The Chediak-Higashi Syndrome: Continuous Suspension Cultures Derived From Peripheral Blood

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The CHEDIAK-HIGASHI SYNDROME is characterized by giant lysosomal inclusions in all circulating leukocytes, an autosomal recessive pattern of inheritance, partial oculocutaneous albinism, and an increased susceptibility to pyogenic infection. Many patients develop a lymphoma-like (accelerated) phase characterized by hepatosplenomegaly, pancytopenia, lymphadenopathy, and generalized lymphocytic and histiocytic proliferation. The mononuclear cell infiltrates of this phase often destroy the architecture of involved organs. The peripheral blood may contain atypical or “immature” lymphoid cells. Investigation of this syndrome and its associated complications, as well as the nature of the heterozygous state has been limited by the paucity of affected individuals and material for study. Tissue culture techniques afford a means of obtaining bulk quantities of material in rare diseases. We now describe the establishment and characteristics of three long-term suspension cultures derived from peripheral blood leukocytes, two from a patient with the Chediak-Higashi syndrome, and the third from his heterozygous father.

Materials and Methods

Subjects

Four patients with the Chediak-Higashi syndrome (3 males ages 17, 16, and 7 years, and one female age 7 years) were studied. Certain of the clinical aspects of three of these patients have been reported previously. The two youngest patients were in the accelerated phase of this disease and were receiving vincristine 1 mg./m²/month. The 7 year old male patient from whom the cell lines were obtained was also receiving 4 mg. prednisone/day. Four parents (demonstrated heterozygotes) and three siblings of one patient, none of whom exhibited any characteristics of this syndrome, were similarly studied.
Table 1.—Attempts to Establish Long-Term Suspension Cultures from Peripheral Blood of Patients with the Chediak-Higashi Syndrome and Their Families

<table>
<thead>
<tr>
<th>Subject</th>
<th>Number of Cultures Attempted</th>
<th>Number (× 10⁸) of Lymphocytes Cultured/10 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (Homozygotes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Male 17 years</td>
<td>7</td>
<td>0.9-10.0</td>
</tr>
<tr>
<td>B. Male 16 years</td>
<td>8</td>
<td>1.0-10.0</td>
</tr>
<tr>
<td>C. Male 7 years*†</td>
<td>3</td>
<td>0.9-6.0</td>
</tr>
<tr>
<td>D. Female 7 years*</td>
<td>6</td>
<td>1.0-5.0</td>
</tr>
<tr>
<td>Parents (Heterozygotes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. Father of C</td>
<td>4</td>
<td>1.0-3.0</td>
</tr>
<tr>
<td>F. Mother of C</td>
<td>3</td>
<td>1.2-1.8</td>
</tr>
<tr>
<td>G. Father of A &amp; B</td>
<td>2</td>
<td>1.5-2.0</td>
</tr>
<tr>
<td>H. Mother of D</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>Siblings (Potential Heterozygotes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Sister of C</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>J. Brother (M) of C</td>
<td>2</td>
<td>1.5-2.4</td>
</tr>
<tr>
<td>K. Brother (R) of C</td>
<td>2</td>
<td>1.3-2.0</td>
</tr>
</tbody>
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* Accelerated (lymphoma-like) phase.
† Two long-term suspension cultures established.
‡ One long-term suspension culture established.

Technics of Establishment and Maintenance of Suspension Cultures

Leukocyte cultures were prepared from heparinized peripheral blood as previously described, and subsequently cultured at 37°C in screw cap prescription bottles containing 10 ml of RPMI 1640 medium supplemented with 20 per cent fetal calf serum. Initial cell concentrations varied from 0.9 to 10.0×10⁸ mononuclear cells per 10 ml. No effort was made to maintain the initial cell concentrations. Medium was changed weekly by gravity sedimentation of the cells in culture, removal of 30–50 per cent of the supernatant culture fluid and addition of a similar amount of fresh medium. Thirty-nine samples of peripheral blood, at least three from each patient and at least one from each other subject, were cultured (Table 1). Cultures were discarded when lymphocytes were no longer visible. Established cultures were transferred into fresh medium every two or three days. Continuous suspension cultures derived from the peripheral blood of patients with infectious mononucleosis and without the Chediak-Higashi syndrome, established and maintained in an identical fashion, were used for comparison.

Cytologic Technics

Smears of cells from the established cultures were stained with Giemsa, Wright’s and a modified Romanowsky stain. In addition, material was stained for acid phosphatase, glycopolipid (PAS), and neutral fats (Oil red O and Sudan black). They were also stained specifically for lysosomes with euchrysin, a supravital fluorescent amino-acridine dye. Phagocytosis was assessed at eighteen hours after the addition of a suspension of sterile 0.8mm diameter polystyrene particles by the examination of Wright’s stained smears of the cell cultures.

Immunoglobulin Determination

Immunoglobulin synthesis was assayed by a previously described radioimmunoelectrophoretic technic utilizing monospecific goat antihuman immunoglobulin sera. Serum immunoglobulin levels were determined by radial immunodiffusion in agar gel.

* Dow Chemical Company.
† Hyland Immuno-plates, Hyland Laboratories, Los Angeles, California, U.S.A.
Viral Cultures and Interferon Assays

Antiviral activity was assayed by a previously described vesicular stomatitis virus (VSV) plaque assay system on WI38 fibroblast cultures. Characterization of this active material as interferon was accomplished as previously described: species specificity was tested with chick embryo fibroblast cells; activity of the inhibitory material against an unrelated virus was determined by challenge of WI38 fibroblasts with 100 TCID50 of Sindbis virus; sedimentability was determined by comparison of the activity in the supernatant fluid and pellet following ultracentrifugation at 105,000 \( \times \) g for ninety minutes; trypsin sensitivity was determined by exposure of the active material to 0.2 mg./ml. trypsin (pH 7.8) for thirty minutes; Actinomycin D sensitivity was determined by pretreatment of the WI38 cells with 2 \( \mu \)g/ml. Actinomycin D prior to addition of the antiviral material. Safety testing of aliquots of the suspension culture lines for the presence of viral agents and endotoxin were performed as previously described and include: inoculation into tissue culture lines WI38, HEK, rabbit kidney, HEP-2, AGMK, RbMK; inoculation into rabbits, guinea pigs, and adult mice and blind passage in suckling mice; and pyrogenicity was assayed in rabbits. In addition, aliquots were cultured for bacteria, bacterial L-forms, fungi, and pleuropneumonia-like organisms.

Results

Three continuous cell lines have been established from peripheral blood of the subjects studied. Two are from the 7 year old male patient in the accelerated phase of the Chediak-Higashi syndrome (Homozygous Lines) and one is from his father (Heterozygous Line). None of the other thirty-six attempts at culture were successful. Increasing acidity of the medium, clumping of cells in suspension, a marked increase in cell numbers, and the ability to continuously pass the cell line were the signs of successful culture. These were apparent by day 48 and day 62 of culture in the Homozygous Lines and day 75 in the Heterozygous Line. No supplemental feeder layers were required, although some cells adherent to glass were seen during the first few weeks of culture. The cell lines have continued in suspension culture for five to nine months.

In the three lines, the cells have a doubling time of twenty-four to forty-eight hours and attain a stable density of 1-3 \( \times \) 10⁶ cells per ml. of culture medium. They grow as free-floating pleomorphic cells with a tendency to clump which can be overcome by gentle agitation. In Giemsa-stained preparations, morphologic variation in the primarily lymphoblastic cells is evident: epithelial and fibroblastic elements are absent. The cells are, in general, more immature than those from cell lines derived from patients with infectious mononucleosis (Fig. 1). Many cells have large, lightly stained, often reticular nuclei with prominent nucleoli and a moderate amount of cytoplasm (Fig. 2); binucleate cells are also present. Cells similar to young lymphocytes with a small to moderate amount of cytoplasm, relatively immature nuclei and no nucleoli as well as very large and sometimes multinucleated cells with abundant cytoplasm and numerous uropodia are occasionally noted. Cytoplasmic vacuolization is extensive in many cells.

Many cells in the Homozygous Lines (Fig. 2) contained large, discrete, 1.0-2.5 micra azurophilic granules in the cytoplasm. A few cells contained a single, very large (5-10 micra) inclusion vacuole with a clear zone which contains azurophilic material. A small percentage of the cells from the
Heterozygous Line (Fig. 3) contained similar, although smaller and less numerous, azurophilic granules. The perinuclear (Golgi) zone was strikingly enlarged in cells of this line, more so than in cells of the Homozygous Lines. This zone in all three Chediak-Higashi lines was large and more prominent than in the lines from other sources used for comparison purposes.

Histochemical staining of the cells in the Homozygous Lines and the Heterozygous Line demonstrated that the abnormal granules or inclusions were positively stained with PAS and oil red O, but not Sudan black. These organelles contained acid phosphatase (Fig. 4 and 5) but not peroxidase. This pattern is similar to that of the giant granules of peripheral blood lymphocytes from patients with the Chediak-Higashi syndrome. Giant brilliant orange fluorescent lysosomal granules were prominent in cells of the Homozygous Lines (Fig. 6B) after vital staining with euchrysine. Many smaller, but still abnormally large granules, as well as many normal sized fluorescent granules were also present in these cells. The majority of fluorescent lysosomal granules present in cells of the Heterozygous Line (Fig. 6C) were of normal size, although many abnormally large fluorescent granules are also present. Both lines clearly differed from cell lines derived from patients without the Chediak-Higashi defect (Fig. 6A).

Biosynthetic studies revealed that cells from both Homozygous Lines incorporated 3H amino acids into molecules with IgG (γ) and IgM (μ) heavy polypeptide chain specificity and type K (κ) light polypeptide chain specificity...
Fig. 2.—Modified Romanowsky stains of tissue culture cells from the Chediak-Higashi syndrome homozygous line (×1920). Note the large granular cytoplasmic inclusions. The vacuoles, although prominent, can be seen in many other cell lines from a variety of sources.

at cell concentrations of 0.5 and 1.2 × 10^5 cells/ml. Cells from the Heterozygous Line at a concentration of 10^6 cells/ml. incorporated detectable amounts of radioactive label into molecules with IgG (\(\gamma\)) and IgM (\(\mu\)) heavy polypeptide chain specificity, but not molecules with light chain specificity. The patient had normal serum levels of IgG (4.5–6.7 mg./ml.) and IgM (0.6–1.0 mg./ml.) but quite low levels of IgA (<0.52 mg./ml.) as determined by radial immunodiffusion while the heterozygote’s levels were entirely normal: IgG 9.0 mg./ml., IgM 4.5 mg./ml., and IgA 1.1 mg./ml.

Antiviral activity, assessed by the VSV plaque assay system, was present in the supernatant from the Homozygous Line in low titer, 4-6 50 per cent plaque reducing units/ml. Such activity was not present in supernatant from the Heterozygous Line at any titer, nor in supernatant fluid from short term leukocyte cultures (seven and fourteen days) of all four patients. The antiviral material exhibited the following properties characteristic of interferon: species specificity (no activity in chick embryo fibroblasts); activity against an unrelated virus (Sindbis); destruction of activity by trypsin; non-sedimentability at 105,000 × g.; and loss of activity following pretreatment of cells with Actinomycin D. No conventional viral agents were recovered in any of the tissue culture and animal systems utilized. The material was also pyrogen free in the rabbit assay system. No bacterial, fungal, bacterial L-forms, or pleuropneumonia-like agents were detected in aliquots of any of the suspension cultures.
Fig. 3.—Cells from the heterozygous line (modified Romanowsky stain, ×1920); note that granules are present, but smaller than in the homozygous line.

Thirty-eight per cent of cells from the Homozygous Line and fourteen per cent of the cells of the Heterozygous Line demonstrated phagocytic capacity as determined by the ingestion of polystyrene particles. Neither specific morphologic characteristics nor the presence or absence of abnormal lysosomal granules appeared to be associated with phagocytic capacity.
Fig. 4.—Tissue culture cells from the homozygous line showing strongly positive staining of the cytoplasmic inclusions with acid phosphatase (×1920).

Fig. 5.—Tissue culture cells from the heterozygous line with positive staining of the cytoplasmic inclusions with acid phosphatase (×1920).
Fig. 6.—Vital staining of the tissue culture cells with euchrysine. (A) Infectious mononucleosis cell line showing granules with typical fluorescence. Note large number of small granules and clumping of many. (B) Homozygous Chediak-Higashi cell line which shows the giant abnormal granules. (C) Heterozygous Chediak-Higashi cell line showing normal size lysosomal granules, but also many abnormally large granules are present.

**DISCUSSION**

All three Chediak-Higashi established cell cultures contain a variety of cells with predominantly lymphoid morphology and without epithelial or fibroblastic components. These cells exhibit the morphologic characteristics of primitive or "blastic" lymphoid cells, typical of cells from other lines derived from hematopoietic tissue suspension culture systems. However, they are unique due to the presence of abnormal granules or "inclusions" and the marked prominence of the perinuclear clear zones. The histochemical staining characteristics as well as the euchrysine staining pattern of these granules provide firm evidence that they are lysosomal in nature, paralleling the lysosomal abnormalities present in vivo. These abnormal giant lysosomes do not
appear to interfere with cellular replication, thus paralleling their lack of interference with phagocytosis and killing of bacteria by cells in which they are present.\textsuperscript{18} The lysosomal abnormalities in the Heterozygous Line are distinct but not as common nor as marked as in the Homozygous Line. The perinuclear clear zone abnormality, however, is greater in the Heterozygous than in the Homozygous Lines, although present in both. This latter type of abnormality suggests that investigation of Golgi zone function may afford evidence concerning the nature of the primary defect responsible for the morphologic abnormalities.

The demonstration of distinct abnormalities in the Heterozygous Line is similar to findings reported in other systems.\textsuperscript{4,19} The histochemical staining pattern of the abnormal lysosomes is similar to that of the abnormal lysosomes in circulating lymphoid cells of patients with Chediak-Higashi syndrome.\textsuperscript{19,20} The capacity of these lines to synthesize immunoglobulins also supports the morphologic evidence that these cells are lymphoid in character and origin. The establishment of a cell culture from the spleen of a patient with the Chediak-Higashi syndrome has been reported.\textsuperscript{3} This culture included fibroblastic cells growing on glass as well as cells with lymphoid characteristics. It is probably the presence of a lymphoid component that accounts for the observed loss of contact inhibition demonstrated by this system during its five months in culture. This same system may also represent “lymphoblastoid transformation” of fibroblastic elements as previously suggested for bone marrow cultures.\textsuperscript{21} Giant lysosome-like organelles were seen by electron microscopy in some cells from the Chediak-Higashi splenic cell line, but no histochemical studies were reported. These cells did not grow when injected into the cheek pouches of two Syrian hamsters and no virus-like particles were detectable by electron microscopy. Cells from a lymph node of a second patient failed to grow in culture. Skin fibroblast cultures have been established from a third patient with the Chediak-Higashi syndrome and also from both her parents.\textsuperscript{19} Abnormally large acid phosphatase granules were present not only in the fibroblasts from the affected subject, but also from both heterozygotes in whom the abnormalities were less pronounced.

The establishment of fibroblast cultures or mixed cell cultures containing fibroblasts occurs regularly independent of the source of cells. However, with the small blood volumes and the technics used in the present study, we have not been able to establish continuous cell cultures from peripheral blood of normal individuals, nor has this been reported from other laboratories.\textsuperscript{22} Establishment of continuous culture lines from both the homozygous patient and his heterozygous father would suggest that they exhibit an increased proliferative potential above the normal range.

The establishment of long-term suspension cultures with lymphoid characteristics derived from peripheral blood has been reported in patients with infectious mononucleosis,\textsuperscript{7,22} a variety of leukemias and lymphomas,\textsuperscript{23-26} as well as from normal individuals.\textsuperscript{17} The establishment of cultures from normal individuals, however, has required special technics involving large numbers of
cells\textsuperscript{17} or exposure to lethally irradiated Burkitt's lymphoma cells containing a Herpes virus-like particle.\textsuperscript{22} Herpes-like viral antigen and a C group chromosomal marker, which have been demonstrated in many of the suspension cultures obtained from leukocytes of normal subjects, may be related to their proliferative potential. The cells of the Homozygous Line produce interferon, as do cells in similar lines derived from patients with infectious mononucleosis\textsuperscript{14} and Burkitt's lymphoma.\textsuperscript{28} However, no inducer of interferon production could be identified nor were any viruses recovered. Whether viral agents are present in vivo in the cells obtained for culture, are introduced as laboratory artifacts, or released from a latent stage upon being subjected to in vitro conditions, is not known. All of the above data would suggest a possible relationship between clinical states of lymphoproliferative activity, latent infection with viral agents, and the potential of such cells to replicate in long-term suspension culture. The lysosomal defect in Aleutian mink (analogous to the Chediak-Higashi syndrome in man) is associated with an increased susceptibility to a slow viral agent (Aleutian agent).\textsuperscript{29} Infection with the Aleutian agent results in widespread lymphoid and plasma cell proliferation and organ infiltration similar to the accelerated phase of the Chediak-Higashi syndrome in humans. The establishment of long-term suspension cultures from peripheral blood in the Chediak-Higashi syndrome may reflect a parallel increased susceptibility to agents inducing a state of altered proliferation. The relationships between this proliferative capacity, the genetically determined lysosomal abnormality, viral agents, and karyotypic alterations is presently under investigation. These cell lines may be of use in further defining the fundamental defect of the Chediak-Higashi syndrome, the nature of the heterozygous state and in addition, may be useful as tools for the investigation of the multiple cell functions in which lysosomes play a significant role.

**Summary**

Long-term suspension cultures have been established from peripheral blood of a patient with the Chediak-Higashi syndrome and his father (a heterozygote). The cells in these lines were principally lymphoblastoid in appearance, synthesized immunoglobulins and interferon, and demonstrated a modest phagocytic capacity. Giant abnormal granules characteristic of the Chediak-Higashi syndrome were present in the Homozygous Lines, and abnormally large granules were also present in the Heterozygous Line. Histochemical and euchrysin supravital staining demonstrated the lysosomal nature of these granules. Striking Golgi zone prominence was present in the Heterozygous Line, and to a lesser extent, in the Homozygous Lines. The potential significance of the establishment of these unique lines for investigations of the Chediak-Higashi syndrome, the nature of the heterozygous state, and role of lysosomes in general is discussed.

**SUMMARIO IN INTERLINGUA**

Culturas suspensional a longe termino esseva establite con sanguine peripheric ab un patiente con le syndrome Chediak-Higashi e ab su patre (un heterozygotico). Le cellulas in iste lineas esseva principalmente lymphoblastoide in apparentia. Illos synthetisava
THE CHEDIAK-HIGASHI SYNDROME

immunoglobulinas e interferon e manifestava un moderate capacitate phagocytic. Gigante granulos anormal characteristic del syndrome Chediak-Higashi esseva presente in le linea homozygotic, sed le granulos presente in le linea heterozygotic esseva etiam anormalmente grande. Tincturation histochimic e tincturation supravital a euchrysin demonstrava le natura lysosomal de iste granulos. Prominente zonas de Golgi esseva note in le linea heterozygotic e—ben que minus frappantemente—in le linea homozygotic. Es commentate le signification potential del establimento de iste unic lineas pro investigationes del syndrome Chediak-Higashi, le natura del stato heterozygotic, e le rob de lysosomas in general.

ACKNOWLEDGMENTS

We would like to thank the Bacteriology Service, Clinical Center, National Institutes of Health, for the numerous attempts to isolate bacteria, bacterial L-forms, pleuropneumonia-like organisms, and fungi from the cell cultures.

REFERENCES


The Chediak-Higashi Syndrome: Continuous Suspension Cultures Derived From Peripheral Blood

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