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Involvement of Natural Killer Cells in Chronic Myeloid Leukemia

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ABSTRACT: *We assessed the involvement of natural killer (NK) cells in chronic myeloid leukemia (CML). We adopted the MAC (morphology antibody chromosomes) method, which allows simultaneous assessment of cell morphology, immunophenotype, and chromosome aberrations in the same mitotic or interphase cells. We examined three patients with CML in chronic phase and two patients with the disease in blast crisis. Patients in the chronic phase of the disease showed no involvement of NK cells, but involvement was detected in one of the patients in blast crisis. In this patient, a proportion of the B cells and lymphoid stem cells was also neoplastic, whereas mature postthymic T cells were normal.*

INTRODUCTION

Chronic myeloid leukemia (CML) is usually detected during its chronic phase, characterized by cells of relatively mature morphology and good response to treatment. In most patients, the disease later enters an accelerated phase and finally advances to blast crisis, characterized by increasing numbers of primitive blast crisis, characterized by increasing numbers of primitive blast cells in bone marrow (BM) and blood. The blasts usually are of granulocytic-monocytic lineage, but erythrocytic or megakaryocytic as well as T- and B-lymphatic blast crises can occur [1, 2]. There are no reports of cases in which the blasts expressed characteristics of natural killer (NK) cells. That lymphoid blast crises may occur and the involvement of T and B cells in some patients [2, 3] suggest that CML is a disease of pluripotent stem cells. Furthermore, recent articles described bilineal T-lymphoid/myeloid and B-lymphoid/myeloid involvement [4, 5]. We assessed the involvement of NK cells (CD56 and CD16) in CML patients whose disease was in chronic phase or in blast crisis predominantly involving lineages other than lymphoid.

For this study, we adopted the MAC (morphology antibody chromosomes) method, which allows simultaneous assessment of cell morphology, immunophenotype and chromosome aberrations in the same mitotic or interphase analysis [6, 7]. The study was performed on BM and recombinant interleukin-2 (rIL-2)- phytohemagglutinin (PHA)-stimulated venous blood cells.

MATERIALS AND METHODS

Patients with CML in Chronic Phase

We studied peripheral blood (PB) samples from three Philadelphia (Ph)-positive CML patients (two men and one woman, age range 37–40 years) in chronic phase (patients 1–3). PB lymphocytes were isolated by centrifugation over Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). Monocyte-macrophages and B cells were depleted by filtration through nylon wool columns [8]. For patient 3, large granular lymphocytes (LGL) were isolated by discontinuous four-step density gradient centrifugation on Percoll (Pharmacia) according to the methods of Julius et al. [8] and Timonen and Saksela [9]. The cells were cultured for 3–5 days in rIL-2–containing (200 U/ml, EuroCetus, Amsterdam, The Netherlands) medium supplemented with a low-molecular-weight B-cell growth factor (BCGF, 1% of culture volume; Cellular Products, Buffalo, NY). The complete culture medium comprised 80% RPMI 1640 20% fetal calf serum, 0.29 mg/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Grand Island, NY).

To demonstrate the presence of NK cells ($CD3^-$, $CD56^+$), we performed phenotypic analysis (after isolating and culturing the cells) with two-color analysis with directly labeled antibodies. The cells (5×10^5) were washed twice and incubated with FITC-conjugated Leu-4 (anti-CD3, Becton Dickinson, Mountain View, CA) and with phycoerytrin (PE)-conjugated Leu-19 (anti-CD56, Becton Dickinson) antibodies for 30 minutes at 4°C. The cells were then washed twice in phosphate-buffered (PBS) and fixed with 3.5% paraformaldehyde in PBS. We used flow cytometry to study fluores-

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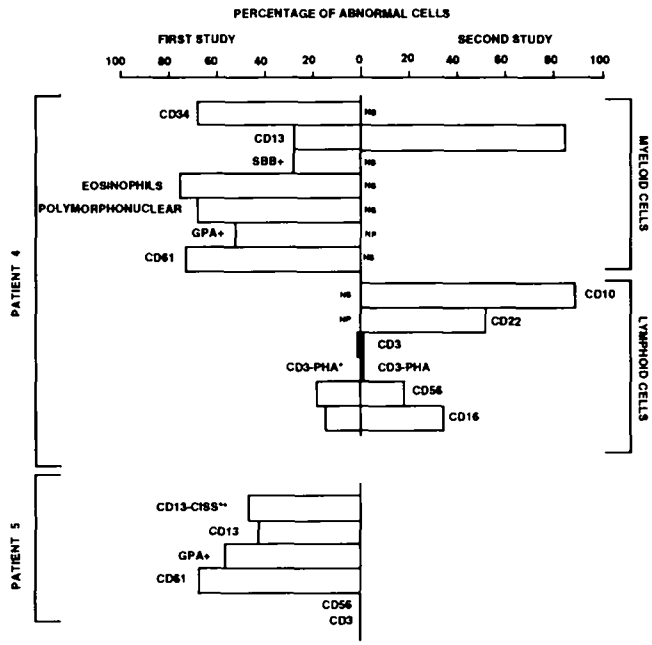

Figure 1 Results of MAC study on cells from patients 4 and 5. Frequency of interphase BM or blood cells with an additional signal in chromosomes 1 and 8, as detected by in situ hybridization using chromosome 1 centromere-specific probe pUC1.77 [14] and chromosome 8 centromere-specific probe generated by PCR [15], respectively. For each lineage, 200–500 cells were analyzed. Immunophenotype was obtained with APAAP with the following monoclonal antibodies: CD34, stem cell (anti-CD34); CD13, granulocytic/monocytic (MY7); CD61, megakaryocytic (Y2/51 provided by D. Mason, M.D., Oxford, England); CD10, lymphoid stem cells (J5); CD22, B-lymphocytes (Leu14); CD3, T-lymphocytes (Leu4); CD56, LGL (Leu19); CD16, LGL (CLB): and GPA, glycophorin A, erythrocytic lineage (anti-GPA, provided by Dr. Griffin, Boston, MA). Granulocytes were also identified by Sudan black B staining (SBB), and eosinophils and mature polymorphonuclear leukocytes were identified by Giemsa staining. *CD3-PHA-CISS (patient 4): 500 metaphases were studied by CISS hybridization with chromosome 22 library probe (Library LL22NS01). No cells with Ph chromosome were noted. **CD13-CISS (patient 5): To detect the Ph chromosome, 500 metaphases were studied by CISS hybridization with chromosome 22 library probe (Library LL22NS01).

cence (FACScan, Becton Dickinson) with normal mouse IgG1 (Coulter, Hialeah, FL) as the control antibody.

To arrest mitotic cells, the cultures were treated with Colcemid 0.1 μg/ml (GIBCO) for 4 hours at 37°C. For MAC analysis, cytospin preparations were made from enriched NK cells as described previously [6]. For immunocytochemical staining, the alkaline phosphatase antialkaline phosphatase (APAAP) procedure described in detail by Keinänen et al. [10] was used. Monoclonal antibodies used to detect NK cells were anti-CD16 (CLB-Fc-gran1, Janssen, Beerse, Belgium) and anti-CD56 (Leu-19, Becton Dickinson). Anti-CD3 (Leu-4, Becton Dickinson) was used to detect T cells.

Chromosome in situ suppression (CISS) hybridization was performed with DNA from a bacteriophage library probe, established from sorted human chromosome 22 [Library LL22NS01, American Type Culture Collection (ATCC) Rockville, MD] [11]. The probe was labeled by nick-translation using biotin-11-dUTP (Sigma Chemical, St. Louis, MO) according to the instructions of the kit supplier (Nick translation kit, Bethesda Research Laboratories, Gaithersburg, MD). CISS hybridization and detection of the hybridized probe with FITC conjugated with avidin (Vector Laboratories, Burlingame, CA) were performed as described in detail previously [12]. The signal was amplified as described by Pinkel et al. [13].

Patients with CML in Blast Crisis

MAC analyses were performed on BM cells cultured for 2 h (patients 4 and 5) and on venous blood cells cultured for 3 days with PHA (0.5 μg/ml, Difco Laboratories, Detroit, MI). Unstimulated cultures (3 days) were also established.

Interphase cytogenetic analysis was performed after the immunophenotype classification of the cells was obtained [6]. Figure 1 shows the antigens used for classification of cell lineages. Because the karyotype abnormalities of the patients included a gain in the centromeric area of chromosome 1 (patient 4) and chromosome 8 (patient 5) (described in case reports), in situ hybridization was performed with a chromosome 1 centromere-specific probe pUC1.77 [14] and a chromosome 8 centromere-specific probe generated by polymerase chain reaction (PCR) [15]. Probe pUC1.77 was labeled by nick-translation using biotin-11-dUTP (Sigma) and a nick-translation kit in the manner recommended by the manufacturer. Digoxigenin-11-dUTP (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany) was incorporated into the chromosome 8 centromere-specific probe during PCR reaction.

Case Reports of Patients in Blast Crisis

Patient 4, a 36-year-old man, was diagnosed as having CML in August 1990. The disease was then in chronic phase. During Summer 1991, the disease progressed to an accelerated phase; in September 1991, the patient received a BM transplant from an unrelated female donor after conditioning with total body irradiation and cyclophosphamide. This produced remission; but 2 months later hematologic analysis suggested relapse. A cytogenetic study of 20 metaphase cells 6 weeks after the transplant showed a chromosome aberration in all cells [47,XY, + i(1)(q10),t(9;22)(q34;q11)], identical to that observed before the transplant. The patient was treated with interferon, and immunosuppressive drugs were discontinued. Blood cell levels returned to normal, except for moderate interferon-induced cytopenia, and in June 1992 a BM aspirate was essentially normal. Three months after initiation of interferon treatment, G-banding analysis showed a normal female karyotype in all 20 metaphase cells studied, but karyotype analysis performed in August 1992 again showed a population of Ph$^+$ cells and clinical progression of the disease was noted in the following month. An overt blast crisis developed gradually, and the patient died in November 1992. The MAC studies we report were performed in August and October 1992.

Patient 5, a 38-year-old man, was diagnosed in July 1991 as having CML in the accelerated phase. Karyotype analysis showed the Ph chromosome, and + 8, + 19, and + 20 as additional abnormalities. Hydroxyurea was administered, and

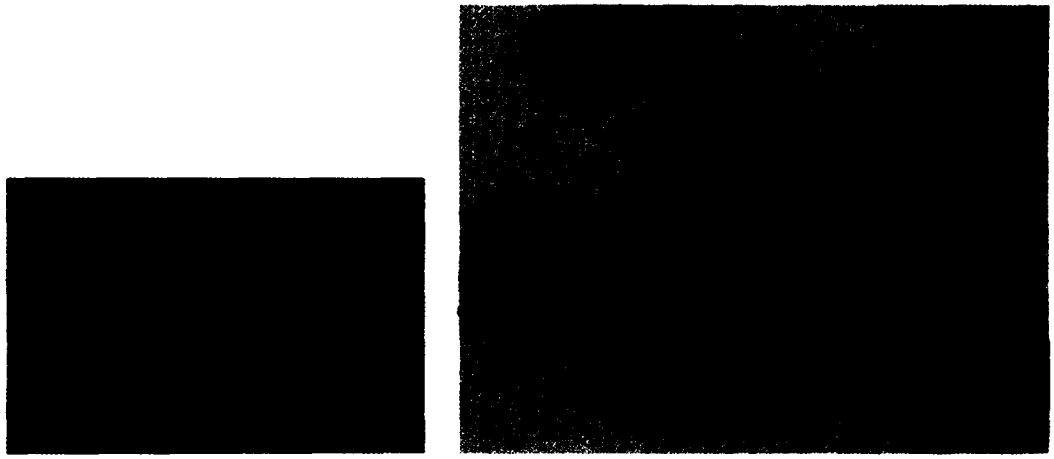

Figure 2 A CD16$^+$ mononuclear NK cell (A) and CD3$^+$ T-lymphoid cells (B) from patient 4. Three hybridization signals (additional isochromosome 1) in the CD16$^+$ cell demonstrate the involvement of NK cells in this patient. Two signals in CD3$^+$ cells indicate that T cells are not involved. A polymorphonuclear cell (arrow) shows three signals expressing abnormality (B) (APAAP immunostaining; chromosome 1 centromere-specific probe, pUC1.77).

a brief chronic phase was achieved in August 1991. However, blasts soon reappeared in the blood and increased to constitute 20% of the differential white blood cell (WBC) count. MAC analysis was performed at that time. The karyotype showed +8 as the only abnormality other than the Ph translocation in 80% of cells analyzed. The patient died in blast crisis in November 1991.

RESULTS

Patients with CML in Chronic Phase (Patients 1–3)

Enriched LGL cultured from three CML patients in chronic phase were studied by immunocytochemical staining and chromosome painting. A chromosome 22 library probe showed that none of the 94 CD56$^+$ cells or the 76 CD3$^+$ cells analyzed had the Ph translocation. Because only eight CD16$^+$ cells were analyzed reliably, we cannot show conclusively that NK cells are not involved in the chronic phase of CML, but none of the eight cells had the translocation.

Patients with CML in Blast Crisis (Patients 4 and 5)

In the 2-hour cultures of BM cells from patients 4 and 5, the karyotypes were 47,XY, + i(1)(q10),t(9;22)(q34;q11) and 47, XY, + 8,t(9;22)(q34;q11), respectively. In situ hybridization with centromere-specific chromosome 1 and 8 alphoid probes showed that the abnormal cells contained three hybridization signals. Some of our results were reported previously in our article describing the MAC technique [6].

Figure 1 shows that both patients 4 and 5 had three signals in chromosomes 1 and 8 in all myeloid lineages [i.e., glycophorin-A$^+$ erythroid, CD13$^+$ granulocytic/monocytic (also mature neutrophils, eosinophils and monocytes) and CD61$^+$ megakaryotic]. Furthermore, CD34$^+$ stem cells contained this abnormality. In patient 4, we were also able to demonstrate the abnormality in CD10$^+$ lymphoid stem cells, CD20/22$^+$ mature B cells, and CD16$^+$ (mononuclear) and CD56$^+$ LGL (Fig. 2).

For patient 4, we could not demonstrate the trisomy in CD3$^+$ mature T lymphocytes in PHA-stimulated or unstimulated cultures. The frequency of cells with three signals was not more than 0.5%. The frequency of false-positive signals in T-lymphocyte control preparations in our laboratory is also approximately 0.5%, indicating that the detection limit for abnormal cells using interphase cytogenetics is 0.5%. For patient 5, the number of B cells was so low (0.1%) that a reliable analysis could not be made. Neither T cells nor LGL had the abnormality (Fig. 1).

DISCUSSION

Earlier morphologic studies have suggested that NK cells are involved in blast phase CML. We have now demonstrated direct involvement of LGL. In the metaphase cytogenetic studies, we used the Ph translocation as a marker of malignancy in all cell types from patients 1–3 and in T lymphocytes from patient 4. For NK cells of patient 5, however, we had to seek another chromosome abnormality as a marker of malignancy because we could not obtain enough NK cell metaphases to perform CISS hybridization with the chromosome 22 probe. The absence of any additional chromosome abnormality in the NK cells of this patient therefore does not preclude their involvement. Neither did we perform interphase cytogenetic studies with cosmid clones [16] because this technique yields many (about 5%) false-positive readings for the Ph translocation. We obtained less than 1% false-positive signals using chromosome 1 and 8 centromere-specific probes. Therefore, if the proportion of leukemic NK cells (patient 5) or T cells (patient 4) was less than 1%, this technique would not detect the abnormality. CISS hybridization, in which the signals are scored on morphologically classified chromosomes, does not yield false-positive signals for the Ph translocation.

In chronic phase CML, the Ph chromosome was not detected in NK cells, yet we cannot conclude that NK cells are

never involved in chronic phase CML. This would require study of more patients and more cells from each patient.

Taking into account that abnormal LGL were not observed in patient 4 until the terminal phase of the disease, they had more attributes in common with B and T lymphocytes than with myeloid cells. Our results therefore suggest that NK cells and T and B lymphocytes share common developmental pathways and that NK cells originate from lymphatic stem cells [17]. The origin of LGL is a matter of controversy.

In the patient with NK cell involvement, the abnormality was detected in $CD10^+$ lymphoid stem cells and mature B cells but not in mature T lymphocytes, suggesting that abnormal T cells are rejected during maturation. In addition to the preferential selection of normal cells over abnormal cells during differentiation, the presence of long-lived lymphocytes and slow lymphopoiesis may offer alternative explanations for the absence of Ph^+ lymphoid cells. Because they develop so slowly, the lymphocytes could not be the progenitors of abnormal stem cells.

Patient 4 was in the terminal phase of the disease, which had already lasted 3 years. He had received a BM transplant and, until death, interferon treatment for relapse. The possible effects of these treatments on the appearance of lymphoid cells should be taken into consideration. Nevertheless, our results indicate that, at least in this patient, CML is a disease of pluripotent stem cells committed not only to myeloid and lymphoid lineages but also to NK cells.

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