Autologous Activated Natural Killer Cells Suppress Primitive Chronic Myelogenous Leukemia Progenitors in Long-Term Culture

By Francisco Cervantes, Bryce A. Pierson, Philip B. McGlave, Catherine M. Verfaillie, and Jeffrey S. Miller

A graft-versus-leukemia effect has been well documented to prevent relapse in chronic myelogenous leukemia (CML) after allogeneic marrow transplantation. One type of lymphocytes that may contribute to this effect are natural killer cells (NK), which, after activation with interleukin (IL)-2, exhibit a broad range of cytolytic activity against allogeneic and autologous cells. We have previously demonstrated that IL-2-activated NK (ANK) can be generated from blood of patients with CML and are benign in origin. Their proliferation and function, however, diminish with disease progression in CML, suggesting a role in tumor surveillance. We studied the effect of IL-2-activated NK (ANK) on normal and malignant primitive and committed progenitors in a novel long-term bone marrow culture (LTBMC) assay. Because ANK destroy marrow stromal layers, the use of classic stroma-dependent long-term cultures is not possible. Therefore, we used the stroma noncontact LTBMC system developed in our laboratory to analyze the effect of autologous ANK cells on primitive hematopoietic progenitors. Autologous ANK (CD56+/CD3-1) were generated from the peripheral blood of 10 patients with chronic phase CML and from six normal individuals by culturing CD56/CD8-depleted mononuclear cells for 14 days in 1,000 U/mL IL-2. At the same time ANK cultures were initiated, sorted normal (CD34+) and CML (CD34+/DR+) marrow populations were plated in Transwell inserts of the stroma noncontact culture. On day 15, hydrocortisone, which rapidly inhibits ANK function, was removed, and autologous ANK were added to the Transwell inserts with fresh LTBMC medium without hydrocortisone but supplemented with 1,000 U/mL IL-2. After 48 hours, the number of colony-forming cells (CFC) was enumerated in methycellulose culture. To determine the effect of ANK on more primitive long-term culture-initiating cells (LTICIC), the IL-2-supplemented LTBMC medium was replaced with fresh hydrocortisone containing LTBMC medium, and cultures were maintained for an additional 5 weeks. We demonstrate that autologous ANK did not suppress normal CFC or LTICIC. In contrast, ANK from eight patients with CML with potent cytotoxicity against NK-sensitive (K562) and NK-resistant (Raji) tumor targets exhibited an ANK dose-dependent suppression of both CFC and LTICIC. Interestingly, ANK from two patients with CML who exhibited diminished cytotoxicity also did not suppress autologous CFC and LTICIC. These studies indicate that ANK with potent major histocompatibility complex unrestricted cytotoxic activity suppress malignant hematopoiesis. This effect was not mediated by soluble factors and was absolutely dependent on direct cell-to-cell contact. We further demonstrate that the β2 integrin receptor is involved in ANK recognition of CML targets. These observations support the use of autologous ANK therapy to prevent relapse of CML after autologous marrow transplantation or use of ANK to purge CML marrow for autologous transplantation.

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greater extent than when normal CD34+/DR- cells are cultured in classic long-term bone marrow culture (LTBMc), where progenitors are plated in direct contact with stroma. Additional studies have demonstrated that the murine stromal cell line (M2-10B4) can replace primary human marrow stroma used in either classic long-term culture or stroma noncontact culture. We hypothesize that modified long-term culture systems where progenitors are suspended above stroma should be amenable to further modifications to test the effect of ANK and other effector populations on normal and malignant LTCIC. We developed a novel culture system in which ANK are coincubated with primitive progenitors but are separated from the stromal feeder. ANK can be functionally inactivated by adding hydrocortisone back to the culture medium, and the long-term culture can be continued to assess LTCIC. Using this assay, we demonstrate for the first time that autologous ANK can suppress malignant CML LTCIC while sparing normal hematopoiesis.

**MATERIALS AND METHODS**

**Study population.** Peripheral blood and bone marrow from 12 patients with chronic-phase Ph-positive CML were studied. Median duration of the chronic phase was 17 months (range, 4 to 43 months); six patients were studied within 1 year from diagnosis. Two patients had been treated with hydroxyurea, one with alpha interferon, eight with both drugs, and one had received busulfan as initial therapy. One had been treated with hydroxyurea, one with alpha interferon, eight with both drugs, and one had received busulfan as initial therapy. All patients and control samples were obtained using guidelines approved by the Committee on Human Subjects in Research at the University of Minnesota (Minneapolis, MN). All patients and control samples were obtained using guidelines approved by the Committee on Human Subjects in Research at the University of Minnesota (Minneapolis, MN).

**Purification of peripheral blood**. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque (Sigma Diagnostics, St. Louis, MO) density gradient centrifugation (30 minutes at 37°C and 400g). When the WBC count was higher than 10 x 10^6/L in patients with CML, PBMC were subjected to counterflow centrifugation elutriation. PBMC resuspended in phosphate-buffered saline (PBS) supplemented with 0.3% bovine serum albumin (BSA) and 0.01% EDTA were injected into an elutriation system with a standard separation chamber (Beckman Instruments, Palo Alto, CA) primed with PBS-BSA-EDTA. Rotor speed and temperature were maintained at 1,950 rpm and 10°C. After loading, 200 mL of effluent was collected at a flow rate of 6 to 16 mL/min. The NK population was further purified by panning PBMC or elutriated PBMC onto MicroCELLector flasks coated with CD5 and CD8 monoclonal antibodies (provided by Applied Immune Sciences, Inc, Santa Clara, CA) as described.16 The resulting nonadherent cells were reincubated into a fresh MicroCELLector flask for a second depletion before use. Some ANK populations were prepared by sorting CD56-/CD3- NK from PBMC after immunomagnetic bead depletion of PBMC using anti-CD3 (Becton Dickinson, Palo Alto, CA) and anti-CD5 (Becton Dickinson) antibodies with the addition of anti-CD19 (Becton Dickinson), MY8 (Coulter Cytometry, Hialeah, FL), and anti-glycoprotein A (AMAC, Westbrook, MD) antibodies for patients with CML.

**Culture of ANK.** Fluorescence-activated cell sorter (FACS)-purified CD56-/CD3- NK were expanded in direct contact with M2-10B4 accessory cells as previously described. Enriched NK populations from CD5/CD8 panning were suspended at a concentration of 0.5 x 10^6/mL in 5 mL of complete culture medium containing 1,000 U/mL of recombinant IL-2 (a gift from Amgen Inc, Thousand Oaks, CA) in horizontal T-25 flasks (Corning Glassware, Corning, NY) at 37°C in 5% CO2 in a humidified atmosphere. Complete culture medium consisted of a 2:1 vol/vol mixture of Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 supplemented with 10% human heat-inactivated AB serum (North American Biologica, Miami, FL), 24 μg/mL/2-mercaptoethanol, 50 μg/mL ethanolamine, 20 μg/mL L-ascorbic acid, 5 μg/mL of sodium selenite (NaSeO3), and 100 U/mL penicillin plus 100 U/mL streptomycin (GIBCO Laboratories, Grand Island, NY). At day 7 of culture, the culture volume was doubled with fresh NK medium, and thereafter, full medium changes were performed twice weekly.

**Cytotoxicity assay.** ANK were tested at day 14 for cytotoxicity against the NK-sensitive cell line K562 (American Type Culture Collection [ATCC], Rockville, MD) and the NK-resistant cell line Raji (ATCC) in a 4-hour [Cr] release assay as previously described. Purification of bone marrow primitive progenitors. Bone marrow was aspirated from the posterior iliac crest and bone marrow mononuclear cells (BMMNC) separated using Ficoll-Hypaque centrifugation. An enriched CD34+ population was obtained as previously described. The resultant cells were labeled with anti-CD34 (phycoerythrin [PE]; Becton Dickinson) antibody for normal individuals and with both anti-CD34 (PE) and anti-HLA-DR fluorescein isothiocyanate (FITC) antibodies for patients with CML. Cells were then sorted on the FACs Star plus laser flow cytometer (Becton Dickinson) selecting CD34+ cells from normal marrow and CD34+/DR- cells from CML marrow as previously described.

**Hematopoietic progenitor culture.** LTBMc of hematopoietic progenitors were established in a stroma noncontact system as previously described. This consisted of 24-well plates containing the murine marrow stroma-derived fibroblastic cell line M2-10B4 (a gift from Dr Connie Eaves, Terry Fox Laboratories, Vancouver, Canada). A collagen-treated Transwell insert (0.4-μm microporous filter) (Costar, Cambridge, MA) was placed above the stromal layers and 5,000 CML CD34+/DR- cells or 10,000 normal CD34+ cells were plated in the upper wells. Complete LTBMc medium consisted of Iscove's Modified Dulbecco's Medium (IMDM) with 12.5% fetal calf serum (FCS), 12.5% horse serum (Terry Fox), 2 mMolar L-glutamine (GIBCO), 100 U/mL penicillin plus 100 U/mL streptomycin (GIBCO), and 10−6 Molar hydrocortisone.

**Incubation of ANK with hematopoietic progenitors.** On day 14 of culture, LTBMc medium was removed from stroma noncontact cultures and replaced with LTBMc medium without hydrocortisone (HC) but supplemented with 1,000 U/mL IL-2. Autologous normal, allogeneic normal, or autologous CML ANK were then added at effector-to-target (E:T) ratios ranging from 60:1 to 2.2:1 to the cultured progenitors present in the Transwell inserts above the stroma. After 48 hours, the cells in some of the Transwell inserts were harvested and plated in methylcellulose culture to determine the number of CFC present, as described previously. When allogeneic ANK were used, 5 x 10^6/mL HC was added to the methylcellulose culture to inactivate ANK. In the remaining wells, the HC-free, IL-2-containing medium was removed, and HC-containing, IL-2-free LTBMc medium was added to inactivate ANK. Progenitors were maintained in culture for an additional 5 weeks and then plated in methylcellulose culture to determine the number of LTCIC (Fig 1). For the antibody blocking experiments, specific antibodies were added to the Transwell insert containing cultured progenitors 30 minutes before the addition of ANK. The antibodies used were as follows: anti-human interferon (IFN)-γ neutralizing antibody, 50 μg/
Effect of HC on normal ANK function. To determine if the presence of HC in LTBMC medium would affect the cytolytic capacity of ANK, studies were performed with ANK derived from eight healthy volunteers. On day 14 of ANK culture, cells were harvested, and 2 × 10⁶ ANK were incubated in LTBMC medium without HC plus 1,000 U/mL IL-2 (LTBMC − HC + IL-2), LTBMC medium with 10⁻⁶ mol/L HC plus IL-2 (LTBMC + HC + IL-2), or standard LTBMC medium (LTBMC + HC − IL-2). Cytotoxicity against an NK-sensitive target (K562) and NK-resistant target (Raji) was evaluated after 18- and 42-hour exposure. Incubation of ANK with HC for 18 hours significantly reduced the cytotoxic activity against Raji targets and less so against K562 targets (Fig 2). However, 42-hour incubation of ANK in the presence of HC markedly inhibited cytolytic activity against both targets (Fig 2). Addition of IL-2 to LTBMC medium containing HC partially preserved cytotoxic activity of ANK, suggesting that IL-2 can only partially reverse the decrement in ANK function induced by HC. This demonstrates that HC has a significant and rapid inhibitory effect on ANK function. Therefore, evaluation of ANK cytotoxicity for LTCIC in LTBMC should not be performed in the presence of HC.

ANK generation and function. Optimal generation of ANK from normal blood requires monocytes as accessory cells. We have previously demonstrated that a CD5/CD8 depletion leads to an optimal starting population of NK precursors and monocytes. However, these methods are not adequate to generate ANK from patients with CML with high WBC counts, because the blood of patients with CML is flooded with myeloid cells that are inadequately removed by Ficoll Hypaque separation. To further enrich for lymphoid cells in patients with CML, counterflow centrifugation elutriation was used at a constant rotor speed of 1,950 rpm and variable flow rate ranging from 6 to 16 mL/min. A flow rate of 8 mL/min significantly enriched for cells falling within the lymphocyte gate on the forward and side scatter by flow cytometry compared with the starting mononuclear fraction (61.3% ± 4.2% vs. 18% ± 5.7%, n = 6; P = .001) and increased the number of cells expressing CD7 (44% ± 4.7% vs. 9.5% ± 4.1%, n = 6; P = .003), an antigen found on both NK and T lymphocytes. In five of six experiments, there was less than 1% residual CD7⁺ cells found in the rotor off fraction. The resulting fraction 8 cells were then depleted of CD5/CD8-positive cells, and ANK were generated using the same methods as previously described for the generation of ANK from normal blood. There was no difference in function between ANK processed with or without elutriation (data not shown).

At day 14 of culture, morphologic examination of Wright-Giemsa-stained cells demonstrated a marked preponderance of cells with the appearance of large granular lymphocytes. Phenotypic analysis of ANK demonstrated a similar number of CD56⁺/CD3⁻ cells from normal donors (95% ± 1.6%) and patients with CML (88% ± 2%). However, the number of CD56⁺/CD3⁻ cells was significantly lower in patients with CML (57.4% ± 4.5%) than in normal donors (79.5%

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**Statistics.** Results from experimental points obtained from multiple experiments were reported as mean ± standard error of the mean (SEM). Significance levels were determined by two-sided Student’s t test analysis.
Fig 2. Effect of HC on ANK function. ANK from eight normal donors were harvested after 14 days of culture and preincubated in LTBMC medium without 10^{-6} mol/L HC but with 1,000 U/mL IL-2 (LTBMC - HC+ IL-2) for 42 hours to serve as the baseline function for each panel (O). The effect of HC on ANK was tested by incubating effectors in medium containing LTBMC + HC + IL-2 (open symbols in A and C) or LTBMC + HC - IL-2 (open symbols in B and D) for 18 (C) or 42 hours (D). All data represent cytotoxicity tested in Cr\(^{51}\) assays against the NK-sensitive K562 target (A and B) and NK-resistant Raji target (C and D). Data are presented as the mean ± SEM at six different E:T ratios.

Poor ANK function was observed in two patients with CML, in whom the CD56\(^{bright}\)/CD3\(^-\) cells represented only 18.1% and 26.8% of the total cells, respectively.

The cytotoxicities of ANK from the seven patients with CML in whom greater than 40% of ANK were CD56\(^{bright}\)/CD3\(^-\) and the six normal donors are presented in Fig 3. There was a small but significant decrease in cytotoxicity against K562 and Raji between ANK derived from normal donors and ANK derived from patients with CML. Two patients with CML, in whom only 18.1% and 26.8%, respectively, of ANK were CD56\(^{bright}\)/CD3\(^-\), exhibited significantly diminished killing of K562 and especially of Raji cells compared with the other CML or normal ANK populations (Fig 3). Differences in cytotoxicity could be correlated (correlation coefficient \(R = .938; P < .001\)) with differences observed in the number of CD56\(^{bright}\)/CD3\(^-\) cells within the ANK population (Fig 4).

Hematopoietic progenitor studies. Because HC rapidly inhibits ANK function and ANK rapidly lose activity if not maintained in IL-2,\(^{19}\) we designed a novel long-term culture assay to test the effect of ANK on primitive progenitor populations (Fig 1). CD34\(^+\) cells from normal donors (containing both CFC and LTCIC) and CD34\(^+/DR\(^+\) cells from patients with CML (containing Ph\(^+\) CFC and LTCIC) were plated in Transwell inserts placed above M2-10B4 stromal monolayers (Fig 1, step 1, 2). After 14 days, HC-containing LTBMC medium was replaced by IL-2-containing, HC-free LTBMC medium, and autologous ANK were added to the Transwell insert (Fig 1, step 3). After 48 hours, the culture medium was removed and replaced with HC-containing LTBMC medium free of IL-2 to inactivate residual ANK function, and long-term culture was continued (Fig 1, steps 4 and 5).

In control experiments, IL-2 alone or IL-2 plus ANK placed in Transwell inserts above M2-10B4 had no detrimen-
The number of absolute colonies derived from the initially plated normal CD34+ cells compared with CML CD34+/DR+ cells maintained in LTBMC alone was not significantly different for either CFC (201 ± 12 vs 189 ± 29) or LTCIC secondary colonies (89 ± 8 vs 96 ± 24). The number of CFC and LTCIC was not affected by the 48-hour removal of HC and addition of IL-2 to normal CD34+ cells (92% ± 4% of control CFC; 102% ± 11% of control LTCIC) or CML CD34+/DR+ cells (93% ± 6% of control CFC; 97% ± 3% of control LTCIC). Finally, addition of autologous CML ANK without removal of HC and without addition of IL-2 did not result in suppression of either CML CFC or LTCIC.

Coculture of normal CD34+ cells with autologous ANK did not affect the numbers of CFC or LTCIC at any of the E:T ratios tested. In marked contrast, when ANK from the seven patients with CML, which exhibited significant killing of K562 and Raji tumor target cells, were incubated with CD34+/DR+ cells, a dose-dependent suppression of the growth of both CFC and LTCIC was observed (Fig 5). Although the data are presented as the E:T ratio based on the number of initial CD34+/DR+ cells plated on day 0 to standardize experiments, there was significant expansion in the Transwell before addition of ANK (12.7-fold expansion; $n = 7$). Therefore, the true 60:1 E:T ratio in the assay is significantly lower (approximately 5:1) and conceivably more physiologic. To further support the observation that suppression induced by CML ANK on progenitors correlated with ANK functional capacity, we demonstrate that ANK from the two patients with CML that killed K562 and Raji poorly also did not suppress the growth of autologous CFC and LTCIC (Fig 5).

CML progenitor killing by ANK is major histocompatibility complex (MHC)-unrestricted and mediated by direct cell-to-cell contact. As the effect of autologous ANK on CFC and LTC-IC was essentially the same, the shorter readout CFC assay was used in subsequent experiments. The mechanism of NK killing is presumed to be MHC-unrestricted. Therefore, allogeneic normal NK should have the same effect on CML progenitors as autologous NK. This was tested directly using a cultured population of allogeneic normal ANK (greater than 90% CD56+bright) devoid of T-lymphocytes (less than 1%) prepared by sorting, which resulted in significant suppression of CML progenitors (Fig 5). The increased killing observed with allogeneic normal ANK
ANK SUPPRESS CML PROGENITORS

Fig 5. ANK suppress autologous malignant progenitors but not benign progenitors. ANK were incubated with normal CD34⁺ (○) or CML CD34⁺/DR⁺ progenitors (open symbols) cultured for 14 days in stroma noncontact culture as described in Fig 1. ANK were added to autologous progenitors in medium without HC and with IL-2 for 48 hours. To evaluate the effect of ANK on CFC, CD34⁺ and CD34⁺/DR⁺ progeny were evaluated after the 48-hour incubation with ANK by plating in methylcellulose colony assay. To evaluate the effect of ANK on LTCIC, LTBMNC medium (+HC - IL-2) was added after the 48-hour incubation and was maintained for 5 weeks and then evaluated for the number of secondary CFC. CFC (left) and LTCIC (right) were analyzed in an autologous setting from normal donors (B, n = 6), patients with CML with good ANK function (C, n = 8), and patients with CML with poor ANK function (D, n = 2). CFC were also evaluated after incubation of CML progenitors with allogeneic normal ANK (◊, n = 7). CML ANK with good function significantly suppressed autologous CFC at all but the lowest E:T ratio (P < .01 for the three highest E:T ratios) and significantly suppressed autologous LTCIC at all E:T ratios (P < .001 for each E:T ratio) compared with normal ANK and autologous progenitors. CML ANK with poor function did not suppress CFC or LTCIC similarly to normal ANK and autologous progenitors. There was a significant difference in suppression between CML ANK with good function and CML ANK with poor function for autologous CFC (P < .05 at three highest E:T ratios) and LTCIC (P < .015 at all but the E:T ratio of 6:1, where P = .99).

Compared with autologous CML ANK was not significant and could be explained by the higher number of CD56⁺bright cells in the normal allogeneic population and corresponding increased cytotoxicity of the normal ANK population compared with ANK from patients with CML (similar to data in Fig 3, data not shown).

The importance of direct contact on the observed ANK suppression of CML progenitors was further evaluated. CML CD34⁺/DR⁺ progenitors were first initiated into long-term culture in Transwells as described above for 14 days and then transferred into four different conditions (Fig 6). In condition A, Transwells containing CML progenitors were transferred to a well containing M2-10B4 conditioned medium (+IL-2) alone in the bottom compartment, and after 48 hours, progenitors were harvested from the Transwell and plated into methylcellulose culture without IL-2. There was no difference in CFC outgrowth from CML progenitors incubated above viable stroma or progenitors incubated in conditioned medium (110 ± 46 CFC v 95 ± 40 CFC, n = 4; P = not significant [NS]). In condition B, Transwells containing CML progenitors were transferred to a well containing M2-10B4 conditioned medium (+IL-2) and ANK (E:T, 60:1) in the bottom compartment. After 48 hours, progenitors were harvested from the Transwell and plated into methylcellulose culture without IL-2. There was no suppression of CML progenitors by soluble factors produced by ANK (Fig 6, condition B).

Direct contact between ANK and CML progenitors was tested by adding ANK to CML progenitor-containing Transwells that were then transferred to a well over viable M2-10B4 (+IL-2). After 48 hours, the ANK-progenitor mixture was harvested from the Transwell and plated into methylcellulose culture without IL-2. In contrast to culture with conditioned medium (condition A) and ANK soluble factors (condition B), when ANK were incubated in direct contact with CML progenitors, near complete CML progenitor suppression is observed (Fig 6, condition C). Finally, CML progenitor containing Transwells were transferred to a well containing conditioned medium (+IL-2) and ANK in the bottom compartment. After 48 hours, progenitors were harvested from the Transwell, mixed with ANK harvested from the bottom compartment, and plated into methylcellulose culture without IL-2. Minimal suppression was observed when contact was prohibited during the 48-hour incubation, but progenitors and ANK were mixed back together before methylcellulose culture (Fig 6, condition D). This observation validates the design of this model where the effects of ANK on progenitors primarily occur during the 48-hour incubation period and not when the ANK-progenitor mixture is plated in methylcellulose culture.

The suppression of CML progenitors by ANK was further evaluated using blocking antibodies against soluble factors (IFN-γ, TNF-α, TGF-β) and ligands/receptors with known importance in cell recognition (Table 1). There was no effect...
on suppression by blocking antibodies used against soluble factors or the corresponding control antibodies during the 48-hour incubation of ANK in direct contact with CML progenitors. However, antibodies against the β2 integrin, with known importance in NK recognition, significantly blocked suppression compared with control antibodies (control IgG, antibody: 73% ± 16% suppression vs anti-β2: 37% ± 11% suppression, n = 3; P = .021). These blocking experiments further corroborate the above studies, demonstrating that suppression of CML progenitors is mediated through direct cell-to-cell contact and soluble factors are not involved under the conditions tested here.

DISCUSSION

The effect of autologous ANK cells on primitive normal and CML hematopoietic progenitors (LTCIC) has been difficult to study in the classic stroma contact Dexter culture, mainly because direct contact between ANK and stroma results in destruction of stroma. Therefore, we developed a novel in vitro assay based on further modifications of the stroma noncontact culture described by our laboratory. In this assay, hematopoietic progenitors and ANK are separated from stromal layers by a 0.4-μm microporous membrane preventing direct contact with the stromal feeder while allowing diffusion of stroma-derived factors sufficient for the in vitro induction of progenitor proliferation and differentiation. A second limitation on the coincubation of ANK cells and primitive hematopoietic progenitors in classical LTBMC is that the HC present in LTBMC medium and required for optimal growth of LTCIC and CFC suppresses ANK function. In agreement with previous studies, we demonstrate that HC significantly inhibits ANK killing of K562 and especially Raji targets. The time course of this interaction is rapid, as an effect is seen as early as 18 hours after incubation, and becomes more pronounced after 42 hours. This finding differs from data reported by Klingemann et al that shows no inhibition of cytotoxicity was observed when fresh Percoll-separated marrow cells were cultured for 7 days with simultaneous addition of HC-containing LTBMC medium and IL-2. We demonstrate that sequential addition of HC to IL-2-activated NK suppresses function. Although IL-2 can partially protect against this loss of function, HC was still significantly suppressive under the conditions tested here. This discrepancy may be explained by differing effects of HC on mature blood NK (used here) versus marrow NK or by differing actions of HC when added to previously cultured, IL-2-activated NK (used here) versus simultaneous addition of IL-2 and HC to resting marrow NK.

Several studies have addressed the effects of various killer cell populations on autologous hematopoietic colony growth using various experimental conditions. Results vary from significant suppression to stimulation of different colony types. These divergent results may be explained by differences in type of effector cell populations studied, such as NK clones, purified CD16+/CD3- cells, HNK1+ NK, and conventional lymphokine-activated killer (LAK) cells. Results may also vary be-
Table 1. Effects of Antibody Blocking on CFC Generation During 48-Hour Coincubation of ANK and CML Progenitors (n = 3)

<table>
<thead>
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<th>Antibody (isotype)</th>
<th>% Suppression of CFC</th>
<th>P*</th>
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<tr>
<td>No antibody</td>
<td>72 ± 16</td>
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</tr>
<tr>
<td>Anti-IFN-γ (Goat IgG)</td>
<td>66 ± 19</td>
<td>NS</td>
</tr>
<tr>
<td>Control goat IgG</td>
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<td>NS</td>
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<td>Anti-TNF-α (rabbit anti-goat IgG)</td>
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</tr>
<tr>
<td>Anti-TGF-β (chicken IgY)</td>
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<td>NS</td>
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<td>Control chicken IgY</td>
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<td>NS</td>
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After 14 days of culture in Transwell inserts over irradiated M2-1084 cells, HC-containing medium was removed, and CML progenitors were placed in IL-2-containing medium. Blocking antibodies of control antibodies were added 30 minutes before the addition of 3 x 10^6 CML progenitors plus ANK were harvested from the transwells and plated in methylcellulose culture in the presence of 5 x 10^-8 mol/L HC to quantify CFC.

* P value compared with no antibody control.

cause differing marrow cell populations have been tested, including BMMNC,26 monocyte-depleted BMMNC,28 monocyte- and T cell-depleted BMMNC,33,27,29 lineage-negative BMMNC,31,34 and progenitors derived from peripheral blood.25 We previously described the effects of IL-2–activated NK on normal committed progenitors present in BMMNC.35 IL-2–activated NK exhibited a dose-dependent, IL-2–dependent inhibition of committed progenitors when coincubated together with BMMNC for the entire 16 days of the methylcellulose culture. This effect was entirely mediated by IL-2–induced TNF-α and IFN-γ and was not the result of direct cell-to-cell contact. When normal IL-2–activated NK were incubated in close contact with committed progenitors for 36 hours, some suppression of burst-forming unit-erythroid (BFU-E) colony growth was observed. However, no suppression of the more primitive multilineage mixed colony-forming unit (CFU-MIX) progenitor was observed. The studies presented here confirm and extend these observations using highly purified CD34+ cells. We demonstrate that ANK cultured for 48 hours in close contact with autologous progenitors do not affect the growth of normal LTCIC.

In contrast, incubation of autologous CML ANK with malignant hematopoietic progenitors resulted in significant suppression of CFC and LTCIC in seven of nine patients with chronic-phase CML. The suppressive effect was observed for committed CFC, thus confirming preliminary studies from our group10 and others.4 In addition, the use of the newly developed assay allows the definitive demonstration of marked suppression of the malignant CML LTCIC present in FACS-selected CD34+/DR+ cells. In contrast with the findings in most CML patients, no suppression was observed in any of the normal subjects, all of whom exhibited potent ANK cytotoxic function, suggesting that ANK recognize CML progenitors but not normal progenitors at the CFC and LTCIC developmental stage. This suppression is absolutely dependent on ANK direct contact with CML progenitors, is not mediated by ANK soluble factors including IFN-γ, TNF-α, or TGF-β, and is not specific to autologous ANK, as allogeneic normal ANK also suppress CML progenitors. We conclude that surface moieties on CML progenitors make them more sensitive to ANK recognition and suppression than normal progenitors, and this effect is at least partially mediated through β2 integrins present on ANK.32 Failure to block suppression with anti–ICAM-1 antibody suggests that other β2 family ligands may be involved33,34. The sparing of normal hematopoiesis in LTBM after coinoculation with autologous ANK is of practical relevance, because in CML the benign progenitors have been demonstrated to be similar in almost all respects, except adhesion,7 to the most primitive progenitors isolated from normal marrow.6,8

Finally, progenitor suppression was correlated with ANK cytotoxicity, as no suppression was observed in the two patients with CML with poorly functional ANK. Failure to generate ANK with good function could be explained by duration of disease and in part by their therapies. One patient who did not generate potent ANK was 25 months from diagnosis at the time of study, consistent with our previous studies, demonstrating that cytotoxicity and CD56+ bright cells within an activated NK population diminish in late chronic phase (greater than 1 year from diagnosis).2 This patient was also the only patient who received prior therapy with busulfan. The other patient was studied within a year from diagnosis of CML but had a concurrent diagnosis of sarcoidosis, was on prednisone at the time of study, and received prior therapy with weekly methotrexate.

To date, bone marrow transplantation remains the only therapeutic measure capable of curing CML.35 However, many patients lack a suitable donor. Autologous transplantation is an alternative form of treatment that is increasingly being performed in this disease.36,39 Although there are data indicating that autologous transplant may result in a substantial prolongation of survival, residual disease leading to relapse in greater than 90% of patients remains to be addressed.40 Posttransplant IL-2 administration to induce a graft-versus-leukemia–like effect at a time of minimal residual disease has been considered a therapeutic possibility.31,45 Because maximal ANK activity may be needed, and because clinical toxicity poses a limitation to the concentration of IL-2 that can safely be given, the administration of ex vivo high-dose IL-2–activated autologous ANK in combination with low-dose IL-2 therapy after transplantation could be an alternative.19

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