Augmentation of Antitumor Immunity by Tumor Cells Transduced With a Retroviral Vector Carrying the Interleukin-2 and Interferon-γ cDNAs

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Therapeutic models using gene transfer into tumor cells have pursued three objectives: (1) to induce rejection of the tumor transduced with therapeutic genes; (2) to induce immune-mediated regression of metastatic disease; and (3) to induce long-lasting immunity to protect against challenge with tumor cells or clinical regrowth of micrometastatic disease. Because in vivo therapy for patients with cancer using gene transfer would, as a first step, attempt to eliminate the existing tumor, we have investigated whether antitumor immunity induced by tumor cells secreting a single cytokine could be increased by cotransfer of a second cytokine gene. To test this approach, CMS-5, a murine fibrosarcoma, was transduced with retroviral vectors carrying interleukin-2 (IL-2), interferon-γ (IFN-γ), or granulocyte-macrophage-colony-stimulating factor (GM-CSF) cDNA alone or IL-2 cDNA in combination with IFN-γ or GM-CSF cDNA. Single cytokine-secreting clones were selected to match levels of cytokine production by double cytokine-secreting clones so that similar amounts of cytokine were secreted. IFN-γ and IL-2/IFN-γ-secreting CMS-5 cells showed increased levels of major histocompatibility complex class I expression compared with IL-2- and GM-CSF-secreting or parental CMS-5 cells. IL-2/IFN-γ-secreting CMS-5 cells were always rejected by syngeneic mice, whereas the same number of CMS-5 cells secreting only one of these cytokines or mixtures of single cytokine-secreting CMS-5 cells were not rejected. In vivo depletion of CD4+, CD8+, or natural-killer effector cell subpopulations showed that CD8+ cytotoxic T cells were primarily responsible for rejection of IL-2/IFN-γ-transduced tumor cells. Our data show the successful use of a single retroviral vector to stably transduce two cytokine genes into the same tumor cell, leading to an increased effect on the in vivo induction of antitumor immunity.

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roviral vectors carrying IL-2, IFN-γ, or GM-CSF genes. These vectors were used to transduce the murine fibrosarcoma cell line, CMS-5. The ability of single cytokine-secreting CMS-5 clones to induce primary rejection of tumor cells was compared with that for tumor cells secreting similar amounts of two cytokines. The immunologic mechanisms involved in the rejection of cytokine-secreting tumor cells and the potential to induce long-lasting systemic antitumor immunity were also investigated.

MATERIALS AND METHODS

Retroviral vector design and conversion of vectors into viruses. The retroviral vector N2 is derived from the genome of Moloney murine leukemia virus and contains the bacterial neomycin resistance (neo) gene as a selectable marker. The sources and restriction enzymes used to obtain DNA fragments encoding the human IL-2 cDNA, the mouse IFN-γ cDNA, the Herpes simplex virus-thymidine kinase (TK) promoter, the major immediate early human cytomegalovirus (CMV) promoter, and the adenosine deaminase (ADA) promoter and the poly-A signal, have been described previously. The murine GM-CSF cDNA was a gift of Genetics Institute, Inc (Cambridge, MA). CMV promoter-, ADA promoter-, and TK promoter-encoding DNA fragments were fused to the human IL-2 cDNA, murine GM-CSF cDNA, or murine IFN-γ cDNA fragment and cloned into different sites of the N2 vector. The TK-IL-2 or TK-IFN-γ fusion products were cloned into the XhoI site present in the polylinker in the 3′ long terminal repeat (LTR) of a modified N2 vector to generate vector constructs DC/TKI2, N/CIFNγ/TIL2 or N/CIL2/TIFNγ. To complete cloning of the vector constructs N/CIL2/TIFNy and N/CIFNy/TIL2, the CMV-IL-2 or CMV-IFN-γ fusion products, respectively, were cloned into the XhoI site present downstream to the neomycin resistance gene. To generate the retroviral vectors DC/AD/R/IFNy, DC/AD/R/GM-CSF, and N/CGM-CSF/RIL2, the ADA promoter was cloned in reverse orientation into the Klenow-modified HmuI site; the murine IFN-γ, human IL-2, or murine GM-CSF cDNA were cloned in reverse orientation into the Klenow-modified MluI site; the murine IFN-γ, human IL-2, or murine GM-CSF cDNA were cloned in reverse orientation into the XhoI site and the poly-A signal was cloned into the Klenow-modified ApaI site of the 3′LTR polylinker. To complete cloning of N/CGM-CSF/RIL2 the CMV-GM-CSF fusion product was cloned into the XhoI site downstream to the neomycin gene coding sequences of DC/AD/R/JIL2. N2/CMV-GM-CSF was generated by cloning the CMV-GM-CSF fusion product into the same XhoI site in the N2 vector. DCA is a previously described vector in which the human ADA minigene was cloned into the 3′LTR of N2. Retroviral vector constructs were converted to the corresponding virus by electroporating vector DNA into a helper-free, ecotropic (GP-E+86: DC/AD/R/IFNy, DC/AD/R/GM-CSF, N2/CMVGM-CSF, N/CIL2/TIFNy, N/CIFNy/TIL2, N/CGM-CSF/RIL2) or amphotropic packaging cell line (GP+envAM12: DC/TKI2, DCA). Colonies were isolated by G418 selection (0.7 mg/mL of Genticin; Gibco Laboratories, Grand Island, NY) and were expanded to produce cell lines, and cell-free supernatant was tested for viral titer.

Tumor cell lines and injection of tumor cells. CMS-5 and CMS-13 are methylcholanthrene-induced, nonmetastatic fibrosarcoma cell lines of BALB/c origin. Both cell lines reproducibly form tumors in syngeneic as well as in immunodeficient nude mice after intradermal (ID) injection. The minimum lethal dose in (BALB/c)b mouse is 2 x 10⁷ cells. Tumor cells were cultured in Dulbeccos modified Eagle’s medium supplemented with 10% fetal calf serum (FCS), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mmol/L L-glutamine. Virus-producer cell lines secreting high titer of virus were used to infect CMS-5 cells. Clonal derivatives of CMS-5 cells were isolated by G418 selection, expanded to cell lines, and used for further analysis. The in vitro growth rate of parental and transfected CMS-5 cells was evaluated by plating 0.25 x 10⁶ cells/mL, in triplicate for each day, and counting viable cells 2, 3, 4, and 5 days after seeding.

Cytokine assay. Secretion of IL-2, GM-CSF, and IFN-γ into supernatants by retrovirally infected tumor cells was determined using appropriate bioassays and confirmed by enzyme-linked immunosorbant assay (ELISA; Genzyme, Boston, MA and Endogen, Cambridge, MA). Supernatant from semiconfluent parental or cytokine-secreting CMS-5 cells was collected after 24 hours and assayed for human IL-2, murine GM-CSF, and murine IFN-γ. IL-2 biologic activity was measured by testing the ability of IL-2-containing preparations to induce thymidine incorporation into DNA of IL-2-dependent human primary lymphoblasts. Concentration of murine GM-CSF was similarly determined in a bioassay using GM-CSF–dependent murine 32D/13 cells. After overnight incubation without GM-CSF, 10⁶ cells were incubated with serial dilutions of test samples and cultured for 6 hours at 37°C, 5% CO₂. After pulsing cultures with 1 μCi of 3H-thymidine, incubation was continued for another 15 hours at 37°C, and cells were counted by liquid scintillation. GM-CSF activity was expressed as counts-per-minute and calculated in nanograms per 24 hours per 10⁶ cells by comparison with a standard curve of recombinant murine GM-CSF. The mouse IFN-γ bioassay was based on the antiviral activity of supernatants as determined by the reduction of cytopathic effects of vesicular stomatitis virus on L cells.

Fluorescence-activated cell analysis. Flow cytometry was performed for quantitative analysis of surface MHC class I and II expression. One million cells were harvested from tissue culture plates by treatment with 5 mmol/L EDTA, washed with fluorescence-activated cell sorter (FACS) buffer (phosphate-buffered saline [PBS] with 2% FCS and 0.02% sodium azide), incubated for 30 minutes at 4°C with culture supernatants of rat hybridoma cell lines My/ 42.3.9.8.HLK (TIB 126) and B2-1-2 (TIB 229). These cell lines, obtained from American Type Culture Collection (Rockville, MD), produce monoclonal antibodies (MoAbs) reactive with a mono- meric determinant on the MHC class I molecule and a polymorphic determinant on the MHC class II molecules of I-Aκ and I-Eκ haplotypes, respectively. After washing twice, cells were incubated with a 1:50 dilution of fluorescein isothiocyanate-conjugated goat antirat IgG (Cappel Laboratories, Durham, NC) for 30 minutes at 4°C in the dark. The cells were washed twice and then analyzed. Fluorescence originating from dead cells was excluded by propidium iodide staining and threshold gating of forward and 90° light scatter signals. Results were confirmed by fluorescence microscopy.

Tumor growth in vivo. BALB/c mice were obtained from a colony bred at Sloan Kettering Institute (New York, NY). Tumor cell injections were performed using freshly prepared tumor cells that were removed from culture plates by trypsinization and washed twice in PBS. Cells were injected ID in the back of 7- to 10-week-old female animals. Tumor growth was measured in millimeters using a caliper and recorded as mean diameter (longest surface length plus width divided by two).

In vivo depletion of effector cell subpopulations. The rat antimouse MoAb GK1.5 (IgG2b) and the rat antimouse MoAb 2.43 (IgG2b) in ascites fluid were used to deplete for CD4⁺ helper cells and for CD8⁺ cytotoxic cells, respectively. The amount necessary for T-cell depletion was determined by weekly IP injections of different dilutions of ascites into BALB/c mice. Splenocytes were analyzed 1 week after injection by flow cytometry. For in vivo studies weekly IP injections of 0.5 mL of 1:50 (2.43) or 1:30 (GK 1.5) diluted ascites
were administered, starting 1 week before tumor cell injection. For natural killer (NK)-cell depletion, 30 μL of rabbit antiasialo GM1 serum (Wako Chemicals, Richmond, VA) was injected intraperitoneally (IP) every 5 days, starting 5 days before tumor cell injection. NK-cell depletions were confirmed by in vitro analysis of spleen cell cytotoxicity against the NK-target cell line YAC-1.

RESULTS

Infection of tumor cells and secretion of cytokines. The structures of the retroviral vectors used to transduce the CMS-5 murine fibrosarcoma cells are shown in Fig 1. Virus-containing, cell-free supernatants of infected packaging cell clones were used to infect NIH 3T3 fibroblasts to determine virus titer. Virus titers of different constructs varied and were correlated inversely with the complexity of the retroviral construct used (Table 1). Viral titers for double cytokine constructs ranged from $10^3$ to $10^5$ Neo colony-forming units (CFU)/mL, those of single cytokine constructs from $10^5$ to $10^6$ Neo CFU/mL. High titer clones were selected to infect CMS-5 fibrosarcoma cells. G418-resistant CMS-5 clones were screened for expression of transfected cytokine genes by measuring IL-2, GM-CSF, and IFN-γ release into the culture supernatant using a bioassay. The results were then confirmed by ELISA. CMS-5 parental cells did not secrete any of the cytokines tested in this study. Clonal isolates of cytokine-gene-transduced CMS-5 cells varied in their ability to express and secrete cytokines, depending on the retroviral vector used. The range of cytokines produced by different clones were between 0 to 170 ng/24 h/10^6 cells for IL-2, 0 to 1,600 pg/24 h/10^6 cells for IFN-γ, and 0 to 400 ng/24 h/10^6 cells for GM-CSF. Single cytokine-secreting clones were selected that after inoculation of one million cells, showed delayed tumor growth but not tumor rejection. IL-2- or IFN-γ-transduced CMS-5 clones DC/TKIL2/CMS5 no. 6 and DC/AD/R/IFNγ/CMS5 no. 13 secreted IL-2 at 53 ng/24 h/10^6 cells (160 U/24 h/10^6 cells) and IFN-γ at 268 pg/24 h/10^6 cells (2 U/24 h/10^6 cells), respectively. To investigate whether secretion of a second cytokine by the same cell would induce tumor rejection, we selected tumor cell clone N/CIL2/TIFNy/CMSS no. 3, which produced 58 ng/24 h/10^6 cells (174 U/24 h/10^6 cells) of IL-2 and 159 pg/24 h/10^6 cells (0.72 U/24 h/10^6 cells) of IFN-γ (Table 2). To analyze in vivo effects of GM-CSF on primary tumor rejection, CMS-5 bulk-transduced cells that secreted high (130 ng/24 h/10^6 cells), medium (80 ng/24 h/10^6 cells), or low (8 ng/24 h/10^6 cells) amounts of GM-CSF and clone N2/CMV-GM-CSF/CMS5 no. 6.1 that secreted 65 ng/24 h/10^6 cells (174 U/24 h/10^6 cells) of IL-2 and 159 pg/24 h/10^6 cells (0.72 U/24 h/10^6 cells) of GM-CSF, and clone N/CIL2/TIFNy/CMSS no. 6, which produced 58 ng/24 h/10^6 cells (174 U/24 h/10^6 cells) of IL-2 and 159 pg/24 h/10^6 cells (0.72 U/24 h/10^6 cells) of IFN-γ (Table 2). To analyze in vivo effects of GM-CSF on primary tumor rejection, CMS-5 bulk-transduced cells that secreted high (130 ng/24 h/10^6 cells), medium (80 ng/24 h/10^6 cells), or low (8 ng/24 h/10^6 cells) amounts of GM-CSF and clone N2/CMV-GM-CSF/CMS5 no. 6.1 that secreted 65 ng/24 h/10^6 cells were selected. Tumor cell clone N/CGM-CSF/RIL2 no. 14.14 secreting 130 ng/24 h/10^6 cells of GM-CSF and 3.3 ng/24 h/10^6 cells (10 U/24 h/10^6 cells) of IL-2 was chosen as a control (Table 2). Secretion of IL-2, IFN-γ, or GM-CSF had no discernible effect on cell morphology. No change in growth rate in vitro of GM-CSF- or IL-2/GM-CSF-secreting CMS-5 cells was observed as compared with that for parental CMS-5 cells. Growth rate in culture was slightly reduced by the secretion of IFN-γ (Fig 2).

MHC expression on IFN-γ-transduced tumor cells. Upregulation of MHC molecules and other molecules involved in antigen processing and presentation by IFN-γ has been shown and is believed to contribute to the observed antitumor effects of IFN-γ. To determine whether the constitutive expression of IFN-γ would lead to effects on MHC class I and II expression, IL-2–, IFN-γ–, GM-CSF–, and IL-2/IFN-γ–transduced CMS-5 cells were assessed by indirect immunofluorescent staining and fluorescence-activated cell analysis (Fig 3). Parental CMS-5 cells expressed MHC class I molecules, but no MHC class II molecules were
Table 1. Viral Titers for the Constructs Used in the Experiments

<table>
<thead>
<tr>
<th>Construct</th>
<th>Titer (Neo CFU/mL)</th>
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<tbody>
<tr>
<td>DC/TKIL2</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>DC/AD/R/IFNγ</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>DC/AD/R/GM-CSF</td>
<td>$5 \times 10^5$</td>
</tr>
<tr>
<td>N2/CVM/GM-CSF</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>DCA</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>N/OIL2/TIFNγ</td>
<td>$5 \times 10^5$</td>
</tr>
<tr>
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<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>N/CGM-CSF/RIL2</td>
<td>$1 \times 10^6$</td>
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detectable. The level of MHC I expression was substantially elevated in cells secreting IFN-γ alone and modestly elevated in those secreting IFN-γ plus IL-2, compared with that for nontransduced fibrosarcoma cells. No de novo expression of MHC class II molecules in IFN-γ-transfected cells was seen. As expected, no effects of IL-2 or GM-CSF secretion were observed on MHC expression (data not shown). 

Augmentation of a specific antitumor response by IL-2 and IFN-γ. To study the in vivo effects of IL-2, IFN-γ, and GM-CSF secretion on primary tumor rejection, cytokine-secreting or parental CMS-5 cells were injected ID into the back of BALB/c mice, and tumor growth was measured. We previously showed that injection of $2 \times 10^5$ unmodified CMS-5 cells into BALB/c mice results in tumor growth in 100% of mice. Therefore, we used $2.5 \times 10^5$ parental CMS-5 cells in CMS-5 control groups. Previous work showed that secretion of IL-2 or IFN-γ alone by CMS-5 cells leads to primary tumor rejection. We have now chosen single cytokine-secreting CMS-5 clones that did not lead to rejection when 1 million cells were injected. However, 1 million CMS-5 cells secreting comparable amounts of both, IL-2 plus IFN-γ, were always rejected within 3 to 4 weeks. One representative experiment is shown in Fig 4. Coinjection of $10^6$ or $5 \times 10^5$ IL-2-secreting CMS-5 cells with the same number of IFN-γ–secreting CMS-5 cells also did not result in tumor rejection (Fig 5). Thus, the cosecretion of IL-2 and IFN-γ from the same tumor cells seems to augment the primary host antitumor responses compared with that for single cytokine secretion.

Because GM-CSF has proven to be a potent inducer of immunity against a challenge of parental tumor cells, we have investigated the direct in vivo effects of GM-CSF-secreting fibrosarcoma cells. CMS-5 bulk-transduced cells secreting high, medium, or low amounts of GM-CSF did not regress at a dose of $2.5 \times 10^5$ cells (Fig 6). Moreover, the results suggest that GM-CSF secretion by CMS-5 fibrosarcoma cells might even have promoted tumor growth. To show continuing cytokine secretion in vivo, GM-CSF-secreting tumors were removed after 3 to 4 weeks and reestablished in culture, and production of cytokine was measured before and after 10 days of culture with G418. GM-CSF secretion before injection corresponded in all cases to the amount secreted after resection of the tumor and after G418 selection (data not shown). These data suggest that GM-CSF production was responsible for the in vivo observed effects. Ongoing in vivo experiments using CMS-5 cells transduced
DOUBLE CYTOKINE RETROVIRAL VECTORS

with vectors carrying the IL-2 cDNA alone or GM-CSF plus IL-2 cDNAs show that the secretion of GM-CSF, in addition to IL-2, has no further effect on tumor growth inhibition compared with that for IL-2 alone (data not shown).

To investigate whether the antitumor activity induced in N/CIL2/TIFNγ/CMS5 injected mice was a systemic, long-lasting tumor-specific response, mice that had previously rejected cytokine-secreting CMS-5 cells were challenged with a lethal dose of parental tumor cells or with non-cross-reacting methylcholanthrene-induced tumor cells of the same genetic background (CMS-13). Mice grew in mice challenged with CMS-13, but not in mice challenged with CMS-5 cells, confirming previous data (data not shown). Thus, CMS-5 cells that secreted IL-2 and IFN-γ induced both immediate antitumor responses as well as a lasting protective immunity against the parental tumor.

Influence of in vivo depletion of effector cell subpopulations on tumor growth. To determine the contribution of different effector cell subpopulations to the rejection of IL-2/IFN-γ-secreting tumor cells, tumor growth was monitored in mice that were selectively depleted of CD4+ cells, CD8+ cells, or NK cells. One representative experiment is shown in Fig 7. Mice were injected with MoAbs to CD4+, CD8+, or NK cells as described in Materials and Methods. The absence of CD8+ T cells prevented the rejection of IL-2/IFN-γ-secreting CMS-5 cells. In contrast, depletion of CD4+ cells did not interfere with the ability of mice to reject transduced tumor cells. NK-cell depletion retarded rejection of IL-2/IFN-γ-secreting tumors compared with that for non-depleted mice. These results suggested that, in the primary rejection of IL-2/IFN-γ-secreting tumor cells, CD8+ cytotoxic T cells were mainly responsible, but NK cells were also induced and that, whereas they were not necessary for elimination of the tumor, they may also influence tumor rejection. CD4+ cells did not seem to play a role in the antitumor activity mediated by IL-2/IFN-γ-secreting CMS-5 cells.

DISCUSSION

In this report, we investigate the mechanisms of primary rejection of tumor cells transduced with cytokine genes presented in single or double cDNA-containing retroviral constructs. Although it is currently not possible to target therapeutic genes to tumor cells in situ, progress in the development of tissue-specific delivery and expression systems makes this a realistic approach in the future. Because in vivo treatment of patients with cancer using gene transfer would try to achieve maximum induction of antitumor immunity by a single transduction step, we have investigated whether the cotransduction of two cytokines into one tumor cell by a single retroviral vector would lead to an increased host antitumor response.

We chose IFN-γ and IL-2 in this study, because these cytokines have previously shown efficacy in inducing primary tumor rejection4-12 and both act on different levels of the immune response. IFN-γ is a cytokine known to upregulate molecules that participate in processing and presentation of antigen and, thus, acts on the levels of the target cells.45-52 IFN-γ also regulates induction of cytotoxic-T cells and activation of macrophages, NK cells, and lymphokine-activated killer (LAK) cells and can induce various other immune regulatory factors.29,33,53,54 IL-2, on the other hand, causes proliferation of cytotoxic-T cells, NK cells, and LAK cells and, thus, primarily acts on the level of the effector cells.54-57 Synergy of IL-2 and IFN-γ might also be anticipated, because recent data show that activation of T cells can be enhanced, if an antigen-nonspecific costimulatory signal, like B7 or IL-2, is delivered by the antigen presenting cell.26,27,58-60 Vaccination with GM-CSF-secreting, irradiated tumor cells can stimulate a potent, long-lasting immunity against a challenge with parental tumor cells.54 We studied the effects of live GM-CSF- and GM-CSF/IL-2-secreting tumor cells on induction of primary tumor rejection.

To this end, we constructed single-cytokine retroviral vectors carrying the murine IFN-γ, murine GM-CSF, or human IL-2 cDNA and double-cytokine retroviral vectors containing both the IL-2 and IFN-γ or IL-2 and GM-CSF cDNA and used those to infect CMS-5, a weakly immunogenic fibrosarcoma. Our data show that retroviral vectors carrying two cytokine genes can be used to stably transfect tumor cells leading to secretion of biologically active pro...
teins over prolonged periods of time. Secretion of low amounts of IFN-γ by fibrosarcoma cells resulted in the upregulation of MHC class I molecules on the cell surface. Thus, immunogenicity could be enhanced because tumor-associated antigens are presented to cytotoxic T cells in context of MHC class I molecules.61,62 The secretion of IL-2 and IFN-γ from the same tumor cell led to the generation of cytotoxic cells capable of rejecting at least 1 million CMS-5 cells, a dose four times higher than the lethal dose of CMS-5 for BALB/c mice. The secretion of similar amounts of IFN-γ or IL-2 alone did not induce an immune response strong enough to cause rejection of 1 million tumor cells. Mixing the same numbers of IL-2- and IFN-γ-secreting CMS-5 cells also did not result in tumor rejection. This phenomenon may be related to the circumstance that, with the double cytokine vector, both cytokines are secreted at a constant concentration per cell until all tumor cells are destroyed and that, in the cell mixture circumstance, one cytokine-secreting population is preferentially destroyed before the other one. These data support the concept that IL-2 and IFN-γ can cooperatively activate cytotoxic functions of the immune system63,64-65 and suggest an advantage to having both cytokines secreted by the same cell. It is possible that in vivo IFN-γ-induced upregulation of molecules involved in antigen processing and presentation is more efficacious in the autocrine situation and that more IFN-γ would be needed in the paracrine situation. Yet, it has to be considered that, on a per-cell basis, only half as much cytokine is produced in a mixture of clones. The question of the advantage of cosecretion of both cytokines by one tumor cell is not definitely answered with the experiments per-

Fig 4. Growth of parental and cytokine-secreting CMS-5 clones in BALB/c mice is shown. Groups of three mice in each category were injected with $2.5 \times 10^6$ parental or $1 \times 10^6$ transduced tumor cells as indicated. Results are expressed as the mean diameter (in millimeters) of tumors. Error bars represent the standard deviation of the mean. Tumor growth was monitored as described in Materials and Methods.

Fig 5. Growth of a mixture of IL-2- and IFN-γ-secreting CMS-5 clones in BALB/c mice is shown. One group of BALB/c mice was injected with a mixture of $5 \times 10^6$ DC/TKIL2/CMS5 cells and $5 \times 10^6$ DC/AD/RIFNγ/CMS5 cells (△); another group was injected with $1 \times 10^6$ DC/TKIL2/CMS5 cells and $1 \times 10^6$ DC/AD/RIFNγ/CMS5 cells (○), as indicated. Results are expressed as the mean diameter (in millimeters) of tumors. Error bars represent the standard deviation of the mean.
formed here. Nonetheless, secretion of both cytokines by a single cell should lead to simultaneous exposure of all effector cells to both cytokines. In contrast, secretion of both cytokines by different tumor cells would be expected to result in exposure of effector cells to variable concentration of the cytokines. Furthermore, the use of a single vector carrying both cytokine genes would simplify gene transfer for the treatment of tumors in vivo requiring only one transduction step.

Unirradiated GM-CSF-secreting, bulk-transduced CMS-5 cells and clonally selected GM-CSF- and GM-CSF/IL-2-secreting CMS-5 cells were not rejected, even at lower cell numbers. To the contrary, the more GM-CSF that was secreted, the faster the tumors grew, suggesting a growth-pro-
moting effect of GM-CSF on CMS-5 cells. Tumor explanta-
tion and reselection with G418 in vitro showed that outgrowth of no or low GM-CSF-secreting CMS-5 cells could not be the reason for the observed tumor growth in vivo. In additional experiments, the GM-CSF-containing vector was used to transduce MBT-2, a bladder carcinoma; TS/A, an adenocarcinoma; and B16, a melanoma. In no case did GM-CSF secretion induce rejection of the transduced tumor cells. Our data are in agreement with published data showing that GM-CSF exerts its potent immunostimulatory effects best if secreted by irradiated tumor cells incapable of proliferation. Dranoff et al.\textsuperscript{24} reported a systemic syndrome of fatal toxicity manifested by leukocytosis, hepatosplenomegaly, and pulmonary hemorrhage in mice injected with live GM-CSF-secreting B16 cells. This phenomenon was attributed to the progressively increasing number of tumor cells expressing GM-CSF in vivo. Human tumors naturally secreting GM-CSF do not appear to be rejected either.\textsuperscript{66} These results and our data underline the importance of carefully selecting the cytokine for each tumor- and gene-therapy approach chosen.

In the absence of cytokytic CD8\textsuperscript{+} T cells, IL-2/IFN-\gamma-secreting CMS-5 tumors were not rejected, confirming the necessity of CD8\textsuperscript{+} cytotoxic T lymphocytes CTLs for elimination of the tumor. These result suggest that, despite the slight growth inhibition of IFN-\gamma-secreting cells in vitro, direct inhibitory effects of IFN-\gamma on tumor growth were not responsible for tumor regression. Although IL-2/IFN-\gamma secretion by tumor cells activated NK cells, as shown by the delayed rejection of tumors in NK-depleted animals, their presence was not necessary to eliminate tumors. CD4\textsuperscript{+} helper-T cells did not seem to contribute to the rejection of IL-2/IFN-\gamma-secreting CMS-5 cells, because tumor rejection was not impaired in CD4-depleted mice. This impairment was probably caused by constitutive secretion of IL-2 and IFN-\gamma by target cells which bypassed the requirement for helper-T cells.\textsuperscript{5} From the experiments presented here, we cannot exclude that other nonspecific effector cells of the immune system contributed to the rejection of neoplastic cells. IFN-\gamma is a potent macrophage activator and, thus, antitumor activity in this system might be partially attributable to cytotastic or cytotoxic effects of activated macrophages. Moreover, IL-2/IFN-\gamma secretion not only leads to rejection of transduced tumor cells but also stimulates a specific, long-lasting antitumor immunity. This could be clinically important if, after primary therapy, not all tumor cells were eliminated; then, immunity might prevent regrowth of micrometastases. Whether or not the immune response induced by double cytokine-transduced tumor cells might be sufficient to eliminate pre-established disease and how this can be achieved needs further investigation.

In summary, double cytokine-gene-carrying retroviral vectors can be used to stably transfect tumor cells, and augmentation of in vivo antitumor activity can be obtained. Also, expression of both cytokines from one vector construct may be of benefit for future clinical applications to deliver both genes at the same time into one tumor cell.

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Augmentation of antitumor immunity by tumor cells transduced with a retroviral vector carrying the interleukin-2 and interferon-gamma cDNAs

FM Rosenthal, K Cronin, R Bannerji, DW Golde and B Gansbacher