitroblue tetrazolium (NBT) upon stimulation (3), so the still more primitive colony-forming unit in culture (CFU-C) progenitor cell would not be expected to do so. Thus, their genetic program for phagocytic function must be examined in their differentiated progeny.

METHODS

The patient is a 13-year-old boy previously reported as a child with Serratia marcescens osteomyelitis (5) and as a CGD patient with typically defective latex-stimulated NBT reduction and bacterial killing, but no detectable carrier state in his mother and sisters (6). We confirmed these findings with more sensitive phorbol myristate acetate (PMA) and opsonized zymosan-stimulated NBT slide tests (7, 8) and cytochrome c reduction (9) assays. Thus, he has either autosomal recessive or newly-mutated X-linked disease. Normal subjects for bone marrow culture included the patient’s 15-year-old sister, a 13-year-old girl with factitious fever, an adult paid donor, and two adults with anemia but no granulocyte abnormalities. Informed consent was obtained in accordance with the human subjects committees of the Children’s Hospital Medical Center and Beth Israel Hospital.

Bone marrow samples (1–2 ml) were aspirated into syringes containing dilute preservative-free heparin in a final concentration of 20 U/ml. The mononuclear cell interface after centrifugation in Ficoll-Hypaque was removed and washed twice with medium 199 (Gibco Laboratories, Grand Island Biological Co., Grand Island, N.Y.) supplemented with 15% fetal calf serum (Microbiological Associates, Walkersville, Md.; lot 92441) and 1% penicillin/streptomycin (Gibco Laboratories).

CFU-C agar colonies. The method of Pike and Robinson (10) was used. Feeder layer contained 1 x 10^6 unwashed peripheral blood leukocytes from the heparinized blood buffy coat of a normal volunteer. Overlayers contained 2 x 10^6 mononuclear bone marrow cells from the patient or controls. Plates were incubated for 14 d in a humidified incubator with 5% CO_2 at 37°C. Cultures of normal marrow produced 54±13 (mean±SEM) colonies (50 or more cells) per plate and those of the CGD patient’s marrow produced 41±4 colonies per plate, with 15–20 plates in each experiment.

Liquid suspension culture. The method of Sumner et al. (11) was modified for use with human cells. Culture medium consisted of 64% NGTC-135 (Gibco Laboratories); 15% fetal calf serum (Microbiological Associates; lot 92441); 10% human serum pooled from 15 normal donors and stored frozen until use; and 1% penicillin/streptomycin. Exogenous colony-stimulating factor was not required for cell growth and proliferation in this system. Cells were added to a final concentration of 4 x 10^6 cells/ml, and 50-ml aliquots were cultured in 75 cm^2 polystyrene flasks (Corning 25116; Fisher Scientific Co., Pittsburgh, Pa.). Cytocentrifuge preparations stained with Wright’s-Giemsa were used for 200 cell differential counts. Cells for functional studies were harvested from flasks on day 6 of culture when maximum numbers of polymorphonuclear cells were found.

Functional studies. All reagents were purchased from Sigma Chemical Co., St. Louis, Mo., unless otherwise noted.

Assays of NBT reduction used a reagent mixture of 0.1 mg/100 ml NBT, 2 g/100 ml human serum albumin (Hyland Diagnostics Div., Travenol Laboratories, Costa Mesa, Calif.), 5 mM glucose, and either 1 µg/ml PMA (Consolidated Midland Corp., Brewster, N. Y.) or 0.4 mg/ml opsonized zymosan, all in phosphate-buffered 0.154 M NaCl, pH 7.4. Zymosan (ICN K&K Laboratories, Inc., Plainview, N. Y.) was opsonized by a 30-min incubation at 37°C with fresh human serum (8). CFU-C colonies were assayed for NBT reduction by overlying each 35-mm agar gel with 2 cm^2 of PMA-containing or PMA-free reagent mixture with or without 1 mM N-ethylmaleimide, an inhibitor of O_2 generation (9). After 20 min at 37°C, the NBT mixture was removed and the gels washed with 0.154 M NaCl and fixed with formalin (40 g/100 ml). Colonies were then examined microscopically for NBT reduction. NBT slides were prepared as previously described from incubations of cell suspensions with the reagent mixture (8, 12).

O_2 generation by cells from liquid suspension culture was measured by a previously described (9) continuous assay of superoxide dismutase-inhibitable cytochrome c reduction. The ingestion assay measured the cellular uptake of opsonized ^14C-labeled E. coli, corrected for adherence by taking the difference between uptake of label in the presence and absence of 1 mM N-ethylmaleimide (8). The degranulation assay measured lysozyme and β-glucuronidase release (expressed as a proportion of the total cellular enzyme content) in response to opsonized zymosan in the presence of cytochalasin B, corrected for nonspecific leakage during parallel incubations without zymosan (13).

All assays were performed in triplicate, along with simultaneous controls run on normal peripheral blood granulocytes prepared as previously described (9). Ingestion and O_2 generation assays were corrected for the number of phagocytic cells present (mature granulocytes, monocytes, and macrophages). At the time of harvesting, liquid cultures of CGD and normal marrow contained 70–75% mature neutrophils, 5–15% monocytes and macrophages, 0.5% eosinophils and basophils; the remainder were lymphoid, erythroid, and immature myeloid cells. Results are expressed as the mean±SEM of three or more replicate determinations.

RESULTS

We used two different in vitro culture methods to grow mature cells from bone marrow precursors of CGD patients and normal controls. Growth in semisolid medium produced discrete granulocyte-macrophage colonies derived from CFU-C progenitor cells (10). In cultures of normal bone marrow, all cells present in these colonies at day 14 of culture generated sufficient O_2 to reduce NBT to dark-blue formazan (Fig. 1, left panel). No NBT reduction occurred in the absence of the stimulant PMA or the presence of the inhibitor N-ethylmaleimide. Colonies grown from CGD marrow failed to reduce NBT (Fig. 1, right panel). Wright’s Giemsa-stained smears of the colonies showed that both CGD and normal cells had undergone normal maturation.

The liquid culture system provided mature myeloid cells “in suspension,” readily available for more complete functional studies. Cultured cells from normal marrow produced O_2 in response to PMA and opsonized zymosan stimulation (Fig. 2, left panel).
Figure 1 NBT reduction by CFU-C colonies. Bone marrow samples were cultured in soft agar medium for 14 d and the resultant granulocyte-macrophage colonies incubated with NBT and PMA and fixed; representative colonies were photographed through the 10 × objective of a light microscope. Left panel: normal marrow (note dense formazan deposits); right panel: CGD marrow.

nearly as well as control granulocytes freshly harvested from normal peripheral blood. The lag time—a function of the time necessary for activation of the respiratory burst (9)—was identical to that of peripheral blood granulocytes: cultured cells 54±5 and 48±5 s for PMA and opsonized zymosan stimulation, respectively; normal granulocytes 59±3 and 44±4 s. In contrast, cultured cells from the CGD patient’s marrow failed to generate detectable O2− in response to either stimulus.

However, as shown in the middle panel of Fig. 2, cells derived from CGD and normal marrows ingested opsonized bacteria equally well. Their function approximated that of control granulocytes from peripheral blood. The right panel of Fig. 2 demonstrates that the cultured CGD cells were not only capable of degranulation, but actually performed that function better than both cultured normal bone marrow cells and normal peripheral blood granulocyte controls. Under the same conditions, peripheral blood granulocytes from another patient with CGD showed 1.3 times the lysozyme release of normal granulocytes.

Discussion

These studies demonstrate that myeloid cells grown by in vitro culture of bone marrow precursors from a patient with CGD manifest the basic metabolic defect of that disease: the absence of respiratory burst activity with the preservation of ingestion and degranulation. Thus, whereas committed granulocyte-macrophage precursor cells themselves cannot be shown to have the functional abnormality seen in more mature cells, they contain and impart the defect to their progeny that develop in vitro. The stem cell origin of CGD is consistent with the genetic nature of this disorder (1, 2) and the known expression of the defect in neutrophils, monocytes, and eosinophils (12). It is also consistent with the reported development of NBT-positive peripheral blood granulocytes in a CGD patient who became chimeric after partially successful bone marrow transplantation (14).

Cultured cells from CGD bone marrow showed supranormal degranulation, a phenomenon we have also observed in studies of CGD peripheral blood granulocytes. This finding may derive from the dual effects of opsonized zymosan as a stimulant of both degranulation and the respiratory burst. The latter activity produces toxic autooxidation (15) that might limit degranulation in normal but not CGD leukocytes. Stossel et al. (16) reported normal kinetics of degranulation in CGD peripheral blood granulocytes, but they used opsonized paraffin oil particles, a less potent stimulus of the respiratory burst, and they measured the specific activity of lysosomal enzymes in phagocytic vesicles rather than total exocytic enzyme release.

The ability to detect NBT reduction in individual CFU-C colonies may prove useful in a variety of clinical situations. For example, an occasional CGD carrier could have an extremely skewed distribution of X chromosome inactivation in her peripheral blood.

Newburger, P. E. Unpublished data.

FIGURE 2 O2− generation (left), ingestion (center), and degranulation (right) by CGD and normal cells in liquid suspension culture. Bone marrow samples were cultured in liquid medium for 6 d, harvested, and assayed for the indicated functions as described in Methods. Results are expressed as percentages of the functional capacities of simultaneous peripheral blood granulocyte controls. Bar heights represent the means, and the error lines the SEM of three replicate determinations for CGD cells and five such assays for normal cells. Horizontal lines for CGD cells in the left panel indicate <1% of normal granulocyte O2− generation.
granulocytes so that she might appear normal (>97% positive) or abnormal (<2% positive) on NBT slide tests. CFU-C colonies should be less likely to show false positive or negative results than individual cells because a 1–2% minority of aberrant cells would not interfere with the correct scoring of each colony as a whole. Bone marrow culture might then distinguish the carrier state (two populations of colonies reflecting the skewed distribution of X-inactivation in progenitor cells) from normal (all NBT-positive) or autosomal CGD (all negative). One could similarly distinguish between complete and chimeric engraftment after bone marrow transplantation for CGD; or with a more rapidly maturing culture system, one might demonstrate engraftment before the appearance of peripheral blood granulocytes in the patient. NBT reduction by clusters and colonies grown from bone marrows of patients with myeloid leukemia might identify those leukemic stem cells able to achieve functional differentiation in vitro.

The finding of virtually normal granulocyte function in cells grown in liquid suspension culture illustrates the utility of this culture system for studying not only myeloid proliferation and differentiation but also disorders of neutrophil function. The technique may prove useful for the dissection of the heterogeneity of CGD and chemotactic disorders and for the study of the specific defects in diseases such as the Chédiak-Higashi syndrome.

ACKNOWLEDGMENTS

We thank Drs. David G. Nathan and Fred S. Rosen for advice and manuscript review, Robert O'Leary for expert technical assistance, and Ms. Catherine Lewis for manuscript preparation.

This work was aided by a Basil O'Connor Starter Research grant from the March of Dimes Birth Defects Foundation, American Cancer Society grant CH51, National Institutes of Health grants CA-26506, CA-26033, RR-00128, and AM-17148, and Research Career Development Award AI-00311 (Dr. Cohen).

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