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*J Immunol* 2002; 168:643-650; doi: 10.4049/jimmunol.168.2.643
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The BCR/ABL Transgene Causes Abnormal NK Cell Differentiation and Can Be Found in Circulating NK Cells of Advanced Phase Chronic Myelogenous Leukemia Patients

Hikaru Nakajima,* Robert Zhao,* Troy C. Lund,* Jeanne Ward,* Michelle Dolan,** Betsy Hirsch, † and Jeffrey S. Miller2gr

NK cells from the blood of chronic myelogenous leukemia (CML) patients are progressively decreased in number as the disease progresses from chronic phase to blast crisis. We hypothesize that BCR/ABL may be directly responsible by interfering with NK cell differentiation. CD34+ HLA-DR+ cells from CML patients were studied for their capacity to differentiate into NK cells. The NK cell cloning frequency was significantly decreased from CML CD34+ HLA-DR+ cells compared with cells from normal donors, yet CD34+ HLA-DR+ cells gave rise to BCR/ABL+ NK cells in some patients. This finding prompted us to further investigate circulating NK cells from the blood of CML patients. CD56+CD3− NK cells were sorted from CML patients and examined by fluorescence in situ hybridization (FISH). In contrast to chronic phase CML, significant numbers of NK cells from advanced phase CML patients were BCR/ABL+, whereas T cells were always BCR/ABL− regardless of the disease stage. To test the effects of BCR/ABL as the sole genetic abnormality, BCR/ABL was transduced into umbilical cord blood CD34+ cells, and NK development was studied. p210-enhanced green fluorescence protein-transduced cells gave rise to significantly decreased numbers of NK cells compared with enhanced green fluorescence protein transduction alone. In addition, the extrinsic addition of BCR/ABL-transduced autologous CD34+ cells suppressed the NK cell differentiation of normal umbilical cord blood CD34+ CD38− cells. This study provides the first evidence that BCR/ABL is responsible for the altered differentiation of NK cells and that the NK cell lineage can be involved with the malignant clone in advanced stage CML. The Journal of Immunology, 2002, 168: 643−650.

The NK cells comprise a small population of lymphocytes defined by surface expression of CD56 and absence of the TCR (CD3−). NK cells do not need to recognize MHC molecules for cytolytic function, and they may play a role in tumor surveillance. In support of this, IL-2-activated NK cells from patients with chronic myelogenous leukemia (CML) suppress autologous primitive CML progenitors in long term culture (1). Despite this, NK cells from patients with CML progressively decrease in number as the disease progresses (2). The progressively decreased cloning frequency and proliferative capacity of CD56+brightCD2−, CD56−dimCD2+, and CD56−dimCD2− NK cell subsets was demonstrated as CML disease stage progresses, suggesting a possible cause for the observed decrease in circulating NK cell numbers. However, an alternative hypothesis is that BCR/ABL+ progenitors have a diminished capacity to differentiate into NK cells, and this accounts for the decreased numbers found in CML patients.

It has long been believed that T cells and NK cells from patients with CML are Philadelphia chromosome (Ph1) negative. Our group studied the origin of NK cells in CML patients using adherent lymphokine-activated killer cell (A-LAK) methods (3). This culture relies on IL-2 to selectively adhere NK cells to plastic from PBMC. These plastic-adherent NK cells can then be expanded into large numbers for analysis. Although the outgrowth from A-LAK cultures diminished as CML progressed, expanded NK cells were always BCR/ABL− and Ph1 negative. However, because the A-LAK culture relied on plastic adherence and proliferation, an abnormal, possible malignant population in CML may have been missed based on the selection method used.

NK cells arise from primitive CD34− marrow progenitors. Using primary human bone marrow (BM) stromal cells, we have shown CD34+ HLA-DR− progenitors give rise to functional NK cells in the presence of IL-2 (4). Furthermore, CD34+ CD7+ cells have a high cloning frequency for NK cell differentiation, suggesting a more committed and defined NK progenitor (5). With the addition of c-kit ligand (KL) and flt3 ligand (FL), the proliferation and the cloning frequency increase (6). If these and other cytokines are added, NK cell differentiation can occur in the absence of stroma (7, 8). Recently, we improved the efficiency of the NK cell assay by use of the murine fetal liver cell line AFT024, developed by Moore et al. (9). Using this feeder cell line, we have shown that NK cells can derive from human primitive progenitors at the single-cell level (6). AFT024 is permissive in that multilineage differentiation can occur into NK cells, B lineage cells, dendritic cells, or myeloid cells when cultures are supplemented with IL-2, KL, FL, IL-7, and one time addition of IL-3 at culture initiation.

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Received for publication March 27, 2001. Accepted for publication November 9, 2001.

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1 This work was supported in part by National Institutes of Health Grants R01-HL-55417 and PO1-CA-65493 and by Grant MO-1-RR00400 from National Center for Research Resources.

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3 Abbreviations used in this paper: CML, chronic myelogenous leukemia; UCB, umbilical cord blood; FISH, fluorescent in situ hybridization; eGFP, enhanced green fluorescence protein; Ph1, Philadelphia chromosome; A-LAK, adherent lymphokine-activated killer cell; KL, c-kit ligand; FL, flt3 ligand; BM, bone marrow; PB, peripheral blood; MSCV, murine stem cell virus.
The presence of benign primitive hemopoietic cells in patients with CML is well documented. CML BM contains a 10-fold higher number of CD34+ “HLA-DR+” cells compared with normal marrow and most are malignant as defined by the presence of the Ph1 and BCR/ABL mRNA in long term culture initiating cell progeny (10). In contrast, a majority of myeloid clonogenic progeny of CD34+ “HLA-DR+” populations are BCR/ABL+ and Ph1 negative. Even though B cells can clearly derive from the malignant clone in CML (11), it is still unclear how BCR/ABL affects the differentiation into other lymphoid lineages. We used CML CD34+ “HLA-DR+” cells, which are most likely to give rise to malignant progeny, to investigate whether NK cell differentiation is altered from BCR/ABL+ progenitors.

Materials and Methods

Umbilical cord blood (UCB), BM, and peripheral blood (PB)

UCB was obtained from full term mothers from local obstetrical units or from the St. Louis Cord Blood Bank (St. Louis, MO). BM and PB were obtained from patients with CML and from normal healthy volunteers. Mononuclear cells were obtained by Ficoll-Hypaque (specific gravity, 1.077; Sigma, St. Louis, MO) density gradient centrifugation. The use of all tissue was approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota. For the purpose of this study, chronic phase CML was defined as being within 3 years from diagnosis with no cytogenetic abnormalities other than Ph1. Advanced phase CML was defined as additional chromosome abnormalities or >5 years from diagnosis.

Purification of cells

NK cells were first enriched from PBMC using the immunomagnetic NK isolation kit (Miltenyi Biotec, Auburn, CA) per the manufacturer’s instructions and then stained with PE-conjugated anti-CD56 Ab (BD Biosciences, San Jose, CA) and FITC-conjugated anti-CD3 Ab (BD Biosciences). CD56brightCD3 and CD56dimCD3 NK cells were sorted on a FACStarPlus flow cytometer (BD Biosciences) and criteria previously described (2). PBMC were stained with FITC-conjugated anti-CD3 Ab (BD Biosciences) to obtain CD3+ T cells. In patients with higher white blood cell counts, myeloid cell depletion using CD15 immunomagnetic columns (Miltenyi Biotec) was performed before NK enrichment. CD34+ cells were enriched from either BM or UCB using the CD34 isolation kit (Miltenyi Biotec). To obtain CD34-“HLA-DR-” or CD34-“CD38-” cells, enriched cells were subjected to 4,6-diamidino-2-phenylindole-dihydrochloride-FITC-Texas Red filter sets (Chroma Technology, Brattleboro, VT). The probe set is configured to reveal two separate red (ABl) signals and two separate green (BCR) signals in a normal (Ph1-) cell. In a Ph1+ cell in which a classic 9;22 translocation is present, the expected pattern with this probe set is one fused signal (yellow or contiguous red and green), one green signal, and only one large red signal. This probe, with its extra signal, decreases the number of potential false positive results that would result from artificial juxtaposition of the labeled genes. Within our laboratory, the control range for Ph1-negative specimens is 0–0.6%.

Stromal cell lines

AFT024 was maintained at 33°C in low glucose DMEM (Life Technologies, Gaithersburg, MD) supplemented with 20% FCS (HyClone, Logan, UT) and 114 μM 2-ME (Bio-Rad, Hercules, CA) and subcultured in 96-well flat-bottom plates precoated with 0.1% gelatin (Specialty Media, La-Valette, NJ). Feeders were irradiated (2000 rad) after cells were grown to confluency before use.

Culture of hemopoietic progenitors

Sorted CD34+“HLA-DR-” cells were plated on AFT024 in limiting dilutions from 1200 to 45 cells/well with 22 replicates per concentration. DMEM (high glucose)-Ham’s F-12-based medium, developed to maximize NK cell growth, was supplemented with 24 μM 2-ME, 50 μM ethanolamine, 20 mg/L L-ascorbic acid, 50 μg/L sodium selenite, 100 μM penicillin, 100 U/ml streptomycin (Life Technologies), and 20% heat-inactivated human AB serum (North American Biologicals, Miami, FL) at culture initiation and reduced 10% for subsequent medium changes. Culture was conducted in 5% CO2 at 37°C for 5–6 wk and medium was half-changed with fresh medium and cytokines once a week. A well was considered positive for NK cells if it contained a distinct, specific CD56-CD3+ population by flow cytometry. The NK cell cloning frequency was calculated as the reciprocal of the concentration of cells that resulted in 37% negative wells using Poisson statistics and the weighted mean method. CD34+“enhanced green fluorescence protein” (eGFP) cells (600/well) were plated on irradiated AFT024 and analyzed after 3 wk of culture with twice weekly medium changes starting at day 7. Cultures were supplemented as indicated with 1000 U/ml IL-2 (a gift from Amon, Thorn- sound Oaks, CA), 10 ng/ml FL (a gift from Immunix, Seattle, WA), 20 ng/ml KL (a gift from Amgen), and 20 ng/ml IL-7 (R&D Systems, Minneapolis, MN). IL-3 (R&D Systems; 5 ng/ml) was added only once at culture initiation.

We designed a coculture assay to test the effect of BCR/ABL+ cells on normal hemopoiesis. Untransduced CD34+“CD38-” cells (300/well) were plated on AFT024 in 96-well plates using medium and cytokines to induce NK cell differentiation. On the day of initial plating, autologous CD34+ cells were prestimulated and transduced with eGFP or p210-eGFP as described below. One day after the last transduction, CD34+“BCR/ABL-” cells were harvested from AFT024 and analyzed for NK cell growth. Transduced progenitors initiated 5 days earlier. Cultures were analyzed 3 wk later. Experiments testing the effect of direct contact between BCR/ABL+ cells and normal progenitors were performed in 24-well plates using Transwell inserts with a 0.4-μm pore size membrane (Costar, Cambridge, MA). Neutralizing Abs against IFN-γ, TGF-β (both at 50 μg/ml; R&D Systems), and TNF-α (1/100; Genzyme, Cambridge, MA) were added to some cultures alone or in combination at culture initiation and with each medium change.

p210-eGFP retroviral vector

The full-length b3a2 BCR/ABL cDNA (a kind gift from Dr. J. Y. Wang, University of California, San Diego, CA; Ref. 12) was cloned upstream from the internal ribosomal entry site of the murine stem cell virus (MSCV)-internal ribosomal entry site-eGFP vector (a kind gift from Dr. W. Pear, University of Pennsylvania, Philadelphia, PA; Ref. 13). The BCR/ABL-containing vector was termed p210-eGFP. The correct sequence of the p210-eGFP construct was determined by DNA sequence mapping. The eGFP as well as the p210-eGFP plasmids (both at 50 μg/ml; R&D Systems), and TNF-α (1/100; Genzyme, Cambridge, MA) were added to some cultures alone or in combination at culture initiation and with each medium change.

Transduction of primary CD34+ cells

CD34+ cells obtained from UCB were cultured in IMDM supplemented with 20% FCS, 20 ng/ml each IL-7, KL, FL, and thrombopoietin (Amgen) for 2 days. CD34+ cells (10⁵) were then placed in collagen-coated Transwells of 6-well plates that had previously been incubated with 50 μg/ml...
recombinant CH296 fibronectin (Takara Shuzo, Otsu, Japan). Two milliliters (200 μl at a time) of p210-eGFP- or eGFP-containing retroviral supernatant were filtered through the Transwell, and cells were cultured with virus for 8 h at 37°C in 5% CO₂ (15). This was repeated two to three times. After 24 h, cells were labeled with anti-CD34-PE (BD Biosciences), and CD34⁺ eGFP⁺ cells were selected by FACS (FACStarPlus with Consort 32 computer) as bulk populations or using the single-cell deposition device on the FACStarPlus for clonal analyses. The progeny of NK cell cultures was analyzed by immunophenotyping. Polyethylene microbeads (30,000/well) were added to each tube before immunophenotyping, so that absolute cell numbers could be calculated based on the ratio with a known number of polyethylene beads. Allophycocyanin-conjugated anti-CD56 Ab (BD Pharmingen, San Diego, CA) and PE-conjugated anti-CD33 Ab (BD Biosciences) determined lymphoid (CD56⁺ cells) vs myeloid (CD33⁺ CD56⁻ ) origin, respectively.

Western blot for p210BCR/ABL

CD56⁺ eGFP⁺ FACS-sorted NK cells (1 × 10⁵) generated from p210-eGFP- or eGFP-transduced UCB CD34⁺ cells were lysed directly in SDS sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 5% 2-ME). After boiling, lysates were subjected to electrophoresis on 8% sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 5% 2-ME). After boiling, lysates were subjected to electrophoresis on 8% sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 5% 2-ME). After boiling, lysates were subjected to electrophoresis on 8%

Statistics

Results for experimental points obtained from multiple experiments are reported as the mean ± SEM. Significance levels were determined by two-sided Student t test analysis.

Results

Progenitors from patients with CML have a diminished NK cell cloning frequency

We designed experiments to determine whether CML progenitors can differentiate along the NK cell lineage using a stromal based, cytokine supplemented NK cell differentiation culture (6). BM was collected from normal donors or CML patients and CD34⁺ HLA-DR⁺ cells were isolated by flow cytometry (Fig. 1A). At the time of sample collection, the BM of all CML patients contained 100% Ph⁺ metaphases by standard G-banding. All chronic phase patients had Ph¹ as a sole chromosome abnormality. Three of four chronic phase patients were <2 years from diagnosis. Four patients were categorized as advanced phase based on additional chromosome changes or morphological blast crisis. One histologically chronic phase patient 6 years from diagnosis who received autologous transplantation 6 mo earlier was categorized as advanced phase. CD34⁺ HLA-DR⁺ cells were plated in limiting dilution on the murine feeder layer stroma, AFT024, with IL-2, FL, KL, IL-7, and IL-3. A well was scored as positive by the presence of CD56⁺ CD3⁻ cells after 5–6 wk of culture and the cloning frequency of the initially plated progenitor population was determined by Poisson statistics.

The cloning frequency of CD34⁺ HLA-DR⁺ cells from normal donors ranged between 2.5 and 4%. In contrast, the cloning frequency from CML patient’s cells was 10–400 times lower (p < 0.001; Table I). The addition of 5 ng/ml IL-3 at culture initiation is optimal for NK differentiation from normal donors (6). However, in some systems, IL-3 inhibits lymphoid development (16) and was therefore removed in some experiments (n = 5). Removal of IL-3 did not increase the cloning frequency of CML progenitors, suggesting that IL-3 inhibition was not the cause of abnormal NK cell differentiation (data not shown).

NK cells from patients with advanced phase CML are part of the malignant clone

Our in vitro studies show that CML progenitors poorly differentiate along the NK cell lineage. The low numbers of NK cell progeny derived from CML CD34⁺ HLA-DR⁺ cells were of mixed clonal origin when analyzed by FISH. The presence of some BCR/ ABL⁺ NK cells differentiating in vitro (from three of five patients studied) prompted additional experiments to determine the clonal origin of NK cells found circulating in vivo. NK cells were isolated from patients with CML into two well-characterized NK cell subsets, CD56⁺brightCD3⁻ and CD56⁺dimCD3⁻. NK cell subsets and simultaneously collected CD³ T cells were analyzed by FISH. T cells were consistently negative for BCR/ABL regardless of the disease stage (<5% positive; Fig. 1B). In patients with chronic phase CML, there was a slight increase in BCR/ABL⁺ NK cells compared with the T cell control. In contrast, almost one-half of the NK cells from both the CD56⁺bright (p = 0.015) and CD56⁺dim (p = 0.04) populations were BCR/ABL⁺ in patients...
with advanced phase CML compared with T cell controls. In one patient who had accelerated disease after autologous transplantation, two samples were analyzed separated by a 2-mo interval. Both samples showed >80% BCR/ABL+ NK cells. On the second sample, a majority of the cells expressed CD2 and CD7 (data not shown), similar to NK cells from normal donors. In addition, essentially all of these BCR/ABL+ NK cells expressed CD94 and a subset expressed the killer Ig-like receptor, KIR3DL1 (Fig. 1C). This finding verifies the lymphoid origin of these cells and shows that CD56 expression is not caused by aberrant expression on malignant myeloid cells (17). The paucity of circulating BCR/ABL+ NK cells in chronic phase CML suggests that BCR/ABL+ may be responsible for the diminished NK cell development and that molecular abnormalities, in addition to BCR/ABL+, may account for the increase in BCR/ABL+ NK cells found in advanced phase CML.

Transduced p210 into normal UCB CD34+ cells interferes with NK cell differentiation

To investigate the role of the BCR/ABL gene as the sole genetic abnormality accounting for decreased NK differentiation, CD34+ UCB cells were transduced with MSCV retrovirus containing eGFP (control) or p210-eGFP. CD34+ eGFP+ and CD34+ p210-eGFP+ cells were sorted by FACS and plated (600 cells/well) on irradiated AFT024 feeders in medium containing IL-2, KL, FL, IL-7, and IL-3. After 3 wk culture, cells were harvested and analyzed for eGFP+ NK cells (Fig. 2A). Because some cord blood NK cells can express CD33 (18), mutually exclusive populations of NK cells were defined as CD56+, and myeloid cells were defined as CD33+ CD56−. Expression of eGFP in NK cells was lower than in myeloid cells by both the control and p210-containing vector, suggesting that the constructs may be differentially regulated between lymphoid and myeloid cells. The control construct containing eGFP alone did not affect NK cell differentiation compared with untransduced cell (data not shown). In contrast, p210-eGFP-transduced progenitors poorly supported NK cell differentiation. The absolute number of NK cells generated from p210-eGFP-transduced progenitors was significantly decreased compared with eGFP-transduced control cells (Fig. 2B). Western blot analysis of sorted eGFP+ CD56+ NK cells generated from p210-eGFP-transduced UCB CD34+ cells shows expression of p210 protein, demonstrating that p210 can be translated through development into CD56+ NK cells (Fig. 3). Transduction of normal UCB CD34+ cells with p210 does not interfere with myeloid differentiation and increases the growth of myeloid cells (Fig. 2C), suggesting that decreased NK differentiation was not due to a general survival disadvantage but rather a selective effect on lymphoid differentiation.

Exogenous addition of p210-eGFP transduced CD34+ progenitors inhibits normal hematopoiesis

The above results suggest that the BCR/ABL gene product (p210) is responsible for decreased NK cell differentiation. However, because AFT024 cultures support both NK cell and myeloid cell differentiation from the same starting population, it is not clear whether p210 acts intrinsically within the undifferentiated cells or whether NK differentiation is extrinsically inhibited by the interaction with BCR/ABL+ cells. To test the extrinsic hypothesis, untransduced CD34+ CD38− UCB progenitor cells were cocultured...
alone, with eGFP, or with p210-eGFP-transduced autologous CD34+ UCB cells under NK differentiation conditions. After 3 wk culture, when CD34+ eGFP+ autologous cells were added, only 2.6% of progeny were eGFP+ and therefore mostly derived from the initially plated CD34+CD38- cells. In contrast, when CD34+ p210-eGFP+ autologous cells were added, 93% of progeny were eGFP+, showing a proliferative advantage of p210-eGFP-transduced cells over untransduced normal cells (Fig. 4A). Addition of eGFP-transduced cells had little effect on normal myeloid or NK cell differentiation, whereas addition of p210-eGFP-transduced cells significantly decreased normal hemopoiesis of both lineages. To determine whether p210 changed the proportion of outgrowth by overwhelming myeloid proliferation, absolute numbers were determined. The addition of eGFP-transduced cells did not affect the absolute number of normal NK cell or myeloid cells compared with outgrowth of initially plated CD34+CD38- cells without exogenous cells. In contrast, compared with eGFP controls, the absolute numbers of both normal CD56+ NK cells as well as CD33+CD56- myeloid cells was significantly decreased by the addition of p210-eGFP-transduced cells (Fig. 4, B and C). Neutralizing Abs against TNF, IFN-γ, and TGF-β increased NK cell outgrowth from control cultures but did not abrogate the suppression by extrinsic BCR/ABL+ cells (data not shown). When normal progenitors were separated from BCR/ABL+ progenitors, suppression was incomplete suggesting that direct contact between normal progenitors and malignant progenitors was required for maximum suppression (Fig. 5). These findings show that BCR/ABL-transduced cells extrinsically suppress normal differentiation and suggest a mechanism by which the malignant clone dominates over normal hemopoiesis as seen in patients with CML.

The above experiments definitively show that BCR/ABL+ cells extrinsically inhibit NK cell differentiation. However, it was still unclear from the progenitor transduction experiments presented in Fig. 2 whether BCR/ABL intrinsically blocks differentiation along the NK cell lineage or whether the inhibition seen from p210-transduced progenitors is all from extrinsic BCR/ABL+ cell interactions. To address this question, we analyzed single CD34+ progenitors transduced with eGFP or p210-eGFP to directly determine cloning frequencies for lymphoid and myeloid progeny. Single eGFP+CD34+ or p210-eGFP+CD34+ cells were deposited onto pre-established AFT024 feeders, and cytokines were supplemented as in the above experiments. The frequency of single cells giving rise to any cell growth was greater from p210+ progenitors than from control progenitors, and p210+ cells give rise to more progeny per single cell (Table II). The cloning frequency of p210+ cells capable of NK differentiation was 8.1%, higher than the 3.2% seen with control cells. Therefore, by limiting the input cells to single progenitors and minimizing their extrinsic interaction with BCR/ABL+ cells, we can exclude a major intrinsic effect of BCR/ABL on the capacity of progenitors to differentiate along the NK cell lineage. In contrast to control progenitors, single p210-eGFP+CD34+ progenitors resulted in a smaller percentage of NK cells, less NK cells per progenitor, and a marked increase in the absolute number of CD33+CD56- myeloid cells. Single p210-eGFP+CD34+ progeny resulting in >100,000 myeloid cells (i.e., more extrinsic suppression) simultaneously gave rise to 341 ± 144 NK cells (n = 16), whereas single-cell progeny resulting in <100,000 myeloid cells gave rise to 408 ± 1126 NK cells (n = 115; p = 0.0016).
primary BCR/ABL normality resulted in decreased NK cell differentiation similar to transduction of UCB CD34+ cells. Exogenously added CD34+ eGFP- or CD34+ p210-eGFP-transduced UCB cells (500 cells) were plated in direct contact with (Contact) or separated from untransduced progenitors (plated in the bottom well) by a Transwell membrane (TW). Each bar shows the mean ± SEM of three replicates for each condition from two separate donors.

Discussion

We demonstrated that BCR/ABL+ progenitors from CML patients have decreased NK cell cloning frequency compared with normal progenitors of the same phenotype. In chronic phase CML, this may explain the predominance of BCR/ABL- NK cells found in blood, albeit in low numbers. As CML becomes more advanced, the presence of circulating BCR/ABL+ NK cells increases, which may be due to secondary molecular abnormalities. Retrovirus transduction of UCB CD34+ cells with BCR/ABL as the sole abnormality resulted in decreased NK cell differentiation similar to primary BCR/ABL+ CML progenitors. We hypothesized that the inhibition of NK cell differentiation by BCR/ABL was from intrinsic mechanisms within the cell or from extrinsic interactions with BCR/ABL+ cells. Single-cell experiments show that p210-transduced progenitors actually give rise to a higher frequency of cells capable of NK cell differentiation than do eGFP-transduced controls. This supports the notion that p210 within an undifferentiated progenitor does not inherently block NK cell differentiation. Although the single-cell NK cell cloning frequency was higher for p210-transduced progenitors, the number of NK cells per single cell was lower than in the eGFP-transduced controls, and NK cell development was lowest from single cells that differentiated into the highest number of myeloid cells. These data and data from Fig. 5 support the conclusion that although soluble factors may play a partial role, the interaction between normal progenitors in direct contact with BCR/ABL+ cells is predominantly responsible for the inhibition of NK cell differentiation from normal progenitors.

The clonal origin of CML lymphoid cells has been extensively studied using different selection and detection techniques with variable results. Cultured and FACS-purified T cells from CML patients have minimal involvement of BCR/ABL (19), whereas B cells have reproducibly been found to be BCR/ABL+ (11) However, some T cells clones from CML patients are BCR/ABL+ by PCR (20), suggesting that the BCR/ABL gene can occur in pluripotent stem cells even though the number of involved T cells is minimal. Our results showing BCR/ABL+ T cells from CML patients is consistent with others and extends the knowledge that T cells mostly stay BCR/ABL+ even after disease progression. For the NK cell lineage, our laboratory previously showed that A-LAK cells generated from CML patients were BCR/ABL- (3). However, this method relied on plastic adhesion and expansion to detect NK cell outgrowth; therefore, malignant NK cells, which may have different adhesion and proliferation characteristics, were likely missed. This is consistent with data by other groups showing that A-LAK cultures are positive for BCR/ABL by RT-PCR (21). FACS and FISH are direct methods to assess BCR/ABL in fresh NK cells without a functional bias and definitively show BCR/ABL involvement of NK cells in vivo. Although T cells and NK cells share a common committed progenitor (22), the differentiation process to respective lineages varies. Thymic influence, both humoral and direct contact with thymic epithelial cells, results in T cell development and clonal selection. In contrast, NK cell differentiation occurs in BM. This may explain the difference of Ph1 involvement of T cells and NK cells in advanced stage CML.

Takahashi et al. (23) studied 12 patients with CML and analyzed lymphoid progenitors and mature lymphocytes by FISH. Of the 12 patients studied, 4 were newly diagnosed and untreated, 3 were in chronic phase and on standard therapy, and 5 of their patients fit our definition of advanced phase based on having CML for >5 years or additional cytogenetic abnormalities (n = 2). They found that 15–70% of B lymphocytes contain the BCR/ABL gene abnormality while no NK cell or T cell populations were involved. The discrepancy between their result and our study is uncertain but may be explained in part by their therapy. All of Takahashi’s advanced patients were treated conventionally; however, four of six of our advanced patients had autologous transplantation before this study. Takahashi also showed that purified CD34+CD7- and CD34+CD7-CD5- NK and T cell progenitors were 80% positive for the BCR/ABL gene rearrangement. The finding of BCR/ABL in

![Table II. Single eGFP CD34+ cells give rise to NK cell and myeloid cell progeny](#)

<table>
<thead>
<tr>
<th>Analysis Parameter</th>
<th>eGFP</th>
<th>p210-eGFP</th>
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<tr>
<td>Single cells yielding any growtha</td>
<td>62/949 (6.5%)</td>
<td>141/791 (17.8%)</td>
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<tr>
<td>Absolute progeny per single cell</td>
<td>12,035 ± 4,363</td>
<td>64,281 ± 6,099</td>
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<tr>
<td>Single cells yielding NK growthb</td>
<td>31,914 (3.2%)</td>
<td>64,791 (8.1%)</td>
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<td>% NK from all single cells with growth</td>
<td>41 ± 5.5</td>
<td>7.2 ± 1.7</td>
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<td>Absolute NK cells per single cell</td>
<td>18,955 ± 8,357 (n = 31)</td>
<td>7,139 ± 1,940 (n = 64)</td>
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<tr>
<td>Single cells yielding myeloid growthd</td>
<td>42,949 (4.4%)</td>
<td>128,791 (16.2%)</td>
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<tr>
<td>% myeloid from all single cells with growth</td>
<td>27 ± 4</td>
<td>66 ± 2</td>
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<tr>
<td>Absolute myeloid cells per single cell</td>
<td>1,658 ± 566 (n = 42)</td>
<td>44,367 ± 4,238 (n = 128)</td>
</tr>
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</table>

a Single eGFP+CD34+ cells derived from five separate UCB units were cultured on AFT024 feeders and cytokines for 26–29 days and then harvested for analysis.

b Single-cell growth determined by the visual presence of >100 cells by light microscopy.

c NK cell growth determined by the presence of CD56- cells by FACS.

d Myeloid cell growth determined by the presence of CD33-CD56- cells by FACS.
progenitors but not in mature lymphocytes is in agreement with our data that show BCR/ABL results in diminished lymphoid differentiation.

Chronic phase CML patients typically have a Ph1 chromosome as the sole cytogenetic abnormality. However, other genetic changes below the detection threshold of routine cytogenetics may exist. These undetectable genetic changes may play a role in the decreased NK cell differentiation seen in CML. To investigate the role of BCR/ABL as a sole genetic abnormality on NK differentiation, we used retrovirus transduction of BCR/ABL using a MSCV construct. One caveat to this experimental design is that BCR/ABL-transduced progenitor cells may differ from CML progenitors in p210 expression. However, in support of this strategy, Zhao et al. (24) demonstrated that BCR/ABL transduction into UCB progenitors results in similar characteristics of primary CML progenitors such as defective adhesion to fibronectin, enhanced migration, delayed apoptosis following withdrawal of cytokines, and increased myeloid colony formation. Our results with the decreased NK cell differentiation in BCR/ABL-transduced progenitors mimic CML progenitors and support this experimental model to study mechanisms of CML hematopoiesis.

Because BCR/ABL-transduced progenitor cells gave rise to decreased NK cells, the involvement of BCR/ABL is definitive. However, the exact mechanism involved in the intrinsic inhibition of NK cell differentiation by BCR/ABL+ cells is unknown. The presence of the p210BCR/ABL tyrosine kinase is essential and sufficient for malignant transformation of hematopoietic cells (25–28). A large number of studies have shown that p210BCR/ABL can bind and activate molecules to alter a variety of functions including cytoskeletal proteins (29), RAS (30, 31), phosphatidylinositol 3'-kinase (32), and Janus kinase/STAT signaling pathways (31). These changes induced by BCR/ABL may be involved in the competitive inhibition of normal hematopoiesis by CML cells. BCR/ABL and G-CSF administration have similar inhibitory effects on NK cell development (33). Both also lead to proliferation and egress of myeloid progenitors into the PB: G-CSF by benign alteration of progenitor adhesion (34); and CML by abnormal adhesion as a result of BCR/ABL rearrangement (35). In addition, production of G-CSF and IL-3 in primary CML CD34+ cells has been detected by RT-PCR and intracellular cytokine staining (36), which may explain the autonomous growth of CML cells. These findings may partially explain the intrinsic inhibition of NK cell differentiation by cytokines produced by BCR/ABL+ myeloid cells as shown here. Reactive oxygen metabolites produced during the respiratory burst in myeloid cells and NADPH oxidase-triggered CML cells may also play a role (37).

At least four groups have shown that transplantation of murine progenitors transduced with BCR/ABL cDNA-containing retroviral vectors creates a myeloproliferative disease resembling chronic phase CML with a latency of 4–6 wk (28). Other groups used transgenic strains of mice expressing BCR/ABL. Most of these transgenic mice developed lymphoproliferative disease such as lymphoma and acute lymphoblastic leukemia (38, 39), suggesting preferential effects on the lymphoid lineage in this model. Whether decreased NK cell differentiation in BCR/ABL-transduced progenitors described here is also seen in the murine models is yet to be determined.

In summary, BCR/ABL results in abnormalities in NK cell differentiation from malignant progenitors by the extrinsic interaction with BCR/ABL+ cells. Our data support the notion that CML progenitors maintain their multipotent stem cell properties and that BCR/ABL cell-cell interactions bias against NK cell differentiation. Other than a few case reports of NK cell blast crisis (40), we are the first to show that NK cells can be part of the malignant clone as CML progresses. This latter finding may be through mechanisms other than BCR/ABL. Further investigation of these mechanisms will result in important insights into fate decisions of uncommitted stem cells to the myeloid vs lymphoid lineage.

Acknowledgments

We thank Brad Anderson for his expertise in flow cytometry and Valerie McCullar for her excellent technical assistance and help with data analysis.

References


in the chronic phase as shown by a combination of fluorescence-activated cell sorting and fluorescence in situ hybridization. Blood 92:4758.


