Correlation of Leukemia-associated Antigens and Ph[1] Chromosome in Fibroblastlike Cells Derived From Bone Marrow

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A search was made for the Philadelphia chromosome (Ph[1]+) and leukemia-associated antigens (LAA) in fibroblastlike cells (FLC) derived from human bone marrow. A variable number of Ph[1]+ cells were observed in seven primary cultures and five monolayer passages of FLC derived from patients with chronic myelogenous leukemia. Three monolayers of FLC from normal bone marrow and three cultures of skin fibroblasts were used as controls. LAA were determined by (1) the antibody-dependent, complement-mediated cytotoxicity assay using a monkey antiserum made to the Ph[1]+ cell line, K-562, and (2) the immunoperoxidase assay using the monkey antiserum and rabbit anti-monkey IgG fraction conjugated with peroxidase. By these criteria, Ph[1]+ FLC were positive for LAA while Ph[1]− FLC were negative. Linear regression analysis indicated that there was a direct correlation between the number (3%–18%) of the Ph[1]+ FLC and the number (12%–30%) of immunoperoxidase-positive cells in the same cultures (r = 0.635) and also between the latter and the amount (18%–66%) of ^{51}Cr released per 10^6 cells (r = 0.834). The data presented herein show that Ph[1]+ FLC possess LAA similar or identical with that found in highly undifferentiated myelogenous leukemia cells. This suggests that the Ph[1]+ FLC may represent the earliest precursor (stem cell?) pool of myelogenous leukemia or stromal cells. The Ph[1]+ FLC bearing LAA detached from the solid surface, which was overgrown by normal FLC that depended on anchorage for growth. Thus leukemic FLC did not survive well in long-term monolayer cultures.

CELLS RESEMBLING FIBROBLASTS constitute the predominant, if not the only, cell population when normal or abnormal bone marrow cells of human origin are grown in suspension culture for variable periods of time. The origin of the FLC has intrigued hematologists because a diffuse proliferation of FLC is seen in the bone marrow of patients with myelofibrosis as well as in association with certain myeloproliferative disorders such as chronic myelogenous leukemia (CML) and agnogenic myeloid metaplasia.

The presence of karyotypic abnormalities, including the Ph[1] chromosome, has been investigated in FLC derived from bone marrow of patients with CML,[1-3] acute myelogenous leukemia with acute myelofibrosis,[4] and other malignant hematologic disorders.[2] The results of these studies have been controversial. Thus the cytogenetic analysis of FLC has shown the absence of chromosome markers in subcultures that may have been present in direct prep-

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arations and in primary cultures of bone marrow cells.[1,3,4] On the other hand, Hentel and Hirschhorn[2] reported the persistence of the Ph^1 chromosome and a C-group trisomy in FLC derived from bone marrow of patients with CML and acute leukemia, respectively.

In view of these discrepancies, we analyzed the FLC cultured from bone marrow of normal individuals and those suffering from CML. To this end, the karyotype of the FLC was examined as it related to the presence of LAA on the membrane surface. Such antigens were defined by use of a monkey antiserum[5] made to the Ph^1+ cell line, K-562,[6,7] which has been shown to give specific cytotoxicity for CML cells after selective absorption with normal hematopoietic cells.[5] The results indicate that FLC with the Ph^1 chromosome are subject to specific cytotoxicity by the antiserum and their antigenic sites are also immunolabeled by a peroxidase-antibody conjugate. These observations imply a direct correlation of the presence of the chromosome and specific antigen(s) related to leukemia, particularly CML.

MATERIALS AND METHODS

Patients. Normal hematopoietic cells and skin biopsies were obtained from children with congenital defects suspected to have chromosomal abnormalities. Bone marrow and skin monolayers were prepared only from those individuals who were found to have normal karyotypes. Leukemia peripheral blood and bone marrow cells were obtained from patients diagnosed to have CML.

Culture of bone marrow cells. Bone marrow samples, usually 0.5 1 ml, were collected in 2 ml Eagle's minimal essential medium (MEM) containing 40 units heparin. They were washed with 10 ml Hank's balanced salt solution, centrifuged, and suspended in MEM containing 15% undialyzed fetal bovine serum and antibiotics. Aliquots were inoculated into several flasks that were cultured at 37°C in a humid atmosphere containing 5% CO_2. Cultures were harvested at 24 and 48 hr to determine the karyotype. Other flasks were incubated until a uniform monolayer developed, which required about 4 6 wk. After incubation, representative samples of each monolayer were kept frozen in liquid nitrogen until analyzed.

Chromosome studies. The karyotype was assessed in cultures of cells incubated 24 hr in MEM containing 15% fetal bovine serum. Colchicine at a final concentration of 10^{-7} *M* was added for the final 3 hr. After incubation, a sample of cells was treated for 10 min with a hypotonic solution containing 0.2% potassium chloride and 0.2% sodium citrate. Another sample was treated with a mixture of 95% of a solution of 0.075% potassium chloride and 5% of the potassium chloride-sodium citrate solution. At the end of the 10 min, a few drops of fixative (absolute methanol-glacial acetic acid, 3:1, v/v) were added to the hypotonic solution and the cells were suspended and centrifuged. Then the supernatant was discarded and the cells suspended in the fixative and centrifuged once again. The procedure was repeated three times, and the fixed cells were spread on wet slides and air dried. These slides were stained with a modification of the Giemsa-trypsin staining method and examined microscopically.[8] In some cases, the acid-saline Giemsa banding technique was used.[9] Long-term cultures were grown in 75-cm^2 plastic culture bottles, the FLC harvested by trypsinization, and their karyotypes determined by the same techniques.

Antiserum. The antiserum was produced by immunization of a monkey with K-562 cells emulsified in complete Freund adjuvant. The antiserum was absorbed with human AB blood type erythrocytes, leukocytes, and normal bone marrow cells. Detailed descriptions of the preparation, absorptions, and specificity of the antiserum have been reported.[5]

Complement. Lyophilized normal guinea pig serum (Colorado Serum, Denver, Colo.) was used as the source of complement. Before use in the cytotoxicity assay, the serum was absorbed with 25-30 $\times$ 10^6 K-562 cells/ml of MEM for 60 min at 20°C with continuous stirring.

Cytotoxicity assay. Trypsinized fibroblasts (4 $\times$ 10^5) were seeded into plastic bottles (75 cm^2 growth area) containing MEM with 15% fetal bovine serum and cultured for 5 days, until a monolayer was formed. The monolayer was labeled with 50 μCi $Na_2{}^{51}CrO_4$ (specific activity

280 mCi/mg Cr; New England Nuclear, Boston, Mass.) added to each bottle. After 2 hr at 37°C in a humidified incubator with 5% CO_2, the medium was discarded and each monolayer was washed three times, each time with 10 ml MEM to remove unbound ^{51}Cr. The control monolayers were as follows: cell control contained 10 ml MEM; complement control contained 10 ml of 1:100 dilution of guinea pig serum in MEM; serum control contained 10 ml of 1:300 dilution of preimmune monkey serum and 1:100 dilution of guinea pig serum in MEM. The experimental monolayers were overlaid with a 1:300 dilution of immune monkey serum and 1:100 dilution of guinea pig complement in 10 ml MEM.

Triplicates of control and experimental bottles were incubated for 2 hr. After incubation, the medium from each bottle was delivered into plastic disposable tubes and centrifuged to eliminate any intact ^{51}Cr-labeled cells floating in the medium. The radioactivity was determined by counting 2-ml aliquots of the supernatant in a gamma spectrometer for 5 min. Then each monolayer was rinsed twice with 5 ml MEM. The FLC were suspended with 5 ml of 0.2% trypsin in MEM for 15 min at 37°C and then transferred to conical tubes for centrifugation at 850 *g* for 10 min. The pellet was suspended in MEM and the cells counted. Finally, the cells were centrifuged and the pellet digested for 16 hr at room temperature with 0.2 ml hyamine hydroxide. After incubation, 1.8 ml of 0.1% Triton X-100 in 0.1 *N* HCl was added to each tube and the radioactivity determined in a scintillation spectrometer. The specific release of ^{51}Cr was calculated as follows:

$$\% \text{ specific release} = \frac{(\text{exp supernate}) - (\text{control supernate})}{\text{total (supernate + pellet)} - \text{average control}} \times 100.$$

The average control includes the mean cpm found in the supernatants of monolayers of FLC to which either MEM, guinea pig serum (complement), or preimmune monkey serum was added. Since each control was run in triplicate, the average control was derived from pooling individual values of a total of nine bottles in each experiment.

Surface antigenic markers. The LAA were detected by use of a monkey antiserum[5] to the K-562 cell line.[6,7] To demonstrate the antigenic determinants, monolayers of FLC derived from normal and leukemic bone marrow samples were grown in large Petri dishes containing three or four glass slides. The cells were cultured as indicated earlier for 5 days. At that time, the slides covered by the monolayers were removed from Petri dishes and fixed in absolute methanol containing 0.3% hydrogen peroxide to inhibit endogenous peroxidase.[10] Since some round and oval cells were always seen in suspension, the medium overlying the slides was recovered and smears were made by use of a cytocentrifuge.

Although FLC, cells in suspension, and K-562 cells do not have myeloperoxidase, other peripheral leukocyte samples from patients with CML used as positive cell controls do have the enzyme. Therefore all cells were treated with the same fixative. After fixation, the slides were air dried, washed with 0.9 *M* Tris buffer (ph 7.6), and incubated with 1:10 dilution of monkey antiserum made to the K-562 cell line. After 30 min at 20°C, the slides were washed free of the antiserum and incubated for 30 min at 4°C with a 1:10 dilution of rabbit anti-monkey IgG (heavy and light chains) fraction conjugated with peroxidase (Cappel Laboratory, Downingtown, Pa.). Finally, the slides were covered with fresh benzidine reagent (75 mg of 3,3'-diaminobenzidine-tetrahydrochloride dissolved in 100 ml 0.05 *M* Tris-HCl, pH 7.6) and then stained with 0.1% toluidine blue for 1 min. The percentage of peroxidase-positive cells, defined as having five or more binding sites, was determined by counting 500 cells in each slide.

Although a single dilution of the antiserum (1:300), a single dilution of complement (1:100), and a fixed incubation time (2 hr) were found optimal under the condition of these experiments, several variations of these three parameters were tested. The immune serum was used at dilutions of 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320. The 1:300 dilution was found to be the cytotoxic titer killing 100% of K-562 cells in the presence of complement during 2 hr incubation. Similarly, the complement was tested at 1:10, 1:40, 1:60, and 1:100. The highest dilution of the complement producing maximum antibody-mediated cytotoxicity for the FLC as determined by the ^{51}Cr release assay was found to be 1:100. Cultures times varied from 1 to 3 hr. Since the reaction was completed between 1.5 and 2 hr, the latter incubation period was used to ensure maximum ^{51}Cr release; the nonspecific release of ^{51}Cr from either K-562 cells[5] or FLC accounted for less than 6%.

RESULTS

The Ph[1] chromosome was found in 5%-100% of the bone marrow cells in short-term (primary) culture and in seven of nine patients with CML. The results are presented in Table 1. Nine long-term (monolayer) cultures made up of FLC were observed at various serial passages, and the Ph[1]+ chromosome was found in five. The percentage of cells with the Ph[1] chromosome was significantly lower in monolayer cultures compared to primary cultures. In one instance (K-1483-5) 92% of the cells showed a Ph[1]+ and 8% a trisomy 17 in the 24-hr culture, whereas only 6% of the FLC had a Ph[1]+ chromosome and an absence of the trisomy 17 on passage 3. Although none of the FLC from the monolayer K-1483-5 had a Ph[1]+ chromosome by the fifth and sixth passages, there was a significant increase in the number of hypodiploid cells. Four of the primary cultures had 5%-22% Ph[1]+ cells, and only three (K-626-3, K-653-2, and K-1928-2) had Ph[1]+ FLC by the sixth or seventh culture passage. In two cases (K-1951-2, K-1955-2), cells of both primary and monolayer cultures were negative for the Ph[1] chromosome.

The presence of LAA in the FLC was determined and the data are presented in Table 1. Monolayers derived from bone marrow and skin of normal individuals were totally devoid of reactivity with anti-K-562 serum by either the cytotoxicity or immunoperoxidase assays. In marked contrast, monolayers containing Ph[1]+ cells gave positive reactions to the assays. A linear regression analysis was made of the data presented in Table 1 in which the percentage of Ph[1]+ FLC in monolayer cultures was compared to the percentage of immunoperoxidase-positive cells in those cultures. The correlation coefficient r was 0.6345 ($p < 0.048$). In addition, the correlation between the percentage of ^{51}Cr

Table 1. Presence of the Ph[1]+ Chromosome and Leukemia-associated Antigens (LAA) in Fibroblastlike Cells (FLC) Derived From Bone Marrow Cultures of Patients With CML

	Primary Cultures			Monolayer Cultures				
Cell Source	Culture No.	No. of Cells Analyzed	Ph[1]+ (%)	No. of Passages	No. of Cells Analyzed	Ph[1]+ FLC (%)	Cytotoxicity* [^{51}Cr Release/10^6 FLC (%)]	Antigenic Determinants:* Immunoperoxidase-positive FLC (%)
Normal skin	K-559-3	—	—	10	40	—	0	0.1
	K-570-3	—	—	7	72	—	0	0.1
	K-590-3	—	—	8	18	—	1	0
Normal bone marrow	K-559-2	30	—	7	31	—	0	0
	K-570-2	30	—	4	62	—	3	0.2
	K-590-2	50	—	5	48	—	0	0
CML bone marrow	K-626-3	63	79	6	32	9	49	12
	K-653-2	30	72	6	50	18	18	18
	K-1928-2	52	6	7	68	3	21	16
	K-2373-2	50	100	5	83	6	66	20
	K-1483-2	15	5	8	52	0	3	0.4
	K-1483-5†	76	92	3	30	6	58†	30
	K-1483-5‡			5	89	0	6‡	0.2
	K-1820-2	50	20	4	70	0	4	1.2
	K-1951-2	30	0	2	22	0	2	0.1
	K-1955-2	25	0	2	25	0	5	3.0

LAA as detected by a monkey immune serum to the Ph[1]+ myelogenous leukemia cell line, K-562. For more details, see text and refs. 5–7.

*Each value is the average of triplicate determinations rounded to the nearest unit. Control values have been subtracted.

†K-1483-5 prior to losing the Ph[1] chromosome.

‡K-1483-5 after losing the Ph[1] chromosome.

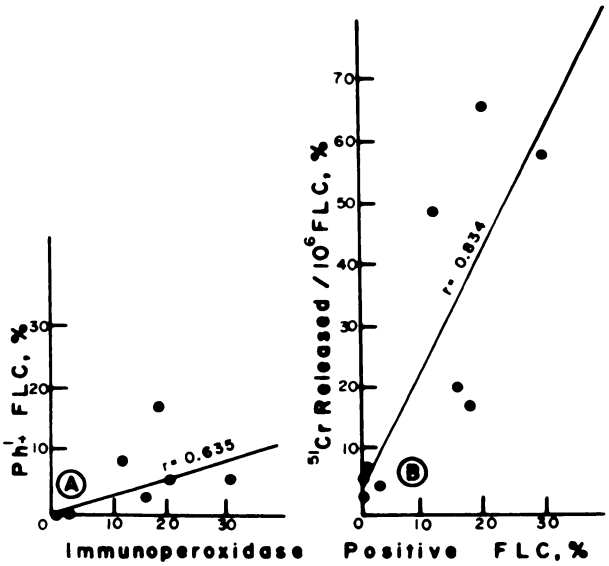

Fig. 1. Linear regression analyses of proportional number of Ph¹+ FLC and number of immunoperoxidase-positive FLC ($p < 0.0486$) and immunoperoxidase-positive cells and amount of cytotoxicity ($p < 0.0027$) in monolayer cultures derived from bone marrow of patients with CML.

release and immunoperoxidase positivity was significant with $r = 0.834$ ($p < 0.0027$). This analysis is presented in Fig. 1 and illustrates the positive correlations.

The monkey antiserum to K-562 cells was not cytotoxic for FLC without the Ph¹ chromosome and did not crossreact with the monolayer K-1483-5 after it had lost the Ph¹ chromosome. The immunoperoxidase-positive sites are illustrated in Figs. 2 and 3. A careful perusal of these monolayers showed that some cells with numerous antigenic sites tended to round up and become detached from the plastic surface (Figs. 2B, 3), whereas the FLC totally attached to the surface appeared to lack reactive sites (Figs. 2A, 2B). The latter appeared as small spots of brown color after reaction with the benzidine reagent. The round and oval mononuclear cells appeared to overlap FLC (Fig. 2C) or were in suspension (Fig. 2D).

DISCUSSION

The data presented herein allow us to conclude that approximately half of the monolayers derived from bone marrow of patients with CML and maintained for several passages in culture retain only a low number (3%–18%) of Ph¹+ FLC. Hentel and Hirschhorn[2] found the Ph¹ chromosome in 14% of the FLC of one patient and in 100% of FLC derived from the bone marrow of another patient with CML. The decrease in the number of cells with the Ph¹ chromosome at various serial passages suggests that the absence[1,3] or presence[2] of the Ph¹ or other chromosomal markers[4] is related primarily to the growth characteristics of the cultured cells and secondarily to the percentage of Ph¹+ cells in the bone marrow sample. Although both normal and Ph¹+ FLC initially depend on anchorage for growth, Ph¹+ cells with a high density of LAA tend to detach from solid surfaces and appear as round or oval cells floating in the medium. Such detachment could indicate pending cell death concomitant with the relatively poor survival of such cells in a mixed population in culture. It is our belief

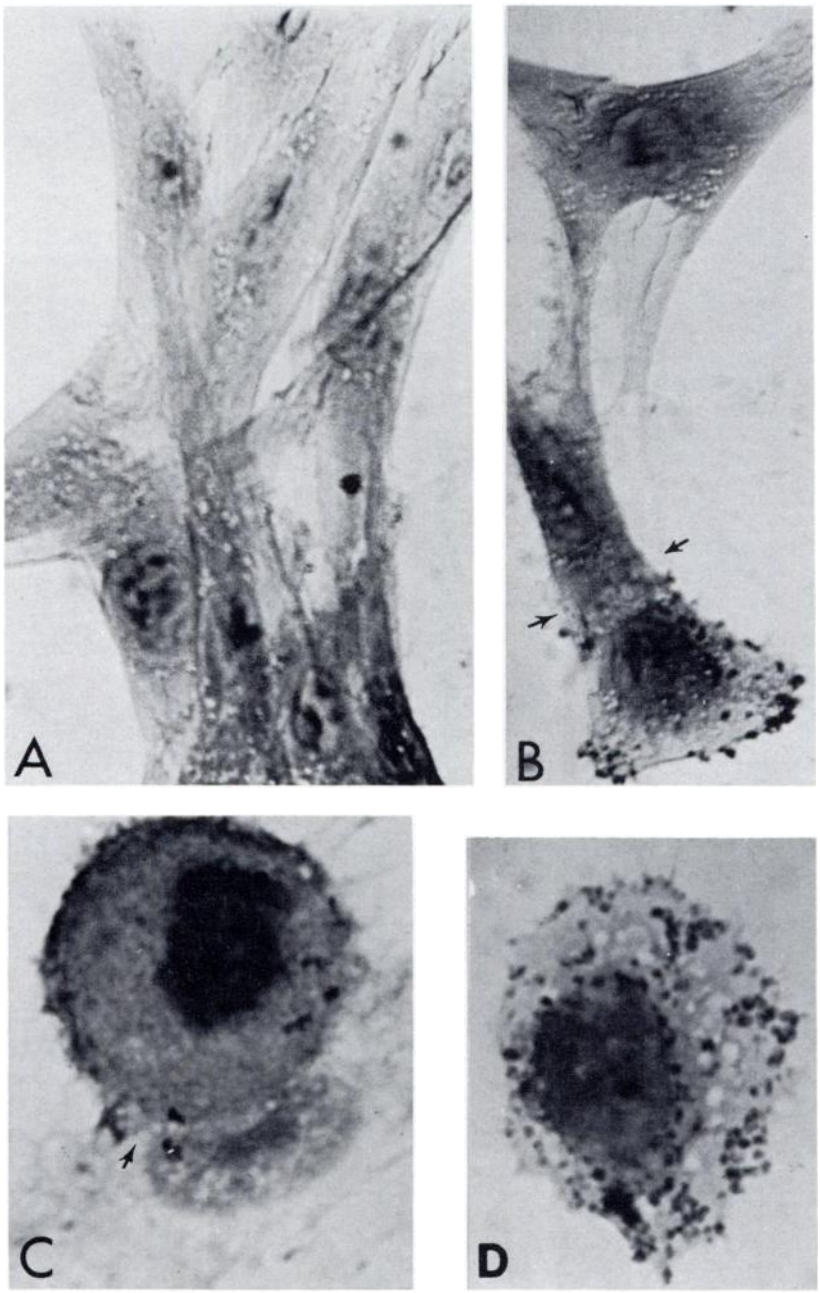

Fig. 2. Composite picture of fibroblastlike cells (FLC) derived from bone marrow cultures of normal individuals and those of patients with CML. (A) Normal FLC attached to plastic surface are devoid of leukemia-associated antigens (LAA). ×1000. (B) Cell on bottom is beginning to detach from plastic surface and shows antigen-antibody combining sites, whereas FLC in middle and on top do not. Note immunoperoxidase precipitates (arrows) between FLC. ×540. (C, D) Cells floating in the medium have either few (C) or several (D) antigenic determinants. ×1000. Round cell overlapping on normal FLC has broken membrane (arrow). Monolayers were incubated with monkey immune serum to K-562 myelogenous cell line, then with rabbit anti–monkey IgG conjugated with peroxidase, reacted with benzidine, and counterstained with toluidine blue.

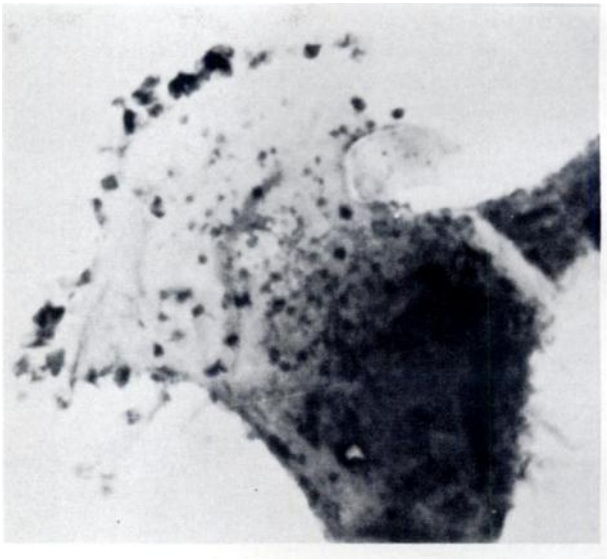

Fig. 3. FLC with several antigenic determinants detaching from plastic, from bone marrow culture of another patient with CML. ×1950.

that the absence of leukemic markers in a given monolayer culture reflects the loss of Ph^1+ cells released from the monolayer. Therefore the monolayer would be gradually populated by normal FLC. Thus normal cells would have a selective advantage in mixed culture, with the eventual elimination of the slower growing leukemic FLC. It is important to note, however, that some leukemic FLC cells can be maintained in serial passage for several weeks or months, as was found in nearly half of the monolayer cultures analyzed. This persistence of Ph^1+ FLC bearing LAA allows further studies on the nature and/or origin of leukemic FLC derived from human bone marrow.

A significant finding in the present study was the positive correlation between the presence of LAA by cytotoxicity or immunoperoxidase assays and the presence of the Ph^1 marker. The LAA were similar to those detected by use of the same immune serum in undifferentiated myelogenous leukemic cells observed in bone marrow and/or peripheral blood of individuals with acute myelogenous leukemia and CML in blastic crisis.[5] This correlation is of the utmost importance because it implies that myelogenous LAA in FLC are directly related to the presence of a specific chromosomal marker, the Ph^1 chromosome. In turn, Ph^1+ FLC may be a unique source of specific myelogenous LAA.

Friedenstein and co-workers[11-13] concluded that in guinea pigs FLC are derived from bone marrow stromal cells.[12] The results of the present studies strongly suggest that Ph^1+ FLC arise from the bone marrow cell population that is diluted by varying numbers of normal FLC.

Current evidence suggests that CML may be considered a clonal type of leukemia involving a pluripotential stem cell, since the Ph^1 chromosome is found in granulocytes, erythrocyte precursors, megakaryocytes, and the monocyte-macrophage complex.[14-19] Although the results of this study do not provide conclusive evidence on the origin of the FLC in monolayer cultures of human bone marrow cells, they represent an additional step in the characterization of Ph^1+ FLC. Such cells may derive from the original Ph^1+ cell clone, or, alternatively, the Ph^1+ FLC may be the precursor cells for the myelogenous leukemia

cell that does not survive in culture in vitro. In the latter instance, the Ph^1+ FLC may be the stem cell for the malignancy.

Pertinent to the present findings of positive correlation between the presence of the Ph^1 chromosome and the reactivity with anti-K-562 serum is our recent report[20] that a specific antigen can be isolated from the K-562 cells that inhibits the antiserum cytotoxicity. Thus it is probable that we have detected the unique LAA of CML that is associated with the Ph^1 chromosome and is relevant to the leukemic state.

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