Lysis of Neuroblastoma Cell Lines by Human Natural Killer Cells Activated by Interleukin-2 and Interleukin-12

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Neuroblastoma is the most common extracranial, solid tumor in children. Despite intensive chemotherapy and bone marrow transplantation, the 5-year projected survival rate is 20% to 25%. In vitro studies have shown enhanced natural killer cell (NK) lysis of tumor cells after exposure of NK cells to interleukin-2 (IL-2). In vivo studies have demonstrated similar immunologic effects but have also revealed severe toxicities associated with the use of IL-2. IL-12 is a newly described cytokine that has several properties, including the ability to act synergistically with IL-2 in generating lymphokine-activated killer cells (LAK) against known tumor targets. We investigated the role of IL-12 in the generation of peripheral blood mononuclear cell (PBMC) lysis of neuroblastoma cell lines. PBMC were activated with IL-12 alone and in combination with IL-2. Whereas IL-12 alone produced only modest enhancement of NK cell cytotoxicity, the combination of IL-2 and IL-12 was most effective in activating NK cell lysis of neuroblastoma cell lines. Further, we showed that large granular lymphocytes were the effector cells involved in target cell lysis. Finally, the CD18 molecule was shown to be critical in the lysis of neuroblastoma cells by activated PBMC.

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MATeRIALS AND METHODS

Tumor cell lines. Tumor cell lines were maintained by standard cell culture techniques and placed in RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 2 mmol/L L-glutamine, 10 U/mL penicillin, 100 μg/mL streptomycin, 5 mmol/L HEPES buffer (GIBCO), and 5 × 10^-5 mol/L 2-mercaptoethanol (Sigma, St Louis, MO). K562 cells, an erythroleukemia cell line, were maintained in suspension. NB100, NB212, and UCH-I cell lines are all adherent neuroblastoma cell lines and were a gift of Diana Worthington-White (University of Florida, Gainesville). The neuroblastoma cell lines were harvested by brief trypsinization with 0.25% trypsin and 0.04% EDTA in phosphate-buffered saline (PBS).

Patient eligibility. Blood samples from three children with neuroblastoma were evaluated. No patient had received chemotherapy in the 2 weeks before evaluation. Informed consent was obtained from each family before removal of blood sample. Patients no. 1 and 2 were receiving multiagent chemotherapy courses. Patient no. 3 was approximately 19 months post autologous BMT. All three patients had stage IV neuroblastoma.

PBMC isolation. Fresh leukocyte buffy coats were obtained from the Department of Pediatrics, Division of Pediatric Hematology and Oncology, University of Florida, Gainesville; the Department of Medical Microbiology and Biochemistry, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida College of Medicine; and the Department of Surgery, the Division of Otolaryngology, University of South Florida College of Medicine, Tampa.

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with PBS. The collected PBMC were then suspended in RPMI-1640 as described above and PBMC were further separated using plastic adherence for removal of monocytes followed by further removal diluted were layered over 14 mL of Ficoll-Paque (Pharmacia, Piscataway, NJ). After centrifugation at 400g for 20 minutes at room temperature, the interface band of PBMC was collected and washed twice with PBS and diluted to a concentration of 5 × 10^6 cells and incubated at 37°C for 1 hour. The cells were washed twice with PBS and diluted to a concentration of 5 × 10^6/mL, then 100 μL of target cells was added to each well of the 96-well plates. After the cells were incubated at 37°C for 5 hours, 100 μL of supernatant was collected, and the radioactive content was measured by gamma scintillation counting. The percentage of ^51Cr release was calculated by the following formula:

\[
\text{% Release} = \frac{\text{Experimental} ^{51}\text{Cr Release} - \text{Spontaneous} ^{51}\text{Cr Release}}{\text{Maximum} ^{51}\text{Cr Release}} \times 100
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Chromium release data were converted to lytic units (LU). One LU was defined as the number of PBMC resulting in 20% lysis of 5 × 10^3 target cells. In other experiments, anti-CD18 antibodies as well as IgG isotype controls were added to separate sets of PBMC and allowed to bind for 5 minutes, just before addition of ^51Cr-labeled NB100 cells. RH1-38 (a generous gift of Dr Robert Hall, Guthrie

from healthy donors at the Southwest Florida Blood Bank or 10 mL of peripheral blood from patients was obtained. Samples were diluted 1:2 in PBS (M.A. Biologics, Walkersville, MD). The cells were layered over 14 mL of Ficoll-Paque (Pharmacia, Piscataway, NJ). After centrifugation at 400g for 20 minutes at room temperature, the interface band of PBMC was collected and washed twice with PBS. The collected PBMC were then suspended in RPMI-1640 medium (GIBCO) containing 5% heat-inactivated human AB serum (Biocell Laboratories, Carson, CA), 2 mmol/L L-glutamine, 10 U/mL penicillin, 100 μg/mL streptomycin, 5 mmol/L HEPES buffer (GIBCO), and 5 × 10^-3 mol/L 2-mercaptoethanol (Sigma). Plastics were purchased from Costar (Cambridge, MA).

NK cell and T-cell isolation. Fresh buffy coats were obtained as described above and PBMC were further separated using plastic adherence for removal of monocytes followed by further removal of adherent cells and B lymphocytes by incubation on nylon wool columns for 30 minutes at 37°C. The cells passing through the columns were then placed on a six-step discontinuous density gradient with a range from 40% to 52.5% Percoll. After centrifugation at 550g for 30 minutes at room temperature the bands of lymphocytes were collected. LGL, obtained from Percoll fractions 2 and 3, were treated with anti-CD5 antibodies (anti-Leu-1; Becton Dickinson, San Jose, CA) and T cells, from fractions 4 through 7, were incubated with anti-CD16 antibodies (anti-Leu-11b; Becton Dickinson) for 40 minutes. The cells were then incubated with ImmunoBeads coated with goat antimouse IgG (Advanced Magnetics, Cambridge, MA). The labeled cells were removed by exposure to a strong magnetic field. Purified LGL and T cells were then counted and diluted to the appropriate concentration in media with 5% AB serum.

Cytotoxic assay. Cytotoxic activity was evaluated using chromium (^51Cr) release assays. Ninety-six-well, U-bottomed microwell plates were prepared with 100 μL, triplicate serial dilutions of PBMC in effector cell:target cell concentrations of 100:1, 50:1, 25:1, and 12.5:1. Recombinant IL-2 (Hoffman-LaRoche, Nutley, NJ) (specific activity 2 × 10^4 U/mg) and IL-12 (Genetics Institute, Cambridge, MA) (specific activity 8.5 × 10^3 U/mg) were added to PBMC in concentrations ranging from 1 U/mL to 100 U/mL. The cells were then incubated at 37°C in 5% CO2 for 18 hours. Target tumor cells were counted and washed before 100 μCi of sodium ^51Cr-chromium (Amersham, Arlington Heights, IL) was added to 2 × 10^6 cells and incubated at 37°C for 1 hour. The cells were washed twice with PBS and diluted to a concentration of 5 × 10^9/mL, then 100 μL of target cells was added to each well of the 96-well plates. After the cells were incubated at 37°C for 5 hours, 100 μL of supernatant was collected, and the radioactive content was measured by gamma scintillation counting. The percentage of ^51Cr release was calculated by the following formula:

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\]
Research Institute, Sayre, PA) and MC-44 (generated in our lab) monoclonal antibodies against CD18 were used for binding to the effector cells. Murine IgG1 (Sigma) and IgG2a were used as controls for RH1-38 and MC44, respectively. The assay was completed as described previously.

RESULTS

Preliminary data (not shown) indicated that the optimal time for activation of cytotoxic activity against tumor target cells was 18 hours. Initial experiments were designed to examine the ability of IL-12, alone and in combination with IL-2, to induce lytic function against NB100, an NK-resistant neuroblastoma cell line. Figure 1 illustrates the data from a representative experiment evaluating lytic activity against NB100. As shown by the media control, PBMC are unable to lyse NB100 tumor cells. Exposure of PBMC to IL-12 alone (10 U/mL) did not increase the cytotoxicity of the PBMC significantly. IL-2 (100 U/mL) activated the PBMC more than IL-12 alone. However, the combination of IL-2 and IL-12 achieved the greatest lysis of target cells (P < .05), and activated PBMC to lyse the otherwise resistant NB100 tumor cell line. LU for this experiment were calculated as described in Materials and Methods. Further data will be presented as LU for clarity.

To fully evaluate the responses of PBMC to IL-2 and IL-12, several doses of cytokines were tested to determine the dose response (Fig 2). Again, IL-2 alone activated PBMCs against both NB100 and K562 in a dose-dependent fashion. When PBMC were incubated with IL-2 at a dose of 10 U/mL, LU achieved when tested against NB100 were 44.4, and when tested against K562 LU were 358.4. When PBMC were incubated with a higher dose of IL-2 (100 U/mL), LU were higher (153.5 for NB100 and 455.8 for K562). IL-12 activation of PBMC was also dose dependent and LU for PBMC incubated with IL-12 at doses of 1 U/mL and 10 U/mL when tested against NB100 were 19.6 and 54.4, respectively, and against K562 were 194.8 and 363.6, respectively. However, in all cases the response to IL-12 alone was less than the response to either IL-2 (100 U/mL) or IL-12 plus IL-2. The combination of IL-12 (10 U/mL) and IL-2 (100 U/mL) most effectively activated the cytolytic capacity of PBMC. LU of PBMC activated by the combination of these two cytokines tested against NB100 were 242.1 and against K562 were 455.8. As illustrated by these data and as expected, the tumor cell line K562 was more sensitive to lysis by activated PBMC than was the NB100 tumor cell line.

In an effort to show that these findings were not limited to the NB100 cell line, NB100 cells and two other neuroblastoma cell lines, NB212 and UCH-1, were tested. Similar patterns of activation were seen with NB212 and UCH-1 (Fig 3). However, NB100 was the most sensitive cell line. PBMC activated by both IL-2 and IL-12 were the only cells able to lyse UCH-1, an otherwise NK-cell-resistant tumor cell line.

The next step was to test PBMC from children with neuroblastoma to see if these cells could be activated by IL-2 and IL-12. None of the patients had been treated with chemotherapy in the 2 weeks before evaluation. PBMC from all three of the children were activated by IL-2 and IL-12 and were able to kill neuroblastoma cells (Fig 4). As seen with the healthy donor cells, PBMC from these children were activated to the greatest extent by the combination of IL-2 and IL-12.

To identify the effector cells responsible for the lysis of these tumor cells, PBMC were further separated into LGL and T cells as described in Materials and Methods. When these two populations of cells were further purified by removal of T cells from LGL using anti-CD5 antibodies and removal of LGL from T cells using anti-CD16 antibodies, and tested against NB100 cells, the LGL were responsible for the lytic activity (P < .05) (Fig 5).

Finally, in light of previous findings showing the critical role of the CD18 signaling adhesion molecule in IL-2-mediated LAK lysis, we questioned the role of CD18 in the IL-12-dependent mechanism of lysis. Addition of anti-CD18 antibodies, RH1-38 and MC44, clearly suppressed lysis of target cells (Fig 6). IgG1 and IgG2a controls did not interfere with PBMC-mediated lysis of NB100. PBMC untreated with antibody achieved similar lysis of NB100 cells as seen in previous experiments. By comparison, after incubation with IL-2 and IL-12 and testing against NB100, PBMC incubated with RH1-38 and MC44 both had LU of ≤10 whereas those incubated with media, IgG1, and IgG2a achieved LU of 76.2, 99.5, and 77.8, respectively.

DISCUSSION

The role of IL-2 in the treatment of neuroblastoma has been evaluated by several researchers. A Pediatric Oncology
Group (POG) phase I trial of IL-2 treatment of 7 children with advanced malignancies (5 children with ALL, 1 with neuroblastoma, and 1 with rhabdomyosarcoma) reported an increase in the in vitro NK cell cytotoxicity in 5 of the 7 patients treated.6 The investigators noted that patients with the lowest initial NK cytotoxicity had the most dramatic increase in function. Negrier et al5 reported a phase II trial of IL-2 and LAK cell reinfusion in 15 children with neuroblastoma. Twelve children were in relapse after chemotherapy and autologous BMT and three had primary refractory disease despite conventional chemotherapy. Mean lytic function of NK cells, when tested against K562, Daudi, and SKNFl cell lines, increased after treatment with IL-2. Clinical toxicity seen in this trial was severe and included two toxic deaths. As with the POG phase I trial, no durable remissions were seen in the treated population. Alvarado et al3 showed an increase in the NK cell cytotoxicity of PBMC from children with neuroblastoma after 18-hour incubation of these cells with rIL-2 (50 U/mL) and testing against neuroblastoma cell lines CHP-126 and SKNSH. Favrot et al2 conducted a phase II trial of continuous infusion IL-2. The study consisted of two parts. In the first part of the trial, nine children with neuroblastoma in relapse after autologous BMT or with primary refractory disease received continuous infusion IL-2 and LAK cell reinfusion. Objective effects were seen in two of the nine patients. One had brief but dramatic decrease in the catecholamine and dopamine secretion and another had transient regression of subcutaneous cranial lesions. All nine children had progressive disease. The second part of the trial evaluated the use of IL-2 alone in two children who had persistent active disease 60 to 120 days after high-dose chemotherapy and BMT (one autologous and one allogeneic). One of these children experienced complete remission (8 months) after the second course of IL-2. The other child had recrudescence of graft-versus-host disease requiring interruption of IL-2 infusion. The patient subsequently developed progressive disease. NK and LAK cell function in these patients increased after IL-2 administration. Truitt et al14 conducted a phase I trial of 14 children with malignancy, six of whom had stage IV neuroblastoma. Again, they found the in vitro NK cell cytotoxicity to be increased in all children who received recombinant IL-2.

Because of the synergy seen with IL-2 and IL-12 in the generation of LAK cells, researchers have examined the role of IL-12 in NK cell activation. Soiffer et al30 evaluated PBMC from 12 patients with metastatic cancer and 13 patients who were within 2 months of allogeneic BMT. These researchers found IL-12 to enhance the cytotoxicity of PBMC and found the combination of IL-2 and IL-12 to be as effective or more effective than IL-2 alone when tested against K562, a known NK-sensitive erythroleukemia cell line.

Recently reported in vitro experience with IL-12 and prior in vitro and in vivo data on IL-2 treatment of neuroblastoma prompted us to evaluate the role of IL-12 in activation of NK cells against neuroblastoma. Neuroblastoma cell lines are NK resistant but the addition of IL-2, IL-12, or the combination activates NK cells and increases target cell lysis. Interestingly, the combination of both IL-2 and IL-12 does this most effectively. The data seen with normal, healthy donors were reproducible with PBMC from children with neuroblastoma. Interestingly, both the PBMC from patients undergoing multiagent chemotherapy and the PBMC from the patient who had undergone BMT were activated by IL-12. This shows that intensive chemotherapy does not blunt the response to these cytokines. The fact that
PBMC from children with neuroblastoma can be activated by IL-2 and IL-12 to kill neuroblastoma cells in a similar fashion to the PBMC from healthy donors suggests a potential therapeutic role for IL-12 in the treatment of neuroblastoma. That PBMC from children receiving multiagent chemotherapy are also activated by these cytokines indicates immunotherapy may possibly be used in concert with chemotherapy.

Clinical trials have shown the severe toxicities associated with IL-2 treatment in the clinical setting. Because the concentration of IL-12 (1 to 10 U/mL) needed to activate NK cells is lower than IL-2 (100 U/mL), and because the combination of the two affords some synergy, the possibility of using lower doses of IL-2 exists.

We further sought to illuminate the question of which cell was acting as the effector cell for NB100 target cell lysis with IL-12 activation. Comparison of purified LGL- and T-cell-mediated lysis implicates the LGL as the effector cell in NB100 target cell lysis. In Fig 5, activation with IL-2 achieves a similar level of lysis that does not achieve a similar level of lysis of target cells as does activation with IL-2 plus IL-12. In other experiments of the same design, this phenomenon was not seen. Perhaps at such high lytic units as seen in the data represented in Fig 5 the synergistic effects of the two cytokines are not seen.

CD18, a signaling adhesion molecule, has been shown to be critical in NK cytototoxic T cell (CTL) lysis of target cells. It has been shown that the functional display of CD18 on the effector cell but not on the target cell is necessary for NK- and CTL-mediated lysis of target cells. Our results indicate that the CD18 molecule is also essential in IL-12-activated NK cell lysis of target cells.

Further investigation of IL-12, its effects on NK cell function, and its potential role in the treatment of neuroblastoma is warranted.

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Lysis of neuroblastoma cell lines by human natural killer cells activated by interleukin-2 and interleukin-12

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