

# Adherent Lymphokine-Activated Killer Cells in Chronic Myelogenous Leukemia: A Benign Cell Population With Potent Cytotoxic Activity

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We generated a homogeneous population of cells with cytotoxic activity termed "adherent lymphokine-activated killer" (ALAK) cells from the peripheral blood of nine patients in the chronic phase of Ph<sup>1</sup> positive chronic myelogenous leukemia (CML). The selective enrichment of CML ALAK cells depended on their propensity to adhere to plastic and proliferate when cultured in the presence of recombinant interleukin-2 (rIL-2) for 14 days. Culture of peripheral blood mononuclear cells under these conditions resulted in growth of a uniform population of cells with morphologic characteristics of large granular lymphocytes. The NKH1<sup>+</sup>/CD3<sup>-</sup> phenotype associated with IL-2-stimulated natural killer (NK) cells was present on 79% ± 9% of cells. Absence of colony formation in conditions promoting the growth of CFU-GEMM indicated that the CML ALAK population was not contaminated with viable hematopoietic progenitors. Cytogenetic analysis of the CML ALAK population revealed 119/120 Ph<sup>1</sup> negative metaphases and 1/120 Ph<sup>1</sup> positive metaphase in six patients. Southern blot analysis of CML ALAK failed to demonstrate a *bcr* gene rearrangement in seven patients known to have a *bcr* gene rearrangement in myeloid cells. Comparison of ALAK popu-

lations derived from peripheral blood of CML patients and normals revealed similar cytotoxicity against the NK-sensitive K562 cell line (104 ± 36 LU v 88 ± 19 LU; *P* = NS) and the NK-resistant Raji cell line (93 ± 26 LU v 98 ± 28 LU; *P* = NS). The proliferative capacity of CML ALAK cells (101 ± 33 fold expansion) exceeds the growth potential of the normal ALAK cells (22.3 ± 3 fold expansion; *P* = .02). Direct comparison of equal numbers of CML ALAK cells and a CML LAK cell population produced by incubation of peripheral blood mononuclear cells in rIL-2 for 14 days without adherence revealed that the CML LAK population had significantly lower lytic activity against K562 and Raji cell lines. We are able to expand CML peripheral blood mononuclear cells to provide a population of ALAK cells with potent cytotoxic activity. The CML ALAK population is relatively homogeneous, not contaminated with viable stem cells, not derived from a malignant lineage, and more cytotoxic than equal numbers of CML LAK cells. Further studies are underway to determine if this ALAK population may be effective in autologous killing of chronic myelogenous leukemia stem cells.

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**A**LTHOUGH LITTLE IS KNOWN about the characteristics and function of lymphoid cell populations with tumor lytic capacity in chronic myelogenous leukemia (CML), several reports suggest that natural killer (NK) function is decreased in chronic phase CML patients.<sup>1,2</sup> Recently, Vujanovic et al developed a method for selective enrichment of recombinant interleukin-2 (rIL-2)-activated antitumor effector cells called "adherent LAK" (ALAK) cells from rat splenocytes<sup>3</sup> or from human peripheral blood mononuclear cells.<sup>4</sup> The method depends on the propensity of activated NK cells to adhere tightly to plastic in the presence of rIL-2. Cultivation of these adherent cells with rIL-2 for 14 days results in the rapid proliferation of a homogeneous population of cells with the morphologic characteristics of large granular lymphocytes and a predominant NKH1<sup>+</sup>/CD3<sup>-</sup> phenotype. ALAK cells derived from normal humans have significantly higher cytolytic activity on a per cell basis and greater proliferative capacity than LAK cells prepared in a conventional fashion.<sup>3,4</sup> The ALAK population in CML patients has not been previously described. Study of ALAK cells and their progenitors may provide information on the mechanisms regulating tumor proliferation in CML and may suggest useful ex vivo or in vivo approaches to the therapy of CML.

## MATERIALS AND METHODS

**Patient population.** Peripheral blood from nine patients with Ph<sup>1</sup> positive CML in chronic phase were studied at 4 months to 2 years from diagnosis. Six patients had received only hydroxyurea therapy, one patient had received hydroxyurea and a short course of nitrogen mustard, and two patients had never received therapy. Medication was stopped at least five days prior to study in all treated patients. Peripheral blood WBC count ranged from 8 to 70,000/ $\mu$ L (median = 27,000/ $\mu$ L) at the time of study. In each case, a control sample obtained from a normal individual was studied. All patient

and control samples were obtained using guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota.

**Production of ALAK and LAK cells.** ALAK cells were produced by a modification of the method first described by Vujanovic et al and Medler et al.<sup>3,4</sup> CML or normal peripheral blood mononuclear cells (PBMNC) obtained by Ficoll Hypaque centrifugation and depleted of monocytes by plastic adherence were suspended in culture medium containing 1,000 IU/mL rIL-2 (Cetus, Emeryville, CA) in horizontal T25 or T75 flasks (Corning Glassware, Corning, NY), for 24 hours, in 5% CO<sub>2</sub> and at 37°C. Culture medium used for rIL-2 incubations consisted of Roswell Park Medical Institute medium (RPMI) 1640 supplemented with 10% human heat-inactivated AB serum (Pel-Freez Biologicals, Rogers, AZ), 2 mmol/L L-glutamine (GIBCO, Grand Island, NY), 0.2 mmol 2 mercaptoethanol and penicillin (1,000 U/mL) plus streptomycin (100 U/mL) (GIBCO). For normal controls and CML patients with peripheral WBC <20,000/ $\mu$ L (*n* = 4), monocyte-depleted PBMNC were suspended at a concentration of 2 × 10<sup>6</sup>/mL as originally described.<sup>4</sup> For CML patients with peripheral WBC >20,000/ $\mu$ L (*n* = 5), cells were suspended at a concentration of 4 × 10<sup>6</sup>/mL in

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order to increase the absolute number of adherent cells per flask. After 24-hour incubation, the supernatant was decanted and all cells not firmly attached to the plastic were removed by washing  $\times 3$  with warm RPMI. In distinction to the original described method, the adherent cells were then refed with 100% complete medium containing rIL-2 at a concentration of 1,000 IU/mL rather than with autologous conditioned media and cultured for up to 14 days. Cell concentration was adjusted to 1 to  $2 \times 10^6$ /mL at each feeding every 36 to 48 hours. At termination of culture, adherent ALAK cells were recovered by washing the flasks with cold RPMI and, if necessary, with phosphate buffered saline (PBS) containing 0.01 mol/L ethylene diamine tetraacetic acid (EDTA).

LAK cells were produced by suspension of CML or normal PBMNC at a concentration of  $2 \times 10^6$ /mL in culture medium containing 1,000 IU/mL rIL-2. Cells were incubated horizontally in T25 or T75 flasks at 37°C in 5% CO<sub>2</sub> in a humidified atmosphere and resuspended every 48 hours at an adjusted concentration of 1 to  $2 \times 10^6$  cells/mL in fresh medium containing rIL-2.<sup>5,6</sup>

**Cytotoxicity assays.** ALAK and LAK populations from CML patients and normal individuals were tested for cytotoxicity against the NK-sensitive K562 cell line and the NK-resistant Raji cell line in a four-hour Cr<sup>51</sup> release assay.<sup>7</sup> Effector-to-target ratios ranged from 20:1 to 0.66:1. All determinations were done in triplicate, and percentage lysis was determined using the following equation:

$$\frac{\text{experimental mean cpm} - \text{spontaneous release mean cpm}}{\text{total release mean cpm} - \text{spontaneous release mean cpm}} \times 100$$

One lytic unit (LU) was defined as the number of effector cells needed to lyse 15% of the targets and expressed as LU per  $10^4$  effector cells.<sup>7</sup>

**Phenotype.** Cell surface antigens were determined by direct staining of the cells with monoclonal antibodies (Becton-Dickinson). Antibodies used were directed at CD3 (Leu 4), CD 16 (Leu 11a) and NKH1 (Leu 19).<sup>8</sup> Phenotypic analyses were performed with a FACS IV flow cytometer (Becton-Dickinson, Mountain View, CA) equipped with a cosort 40 computer.

**Hematopoietic progenitor assay.** ALAK cells were plated in triplicate at a final concentration of  $2.5 \times 10^5$ /mL in methylcellulose (final concentration 1.12%) with Iscove's modified Dulbecco media (IMDM). Culture medium was supplemented with 30% fetal calf serum (GIBCO), 5% phytohemagglutinin leukocyte conditioned medium (PHA-LCM), 1.5 U human urinary erythropoietin (Terry Fox Laboratories, Vancouver, British Columbia), and  $10^{-5}$  mol/L 2 mercaptoethanol. Cultures were incubated in four well plates (Nunc-clone) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 14 days then assessed for evidence of BFU-E or CFU-GEMM formation as described.<sup>9</sup>

**Cytogenetic analysis.** Cells derived from ALAK cultures were subjected to a two-hour colcemid incubation followed by lysis with hypotonic KCL and fixation in acid/alcohol as previously described.<sup>10</sup> In each of six cases described, 20 metaphases were analyzed by skilled technicians in a reference laboratory after QFQ or GTG banding.

**Detection of bcr gene rearrangement.** High molecular weight DNA was extracted from myeloid cells and day-14 ALAK cells using the nuclei extraction technique.<sup>11</sup> Gene rearrangement studies employed the gel transfer technique described by Southern,<sup>12</sup> with transfer of Bgl II-digested DNA to a nylon membrane. A 1.95 Bgl II Hind III genomic DNA probe for the *bcr* containing exon 1 of this region<sup>13</sup> was a gift from Dr. David Leibowitz. This probe was radiolabeled by nick translation<sup>14</sup> and the filter hybridized according to the manufacturer's recommendations. Following hybridization, the filter was washed (final wash in  $0.1 \times$  SSC, 0.5% SDS at 65°C, for 60 minutes) and exposed to x-ray film for 24 hours at  $-70^\circ\text{C}$ .

**Statistical analysis.** Results of experimental points obtained from multiple experiments were reported as the mean  $\pm$  1 standard error of the mean (SEM). Significance levels were determined by two-sided student's *t*-test analysis.

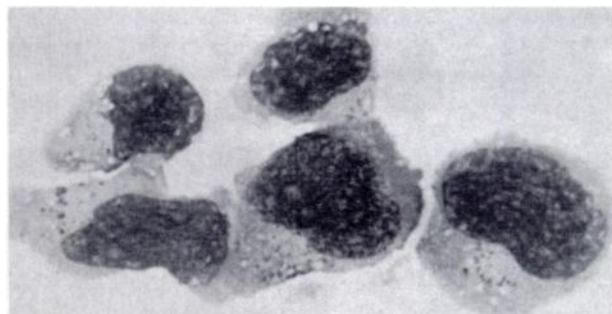
## RESULTS

Morphologic examination of the Wright-Giemsa-stained ALAK cells after 14 days of culture demonstrated a strikingly homogeneous population of large lymphocytes (Fig 1). Nuclei were large and contained an open chromatin pattern with multiple prominent nucleoli. The cells have abundant blue cytoplasm containing large, dark staining granules. Cells bearing morphologic characteristics of myeloid lineage were not seen. CML ALAK and normal ALAK were morphologically identical.

Phenotypic analysis of cells at day 14 of ALAK culture (Fig 2) revealed that the majority were NKH1<sup>+</sup>/CD3<sup>-</sup> both in CML patients (79%  $\pm$  7%) and in normal individuals (78%  $\pm$  4%). Few NKH1<sup>-</sup>/CD3<sup>+</sup> cells (CML = 10.8%  $\pm$  8%; normal = 12.8%  $\pm$  4.5%) or NKH1<sup>+</sup>/CD3<sup>+</sup> cells (CML = 2.5%  $\pm$  0.6%; normal = 1.8%  $\pm$  0.3%) could be found in the ALAK population derived either from CML or normal peripheral blood.

CML (n = 9) and normal (n = 17) ALAK cells have equivalent cytotoxicity against the NK-sensitive K562 cell line and NK-resistant Raji cell line. The CML ALAK cell cytotoxicity was  $104 \pm 36$  LU and normal ALAK cell cytotoxicity was  $88 \pm 19$  LU (*P* = not significant [NS]) against the NK sensitive targets, while for the NK-resistant targets CML ALAK cell cytotoxicity was  $93 \pm 26$  LU and normal ALAK cell cytotoxicity was  $98 \pm 28$  LU (*P* = not significant [NS]). The percent lysis of CML and normal ALAK cells against either K562 or Raji cell lines at each effector to target ratio was totally comparable (Fig 3).

We obtained an index of ALAK cell proliferation by calculating the total number of ALAK cells obtained after 14 days of culture divided by the number of CD16<sup>+</sup>/CD3<sup>-</sup> cells (determined by two-color FACS analysis) present in the cell population used to initiate the cultures at day 0. This method, adapted from Medler et al,<sup>4</sup> was used because the cells recovered after 14 days of culture are not derived from the total number of cells plated at day 0, but from the adherent CD16<sup>+</sup>/CD3<sup>-</sup> NK fraction retained after decant-



**Fig 1.** Wright-Giemsa-stained cytopsin preparations of an ALAK cell population derived from CML peripheral blood obtained after 14 days of culture in 1,000 IU rIL-2.

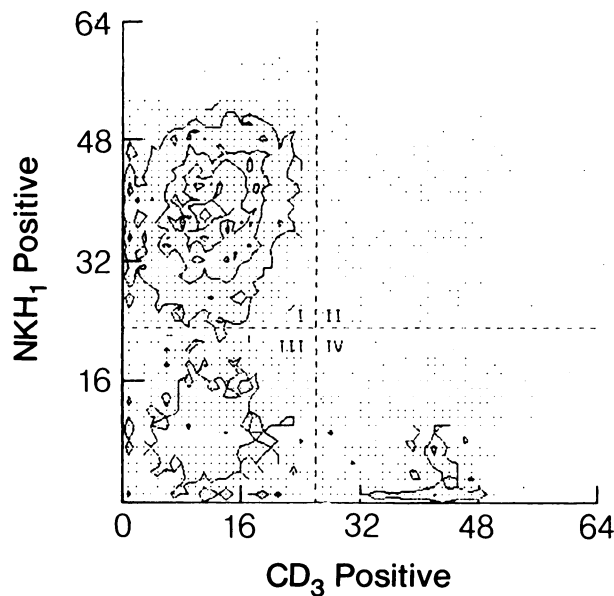


Fig 2. Two-color FACS analysis of a CML ALAK cell population: 78% NKH1 (leu 19) positive cells (Y axis), and 8% CD3 (leu 4) positive cells (X axis).

ing the nonadherent population at 24 hours of culture.<sup>4</sup> Proliferation (Fig 4) of the CML ALAK cell cultures ( $101.2 \pm 33$  fold expansion) exceeded the proliferation found in the normal ALAK cultures ( $22 \pm 3$  fold expansion) ( $P = .02$ ).

To determine if cells recovered from 14-day ALAK cultures were contaminated with hematopoietic progenitors of myeloid origin freshly recovered ALAK cells were plated in methylcellulose under conditions designed to promote growth of BFU-E and CFU-GEMM. No recognizable myeloid colonies were demonstrated in hematopoietic progenitor culture of either CML or normal ALAK cells.

The CML ALAK population was examined for evidence of a malignant lineage. Southern blot analysis failed to reveal a *bcr* gene rearrangement in the ALAK cells of seven patients with a known *bcr* gene rearrangement in the myeloid cell population (Fig 5). Cytogenetic analysis of ALAK

metaphases from six of the Ph<sup>1</sup> positive CML patients was performed. In five cases, 20 of 20 metaphases derived from the ALAK population were Ph<sup>1</sup> negative. In one case, 19 of 20 metaphases were Ph<sup>1</sup> negative and 1 of 20 metaphases was Ph<sup>1</sup> positive. In this case Southern blot analysis of the ALAK population failed to reveal a *bcr* gene rearrangement, and CFU-GEMM culture of ALAK cells did not reveal growth of hematopoietic progenitors. This patient was studied within 6 months of diagnosis and had no clinical or laboratory features consistent with accelerated disease.

Using conventional culture methods,<sup>5,6</sup> we generated a population of LAK cells from the peripheral blood in four CML patients and compared them with the day-14 ALAK cell population. While  $79\% \pm 7\%$  of day-14 ALAK cells were NKH1<sup>+</sup>/CD3<sup>-</sup>, or activated NK cells, only  $20\% \pm 7\%$  of the CML LAK population bore this phenotype. The lytic activity of CML LAK cells against the NK-sensitive cell line K562 was only  $50\% \pm 13\%$  of that produced by CML ALAK cells ( $P = .01$ ), and the lytic activity of CML LAK cells against the NK-resistant cell line Raji was only  $57\% \pm 17\%$  of that generated by CML ALAK cells ( $P = .03$ ). Although the total number of cells obtained from 20 mL of CML blood after a 14-day culture period is similar in the ALAK system ( $69 \pm 27 \times 10^6$  cells) and LAK system ( $50 \pm 12 \times 10^6$  cells) ( $P = NS$ ), the total number of NKH1<sup>+</sup>/CD3<sup>-</sup> cells associated with the more cytotoxic activated NK cells is significantly higher in the ALAK cultures ( $68 \pm 29 \times 10^6$  cells) than in the LAK cultures ( $14 \pm 6 \times 10^6$  cells) ( $P = .03$ ).

#### DISCUSSION

The peripheral blood of chronic phase CML patients is flooded with myeloid cells of malignant lineage, and the cytotoxic capacity of peripheral blood cells as measured by endogenous NK function is severely diminished.<sup>1,2</sup> Despite these limitations it is possible to generate a homogeneous population of highly cytotoxic lymphocytes termed ALAK cells from the peripheral blood of such patients.

We made several modifications of the published techniques<sup>3,4</sup> in order to generate an ALAK population from CML peripheral blood. In cases where the peripheral blood WBC count was greater than 20,000/ $\mu$ L the starting concen-

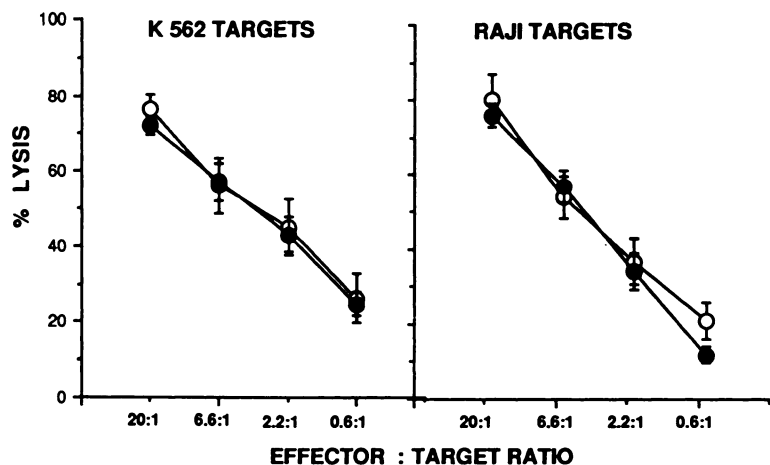


Fig 3. Cytotoxicity of CML (open circle) (N = 9) and normal (closed circle) (N = 17) ALAK cells against an NK-sensitive (K562) target cell line and an NK-resistant (Raji) cell line. All cultures were assessed for cytotoxicity between day 12 and day 14 of growth. Cytotoxicity data are given as mean  $\pm$  SEM percent lysis at four different effector:target ratios. Differences are not significant.

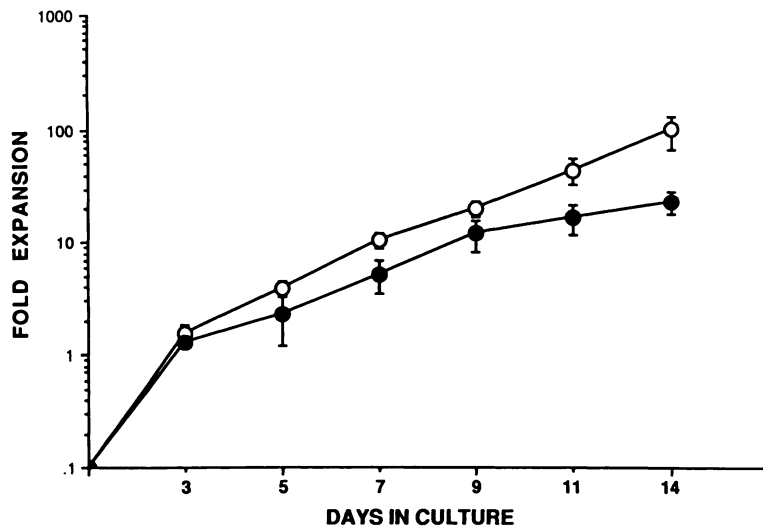


Fig 4. Expansion of ALAK cells obtained from the peripheral blood of CML patients (open circle) (N = 9) and normal controls (closed circle) (N = 17) cultured in the presence of 1,000 IU rIL-2 for 12 to 14 days. Data are given as mean  $\pm$  SEM,  $P = .02$

tration of monocyte-depleted peripheral blood mononuclear cells was increased from 2 to  $4 \times 10^6$ /mL. Use of a higher cell concentration than suggested in the originally described method resulted in more efficient production of ALAK cells, presumably because the starting concentration of ALAK progenitors in CML peripheral blood was diluted by myeloid cells. In a second modification, we performed the 14-day culture with complete media alone rather than with supplemental autologous conditioned media to avoid the potential suppressive effects of malignant cell byproducts found in autologous supernatant.<sup>15</sup>

Culture under these conditions for 14 days results in the generation of a uniform population of cells with morphologic features of large granular lymphocytes. The ALAK population contains a high proportion of cells bearing the NKH1<sup>+</sup>/CD3<sup>-</sup> phenotype commonly associated with rIL-2-stimulated NK cells. Neither morphologic evidence of the myeloid population prevalent in the peripheral blood, nor evidence of myeloid progenitors was found in the ALAK population. Absence of *bcr* gene rearrangement, and virtual absence of

the Ph<sup>1</sup> in the ALAK population strongly suggest that these cells are derived from a benign lineage.

Measurement of cytotoxic capacity of the CML ALAK cells demonstrated very high cytotoxicity not only against the NK-sensitive K562 cell line, but also against the NK-resistant Raji cell line. Cytotoxicity of CML ALAK cells was equivalent to that of normal ALAK cells, suggesting that a cell population with potent cytolytic activity can be generated from CML peripheral blood despite massive contamination of the starting population with malignant myeloid cells, and despite a known deficit in number and function of NK cells in CML peripheral blood.<sup>1,2</sup>

The ability to generate cells with LAK activity from the blood of CML patients has been described.<sup>5</sup> However, comparison of CML ALAK cells and CML LAK cells demonstrates that the CML ALAK population contains a significantly higher number of NKH1<sup>+</sup>/CD3<sup>-</sup> cells associated with an activated NK cell fraction. The lytic activity found in this ALAK population is significantly higher than the cytotoxicity found in the same number of LAK cells.

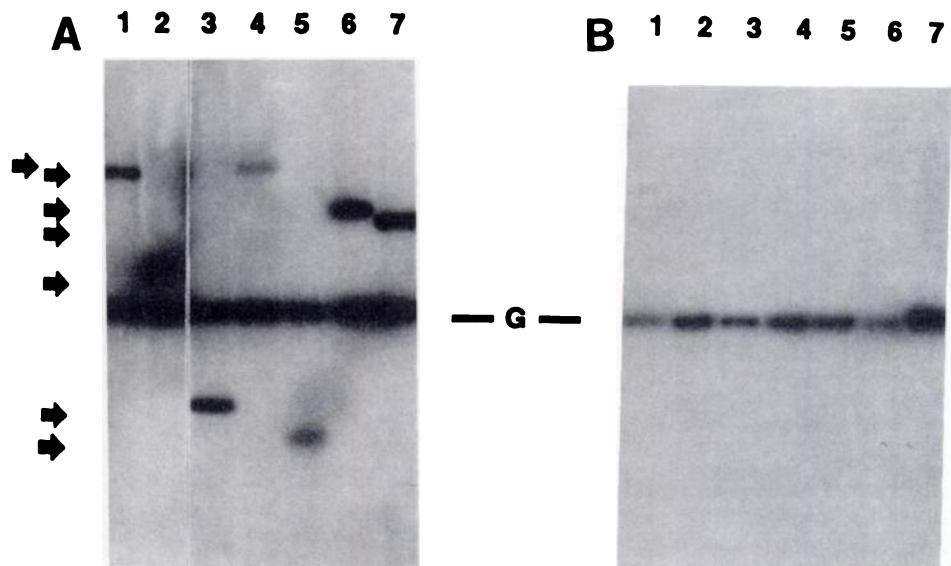


Fig 5. Southern blot analysis of CML myeloid cell (A), and CML ALAK cell (B) DNA. Genomic DNA was digested with Bgl II and probed with a 1.95 Bgl II-Hind III probe. The arrows at the left side show the *bcr* gene rearrangement present in the myeloid cells of each patient. The line marked "G" between panel A and panel B shows the germline DNA band position.

We observed a significantly greater growth capacity for the adherent population in the ALAK cell cultures derived from CML peripheral blood than for ALAK cells derived from normal individuals cultured under identical conditions. This difference in proliferative capacity suggests intrinsic differences between CML and normal ALAK cell progenitors. Further exploration of these differences may lead to a better understanding of tumor cell control mechanisms in CML, and possibly in other malignant conditions.

Several studies have demonstrated that presumably benign Ph<sup>1</sup> negative stem cells coexist with the malignant Ph<sup>1</sup> positive stem cells in some CML patients.<sup>16</sup> Attempts to select the coexistent benign myeloid stem cell population with chemotherapy,<sup>17</sup> interferon therapy,<sup>18</sup> or hematopoietic

progenitor culture<sup>19,20</sup> have been described. If the nonmalignant, highly cytotoxic ALAK cell population can be shown to kill Ph<sup>1</sup> positive hematopoietic progenitors while sparing coexistent Ph<sup>1</sup> negative stem cells, the ALAK population may prove useful for autologous ex vivo or in vivo therapy of CML. Further studies investigating the mechanisms of cell killing by CML ALAK are underway in our laboratory.

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