Absence of Thymus-derived Lymphocyte Markers in Myelogenous Leukemia (Ph\(^1\)+) Cell Line K-562\(^1\)

Carmen B. Lozzio, Bismarck B. Lozzio, Wen-Kuang Yang, Albert T. Ichiki, and Elena G. Bamberger

University of Tennessee Memorial Research Center, Center for Health Sciences, 1924 Alcoa Highway, Knoxville 37920 [C. B. L., B. B. L., A. T. I., E. G. B.], and Biology Division, Oak Ridge National Laboratory, Oak Ridge (W-K. Y.), Tennessee 37820

**SUMMARY**

The myelogenous leukemia cell line K-562 with a Ph\(^1\)+ chromosome, derived from a patient with chronic myelogenous leukemia in terminal blast crisis, is not a bone marrow-derived lymphoblastic cell line, because the cells neither produce immunoglobulins nor possess complement receptors. Since it has been suspected that blasts found in some patients with chronic myelogenous leukemia in blastic crisis might be thymus-derived cells, we have studied several parameters to demonstrate that K-562 cells are not thymus-derived lymphoblasts. The results of this study show: (a) no cross-reactivity of antisera to K-562 cells with normal human thymocytes; (b) lack of cytotoxicity of a specific horse anti-human thymocyte globulin for K-562 cells; (c) failure of the treatment of K-562 cells with bovine thymosin to induce antigenic determinant and erythrocyte rosette receptors on K-562 cells; (d) presence of receptors for the Fc portion of immunoglobulin G; (e) absence of terminal deoxynucleotidyl transferase; and (f) cytotoxicity of monkey antiserum to K-562 cells for malignant thymus-derived cells (Molt-4). However, absorption with Molt-4 cells abolished the cross-reactivity with Molt-4 cells, whereas 60% of the antibody to K-562 cells remained in the immune serum. Studies of DNA polymerase activities revealed that K-562 cells have levels of polymerase \(\alpha\) and \(\beta\), like other proliferating cells, and an RNA-dependent DNA polymerase activity, presumably representing polymerase \(\gamma\).

**INTRODUCTION**

Current evidence suggests that CML\(^2\) may be considered a clonal type of leukemia involving a pluripotential stem cell, because the Philadelphia (Ph\(^1\)) chromosome, derived from a patient with chronic myelogenous leukemia is found in granulocyte and erythrocyte precursors, megakaryocytes, and probably in monocytes \((6, 29)\). Much discussion has centered lately on the terminal blastic crisis of CML as one common cause of death in CML patients. It has been postulated that CML may be a "preleukemic" condition that ends in a terminal blastic crisis indistinguishable from acute myelocytic leukemia \((20)\). It has also been suggested that some patients with CML might have a terminal lymphoblastic rather than a myeloblastic crisis \((3)\).

Recently, we have described a unique cell line \((K-562)\) that contains 100% Ph\(^1\)+ myelogenous leukemic blasts, obtained from a patient with CML in a terminal blastic crisis \((17)\). These cultured K-562 blasts are not B-cells, because they neither produce immunoglobulins nor possess complement receptors. The cells are free of EBV genome, EBV-associated nuclear antigen, and leukemia-associated nuclear antigen, and lack other lymphoid cell markers characteristic of T- and B-cells \((10, 15, 26)\). Further evidence that the highly undifferentiated cells of K-562 are not T-lymphoblasts is presented in this report. This conclusion is based on the results of studies investigating: (a) the cross-reactivity of heteroantisera to K-562 cells for T- and B-cells; (b) the cytotoxicity against K-562 of an antibody specific for human thymocytes; (c) the induction of antigenic receptors for the antibody to thymocytes and of rosette formation by treatment of cells with bovine thymosin; (d) the presence of Fc receptors; and (e) the activities of DNA polymerases and of TdT in K-562 cells.

**MATERIALS AND METHODS**

**Human Cell Lines.** The K-562 cell line was established, maintained, and characterized at the University of Tennessee Memorial Research Center \((17)\); it has reached its 300th passage after more than 5 years in culture. The karyotype of K-562 cells shows the Ph\(^1\) chromosome and a translocation t\((15;17)\). These cells are free of Mycoplasma and EBV genome \((17)\).

The CCRF-SB cell line was developed by Foley et al. \((9)\) from the peripheral blood cells of a patient with ALL. These cells are EBV-associated nuclear antigen positive, possess complement receptors and surface immunoglobulins, and have most of the properties of B-cells \((1, 9, 12, 13)\). The cells in frozen ampuls were kindly provided by Dr. T. G. Tachovsky, Wistar Institute, Philadelphia, Pa. These cells were maintained through the Molecular Anatomy Program at Oak Ridge National Laboratory, Oak Ridge, Tenn. The CCRF-SB cells were cultured in MEM, pH 6.8 to 7.2, with 10% FCS for a short time during which the experiments were carried out.

Another lymphoblastic cell line (Molt-4) was kindly given by Dr. J. Minowada of RPMI, Buffalo, N. Y., and was main-
tained in RPMI Medium 1640 supplemented with 10% FCS. The Molt-4 cell line was developed by Minowada et al. (19) from the peripheral blood of a patient with ALL. The Molt-4 cells are a subpopulation of T-cells that form rosettes with sheep erythrocytes and have no detectable immunoglobulins, complement receptors, or EBV genome (19).

Human Thymocytes. A normal thymus was removed from a 3-year-old boy during open heart surgery. Another thymus was obtained 6 hr after death from a 3-day-old child who had died as a result of multiple congenital malformations. Each thymus was gently teased and filtered through a fine nylon mesh to eliminate gross aggregates. Thymocytes were suspended in MEM containing 10% FCS and made up to the desired concentration. Cell viability was determined by use of the trypan blue dye exclusion test. Thymocytes were 80 to 90% viable when assayed.

Heteroantisera to Myelogenous Leukemic Cells. The development and characterization of a rabbit antiserum cytotoxic for cells from the K-562 cell line have been reported previously (30). Similarly, an antiserum cytotoxic for the K-562 cells was raised in a nonhuman primate (16). Heteroantisera were first absorbed on peripheral blood cells of AB type and then on bone marrow cells from normal humans. The absorbed antiserum to K-562 cells contained antibodies to 2 immunoelectrophoretically distinct FCS constituents, which were removed by affinity chromatography as reported previously (30). The monkey serum obtained after the 2nd and 3rd immunization schedules had a titer of 1:560 (50% cell death). The titer of rabbit sera ranged from 1:60 and 1:120 throughout the period of immunization. Heteroantisera used in the present study were from the 2nd and 3rd immunization schedules.

Anti-Human Thymocyte γ Globulin. This reagent was obtained from the Hypersensitivity Diseases Division of the Upjohn Co., Kalamazoo, Mich. Lot 17900-2 was made available through the courtesy of Dr. W. J. Wechter. This lot was absorbed on RBC stroma and human plasma proteins and has been found to be specific for human thymocytes by Dr. W. Hijmans, Radiobiological Institute, Rijswijk (ZH), Holland. When tested in our laboratory, this antiserum was found to have complement-dependent cytotoxicity for normal human thymocytes with an end point of 1:500.

Complement. Lyophilized normal guinea pig serum (BioQuest, Cockeysville, Md.) was used as the source of complement. Prior to use in the cytotoxicity assays, the complement was absorbed on 25 to 30 × 10⁶ appropriate target cells/ml of serum for 60 min at 20°C to remove nonspecific antibodies.

Cytotoxicity Tests. The trypan blue dye exclusion method, and the ⁵¹Cr release assay were used as described previously (30).

Bovine Thymosin. A protein fraction (Fraction V) having thymosin activity was isolated from bovine thymuses following the method of Hooper et al. (11). Thymosin activity was determined by the E-rosette assay using peripheral leukocytes from an ALL patient who had 5 to 8 spontaneous E-RFC in circulation. Thymosin was diluted in RPMI medium 1640 at the rate of 2.5 mg/ml. This solution was 2-fold serially diluted to a final concentration of 62.5 μg/ml. Incubation of the peripheral leukocytes from the ALL patient with 6 different concentrations of thymosin for 2 hr increased the number of E-rosettes up to 3-fold.

E-Rosette Assay. K-562 cells were made up to a final concentration of 5 × 10⁶ cells/ml with RPMI medium 1640 containing 10% FCS. The T-cell rosette formation was detected by the methods described in the WHO report (31) with sheep and chicken RBC. EA-rosettes were prepared with sheep RBC. For EA-rosettes, RBC were sensitized with subagglutinating dilutions of a rabbit antibody to sheep RBC. In addition, 1 × 10⁶ K-562 cells were incubated with 1 mg of thymosin at 37°C for 20 min. Then, the medium was changed, RBC were added to the cell preparation, and the mixture was incubated for 20 hr at 4°C. The percentage of RFC, i.e., cells with 3 or more RBC, was determined by counting 200 to 300 cells under phase-contrast microscopy.

TDT and DNA Polymerase Activities. Analyses of the deoxynucleotide-polymerizing enzymes generally followed the procedures published elsewhere (4, 7, 8, 22–25, 28).

Leukemic cells were harvested in their logarithmic phase of growth in culture, washed 3 times with phosphate-buffered saline, and used immediately. For subcellular fractionation, the cells were homogenized in 10 mm Tris-Cl (pH 8.0) and 1 mm MgCl₂. The homogenate was immediately adjusted to contain 250 mM sucrose, 50 mM KCl, and 3 mM dithiothreitol. The homogenate was then subjected to differential centrifugation at 800 × g for 10 min, 7,000 × g for 10 min, and 198,000 × g for 60 min to obtain “nuclear,” “mitochondrial,” and “microsomal” pellets and the supernatant fractions. Enzyme extraction was made by homogenizing the whole cells or by suspending subcellular fractions in a hypertonc solution [250 mM sucrose, 50 mM Tris-Cl (pH 8.0), 500 mM KCl, and 3 mM dithiothreitol] and by subsequent centrifugation at 198,000 × g for 60 min. The supernatant fluid was used for direct enzyme measurement and sucrose gradient sedimentation analysis. The enzyme extraction was also done in the hypertonc solution containing 0.5% Triton X-100.

For sedimentation analyses, the extract (0.15 ml) was layered on top of 3.8 ml solution containing 10 to 30% sucrose linear gradient [50 mM Tris-Cl (pH 8.0), 500 mM KCl, and 5 mM dithiothreitol]. The gradient included 0.1% Triton X-100 when extracts were analyzed for RNA-dependent DNA polymerase. Centrifugation was performed at 50,000 rpm (246,000 × g) for 14 hr at 40°C in a Spinco SW 56Ti rotor. Fractions of 0.2 ml were collected from the bottom of the tube and assayed for enzyme activities.

Determination of deoxynucleotide-polymerizing enzyme activities was carried out in a basic reaction mixture containing 30 mM Tris-Cl (pH 8.0 at 22°C); 50 to 100 mM KCl; bovine serum albumin, 0.17 mg/ml; 2 mM dithiothreitol; appropriate concentration of Mg²⁺ and/or Mn²⁺; appropriate deoxynucleoside triphosphates at 0.1 mM with [³H]dGTP included at 17.5 μCi/ml (or [³H]dATP at 42.5 μCi/ml for terminal transferase assays); synthetic template, 20 μg/ml, with or without appropriate primer (or “activated” calf thymus DNA, 100 μg/ml); and the enzyme sample. Divalent metal contents of the reaction varied as follows: 8 mM Mg²⁺ for activated DNA and (rA)₅(dT)₅; 0.5 mM Mn²⁺ plus 2 mM
Results

Cross-reactivity of Heteroantisera to K-562 Cells with Lymphoblasts. Preimmune rabbit and monkey sera were not cytotoxic for the cell populations tested. Similarly, neither the rabbit nor the monkey anti-K-562 sera were cytotoxic for normal human thymocytes (Table 1) or a B-cell line of lymphoblasts (CCRF-SB). When T-cell lymphoblasts (Molt-4) were used as target cells, the rabbit anti-K-562 serum was not significantly cytotoxic. The monkey immune serum was cytotoxic for nearly all Molt-4 cells tested. Absorption of the monkey anti-K-562 with 2 × 10⁶ Molt-4 cells removed all cytotoxicity for the Molt-4 cell line, whereas significant cytotoxicity remained for K-562 cells. Both rabbit and monkey antisera were very cytotoxic for K-562 cells used as antigen.

Cytotoxicity of Anti-Human Thymocyte Globulin. The horse anti-human thymocyte IgG was tested on normal thymocytes, K-562, and Molt-4 cells prior to and after incubation with thymosin at the ratio of 0.5 and 1 mg of protein per 10⁶ cells. Thymosin was dissolved in MEM supplemented with 15% FCS; the cells were incubated in the medium containing thymosin for 2 hr at 37° in a CO₂ incubator. The results are shown in Chart 1. The horse IgG was highly cytotoxic for normal thymocytes; the titer was slightly modified by preincubation of thymocytes with thymosin. In marked contrast, the anti-human thymocyte globulin has little cytotoxicity for malignant T-cells (Molt-4) and none at all for K-562 cells. The treatment with thymosin did not change the effect of horse IgG on K-562 cells, whereas it drastically enhanced the complement-dependent cytotoxicity of the antithymocyte serum for Molt-4 cells. This experiment was repeated 3 times with similar results.

E-Rosette Formation. Very few E-rosettes (4 to 6%) were seen with Molt-4 cells. Incubation with thymosin for 16 to 24 hr increased the number of E-rosettes to 12%. On the other hand, not a single E-rosette was observed with K-562 cells prior to or after incubation with 1 mg of thymosin per 10⁶ cells, thus demonstrating that these highly undifferentiated cells do not possess E-receptors.

EA-Rosette Formation. The presence of Fc receptors on K-562 cells was investigated by measuring the binding of the cells to sheep RBC sensitized with the IgG fraction of anti-sheep RBC rabbit antiserum. To determine whether the number of antibody molecules bound to the RBC affected the rosette-forming capacity of K-562 cells, varying concentrations of anti-sheep RBC IgG were used to sensitize the sheep RBC. The concentration of sensitizing antibodies did not affect the number of RFC (Chart 2). As many as 86% of K-562 cells were RFC. This percentage dropped precipitously with suboptimal amounts of sensitizing antibody.

Terminal Transferase and DNA Polymerase Activities. Enzyme activities were determined on passages 23, 121, and 200 of the K-562 cell line. The Molt-4 cell line was studied in parallel for comparison. The results of sucrose gradient sedimentation analysis on high-salt extracts from total cells, from the nuclear fractions, and from cytoplasmic pellet fractions of the 2 cell lines are shown in Chart 3. Terminal transferase activity was demonstrated in the extracts of Molt-4 cells (Chart 3, D and E), whereas no TDT activity was detected in the K-562 cell extracts (Chart 3, A and B). In contrast, examination of the same gradient fractions for activated DNA-dependent DNA polymerase activities (Chart 3, A and D) revealed that both K-562 cells and Molt-4 cells gave profiles of DNA polymerase α and β, as usually expected of proliferating cells.
Our studies of terminal transferase used various published procedures: (a) 1 M NaCl extraction of "chromatin" fraction; (b) assay for (rA)<sub>4</sub> (dT)<sub>4</sub>-primed dTMP incorporation in reactions containing a 0.3 M NaCl (25) preparation of supernatant of cell homogenate by high-speed centrifugation; (c) assay for polydeoxyadenylate-primed dGMP incorporation and detergent extraction of the cells; and (d) assay of oligo deoxythymidylate-primed dGMP incorporation. All these procedures consistently gave positive TOT findings with Molt-4 cells but negative results with K-562 cells, respectively. In one experiment, an extract was made from a mixture of K-562 cells and Molt-4 cells; no apparent inhibition of TOT activity was observed.

In addition to DNA polymerase α and β, an (rA)<sub>4</sub> (dT)<sub>4</sub>-dependent dTMP-polymerizing activity was observed in the present study. This activity was detected in the combined detergent-high-salt extracts, but not in the plain high-salt extracts, of the cytoplasmic particulate fraction and appeared to be present at higher content in the K-562 cells than in the Molt-4 cells. The template primer preference and its apparent sedimentation rate (Chart 3, C and F) suggests that this activity may correspond to DNA polymerase γ.

DISCUSSION

The results of this study indicate that the undifferentiated blasts of the cell line K-562 are neither T- nor B-lymphoblasts. Therefore, the success in developing this unique cell line was not due to lymphoblastic conversion of the CML at the stage of terminal blastic crisis. Of course, this observation does not rule out the possibility that other CML cases might end in a lymphoblastic crisis (3). It proves, however, that permanent cell lines may be established from samples of undifferentiated blasts in the absence of lymphoid cells.

The studies with the heteroantisera to K-562 cells showed a lack of cross-reactivity with normal thymocytes and a B-cell line from a patient with ALL. This observation suggests that normal T- or malignant B-cells do not have antigenic receptors similar to those of K-562 cells. Although the monkey antiserum was very cytotoxic for a malignant T-cell line (also derived from a patient with ALL), it had no complement-dependent cytoxicity for normal human thymocytes. Therefore, it appears that surface antigens shared by the
Molt-4 and K-562 cell lines were peculiar to these 2 malignant cells but not to normal T-cells. The antigens shared by blasts in culture are probably of fetal or embryonic type, because absorption with 1st-trimester whole human embryo cells diminished the cytotoxicity of the anti-K-562 serum by nearly 50% (30). Antisera raised against ALL blasts also detect antigens present in the myelocytic and normocytic series from human fetal liver cells (5). The results also suggest that either Molt-4 cells have a small proportion of cells with antigens similar to those of K-562 cells or have many cells with a low density of antigen. The fact that the cytotoxicity of the immune serum for Molt-4 was completely removed by absorption with 2 × 10^8 Molt-4, whereas still more than 50% of the antibody to K-562 cells remained in the monkey serum, would suggest that some undifferentiated leukemia cells in long-term cultures share antigenic determinants and also possess specific surface antigens. In this context, absorption with 10^8 K-562 cells (one-half as many as Molt-4 cells) remove all antibody to K-562 and Molt-4 cells that is present in 1 ml of the monkey immune serum.

The lack of cytotoxicity of a horse anti-human thymocyte globulin for K-562 cells and the failure of the treatment with thymosin to induce antigenic and E-rosette receptors on K-562 cells ruled out other T-cell markers that might not be fully expressed on the surface of K-562 cells. In contrast, the treatment of Molt-4 cells with thymosin resulted in a moderate increase of the number of E-RFC and a marked enhancement of the cytotoxicity of the horse anti-human globulin for Molt-4 cells. Thus, the incubation with thymosin somehow modified the membrane of Molt-4 cells either by making available many more cells with antigen receptors (derepression) or by increasing the density of antigens on T-cells already possessing this type of surface marker.

The absence of TDT further indicates that K-562 cells are not immature T-cells. Confirming a previous report (24), TDT activity was demonstrated in Molt-4 cells used as control. Since the enzyme was detectable after an extract of K-562 cells was added to that of Molt-4 cells, the presence of an inhibitor of TDT activity in K-562 cells can also be ruled out. Our results are in agreement with those of others (7, 8) who have detected a very low TDT activity (< 2 units/10^8 cells/hr) at logarithmic phase growth of K-562 cells. This level of activity is typical of the TDT activity seen in about 50% of patients with CML in blastic crisis (Dr. M. S. Coleman, Department of Biochemistry, University of Kentucky, Lexington, Ky. [personal communication]).

In addition to DNA polymerase α and β activities usually found in proliferating cells (7) and lymphocytes stimulated by phytohemagglutinin (18), and (Rα)n-(dT)n-dependent dTMP-polymerizing activity was observed in the present study. This activity appeared to be higher in the K-562 than in Molt-4 cells. The template primer preference and its apparent sedimentation rate suggest that this activity may correspond to DNA polymerase γ observed in other cells (23). In the same context, Dr. R. Gallagher of the National Cancer Institute, Bethesda, Md. (personal communication) found no viral RNA-instructed DNA polymerase (reverse transcriptase) activity in the K-562 cell line.

The presence of Fc receptors has been observed in normal and malignant reticuloendothelial cells as well as certain nonlymphoreticular tumors (27). Also, it has been ob-
C. B. Lozzio et al.


Absence of Thymus-derived Lymphocyte Markers in Myelogenous Leukemia (Ph \(^+\)) Cell Line K-562

Carmen B. Lozzio, Bismarck B. Lozzio, Wen-Kuang Yang, et al.

*Cancer Res* 1976;36:4657-4662.

Updated version  Access the most recent version of this article at:  
http://cancerres.aacrjournals.org/content/36/12/4657

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.