Fibroblasts Genetically Engineered to Secrete Interleukin 12 Can Suppress Tumor Growth and Induce Antitumor Immunity to a Murine Melanoma in Vivo


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ABSTRACT

Interleukin 12 (IL-12), a disulfide-linked heterodimeric cytokine produced primarily by macrophages, is composed of light (p35) and heavy (p40) chains. It binds to a receptor on T-cells and natural killer cells, promoting the induction of primarily a Tn response in vitro and in vivo. To determine whether paracrine IL-12 secretion can alter tumor cell growth or promote antitumor immunity, we have developed a delivery system using genetically engineered fibroblasts in murine tumor models. NIH3T3 cells were stably transduced to express 100–200 units/10^6 cells/48 h of IL-12 using expression plasmids carrying both the murine p35 and p40 genes of murine IL-12. The effects of paracrine secretion of IL-12 on tumor establishment and vaccination models were examined using the poorly immunogenic murine melanoma cell line (BL-6) in C57BL/6 mice. To determine the effects of IL-12 on tumor formation, nonirradiated BL-6 cells were inoculated s.c. into C57BL/6 mice admixed with NIH3T3 cells transduced with both subunits of mIL-12 (3T3-IL-12) or with cells transfected with only the neomycin phosphotransferase gene (3T3-Neo). Compared to mice given injections of BL-6 alone, the day of emergence of detectable tumors was significantly delayed in mice given injections of BL-6 admixed with 3T3-IL-12, but not in mice with BL-6 admixed with 3T3-Neo. Effectiveness in this system was related to the amount of IL-12 expressed by the 3T3-IL-12. To determine the ability of locally secreted IL-12 at the tumor site to induce antitumor immunity, 10^6 irradiated tumor cells mixed with 3T3-IL-12 or 3T3-Neo were injected as a vaccine, and the response to a tumor challenge was subsequently examined. With a tumor challenge of less than 1 x 10^5 nonirradiated BL-6 cells, significant delay of establishment of tumor was noted with a relatively small amount of IL-12 secretion (1.2 units/5 x 10^6 cells/48 h). Larger amounts of secreted IL-12 provided no additional therapeutic benefit. Histological examination of tumor inoculum with 3T3-IL-12 secreting a high level of IL-12 showed peritumoral accumulation of macrophages, a characteristic capsule around the tumor composed of palisades of fibroblasts, and decreased numbers of CD4+ cells in the tumor. These results suggest that local delivery of IL-12 inhibits tumor growth in a dose dependent manner but leads to the development of an antitumor immune response when IL-12 is expressed at the tumor site at the relatively small amount indicated above. These results suggest that IL-12, like IL-2, -4, -6, and -7 and granulocyte-macrophage colony-stimulating factor, can induce an immune response against poorly immunogenic tumors.

INTRODUCTION

IL-12,^1^ formerly termed natural killer cell-stimulatory factor (1) or cytotoxic lymphocyte maturation factor (2), is a disulfide-linked heterodimeric cytokine composed of a M, 35,000 light chain (p35) and a M, 40,000 heavy chain (p40) (1, 2). The two complementary DNAs encoding the p35 and p40 chains of IL-12 from both the mouse and humans have been cloned. Unlike most other cytokines, simultaneous transfection of mammalian cells with two different genes is necessary for the production of biologically active IL-12 (3, 4). This cytokine exerts a variety of biological effects on human T-cells and NK cells in vitro. These include the ability to synergize with IL-2 in augmenting allogeneic CTL response (5), lymphokine-activated killer cell activity (2), and IFN-γ production from peripheral blood lymphocytes (1, 6). IL-12 also directly stimulates the production of IFN-γ and other cytokines from peripheral blood T-cells and NK cells (6, 7), enhances the lytic activity of NK cells (1, 7), and promotes the expansion of activated NK cells and activated T-cells (CD4+ and CD8+ subsets) (8). In addition to these functions, IL-12 has been shown to induce primarily a Tn response in vitro (9), as well as in vivo. Recent results of the administration of recombinant murine IL-12 in normal mice showed that IL-12 enhances NK and CTL activity and induces IFN-γ production in vivo (10). These activities suggest that IL-12 alone, or in combination with IL-2, might have antitumor effects. Since IL-12 is secreted only by “professional” antigen presenting cells (i.e., macrophages and B-cells), it is possible that local secretion of IL-12 at the site of tumor might induce an immune response similar to that which occurs during an immune response to bacterial pathogens. Thus, IL-12 may be a suitable cytokine for gene therapy as part of a tumor vaccine. Local secretion of each of the prototypic T-cell growth factors, IL-2 and IL-4 (11–13), has been previously achieved through the introduction of genes directly into tumor cells. Unlike the systemic injection of recombinant cytokines, direct transfection of cytokine genes into tumor results in the abrogation of tumor establishment and, in many cases, prolonged antitumor immune reactivity. This strategy, however, has many obstacles precluding successful clinical application. Tumor cells may be difficult to culture and transfet, and selection for transfected cells may require prolonged culture and alter expression of nominal tumor antigens. To avoid these potential disadvantages, we have developed an alternative approach to obtain paracrine cytokine secretion using transfected fibroblasts to express bioactive IL-12. The advantages of this strategy are that fibroblasts: (a) can produce physiologically relevant levels of cytokine after transfection; (b) are readily available to culture, transfet and select; and (c) are less likely to migrate to other sites compared to tumor or lymphoid cells. In the present study, we generated a murine fibroblast cell line expressing bioactive IL-12 by cotransfection of two plasmids, carrying either the p35 or p40 subunits. This fibroblast cell line was delivered admixed with BL-6, a poorly immunogenic murine melanoma cell line derived from B16 (14), in a tumor establishment or immunization model in the mouse. We report that paracrine secretion of IL-12 delays tumor formation and promotes antitumor immunity.

MATERIALS AND METHODS

Expression Plasmids and Transfection of Murine Fibroblasts

Murine p35 or p40 subunits of IL-12 were subcloned into BL-pSV plasmids which are then driven by an SV40 promoter (termed BL-pSV35 and BL-
pSV40 (15). JC125-Neo (carrying the bacterial neomycin phosphotransferase gene also driven by the SV40 promoter) was constructed and kindly provided by Dr. J. Cohen, University of Pittsburgh. NIH3T3 cells were cotransfected with these three expression plasmids using standard calcium phosphate techniques (16). To obtain stably transfected clones (3T3-IL-12), transfected cells were grown in G418 containing medium (0.75 mg/ml) for 14 days, and resistant clones propagated separately, with subsequent determination of IL-12 bioactivity in the culture supernatant. Control NIH3T3 cells containing only the neomycin phosphotranspherase gene (3T3-Neo) were prepared by transfection with only JC-125 using the same procedures.

IL-12 T-Cell Growth Factor Bioassay

To determine the bioactivity of IL-12 produced by transfectants, the proliferative response of day 4 human PHA-activated lymphoblasts was measured as described previously with minor modifications (2). In our assay, recombinant IL-2 and anti-human recombinant IL-2 antisera were not used, and the samples were added to cells on day 4 after PHA stimulation. Results were extrapolated from a standard curve using a defined dose of recombinant human IL-12 from Hoffrnann-La Roche (specific activity, 1.2 × 10⁸ units/mg protein).

Units of IL-12 activity were based on previous titrations in the T-cell growth factor assay (2).

PCR for IL-12

Expression of mRNA for both p35 and p40 subunits in transfected clones was confirmed using PCR. mRNA was extracted from approximately 1 × 10⁷ transfected NIH3T3 cells using a single step RNA extraction method with RNAzol (Tel-Test, Inc., Friends Wood, TX). Complementary DNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer) using this mRNA sample. The sets of 5' and 3' primers for PCR were 5'-CTGTGCAAGTCTCCACCTC-3' and 5'-GAGGTTGACTGTGAGCTCC-3', and CAGGGAACACTGTCACCAC for p35 and GAGGTTGACTGTGAGCTCC-3', and CAGGGAACACTGTCACCAC for p40. The expected lengths of the PCR products were 668 and 894 base pairs for p35 and p40, respectively. PCR was performed for 35 cycles. Each cycle consisted of 95°C for 1 min, 64°C for 2 min, and 72°C for 3 min. DNA products were separated on agarose gels, stained with ethidium bromide, and detected under UV light.

Preparation and Assay of Fibroblasts Expressing IL-2 (3T3-IL-2)

NIH3T3 cells were also infected with the retroviral vector carrying human IL-2 (GNL/IL-2, provided by Genetic Therapy, Inc., Germantown, MD) using Polybrene (Sigma Chemical Co., St. Louis, MO) at a concentration of 8 μg/ml. The stably infected cells were selected using G418 as described for 3T3-IL-12, and the G418-resistant colonies were pooled and used in murine experiments. Expression level of fibroblasts (3T3-IL-2) were examined using the same 4-day human PHA blast assay.

Murine Studies

IL-12 Testing in Establishment of the Murine Tumors. BL-6 cell is an aggressive and poorly-immunogenic clone (14) derived from a B16 murine melanoma tumor (generously provided by E. Gorelik, University of Pittsburgh). Syngeneic C57BL/6-H-2k mice (The Jackson Laboratory) in which the BL-6 arose were used for tumor inoculation. The mice were ear tagged and randomized prior to inoculation with tumor. Tumor establishment was determined whether IL-12 can directly affect the growth rate of BL-6 cells, murine recombinant IL-12 protein was added to the culture supernatant of BL-6 cells, the number of BL-6 cells cultured in media alone or containing 250 units/ml of IL-12 were 1.7 ± 0.1 × 10⁶ and 1.6 ± 0.2 × 10⁶, respectively, 48 h after plating and were not significantly different from each other. This is consistent with previous observations that conventional IL-12 receptors are not expressed on cells other than T-cells and NK cells. However, it remains to be
with 10% or less 3T3-IL-12 showed only a marginal delay. The 3T3-IL-12 containing varying percentages of 3T3-IL-12. Animals BL-6 cells were inoculated with 1 x 10^5 3T3 cells (3T3-Neo + 3T3-IL-12) after 10 passages, the local secretion of IL-12 can affect tumor establishment, 1 x 10^6 tumor cells. Within all experiments, the days of tumor emergence in Group II (tumor cells admixed with 3T3-Neo) had palpable tumors prior to day 36 after tumor challenge. With 1 x 10^6 BL-6 challenge (Fig. 3B), the date of emergence of palpable tumors in Group III (100% 3T3-IL-12) was significantly delayed compared with that in Group I (control) (P = 0.01). The date of emergence of palpable tumors in Group III (100% 3T3-IL-12) was only marginally delayed compared with that in Group I (control), but not significantly (P = 0.164). However, all animals developed palpable tumors by day 36 after tumor challenge. With 1 x 10^5 BL-6 cells (Fig. 4A), the date of emergence of palpable tumors in Group II (1% 3T3-IL-12) was insignificantly delayed compared with that in Group I (control) (P = 0.027), but that in Group III (100% 3T3-IL-12) was not (P = 0.853). One animal in Group II was completely free from tumor after day 40. These results suggest that low doses of IL-12 can induce a greater degree of antitumor immunity compared to relatively high doses of IL-12. This is contrary to the establishment model where the best antitumor effect was observed in the mice which received the highest amount of 3T3-IL-12. Similar results were obtained in another experiment, and suppression of tumor growth after immunization was also observed using tumor cells transfected with a retroviral vector expressing both subunits (data not shown).

Emergence of Detectable Tumor Following Challenge with Non-irradiated BL-6 Is Delayed in an Immunization Model. The previous results demonstrated that local expression of IL-12 delays tumor establishment in vivo. To determine if IL-12 can induce antitumor immunity, C57BL/6 mice were immunized with irradiated BL-6 tumor cells mixed with either 3T3-IL-12 or 3T3-Neo cells. The mice received two immunizations and were challenged on day 14 with different doses of tumor cells. With injection of 1 x 10^6 BL-6 tumor cells (Fig. 3A), the date of emergence of palpable tumors in Group II (1% 3T3-IL-12) was statistically delayed compared with that in Group I (control) (P = 0.01). The date of emergence of palpable tumors in Group III (100% 3T3-IL-12) was only marginally delayed compared with that in Group I (control), but not significantly (P = 0.164). However, all animals developed palpable tumors by day 36 after tumor challenge. With 1 x 10^5 BL-6 cells (Fig. 4A), the date of emergence of palpable tumors in Group II (1% 3T3-IL-12) was insignificantly delayed compared with that in Group I (control) (P = 0.027), but that in Group III (100% 3T3-IL-12) was not (P = 0.853). One animal in Group II was completely free from tumor after day 40. These results suggest that low doses of IL-12 can induce a greater degree of antitumor immunity compared to relatively high doses of IL-12. This is contrary to the establishment model where the best antitumor effect was observed in the mice which received the highest amount of 3T3-IL-12. Similar results were obtained in another experiment, and suppression of tumor growth after immunization was also observed using tumor cells transfected with a retroviral vector expressing both subunits (data not shown).

Local IL-12 Production Is Associated with a Histological Pattern of Tumor Necrosis, Macrophage Accumulation, Encapsulation by Fibroblasts, but not with Enhanced T-Cell Infiltration. Tissue samples of the inoculated tumors with various percentages of 3T3-IL-12 were examined histologically. In hematoxylin and eosin staining, the tumor cells inoculated with 3T3-Neo showed invasive growth into the muscle layer without encapsulation (Fig. 4A, arrowheads). Tumor cells inoculated with high amounts of 3T3-IL-12 showed reduced tumor volume and necrosis associated with encapsulation (Fig. 4B, arrowheads). Along with this capsule of palisading fibroblasts, macrophages were observed in low frequencies surrounding the tumor at day 8 post-tumor inoculation (Fig. 4C) and these cells were confirmed to be inflammatory cells including macrophages by electron microscopy (Fig. 4D). Infiltration by either macrophages or lymphocytes was not enhanced by IL-12 production within the tumor. Existence of this characteristic encapsulation was confirmed by the observation on the immunohistochemical staining with an anti-collagen type III antibody even in tumor inocula which were mixed with 100% of 3T3-IL-12 (Fig. 4E). Tumor inocula mixed with 1% of 3T3-IL-12 exhibited this capsule, but it was thicker in tumors inoculated with 100% 3T3-IL-12 (data not shown). To examine lymphocyte infiltration into the tumor more closely, immunostaining was performed using anti-T-cell receptor or anti-CD4 antibody. The tumor inoculum mixed with 3T3-Neo (Fig. 4F) or 1% of 3T3-IL-12 (Fig. 4G) displayed some CD4+ tumor infiltration lymphocytes, but the tumor inoculum mixed with 100% 3T3-IL-12 (Fig. 4H) showed a somewhat decreased number of CD4+ cells. The same tendency was observed with T-cell receptor staining (data not shown). In electron microscopic observation, no significant characteristics were noted determined whether other cell types express conventional or novel IL-12 receptors. This will become clear with cloning of the gene(s) for the receptor(s).

IL-12 Delays Emergence of Detectable Tumor after Intradermal Injection of BL-6 in Establishment Model. To determine if local secretion of IL-12 can affect tumor establishment, 1 x 10^6 tumor cells were inoculated with 3T3-IL-12 or 3T3-Neo (Fig. 2A). All mice in both Group I (tumor inocula consisted of tumor cells alone) and II (tumor cells admixed with 3T3-Neo) had palpable tumors prior to day 10, but mice in Group III (tumor cells admixed with 3T3-IL-12) were tumor free at that time. Tumor establishment was delayed in the Group III mice for at least 10 days. The difference in time to tumor emergence between Group III and Groups I or II was statistically significant (P = 0.003 for both). However, all of the mice in Group III ultimately developed detectable tumors by day 22. The same general findings were found in experiments using 5 x 10^5 and 1 x 10^4 tumor cells and repeated experiments using 1 x 10^6 tumor cells. Within all experiments, the days of tumor emergence in Group II (3T3-IL-12) were not significantly later than those in Group I (tumor only).

Varying numbers of 3T3-IL-12 were inoculated with BL-6 (Fig. 2B). In this particular experiment, the 3T3-IL-12 after 10 passages, expressing 100 units/10^6 cells/48 h in culture, was used. One x 10^6 BL-6 cells were inoculated with 1 x 10^6 3T3 cells (3T3-Neo + 3T3-IL-12) containing varying percentages of 3T3-IL-12. Animals inoculated with BL-6 cells and repeated experiments using 1 x 10^6 tumor cells. Within all experiments, the days of tumor emergence in Group II (3T3-IL-12) were not significantly later than those in Group I (tumor only).

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Fig. 2. Delivery of IL-12 by fibroblasts significantly delays tumor establishment. Emergence of detectable tumors after inoculation of BL-6 (BL-6, $1 \times 10^6$; NIH3T3-Neo or IL-12, $5 \times 10^5$ cells/animal). Each experiment was repeated twice with similar results. A, emergence of palpable tumors after inoculation of tumor cells alone (Group I), tumor cells mixed with 3T3-Neo (Group II), or IL-12 (Group III). The figures show representative results from each experiment ($P = 0.003$; Group III versus Group I or II). B, emergence of detectable tumors after inoculation of BL-6 mixed with graded amount of 3T3-IL-12 in 3T3-Neo. Numbers of inoculated BL-6 cells and total 3T3 were $1 \times 10^4$ and $1 \times 10^5$ (cells/animal) in every group ($P = 0.016$; BL-6 + 3T3 (100% 3T3-IL-12) versus BL-6 + 3T3 (100% 3T3-Neo)).

DISCUSSION

To activate a local antitumor immune response which might also promote systemic immune protection specific to the tumor cells, regional application of cytokines have been examined initially using the peritumoral or perilymphatic injection of cytokines (17, 18) and more recently using constitutive secretion from tumor cells transfected with cytokine genes. After the successful tumor growth inhibition and immune protection using IL-2 or IL-4 gene transfection into tumor was observed (11–13, 19), genes for a variety of other cytokines were tested in the same manner. Other than these prototypic Th1 (i.e., IL-2) and Th2 (i.e., IL-4) cytokines, local secretion of IFN-γ (20, 21), TNF-α (22, 23), granulocyte colony-stimulating factor (24), GM-CSF (25), IL-6 (26), and IL-7 (27) have been reported to induce tumor rejection in mice. However, only GM-CSF, IL-4, and IL-6 have been reported to be effective in the poorly immunogenic murine melanoma tumor cell line B16 (25), which is the parental cell line of BL-6 that we used for the present study (14).

We have developed an alternative method for delivery of cytokine locally at the site of the tumor. Genetically engineered murine fibroblasts can stably produce bioactive IL-12 following transfection of the two expression plasmids for p35 and p40 subunits of murine IL-12 and serve as the vehicle for targeted delivery of cytokine. This system differs from most of other cytokine gene therapy models (11, 12, 25, 28) which utilize transfected tumor cells themselves. Considering the difficulties of prolonged culture and transduction of human primary tumors in vitro, the possibility of selecting only some tumor variants except the encapsulation. This fibrous capsulation was also observed in the tumor inoculum without irradiation (data not shown).
in culture, and in contrast the ready availability of autologous fibroblasts, the use of fibroblasts for cytokine administration is an attractive, alternate method for clinical application of gene therapy. For IL-12, which requires the expression of two genes in the same cell, this system has significant advantages for initiating studies of the effect of paracrine secretion on tumor growth.

Establishment of palpable tumor of BL-6 was significantly delayed (compared with controls) when inoculated admixed with 3T3-IL-12, and such differences were enlarged in the group which received higher amounts of 3T3-IL-12. Paracrine secretion of IL-12 suppressed the growth of the murine melanoma cell line BL-6, in a dose-dependent manner. Thus, IL-12 appears to be a potent cytokine that is able to suppress the growth of this poorly immunogenic tumor cell line. These results are comparable to recent experiments demonstrating tumor suppression of various tumor types after systemic administration of recombinant murine IL-12 protein in murine models (29).

Fig. 4. Prominent capsule formation around tumors is associated with local IL-12 delivery. Histological examination of the tumor inocula mixed with 3T3-Neo or 3T3-IL-12 at 8 days after the inoculation. H & E staining of BL-6 tumor with 3T3-Neo (A) showed invasion into the muscle layer (arrowhead) and no apparent capsule formation surrounding the tumor (×150), (B) but that with 3T3-IL-12 showed a capsule formation (arrowhead) (×150). (C) In some part of the peritumor site, macrophages were observed (×150). (D) These surrounding cells were confirmed to be inflammatory cells including macrophages and neutrophils (arrowheads) by electron microscopic observation (×1500). (E) Immunohistochemical staining of collagen showed thick layer of collagen at the place corresponding to the capsule (×10). Immunohistochemical staining of CD4+ cells in the tumors. The tumor inoculum mixed with 3T3-Neo (F) or 1% of 3T3-IL-12 (G) displayed some CD4+ tumor infiltrating lymphocytes, but the tumor inoculum mixed with 100% 3T3-IL-12 (H) showed a modest decrease in the number of CD4+ cells (F, G, and H, ×150).
prolong the survival of tumor-bearing mice even when IL-12 treatment is started 14 days after the tumor inoculation. The maximum difference in the emergence of palpable tumor in the establishment experiments in this study was 15 days which is less than the results of IL-12 systemic injection (0.1 to 1.0 µg/animal/day, 7 days of treatment). This significant difference may be related to the nature of our system in this study. Although NIH3T3 cells are convenient for the initial investigation of the antitumor effect of IL-12, they are not the ideal vehicle in vivo for this purpose since they are allogeneic to the C57BL/6 mouse. These cells themselves are targets for immune rejection, and the relative amount of expressed IL-12 per tumor cell decreased over time after tumor inoculation. When 1 × 10⁶ TS/A cells (H-2d, kindly provided by Dr. Guido Forni, Turin, Italy) were inoculated in the allogeneic host, C57BL/6-H-2b cells were rejected within 14 days (data not shown). Based on this result, it is likely that 3T3-IL-12, which does not grow or form palpable tumor in vivo, was rejected rapidly by the host and almost certainly within 2 weeks. It is also possible that an allogeneic response has been induced by the fibroblasts which contributed to the induction of the antitumor effect of IL-12. However, when comparing the groups with BL-6 alone and BL-6 administered with NIH3T3 cells in this study, we did not observe suppression of tumor growth in the latter group. This suggests that the tumor-suppressive effects observed are due largely to IL-12 and not critically dependent on an allogeneic immune response directed at NIH3T3 fibroblasts.

Since all mice inoculated with BL-6 and 3T3-IL-12 eventually developed palpable tumors in the establishment model, we had no opportunity to examine whether paracrine secretion of IL-12 from live tumor cells can stimulate protective immunity against a subsequent tumor challenge. Thus, we used irradiated BL-6 tumor cells mixed with a varied amount of 3T3-IL-12 for immunization. Despite our inability to demonstrate complete tumor inhibition using live cells, we found that tumor formation was significantly suppressed or inhibited after immunization using irradiated tumor cells mixed with 3T3-IL-12 in our studies. Based on previous immunization studies with B16 melanoma cells or with the BL-6 subline that we used in this study, immune responses are almost never observed except when the tumor were transsected with GM-CSF, IL-4, or IL-6 (25). Moreover, BL-6 cells are so poorly immunogenic and invasive (14) that they can grow in a fully allogeneic mouse strain when 5 × 10⁶ cells are given i.d. (data not shown). Similar experiments with 3T3-IL-2 to deliver this cytokine failed to show significant influence on tumor establishment of BL-6 and supports those observations. Surprisingly, these effects on tumor suppression in this model were more evident in groups of mice which received irradiated tumor cells mixed with a lower percentage of 3T3-IL-12. This suggests that the optimal IL-12 secretion levels for inducing immunity, most likely mediated by memory T-cells specific for tumor, may be different from those for obtaining maximal tumor suppression in establishment model.

Our histological examination supports the hypothesis that IL-12 exerts tumor suppression through a different mechanism compared with other cytokines that have been tested previously. After the inoculation of IL-2-transfected tumor, an infiltrate of the tumor with neutrophils was observed (28). With IL-4 transfected tumor, eosinophils and macrophages are observed (13). A quite different “tumor environment” was observed for IL-12 when compared with tumors transfected with these cytokines. In our experiments, we observed accumulation of a modest number of macrophages and encapsulation of the tumor cells when inoculated with high percentage of 3T3-IL-12. These capsules were observed even around the tumor inoculated with 1% of 3T3-IL-12 but were less prominent when compared with those with 100% of 3T3-IL-12. However, in the tumor inoculum mixed with 100% 3T3-IL-12, no apparent lymphocytic infiltrate was observed, and there was a decrease in the number of CD4+ tumor infiltration lymphocytes compared to that of the tumors admixed with 3T3-Neo. IFN-γ, possibly produced by tumor-associated T-cells or NK cells stimulated by the IL-12, could be responsible in part for the effect. IFN-γ treatment induces multinucleated giant cells from concanavalin A-stimulated human monocytes in vitro (30) and may have a similar effect in vivo.

The mechanism of antitumor effect of IL-12 is currently under investigation. Primary or secondary vascular effects could be considered as possible nonimmunogenic mechanisms of tumor growth suppression. In squirrel monkeys, only two animals given high dose IL-12 protein developed pulmonary edema of over 20 treated. IL-12, however, does not appear to promote vascular leak in mice when it is given systemically in vivo in mice or in cynomolgus monkeys (29) as IL-2 and IL-4 do (31). When IL-12 is administered i.p., we have demonstrated that anti-IFN-γ antibody, but not TNF-α antibody administration was able to partially but not totally abrogate tumor suppression. Brunda et al. (29) have also suggested the involvement of CD8+ cells, but the precise mechanism of tumor suppression and induction of the tumor immunity requires further study. Systemic application of IL-12 can suppress tumor growth and prolong the survival of tumor-bearing mice in our studies. The B16F10 murine melanoma cell line is particularly virulent, and all the tumors in our experience with systemic IL-12 protein administration eventually progressed, resulting in death of the animals. Thus, it appears that systemic IL-12 treatment initially suppresses tumor growth rather than inducing tumor-specific immunemediated rejection. The only way to induce protective immunity against poorly immunogenic tumors like BL-6 may be to obtain local expression of IL-12 as demonstrated in the present study. Since we have recently shown that systemic administration of IL-12 induces tumor regression and is associated with nitric oxide production in vivo, nitric oxide could also be a mediator of the antitumor effect of IL-12. The production of nitric oxide mediates murine macrophage tumoricidal activity (32) and INF-γ plus TNF-α or IL-2 promote nitric oxide production by these cells (32, 33). Given that production of these cytokines from T-cells and NK cells can be induced by IL-12 in vitro (6, 7) and in vivo (10), and our histological observation showing accumulation of macrophages around the tumor margins when mixed with 100% 3T3-IL-12, it is possible that this pathway may be important in limiting tumor growth.

With regard to the induction of protective immunity against tumor by IL-12, one might speculate, based on our results in the immunization model, that only a relatively low level of IL-12 can successfully induce T-helper responses which are important in generating CD8+ antitumor T-cells (9). A similar observation has been reported for IL-4. Bosco et al. (18) showed that only low dose of IL-4 injected peri-lymphatically in tumor-bearing mice inhibits the growth of tumors and induces a tumor-specific immune memory. Although IL-12 was reported to enhance specific CTL response to allogeneic splenocytes in a dose-dependent manner (10), Orange et al. recently showed only low levels of IL-12 were required to protect animals from lymphocyte choriomeningitis virus infection. They also showed that higher application of IL-12 does not promote, but rather inhibits, viral-specific CTL lytic capacity per spleen and also inhibits the virus-induced expansion of CD8+ cells in spleen, peripheral blood, and lymph nodes. Since shedding of HLA class II antigen and intercellular adhesion molecule-1 from melanoma cells can be induced by INF-γ and TNF-α (34, 35), IL-12 might possibly be responsible for promoting a similar phenomenon, thereby altering effective antigen presentation by tumor cells.

5 T. Anderson, Hoffmann-La Roche, personal communication.

6 J. S. Orange, S. F. Wolf, and C. A. Biron. Effects of IL-12 on the response and susceptibility to experimental viral infections, submitted for publication.
The mechanism of the characteristic capsule formation is also not understood. Among the cytokines released from NK or T-cells activated by IL-12, TNF-α might be at least partially responsible for this capsule formation. Piquet et al. (36) reported that s.c. perfusion of TNF-α for 7 days induced the formation of a tissue mass composed mainly of fibroblasts, collagen, and capillaries along with massive tissue necrosis. This possibility will be formally examined using neutralizing anti-TNF-α antibody administration.

Our current system uses allogeneic 3T3 cells which could induce a C57BL/6 allogeneic response against these cells. They could recruit cells responsible for inducing immune reaction or to stimulate secretion of other cytokines (i.e., IL-1, -2, -4, -6). Direct interpretation of our results is somewhat complicated by these conditions. However, it is clear that the amount of IL-12 is critical for obtaining maximum antitumor immune response. Development of efficient vectors for transfection of both subunits of IL-12, which we have already constructed, will enable us to test syngeneic fibroblasts or tumor cells as vehicles for targeted delivery of IL-12. This might help us gain a clearer understanding of the mechanism by which IL-12 regulates tumor growth.

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