Targeted Interleukin-2 Therapy for Spontaneous Neuroblastoma Metastases to Bone Marrow

Holger N. Lode, Rong Xiang, Nissi M. Varki, Carrie S. Dolman, Stephen D. Gillies, Ralph A. Reisfeld*

Background: Advanced (stage 4) cases of neuroblastoma, a childhood cancer of the nervous system, are associated with high relapse rates, even after intensive chemotherapy, radiotherapy, and autologous bone marrow transplantation. Therefore, the use of monoclonal antibodies directed against the neuroblastoma tumor marker disialoganglioside GD2 (GD2), in combination with recombinant human interleukin 2 (rhIL-2), is under clinical investigation. We hypothesize that targeted cytokine immunotherapy with a recombinant anti-GD2 antibody–interleukin 2 fusion protein (ch14.18–IL-2) is superior to a combination of ch14.18 and rhIL-2. Our purpose was as follows: 1) to develop a syngeneic model for murine neuroblastoma that expresses GD2 and features both experimental and spontaneous metastases to bone marrow and liver, and 2) to assess anti-GD2-targeted IL-2 therapy in this model. Methods: A hybrid neuroblastoma cell line was used to generate the GD2-positive NXS2 cell line. Bone marrow and liver metastases were quantified by reverse transcription–polymerase chain reaction for tyrosine hydroxylase and by organ weight or counts of macroscopic tumor foci, respectively. All P values reported are two-sided. Results: Injection of NXS2 cells resulted in disseminated bone marrow and liver metastases exhibiting stable, but heterogeneous expression of GD2. Treatment with fusion protein (10 µg/day for 6 days) effectively suppressed growth of both experimental and spontaneous metastases to bone marrow and liver (P<.001). In contrast, a mixture of rhIL-2 and ch14.18 at equivalent dose levels was inefficient. Only mice treated with ch14.18–IL-2 showed a twofold prolongation in life span (P<.001). Conclusion: Targeted IL-2 therapy with a ch14.18–IL-2 fusion protein elicits an effective antitumor response. Our data suggest that a study of ch14.18–IL-2 as an adjuvant treatment in patients with minimal residual disease may be of value. [J Natl Cancer Inst 1997;89:1586–94]

The effective treatment of stage 4 neuroblastoma (1,2) is one of the major challenges in pediatric oncology. Neuroblastoma is one of the most common solid tumors in children (3), and more than 50% of these children initially present with nonresectable
primary tumors and disseminated metastasis to distant organ sites, predominantly bone marrow. The overall survival rate of patients has not been significantly improved during the last 20 years, despite the introduction of radiotherapy and/or high-dose chemotherapy followed by allogeneic or autologous bone marrow transplantation (3). Novel immunologic approaches in adjuvant settings with murine and human/mouse chimeric antibodies directed against disialoganglioside GD2, extensively expressed on neuroblastoma cells, resulted in a response rate of more than 50% in phase I and II clinical trials. These clinical responses include several long-term and complete remissions of stage 4 disease among patients (4–7). The rationale for this approach is provided by the extensive expression of GD2 on neuroblastoma and its restricted presence on such normal tissues as the peripheral and central nervous system, which is protected from immunoglobulins by the blood brain barrier. Anti-GD2 antibodies facilitate antibody-dependent cellular cytotoxicity in vitro, a process mediated primarily by Fc-receptor-bearing effector cells such as monocytes, macrophages, and natural killer (NK) cells (8). Antibody-dependent cellular cytotoxicity can be increased after stimulation with immunomodulators, such as recombinant human interleukin 2 (rhIL-2) (9). This cytokine was characterized as a T-cell growth factor, which stimulates a broad range of effector cells, including T and B lymphocytes, monocytes, macrophages, and NK cells. Furthermore, rhIL-2 was also shown to generate an activated lymphocyte population of lymphokine-activated killer cells (10,11) in vitro and in vivo, which can effectively mediate antibody-dependent cellular cytotoxicity (12). Therefore, rhIL-2 and lymphokine-activated killer cells were applied in clinical trials that produced encouraging antitumor responses in some patients with melanoma (13) and renal cell carcinoma (14). However, in neuroblastoma, systemic treatments with rhIL-2 resulted in only modest regression of metastases (15). The promising data from clinical trials with anti-GD2 monoclonal antibodies and the experience from systemic IL-2 therapies led to a first phase I/IIb trial by use of combination therapy with a murine monoclonal antibody against GD2 (14G2a) with rhIL-2 in patients with refractory neuroblastoma who showed antitumor activity in some cases (16).

A novel principle of immunotherapy is to increase cellular immune responses against cancers by achieving cytokine concentrations in the tumor microenvironment sufficient to stimulate effector cells to elicit antitumor responses. This can be accomplished either by cytokine gene therapy or antibody–cytokine fusion proteins. The first approach uses patient-specific, ex vivo genetic modification of autologous tumor cells or fibroblasts to express various cytokines and is currently under active clinical investigation in a variety of cancers (16). Once produced by tumor cells, such cytokines can induce a local inflammatory response, which results in elimination of the transduced tumor cells and, in some cases, induces a systemic immune response effective against distant metastases. Preliminary results of a clinical trial with autologous neuroblastoma cells transduced with the IL-2 gene indicated a relatively low response rate, with one complete and one partial response as defined by the International Neuroblastoma Response Criteria (2) among 14 patients (17). We recently demonstrated the feasibility of an alternative approach to cancer immunotherapy that resulted in effective concentrations of cytokines in the tumor microenvironment combined with a technically simple modus operandi (18–20). Thus, recombinant fusion proteins consisting of tumor-specific antibody and cytokines combine the unique targeting ability of antibodies with the multifunctional activities of cytokines. The ongoing clinical efforts in combining rhIL-2 with anti-GD2 immunotherapy for neuroblastoma provided a rationale to evaluate the efficacy of a recombinant anti-GD2–IL-2 fusion protein in a pathophysiologically relevant, preclinical model of murine neuroblastoma with either experimental or spontaneous metastases to liver and bone marrow.

Our previous preclinical findings demonstrated that a ch14.18–IL-2 fusion protein can suppress the growth of experimental hepatic metastasis in a xenograft model of human neuroblastoma, supplemented with human lymphokine-activated killer cells, more effectively than mixtures of ch14.18 and rhIL-2 at equivalent dose levels (18,19). Here, our purpose was as follows: 1) to develop a syngeneic model for murine neuroblastoma that naturally expresses GD2 and features both experimental and spontaneous metastases to bone marrow and liver, and 2) to determine the antitumor efficacy of GD2-targeted IL-2 therapy in this model.

**Materials and Methods**

**Mice**

Syngeneic female A/J mice were obtained at 8 weeks of age from The Jackson Laboratory (Bar Harbor, ME). They were housed in our institution under specific pathogen-free conditions in groups of four mice per cage. Mice were fed ad libitum on standard mouse laboratory chow. Animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Six to eight mice were randomly assigned to treatment and control groups. A total of 192 mice was used for these studies.

**Cells**

The murine NX31T28 cell line was created by hybridization of the GD2-negative C1300 murine neuroblastoma cell line (A/J background) with murine dorsal root ganglionic cells from C57BL/6J mice and subcloned for high dopaminergic secretion. This cell line was provided by L. Greene (Columbia University, New York) and propagated, as described previously (21). The NXS2 subline was generated by the selection of NX31T28 cells with high GD2 expression. Briefly, after incubation with anti-GD2 monoclonal antibody 14G2a and anti-mouse IgG2a-fluorescein isothiocyanate (Pharmingen, San Diego, CA) or anti-mouse IgG2a magnetic microbeads (Milenyi, Palo Alto, CA), cells were sorted for high GD2 expressors by fluorescence and magnetic-activated cell sorting, respectively. The resulting NXS2 subline was maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum at 5% CO2 and 37 °C. The murine neuroblastoma cell line, TB2, was provided by M. Ziegler (Children’s Hospital Medical Center, Cincinnati, OH) and maintained, as described previously (22). Major histocompatibility complex class I antigen expression was determined with anti-H2K and anti-H2K monoclonal antibodies (Pharmingen). Expression of murine neuronal cell adhesion molecule L1 was assessed with antibody 74–5H7, which was provided by V. Lemmon (Case Western Reserve, Cleveland, OH).

**Antibody and Antibody–IL-2 Fusion Protein**

Mouse–human chimeric anti-GD2 antibody ch14.18 was constructed by joining the complementary deoxyribonucleic acid for the variable region of the murine antibody with the constant regions of the γ1 heavy chain and the κ light chain, as described previously (23). The ch14.18–IL-2 fusion protein was constructed by fusion of a synthetic sequence coding for human IL-2 to the carboxyl terminal of the human Cys1 gene, as described (24). The fused gene was introduced into the vector pSHL2, which encodes the dihydrofolate reductase gene. The expression plasmid was introduced into Sp2/0-Ag14 cells by protoplast...
fusión and selected in the presence of increasing concentrations of methotrexate (100 nM to 5 μM). The specific IL-2 activity of the ch14.18–IL-2 fusion protein was determined in bioassays, as described previously (18, 25): ch14.18–IL-2 (1 μg) was found to be equivalent to 3000 IU rhIL-2.

**Induction of Experimental and Spontaneous Metastases**

NXS2 cells were harvested by trypsinization and washed three times by centrifugation (5 minutes, 500g, room temperature). Cells were used for tumor induction only if their viability exceeded 95%, as determined by trypan blue staining. Experimental metastases were induced by tail vein injection of 1 × 10^6 to 1 × 10^7 NXS2 cells in 200 μL phosphate-buffered saline (PBS; pH 7.4). Mice were killed for evaluation after they became moribund with a distended abdomen. Spontaneous metastases were induced by the subcutaneous injection of 1 × 10^6 NXS2 cells (suspended in 100 μL PBS; pH 7.4) into the left lateral flank. Tumor growth was monitored with calipers and tumor volumes were calculated using the formula, width/2 × width × length. Sixteen days after injection, tumors were surgically removed under aseptic conditions after general anesthesia with 1.8 mg ketamine intraperitoneally and halothane inhalation. Nineteen days after surgery, the mice were killed and examined for metastases to distant organ sites. Kidneys, ovaries, adrenal glands, and livers were routinely examined macroscopically. The number of metastatic foci on the liver surface was counted by eye. Lungs were placed in Bouin’s fixative and then examined microscopically under low magnification for tumor foci on their surface. For evaluation of bone marrow metastasis, the bone cavities of both femurs and tibiae of each mouse were flushed with 3 mL PBS. The cell pellet was used for total ribonucleic acid (RNA) isolation and subsequent reverse transcription–polymerase chain reaction (RT-PCR) for the detection of tyrosine hydroxylase. Specimens for histologic examinations and immunohistochemistry were obtained from organs that were macroscopically affected by metastatic disease. Blood samples for assay of serum catecholamines were obtained by terminal cardiac puncture. Serum catecholamines and immunohistochemistry were obtained from organs that were macroscopically affected by metastatic disease. Blood samples for assay of serum catecholamines were obtained by terminal cardiac puncture. Serum catecholamine levels were assessed by reversed-phase liquid chromatography and electrochemical detection (The Corning Nichols Institute, San Juan Capistrano, CA).

**Immunohistology**

Frozen sections of livers and lymph nodes were fixed in cold acetone for 10 minutes, followed by removal of endogenous peroxidase with H_2O_2 and blocking with 10% species-specific serum in 1% bovine serum albumin/PBS. Monoclonal antibody ch14.18 was overlaid onto serial sections at 10 μg/mL and slides were incubated in a humid chamber for 30 minutes. With PBS washes between each step, a biotinylated goat anti-human IgG antibody was applied for 10 minutes, followed by streptavidin-labeled peroxidase for 10 minutes.

**Biodistribution**

The ch14.18–IL-2 fusion protein was labeled with ^125I, as described (26). Subcutaneous tumors of similar sizes were induced by bilateral injection of GD_2-positive NXS2 (5 × 10^6) and GD_2-negative TBJ cells (1 × 10^6) into the left and right flank of each mouse, respectively, resulting in tumors of 35–40 mg within 6 days. Experimental metastasis was induced by intravenous injection of 1 × 10^6 NXS2 cells and analyzed on day 14. Mice were given an intravenous injection of 5 μCi (1 Ci = 37.5 GBq) of ^125I-labeled ch14.18–IL-2 fusion protein. Groups of four mice each were killed at 4, 8, and 24 hours after injection. Tumors and major organs were removed, weighed, and subsequently assayed in a γ counter for ^125I activity.

**Reverse Transcription–Polymerase Chain Reaction**

Total cellular RNA was isolated with a silica gel membrane-binding procedure, Rneasy (Qiagen, Chatsworth, CA). RNA content and purity were determined by OD260/280 readings. Synthesis of complementary DNA (cDNA) was accomplished with 1 μg RNA in the presence of Moloney murine leukemia virus reverse transcriptase, SuperScriptII (Life Technologies, Inc. [GIBCO BRL], Grand Island, NY), according to the manufacturer’s guidelines. The cDNA was heated at 100°C for 3 minutes and chilled on ice. A cDNA equivalent of 100 ng was used in a 25 μL PCR reaction mixture, which contained 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 0.2 mM deoxynucleotide triphosphate, 2.5 U of Taq DNA polymerase (Life Technologies, Inc.), and 0.5 μM sense and antisense oligonucleotide primers for amplification of murine tyrosine hydroxylase (TH). For the detection of tyrosine hydroxylase, the PCR was adjusted at low and high sensitivity. For low sensitivity, amplification was done with sense 5'-TCTCACATTGTGAAGGAAACG-3' and antisense 5’-CCCCATTCTGTTTTACACCAGC-3’ for 36 cycles (15 seconds, 96°C; 30 seconds, 55°C; and 90 seconds, 72°C), leading to a 325-base-pair (bp) fragment designated TH1. High sensitivity was achieved by nested amplification of 1.5 μL TH1 after 20 cycles using sense 5’-AGTACATCCGTACATGC-3’ and antisense 5’-GAGATGAAATCAGTCCG-3’, followed by a 295-bp fragment. The specificity of all fragments was verified by sequencing.

**Statistical Analysis**

The statistical significance of differential findings between experimental groups of mice was determined by the two-tailed Student’s t test. The nonparametric Wilcoxon rank sum test was used to assess the statistical significance of metastatic scores and life span. Findings were regarded as significant if two-tailed P values were <.01.

**Results**

**Characterization of NXS2 Hybrid Neuroblastoma Cells**

Increased, but heterogeneous, GD_2 expression of NXS2 cells was demonstrated by fluorescence-activated cell sorter analyses (Fig. 1). However, this GD_2 expression of NXS2 cells was highly stable in cell culture at the same level over 40 passages or 120 generations. The average number of binding sites determined by saturation-binding assays with either ch14.18 antibody or ch14.18–IL-2 fusion protein was the same, i.e., 1.1 × 10^6 sites per cell. The two parental cell types C1300 and dorsal root ganglionic cells used for hybridization originated from two H2 incompatible mouse strains, A/J and C57BL/6J, respectively. Determination of major histocompatibility class I antigen expression on NXS2 cells indicated it to be that of A/J mice, i.e., H2Kb positive/H2Kb negative. In contrast to C1300 parental cells, ultrastructural analyses of NXS2 cells revealed the presence of large numbers of dense core vesicles, which are electron microscopic correlates of catecholamine storage vesicles. Furthermore, a high expression of the neuronal cell adhesion molecule L1 was found on the NXS2 cells (data not shown). Both of these findings represent common features of human neuroblastoma cells.

**Spontaneous and Experimental Metastasis Model**

Spontaneous metastasis to the major metastatic sites in neuroblastoma, such as bone marrow, liver, lymph nodes, and adrenal glands, resulted from subcutaneous tumor growth of 10^6 NXS2 cells (Table 1; Fig. 2). Metastasis to distant organ sites was determined 19 days after surgical removal of the primary tumor. All mice revealed metastases to bone marrow, liver, and lung. Metastases to the left axillary draining lymph node indicated a lymphatic route of the metastatic process. Spontaneous metastases to ovary, adrenal gland, and kidney were found in 20%–33% of mice given an injection of 1 × 10^6 NXS2 cells. The morphology varied from single focal lesions of 1–2 mm in diameter to massive organ-replacing tumors, as shown in Fig. 2. Lumbar and aortic lymph nodes were less frequently affected by spontaneous metastases. The histology of metastases to bone marrow ranged from complete suppression of hematopoietic marrow to focal metastases surrounded by normal marrow (data not shown).
Occasionally, tumor cells were observed to infiltrate the bone. Once established, tumor growth extended into surrounding normal tissues, as observed in adrenal glands, ovaries (Fig. 2), and lungs (data not shown). A rather infiltrative pattern was usually observed in kidney metastases (Fig. 2). Thus, spontaneous metastasis of NXS2 cells involved the organ sites typical for metastatic neuroblastoma in humans.

Analysis of GD2 expression on spontaneous bone marrow, liver and lymph node metastases by FACS and immunohistochemistry demonstrated the presence of this disialoganglioside at all metastatic sites investigated (Fig. 1). The staining pattern of GD2 observed on metastatic lesions was heterogeneous, presumably reflecting the heterogeneous GD2 expression by NXS2 cells in culture (Fig. 1). Intravenous injection of NXS2 hybrid neuroblastoma cells into syngeneic A/J mice resulted in experimental metastases to distant organ sites as shown in Table 1. Metastases to bone marrow, liver, and lung occurred in all mice within a 1.5-log range of intravenously injected NXS2 cells. A decrease in tumor cell inoculum resulted mainly in a concomitant decrease of metastases to adrenal glands, ovaries, and kidneys. Rarely observed metastatic locations occurred particularly with higher numbers of injected tumor cells and included urinary bladder causing hematuria, ovarian duct, mesenteric/inguinal lymph nodes, and a spinal metastasis causing paraplegia.

Assessment of Bone Marrow Metastasis Levels by RT–PCR

A staging system for bone marrow metastasis was developed by using RT–PCR of tyrosine hydroxylase, a specific transcript of catecholaminergic cells commonly used for detection of neuroblastoma cells in humans. Two different sensitivities for detection of NXS2 cells in naive bone marrow were established with a single and nested RT–PCR approach, respectively. In reconstitution experiments, the low sensitivity RT–PCR represented by the TH1 fragment indicated bone marrow contamination at reciprocal ratios of $10^1$ and $10^2$ (Fig. 3, A). High sensitivity PCR by nested amplification increased the detection threshold to $10^3$ NXS2 cells in $10^6$ naive bone marrow cells, as indicated by the presence of the TH2 fragment (Fig. 3, B).

<table>
<thead>
<tr>
<th>Table 1. Frequency of metastasis to distant organ sites after injection of NXS2 hybrid neuroblastoma cells</th>
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<tbody>
<tr>
<td><strong>Spontaneous§,‡</strong></td>
</tr>
<tr>
<td><strong>Inoculum</strong> ^*^ ............................................ <strong>10^6</strong></td>
</tr>
<tr>
<td><strong>Time after inoculation</strong> ^§^ ............................................ <strong>35 days</strong></td>
</tr>
<tr>
<td>Bone marrow[^¶]</td>
</tr>
<tr>
<td>Liver[^¶]</td>
</tr>
<tr>
<td>Hemorrhagic ascites[^¶]</td>
</tr>
<tr>
<td>Lung[^#]</td>
</tr>
<tr>
<td>Adrenal glands[^¶]</td>
</tr>
<tr>
<td>Ovaries[^¶]</td>
</tr>
<tr>
<td>Kidneys[^¶]</td>
</tr>
<tr>
<td>Spleen[^¶]</td>
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<td>Draining lymph node[^¶]</td>
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^*Inoculum was applied subcutaneously and intravenously to induce spontaneous and experimental metastases, respectively.
†Values in columns = percent of mice with metastases and number of mice with metastases/total mice tested.
‡Spontaneous metastasis was assessed 19 days after removal of the primary tumors ranging from 200 to 700 μL in size.
§Mice were killed after they became moribund with a distended abdomen due to liver enlargement and hemorrhagic ascites.
¶Bone marrow metastasis was detected by tyrosine hydroxylase reverse transcription-polymerase chain reaction.
#Metastatic disease to the various organ systems was determined macroscopically.
¶Metastasis to lungs was determined under a low magnification microscope after placement in Bouin’s fixative.
The biodistribution of 125I-labeled ch14.18–IL-2 fusion protein in A/J mice bearing two subcutaneous tumors on separate flanks, either induced with GD2-positive NXS2 or GD2-negative TBJ cells, is shown for different time points in Fig. 4, A. 125I-labeled fusion protein was injected 6 days after induction of the subcutaneous tumors, which had reached average weights of 32 ± 9 mg standard deviation (TBJ) and 36 ± 8 mg standard deviation (NXS2), respectively. The highest amount of radioactivity was detected in the GD2-positive NXS2 subcutaneous tumor 8 hours after intravenous injection. At later time points, the selectivity of tumor localization increased as 125I-labeled ch14.18–IL-2 was eliminated more slowly from GD2-positive NXS2-induced subcutaneous tumors than from GD2-negative TBJ tumors or surrounding tissues, such as skin and muscle. Therefore, the ratio of radioactivity detected in the NXS2-induced tumor increased twofold after 24 hours, as compared with the TBJ tumor or the surrounding tissue. The total amount of radioactivity detected in NXS2-induced tumors after 24 hours remained at 60% of its peak level after 8 hours versus 38% in TBJ-induced tumors. A second series of biodistribution experiments was performed in A/J mice bearing experimentally induced lung and liver metastases 14 days after intravenous injection of 10^6 NXS2 cells. The amount of radioactivity detected in hepatic and pulmonary metastases versus naive livers and lungs 8 hours after intravenous injection of 125I-labeled ch14.18–IL-2 revealed a preferential localization of the fusion protein within the tumor-bearing organs (Fig. 4, B). Skin, which is unaffected by the metastatic process, is shown as a control organ for both groups of mice.

**Treatment of Tumor-Bearing Mice With ch14.18–IL-2 Fusion Protein**

The efficacy of the ch14.18–IL-2 fusion protein in suppressing the growth of bone marrow metastasis was assessed by injecting 1 x 10^6 NXS2 cells into the lateral tail vein of A/J mice, followed 24 hours later by daily intravenous injections of either PBS, a mixture of ch14.18 and rhIL-2, or ch14.18–IL-2 fusion protein for 6 days. All mice treated with the ch14.18–IL-2 fusion protein lacked signals in either the high- or low-sensitivity tyrosine hydroxylase RT–PCR assays, indicating no detectable tumor cells among 10^6 bone marrow cells (Fig. 3, C and D). Amplification of murine glyceraldehyde-3-phosphate dehydrogenase, used as a control template for bone marrow without tyrosine hydroxylase signals, is also shown in Fig. 3, E. In contrast, all mice treated either with PBS or a mixture of ch14.18 and rhIL-2 developed bone marrow metastases with a positive signal at either low (TH1)- or high (TH2)-sensitivity PCR. One mouse among the mixture group (No. 7) showed only a TH2 signal (Fig. 3, C), indicating a reduction in bone marrow metastases below the 1:10^2 threshold. Applying the staging system based on tyrosine hydroxylase RT–PCR, all mice in the ch14.18–IL-2 fusion protein-treated group were stage 0. In the ch14.18+rhIL-2 mixture group, one mouse developed stage 1 disease and five developed stage 2, whereas all mice treated with PBS had stage 2 bone marrow metastases.

The results of treating experimental hepatic metastases are summarized in Table 2. The ch14.18–IL-2 fusion protein completely suppressed all metastases to the liver in contrast to mice receiving either ch14.18 and rhIL-2 mixtures or PBS. As shown in Fig. 5, in all mice treated with PBS, most of the liver tissue was replaced by the tumor, resulting in a more than threefold increase in liver weight. Tumor foci were confluent and therefore not individually countable (>250). Mice receiving mixtures of ch14.18 and rhIL-2 revealed a reduction in liver weight and number of tumor foci, as compared with PBS-treated mice (Fig. 5; Table 2). However, this difference was not reflected in a twofold prolongation of life span that was only achieved for mice treated with the ch14.18–IL-2 fusion protein (Table 2). Importantly, differences in numbers of metastatic foci between the fusion protein group and all control groups were statistically significant P<.001 (Table 2). Results of serum catecholamine detection are shown in Table 2. Dopamine, a tumor marker commonly used for neuroblastoma, was found at very high levels in PBS-treated mice and correlated with the tumor load in each treatment group. However, dopamine levels in mice treated with fusion protein returned to essentially the same values as those found in naive mice (0.4 ± 0.2 ng/mL standard deviation, n = 4).
The antitumor activity of the ch14.18–IL-2 fusion protein was further assessed by treating spontaneous bone marrow and liver metastasis. Naive bone marrow cells were reconstituted with NXS2 cells at reciprocal ratios of $10^4$ to $10^8$. Negative controls are shown in lane 1 (A + B) and lane 2 (B). Low- and high-sensitive detection is indicated by the presence of the TH1 (A) and TH2 (B) fragment, respectively. Analysis of bone marrow metastases induced by intravenous injection of $10^6$ NXS2 cells in mice treated with fusion protein and control mice (C through E). Treatment was initiated 24 hours after tumor cell inoculation and consisted of six daily injections with phosphate-buffered saline (lanes 1 through 6), $10 \mu$g ch14.18 + 30,000 IU rhIL-2 (lanes 7 through 12), and $10 \mu$g ch14.18–IL-2 (lanes 13 through 18). Positive signals with low- and high-sensitivity tyrosine hydroxylase RT–PCR are indicated by the presence of a TH1 (C) or TH2 (D) signals, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with samples lacking a high-sensitivity tyrosine hydroxylase signal (E).

Fig. 3. Sensitivity of NXS2 cell detection in naive bone marrow by tyrosine hydroxylase reverse transcription–polymerase chain reaction (RT–PCR) and effect of ch14.18–IL-2 fusion protein on experimental bone marrow metastasis. Naive bone marrow cells were reconstituted with NXS2 cells at reciprocal ratios of $10^4$ to $10^8$. Negative controls are shown in lane 1 (A + B) and lane 2 (B). Low- and high-sensitive detection is indicated by the presence of the TH1 (A) and TH2 (B) fragment, respectively. Analysis of bone marrow metastases induced by intravenous injection of $10^6$ NXS2 cells in mice treated with fusion protein and control mice (C through E). Treatment was initiated 24 hours thereafter, consisting of daily intravenous injections of PBS, ch14.18 antibody plus rhIL-2, or ch14.18–IL-2 fusion protein for 6 consecutive days. Only treatment with the ch14.18–IL-2 fusion protein resulted in a lack of detectable

Fig. 4. A) Biodistribution of ch14.18–IL-2 fusion protein within subcutaneous tumors. $^{125}$I-labeled fusion protein (5 $\mu$Ci) was injected intravenously into four AJJ mice bearing two subcutaneous tumors induced either by GD2-negative TBJ or GD2-positive NXS2 cells. Organ-specific activity was detected after 8 hours (black bars) and 24 hours (white bars) following an intravenous injection. * = $P$ value obtained by comparison of subcutaneous tumors at 8 and 24 hours (* = $P<.001$). B) Biodistribution of $^{125}$I-labeled ch14.18–IL-2 fusion protein (5 $\mu$Ci) in naive and tumor-bearing mice 8 hours after an intravenous injection. $^{125}$I-labeled fusion protein (5 $\mu$Ci) was injected intravenously into naive and tumor-bearing mice 14 days after an intravenous injection of $10^6$ NSX2 cells. Organ-specific activities are given in percent of injected dose per gram of tissue. Each value represents the mean and standard deviation for four tumor-bearing mice (black bars) versus naive mice (white bars). * = $P$ values obtained by comparison of naive and tumor-bearing mice (* = $P<.1$; ** = $P<.01$).
bone marrow or liver metastases in all mice (Table 3). The superior treatment effect of the fusion protein was also observed in adrenal glands, ovaries, and kidneys, which were found to be macroscopically free of metastases only in fusion protein-treated mice (data not shown). This result was not achieved in control mice, either treated with an equivalent dose of ch14.18 and rhIL-2 or PBS (Fig. 2).

**Discussion**

Recombinant antibody–cytokine fusion proteins combine the unique targeting ability of antibodies with the multifunctional activity of cytokines. The idea of using the specific targeting of certain molecules to direct therapeutics to the tumor microenvironment is not new, since it was already proposed by Ehrlich (27) almost a century ago. The novelty of our approach lies in its attempt to induce a tumor-specific cellular immune response by using a fusion protein to direct IL-2 to the tumor microenvironment.

Here, we demonstrate the therapeutic advantage of the recombinant anti-GD2 ch14.18–IL-2 fusion protein over a mixture of equivalent amounts of this cytokine with ch14.18 antibody in effectively suppressing the growth of disseminated experimental and spontaneous metastases to bone marrow and liver. This was accomplished in a novel, syngeneic model for murine neuroblastoma that naturally expresses GD2 and features both experimental and spontaneous metastases to bone marrow and liver. Animal models of consistent and reproducible spontaneous metastasis are relatively rare following subcutaneous tumor growth of human or animal tumor cells in rodents (22,28). Consistency and low variability in the occurrence of metastases in such models remain crucial for reproducible studies of tumor treatment. These features were demonstrated by our model involving NXS2 murine neuroblastoma cells that metastasized spontaneously to bone marrow and liver in all mice.

In contrast, the murine neuroblastoma cell line C1300 is unable to form spontaneous metastasis altogether (29). Thus, it could only be used for the study of experimental metastases to liver, lung, kidney (30), and bone marrow (31) after injection into the tail vein of A/J mice. Two subclones of the C1300 cell line, Neuro 2A and TBJ, were reported to metastasize spontaneously to distant organ sites, including liver or liver and lung, respectively (22,30). However, neither cell line was found to metastasize spontaneously to bone marrow, the most prevalent site of metastasis in human neuroblastoma. More important, C1300 cells and their subclones Neuro 2A and TBJ lack the expression of the disialoganglioside GD2, which is the most commonly expressed tumor antigen of human neuroblastoma (2).

Our novel, syngeneic neuroblastoma model in A/J mice is pathophysiologically similar to human neuroblastoma, as it naturally expresses disialoanganglioside GD2 and metastasizes both experimentally and spontaneously to liver and bone marrow. Consequently, this is the first effective model for the study of anti-GD2 immunotherapies in fully immunocompetent mice. The similarity of our syngeneic animal model to human neuroblastoma is quite evident, as it features dopamine production, presence of dense core vesicles and expression of the neuronal cell adhesion molecule L1. Also, the organs involved in the metastatic process in our model include bone marrow, liver, adrenal glands and lymph nodes and reflect the pathophysiologic characteristics of this cancer in man. In fact, two thirds of patients with neuroblastoma initially present with bone marrow metastases (2), and most have minimal residual disease to the bone marrow after treatment with conventional therapy proto-

### Table 2. Effect of anti-GD2–interleukin 2 (IL-2) fusion protein therapy on dissemination of hepatic neuroblastoma metastases, the tumor marker dopamine, and survival*

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>Liver metastasis, No. of foci‡</th>
<th>Liver weight, mg§</th>
<th>Dopamine, ng/mL¶</th>
<th>Median survival, days∥</th>
<th>Range, days∥</th>
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<td>PBS</td>
<td>&gt;250, &gt;250, &gt;250, &gt;250, 240, 115</td>
<td>3036 ± 547</td>
<td>9.8 ± 0.8</td>
<td>24</td>
<td>21–28</td>
</tr>
<tr>
<td>ch14.18+IL-2 mixture</td>
<td>174, 134, 105, 102, 91, 83</td>
<td>1621 ± 277</td>
<td>3.4 ± 0.3</td>
<td>27</td>
<td>24–34</td>
</tr>
<tr>
<td>ch14.18–IL-2</td>
<td>0, 0, 0, 0, 0, 0</td>
<td>895 ± 101</td>
<td>0.6 ± 0.1</td>
<td>52</td>
<td>44–63</td>
</tr>
</tbody>
</table>

*Experimental metastasis was induced by intravenous injection of 10⁶ NXS2 cells.
†Treatment was initiated 24 hours after tumor cell inoculation and consisted of six daily intravenous injections of phosphate-buffered saline (PBS), 10 μg ch14.18 + 30000 IU recombinant human IL-2, and 10 μg ch14.18–IL-2 fusion protein.
‡Mice were killed 21 days after tumor cell inoculation. Livers were examined macroscopically for tumor foci. Serum samples were obtained by cardiac puncture and analyzed for dopamine. Differences in numbers of metastatic foci, liver weights, and dopamine levels between the fusion protein group and all control groups were statistically significant (P<.001). Liver weights and dopamine levels are expressed as average ± standard deviation of six mice.
§Survival studies were done in experimental groups of six mice each. Differences in survival between mice treated with ch14.18–IL-2 fusion protein versus PBS or the mixture of ch14.18 and rhIL-2 were statistically significant (P<.001). All mice were followed until death occurred.
applications of IL-2 in patients with neuroblastoma

The effectiveness of the ch14.18–IL-2 fusion protein in suppressing the growth of disseminated metastases of neuroblastoma to both bone marrow and liver is remarkable in view of the heterogeneous expression of the target antigen GD2. In fact, analysis of the biodistribution of 125I-ch14.18–IL-2 revealed its preferential location in GD2 antigen-positive tumors and metastases to liver. Therefore, it seems reasonable to propose that antibody–cytokine fusion proteins can deal effectively with the problem of the naturally occurring, heterogeneous expression of target antigens, since these molecular constructs apparently activate effector cells more efficiently than combinations of antibody and cytokine. The mechanisms involved in the antitumor effect of the ch14.18–IL-2 fusion protein in our model are not entirely clear. However, this effect is likely due to the induction of local inflammatory, cellular antitumor responses involving cytotoxic tumor-infiltrating lymphocytes (24) and/or NK cells (25), which are both known to elicit antitumor effects independent of antibody-mediated cellular cytotoxicity (38,39). In fact, the ch14.18–IL-2 fusion protein was previously shown to induce a CD8+ T-cell response that effectively eradicated experimental hepatic and pulmonary metastases of murine melanoma (36), in spite of the heterogeneous expression of GD2 target antigen used for cytokine delivery to the tumor microenvironment (37).

A comparable situation may exist with NK cells. Increased numbers of such effector cells were observed after systemic applications of IL-2 in patients with neuroblastoma (12), and antitumor responses of NK cells were reported to occur independently of their Fc-receptors (38,39). Also, links between both NK and T cell-mediated responses may be involved in eliciting the most effective antitumor response (40). Interestingly, our preliminary data in scid/scid and scid/beige mice revealed that the ch14.18–IL-2 fusion protein was also effective in suppressing NXS2 tumor growth in scid/scid mice, which are deficient in mature T cells, but contain NK cells as well as macrophages and granulocytes. The effect of the ch14.18–IL-2 fusion protein was only abrogated in scid/beige mice, lacking both T and NK cells. These preliminary observations support our contention that an NK cell-mediated mechanism is induced by the ch14.18–IL-2 fusion protein in our neuroblastoma model.

In summary, we demonstrate here that the ch14.18–IL-2 fusion protein can effectively suppress dissemination and growth of both spontaneously and experimentally induced syngeneic neuroblastoma metastases to liver and bone marrow in A/J mice and significantly prolong the life span of these mice. This was accomplished in a newly developed metastasis model of disseminated murine neuroblastoma that naturally expresses GD2 heterogeneously and thus mimics clinical stage 4 disease of human neuroblastoma. On the basis of the previous clinical experience of treating neuroblastoma with combinations of anti-GD2 antibodies and rhIL-2, our data suggest that further study is warranted of the ch14.18–IL-2 fusion protein as an adjuvant treatment in neuroblastoma patients with minimal residual disease.

References

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Notes

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