Human Chronic Myelogenous Leukemia Cell-Line With Positive Philadelphia Chromosome

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A cell-line derived from a patient with chronic myelogenous leukemia (CML) is described. The new cell-line, which has over 175 serial passages in a 3½-yr period, has the following characteristics: (1) CML cells started to proliferate actively since they were first incubated in culture media. A threefold increase in the total number of cells was observed during the first seven passages; the cell population increased by a factor of 10 to 20 every 7 days from passage 8 through 85; from 20 to 40 times from passage 86 through 150, and more than 40 times after 150 passages. (2) The majority of the nononucleated cells are undifferentiated blasts. (3) The karyotype of all the cells examined show the Philadelphia (Ph') chromosome and a long acrocentric marker plus aneuploidy. The Giemsa-banding studies identified the Ph' chromosome as a terminal deletion of the long arm of chromosome 22:del(22)(q12) and the long acrocentric marker as an unbalanced reciprocal translocation of one chromosome 17 and the long arm of one chromosome 15. (4) The CML cells do not produce immunoglobulins, are free of mycoplasma, Epstein-Barr virus, and herpes-like virus particles. (5) CML cells have no alkaline phosphatase and myeloperoxidase activities and did not engulf inert particles. (6) Cultured CML cells provide a constant source of a specific antigen. This CML cell-line represents a unique source of CML cells with meaningful indicators of malignancy for clinical and experimental studies.

Attempts to establish permanent cell-lines derived from the blood of patients with chronic myelogenous leukemia (CML), having the Philadelphia (Ph') chromosome marker, have been consistently unsuccessful, and cultures showing the Ph' chromosome for several months have lost the Ph' chromosome after serial subcultivation for longer periods of time. In all these cultures, the leukemic cells were replaced by immunoglobulin-producing (lymphoid) cells. Recently, the growth and maturation of CML cells was studied in liquid media. Bone marrow and peripheral blood cells from seven patients with Ph'-positive CML were cultured in diffusion chambers for up to 45 days. The Ph' chromosome was found in only two cultures. The Ph' chromosome was also found in short-term soft-gel media cultures.

Preliminary reports from our laboratory showed persistence of the Ph' chromosome 20 mo after establishment of a CML cell-line from a pleural effusion of a terminal case in blastic crisis. These cells have continued proliferating very rapidly and maintain their positive Ph' chromosome and a long acrocentric rearrangement after continuous culture for 3½ yr and over 175 serial passages.

The purpose of this report is to describe the origin, culture method, and characteristics of this unique cell-line.
MATERIAL AND METHODS

Case History

The cell line described herein was derived from leukemic cells obtained in December 1970 from a pleural effusion of a 53-yr-old female suffering from CML for about 4 yr. She had been treated with busulfan for 3 yr and pipobroman for 1 yr. In October of 1970, all peripheral blood cells showed a positive Ph chromosome and a clonal evolution of the leukemia with a new cell-line of 45 chromosomes and a translocation D/E. In November of 1970, a splenectomy was made. Soon after surgery the patient underwent blast crisis, and the malignant cells accumulated as tumor masses of highly undifferentiated cells through most tissues of the body and produced pleural effusion. Nine days prior to the patient’s death a sample of the pleural fluid was obtained and used for chromosome analysis in short-term cultures, and to start the long-term cultures. A positive Ph chromosome and a long acrocentric chromosomal rearrangement were observed in all cells. The aggressiveness and extensiveness of the malignant infiltrate suggested marked alteration of the original leukemic cells, and the presence of a positive Ph chromosome in addition to the lack of structural patterns of the tumor masses provided evidence of the myeloid origin of these neoplastic cells.

Establishment of a CML Cell-line in Suspension Cultures

The pleural fluid was collected with heparin and aliquots containing 15–20 × 10⁶ cells each were diluted with Eagle’s minimal essential medium (MEM) plus additional amino acids and 15% fetal bovine serum. All cultures were incubated at 37°C in a humidified atmosphere with constant flow of 5% CO₂ in air. The cells grew as a free-floating cell suspension since they were first incubated in liquid cultures. Serial subcultivations of aliquot samples were carried out weekly after determination of the total number of cells per culture. The cell-line was named CML-1, K-562.

Freezing and Thawing of Cultured Cells

Samples of cells were frozen at various passages using Eagle’s medium supplemented with 5% glycerol. The cells were frozen at 1°C per min and the ampules stored in liquid nitrogen. The ampules were thawed rapidly, and the cells were suspended in culture medium after determination of their viability with 0.5% trypan blue.

Colony Growth in Agar-gel and Isolation of Cell Clones

The method of Robinson and Pike was followed with slight modifications. The plates, made up of only one layer of CML cells mixed with 3 ml of semisolid agar diluted with McCoy’s 5A medium were incubated at 37°C under controlled pH and humidity for 12–14 days. The number of colonies per plate and the plating efficiency of the CML cell-line were determined at various passages. The plating efficiency was also investigated in plates containing a feeder layer of peripheral leukocytes (2 x 10⁶) from normal humans.

The agar-gel method was used also to isolate clones of cells derived from single cells. The clones were isolated by picking individual colonies which were transferred to Leighton tubes with 1 ml of medium. Each colony grew as a suspension culture and was transferred to culture bottles with increased amounts of media until the cell density was similar to the original cell line K-562. Each clone was maintained by weekly passage. After chromosome analysis of all the clones, samples from each clone were stored frozen in liquid nitrogen.

Cytogenetic studies

Chromosome preparations were made at various passages according to the procedure of Moorhead et al. These smears were stained with Giemsa dye after hydrolysis with 1 N HCl. Chromosome analyses were also performed in smears stained by the Sumner and Evans’ Giemsa-bandung technique, and by the fluorescence method of Caspersson et al.

The length of the cell cycle was determined at various passages by the technique of labeled mitosis of Quastler and Sherman. A suspension containing 10⁵ cells/ml was pulse labeled for
15 min with 1 μCi/ml of 3H-thymidine (specific activity, 1.9 Ci/mM: Schwarz/Mann, Orangeburg, N.Y.). These cells were centrifuged, rinsed with balanced salt solution, and resuspended in fresh culture medium without the isotope. Samples of 10 ml were incubated at 37°C until fixation at 30 min, 1,2,3,4,5,7,9,11,13,15,17,19,21,23, and 25 hr after pulse labeling with 3H-thymidine. The radioautographs were exposed for 10 days, developed with D 11. stained with Giemsa dye. The per cent of labeled versus unlabeled metaphases was determined.

**Cytology**

The morphology of CML cells growing either in liquid or semisolid media was studied periodically on smears stained with Giemsa dye.

**Enzyme Studies**

The Graham-Knoll peroxidase reaction for general differentiation and cytochemical demonstration of alkaline phosphatase according to Gomori technique were performed on smears of normal peripheral leukocytes (control) and cultured CML cells.

**Viral Testing**

Cultured cells were tested for Epstein-Barr virus (EBV) genome and EBV-associated nuclear antigen referred to as ENBA. These tests were kindly made by Dr. Gertrude and Dr. Werner Henle of the Division of Virology, Children's Hospital, University of Pennsylvania, Philadelphia, Pa. Mycoplasm (PPLO) contamination was tested using media and supplements from Microbiological Associates, Bethesda, Md., following the method of Hayflick and Stanbridge, and Hayflick. The media and cells were monitored for PPLO continuously. Tissue culture reagents were also tested for mycoplasma before use following the procedures of Barile and Kern.

**Immunoglobulin (Ig) Determination**

The technique of Mancini et al. was followed throughout with slight modifications. Each plate contained agar impregnated with goat monospecific antiserum. Cells, cultured for 7 days, were centrifuged. The concentration of Ig was determined in nonconcentrated and 10 x concentrated media. The media were concentrated by ultrafiltration. A cell pellet containing 20 x 10^6 CML cells was washed once with cold saline disrupted by sonic vibrations, and an aliquot assayed for Ig.

**Phagocytosis**

The ability of CML cells to ingest polystyrene particles was determined following the technique of Cline and Lehrer. The culture medium was the same used to grow the cells in suspension. Polystyrene particles (1.3-1.7 μ in diameter) were added to tissue culture medium at the rate of 1 mg/ml. Five milliliters of the suspension were mixed with either 2 x 10^6 CML or normal leukocytes (control) contained in 5 ml of medium. The cells and polystyrene particles were incubated for 1 hr at 37°C with shaking. Intracellular particles were studied by phase-contrast microscopy.

**RESULTS**

**Growth Characteristics in Free-floating Suspension Cultures**

The CML cells started to proliferate actively as soon as they were incubated in culture medium. No latent period was observed, but the rate of growth varied at various passages as follows: one to three times increase per passage during the first seven passages, 10-20 times from the 8th to the 85th, 20-40 times from the 86th to the 150th, and 40-70 times from the 150th to the 175th passage. A corresponding decrease in the mean generation time was observed. This was approximately 50 hr during the first seven passages, and 10-12 hr after passage...
85. The decrease in the total cell cycle was probably due to a shortened G₁ stage as seen in cells studied at passage 96. At this passage, the total cycle lasted 12 hr, including a period of DNA synthesis (S) of 8 hr, a postsynthetic stage G₂ of 2 hr, and a G₁ plus mitotic period of 2 hr.

**Cytologic Features of CML Cells**

The morphologic characteristics of CML cells at different passages of the culture were those of highly undifferentiated cells of variable size (Fig. 1A). The majority of the round or oval cells measured 14-17 μ in diameter, approximately 25% of these cells had 20–35 μ. 10% had 7–8 μ, and 5% 40–55 μ. Polyploid cells were frequently seen. There was not a sharp delimitation between the nucleus and cytoplasm in the majority of the cells. The cytoplasm was intensely basophilic, devoid of granules, and contained few small vacuoles. Atypical promyelocytes with eccentric nucleus and scanty azurophilic granules in the cytoplasm account for less than 2% of the cell population. Several cells, with a diameter of 20 μ or over, had deeply basophilic cytoplasmic buds which in some cells were extremely large: one-third of the cell diameter. The nuclei were round, slightly indented, and, occasionally, of semi-moon-like shape during interphase. One to four nucleoli were easily discernible in each nucleolus, regardless of the cell size. Large-sized nucleoli were often uncovered so that their blue-violet color was very prominent. The chromatin was irregularly granular or formed fine laced threads which stained reddish violet with Giemsa. Numerous mitotic figures and binucleated cells were also seen. Another distinct cell type was characterized by a pale blue cytoplasm and a large nucleus, containing two to six blue nucleoli within long skeinlike threads of chromatin. These cells developed and proliferated particularly well on agar (Fig. 1B).

**Growth and Colony-forming Ability in Agar**

The number of colonies was directly proportional to the number of cells plated, as demonstrated in triplicate plates simultaneously made with 250, 500, and 1000 CML cells. Small round colonies containing less than 100 cells were seen by day 5 of incubation, and colonies containing more than 2000 cells were seen on day 14 of culture. The plating efficiency was between 50% and 60%, with an average of 582 ± 67 (1 SD) colonies in the plates inoculated with 1000 cells without a feeder layer. In general, colonies arose from single cells, but occasionally groups of two to three cells were present and originated even larger colonies than those described above.

In five experiments, a feeder layer of normal peripheral leukocytes was added to the hard agar underlayer, and CML cells were added on top with the soft agar layer. The plating efficiency was reduced by a factor of 5–6, and no effect on CML cell differentiation and maturation was observed.

The morphology of all cells in 156 colonies examined was nearly identical (Fig. 1B–D). Two main highly undifferentiated cell types proliferated but did not differentiate into more mature forms of the granulocytic series. All cells growing on agar were very large (40–60 μ in diameter) and exhibited numerous vacuoles in the narrow band of deeply basophilic cytoplasm surrounding the nucleus.
Fig. 1. (A) Composite photograph showing chronic myelogenous leukemia (CML) cells growing in 7-day-old suspension cultures. (B-D) CML cells developing in semisolid agar in a 15-day period. A small part of a large colony is seen in (D). Both liquid and agar cultures were made on passage 168 of the CML cell line K-562. Giemsa stain. × 540.
Cytogenetic Studies of the Patient Cells in Short-term Cultures

A description of the cytogenetic studies of the patient's cells is given herein as a control for the cultured CML cells to show their origin in clones of cells with abnormal karyotypes. A positive Ph' chromosome was seen in all the metaphases studied from peripheral blood in October 1970. Two modal numbers of chromosomes were observed: 28 cells had 45 chromosomes and 22 cells had 46 chromosomes. The cells with 46 chromosomes had a Ph' chromosome in an otherwise normal female karyotype, and the cells with 45 chromosomes had a positive Ph' and had lost one member of the group D (13-15) and one of group E (17-18) with apparent gain of one submetacentric of group C (6-12-X) (Fig. 2), which was interpreted as a reciprocal translocation D/E.

Fig. 2. Karyotype 45,XX,-D, -E, +t(D;E) observed in 24-hr cultures of peripheral blood sustained from the patient 2 mo before the long-term cultures were started. Note the absence of one chromosome from group D and one from group E (probably a number 17) and the presence of an extra C-like chromosome. The arrow indicates this chromosome, which was interpreted as a translocation between one chromosome of group D and one of group E. The Philadelphia chromosome (Ph') is also identified by an arrow. Fifty per cent of the cells cultured from peripheral blood had this karyotype and the other 50% had a karyotype 46,XX,Ph' +.
Fig. 3. Karyotype of an hypodiploid cell showing a long acrocentric marker and a positive Ph' chromosome. This marker and the Ph' chromosome were observed in all the cells analyzed in short-term cultures of 24 hr from the same pleural effusion used to start the long-term cultures.

The cells from the pleural effusion, obtained in December 1970, showed marked aneuploidy with a large proportion of hypodiploid cells. Ninety-two of one hundred cells in metaphase had a chromosome number varying from 40 to 45 chromosomes. All the cells had a Ph' chromosome and a long acrocentric chromosome significantly larger than the other members of group D (13–15) (Fig. 3) and a consistent loss of one chromosome of group E (16–18).

Cytogenetic Studies in Long-term Cultures of the CML Cell-line at Various Passages

Chromosome analyses of the cultured CML cells were performed at passages 5, 10, 20, 40, 90, 110, 144, and 171. One hundred cells were studied, and five to
ten karyotypes were analyzed from each passage. These studies revealed that all the cells contained at least one Ph' chromosome, plus the long acrocentric marker described in the short-term cultures of the pleural effusion. A small percentage of cells with three or four Ph' chromosomes was seen, but they were not directly related to either the age of culture or the age of the cell-line K-562.

Changes in the modal number of chromosomes, as well as appearance of new clones with additional chromosome markers, were observed at different pas-

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Fig. 4. Karyotype of a pseudodiploid cell with 46 chromosomes stained with the G-banding technique. This cell was observed in long-term cultures of the cell-line CML K-562 at passage 110. The banding pattern shows that the long acrocentric chromosome is probably a translocation between one chromosome 17 and one chromosome number 15, and the Philadelphia chromosome is a deletion of half of the long arm of one chromosome number 22. Note the presence of an extra chromosome number 1 and one number 22 and the absence of one chromosome number 11 and one number 12.
sages. By the passage 5, the cells maintained their hypodiploid mode (40–45), and the majority of the cells have lost one group C chromosome (6-12-X), while the minority of the cells have lost a chromosome number 1, or one of the chromosomes of group D (13–15), E (16–18), or F (19–20). On passages 10 and 20, the majority of the cells had an hyperdiploid modal number of 50–52 chromosomes. At passage 84, two sublines were started by performing serial passages at different days of the week. At passage 110, karyotypes of the two sublines were studied and revealed a predominant near triploid mode (69–73) in one subline and a near tetraploid mode (90–96) in the other subline. A new marker chromosome appeared in the near tetraploid subline. These cells had a chromosome number 2 with a significant elongation of the long arm (2q+) in addition to the long acrocentric and one to four Ph' chromosomes.

To demonstrate if cells growing on agar had the same chromosome markers as those cultured in liquid medium, several individual colonies were removed from the agar and cultured in suspension. The cells isolated from semisolid agar showed the same Ph' chromosome and the marker present in the blasts proliferating in the pleural effusion before the suspension cultures were started.

Ten clones were isolated: seven were aneuploid with a predominant near triploid mode (67–73), two had two modal numbers, and one clone had hyperdiploid cells with a modal number of 52 chromosomes. Repeat chromosome analyses of the hyperdiploid clone showed that near triploid cells had overgrown the hyperdiploid cells 4 mo after the clone was established. All the karyotypes analyzed from these clones had one to four Ph' chromosomes and a long acrocentric marker.

Chromosome analyses with the G-banding technique (Fig. 4) were performed at passages 110 (January 1973), 144 (September 1973), and 171 (April 1974) of the original line. The hyperdiploid clone was studied at passages 8 and 21 after cloning. Q bands were investigated at passage 144. The G- and Q-banding studies demonstrated that the Ph' chromosome is a terminal deletion (del) of the segment distal to band 12 of the long arm (q) of one chromosome number 22. Therefore, the Ph' chromosome may be designated as a del(22) (q12), according to the nomenclature established at the Paris Conference. The banding studies also showed that the long acrocentric marker is an unbalanced reciprocal translocation between one chromosome 17 and one chromosome number 15 (Fig. 5). This translocation involved the breakage of the long arm (q) of chromosome 17 at the band number 24 and the long arm of chromosome 15 at band number 21. Therefore, the translocation is reported as t(15;17) (q21;q24) according to the Paris Conference. This translocation originated in vivo as the result of clonal evolution and persisted in vitro for over 175 serial passages. Other chromosomes, including chromosome number 9, had normal G-bands in contrast to reports from other cases with CML, but new rearrangements appeared as the result of in vitro clonal evolution.

In summary, the banding studies have identified several chromosomal struc-
Fig. 5. Partial karyotype of six mitotic cells from passage 171 of the CML cell-line stained with the Giemsa-banding technique and diagrammatic representation of the Giemsa bands. The first row shows the translocation of the distal half of the long arm of one chromosome 15 to the end of the long arm of chromosome 17. This translocation was seen as a marker in all the cells studied in the short-term cultures and in all the cells analyzed at various passages of this cell line. The normal chromosomes numbers 17, 15, 21, and 22 are shown on the second, third, fourth, and fifth rows. The law row demonstrates the terminal deletion seen in all the Ph chromosomes.

The modal number of chromosomes has changed in vitro with spontaneous evolution of new sublines and clones with faster growth rates and increased numbers of chromosomes. Thus, clones containing two cell types are overgrown by the cell type with the largest chromosome number. For this reason, repeated cloning of the original cell-line in agar plates and selective isolation of clones with pseudodiploid karyotypes are required to maintain a source of pseudodiploid CML cells with characteristic chromosome aberrations available for experimental use.
Enzyme Studies

Normal granulocytes exhibited a strongly positive peroxidase and phosphatase reaction, whereas cultured CML cells had consistently negative reactions in ten slides containing several hundred cells each.

Viral Antigens

The CML cell-line was found to be free of the EBV-associated nuclear antigen which is present in most lymphoblastoid cell-lines carrying the EB viral genome. Routine tests for mycoplasma were always negative.

Immunoglobulins

A search for Ig in the CML cells and culture media failed to reveal any detectable amount of IgA, IgG, and IgM (Fig. 6).

Phagocytic Activity

Monocytes were more efficient than neutrophils to engulf polystyrene particles. After 60 min of incubation, a greater number (six or more) of polystyrene

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Fig. 6. Quantitative determination of immunoglobulins (Ig) by single radial diffusion. (A–C) Reference sera: IgG, 4, 10, and 20 μg/ml; IgM, 0.32, 0.80, and 1.60 μg/ml; and IgA, 0.8, 2.0, and 4.0 μg/ml. (D–F) Test samples: (D) Undiluted culture medium, (E) tenfold concentrated culture medium, and (F) disrupted CML cell suspension containing 20 × 10⁶ cells/ml. Media and cells were from 5-day-old cultures of the CML cell-line K-562. No detectable amounts of IgG, IgM, and IgA could be demonstrated in the media and the CML cell pellet at several passages of the culture.
were seen in monocytes, whereas two to five particles were observed in neutrophils and eosinophils. In marked contrast, CML cells cultured in liquid or semisolid media did not ingest polystyrene particles. Also, none of the large CML mononuclear cells developing in semisolid medium phagocytize agar.

**DISCUSSION**

We described herein a unique cell-line derived from a pleural effusion of a patient with CML. As far as we are aware, the CML-1, K-562 is the first permanent cell-line available with a persistent positive Ph' chromosome after prolonged in vitro cultivation. We believe that the successful establishment of this cell-line is due to the active proliferative capacity of highly undifferentiated cells obtained from the patient at the terminal stage of an acute blastic crisis. These cells were derived from a clone with a Ph' chromosome and a new chromosome rearrangement involving the long arm of one chromosome 17. Rearrangements of the long arm of chromosome 17 have been reported in CML patients in blastic crisis and might be another cause of the increased growth rate.

The leukemic origin of the cultures derived by others from CML leukemic blood may be the result of differential in vitro proliferative capacity of normal and leukemic cells. Indeed, the critical factor could be the absence of normal cells in the pleural effusion used to start our cultures. The malignant CML blasts (K-562) proliferated well in suspension cultures and retained the original karyotype because they were derived from a clone of malignant cells with high proliferative capacity which were already growing as a cell suspension in the pleural fluid and were not overgrown by the proliferation of normal cells, as has occurred with most permanent cell-lines established from human leukemic blood.

The myeloblastic cell-line (K-562) described herein differs from the lymphoblastoid cell lines described by others (see reference 4) in many characteristics. The CML cells have a positive Ph' chromosome, they lack immunoglobulins, they are negative for EBV or herpes-like virus, and began to proliferate actively as soon as the suspension cultures were started. In contrast, lymphoblastoid cell-lines established at other laboratories began to proliferate rapidly after a latent period of 4–15 wk, during which the number of cells decreased significantly. After establishment of the lymphoblastoid cell-line, herpes-like virus particles were detected by electron microscopy, and most cell-lines carried the EBV genome. It is believed that an interaction between EBV and human diploid cells is essential for the establishment of a permanent lymphoblastoid cell-line. Also, the majority of these cell-lines synthesize immunoglobulins. Preliminary electron microscopic examinations of CML cells (K-562) fail to show herpes-like virus particles. Therefore, the absence of EBV and herpeslike virus and the failure of this CML cell-line to produce immunoglobulins are three important differential characteristics that indicate that the line (K-562) is not another culture of lymphoblastoid cells. Furthermore, the presence of the Ph' chromosome conclusively demonstrates that this cell-line is of CML origin.

As expected, the undifferentiated CML cells were devoid of alkaline phospha-
tase and peroxidase activity, and lacked the capacity of ingesting inert particles, a property of more differentiated granulocytic cells and macrophages.

The CML cell-line has been found to be a continuous source of a specific antigen. Work in progress indicates that a rabbit anti-CML K-562 serum adsorbed on normal granulocytes is cytotoxic for cultivated CML cells and for CML cells obtained from untreated patients with this disease. The cross-reactivity of the rabbit anti-CML serum with cultured CML cells and those obtained from patients with CML before treatment suggests a common antigen, which may be of value for the detection, prognosis, and/or immunotherapy of CML.

In summary, this new CML cell-line derived from undifferentiated myelogenous leukemic cells with karyotypic clonal evolution is the first human CML cell-line available which has retained meaningful indicators of malignancy: chromosome aberrations, cloning efficiency on agar, and a specific antigen(s) after prolonged cultivation. Therefore, it represents a unique source of human CML cells for experimental and clinical studies.

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