[CANCER RESEARCH 54, 182–189, January 1, 1994]

Fibroblasts Genetically Engineered to Secrete Interleukin 12 Can Suppress Tumor Growth and Induce Antitumor Immunity to a Murine Melanoma *in Vivo*[1]

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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 T_H1 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 transfected to express 100–240 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 transfected 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 1×10^5 nonirradiated BL-6 cells, significant delay of establishment of tumor was noted with a relatively small amount of IL-12 secretion (1.2 units/5×10^5 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,[3], formerly termed natural killer cell-stimulatory factor (1) or cytotoxic lymphocyte maturation factor (2), is a disulfide-linked heterodimeric cytokine composed of a M_r 35,000 light chain (p35) and a M_r 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 INF-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 T_H1 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 transfect, 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, transfect 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-

Received 9/21/93; accepted 10/28/93.

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[1] This work was supported in part by USPHS Grant PO1 CA59371 to M. T. L. H. J. Z. was supported by a grant from Genetic Therapy, Inc.

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[3] The abbreviations used are: IL-12, interleukin 12; NK cells, natural killer cells; IFN-γ, γ-interferon; CTL, cytotoxic T-lymphocyte(s); PHA, phytohemagglutinin; PCR, polymerase chain reaction; TNF-α, tumor necrosis factor α; GM-CSF, granulocyte-macrophage colony-stimulationg factor.

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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 antiserum 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 Hoffmann-La Roche (specific activity, 1.2×10^8 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 1×10^7 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 TTGCTGGTGTCTCCACTC and ATGGTCACGACGCGGGTG for p35 and GAGGTGGACTGGACTCCC and CAGGGAACACATGCCCAC 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-2^b 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 by palpation in a blinded experiment with a treatment group consisting of 4–5 mice/group. One $\times 10^6$ BL-6 tumor cells were inoculated i.d. in the shaved area of the left flank. Tumor inocula consisted of tumor cells alone (Group I), tumor cells admixed with 3T3-Neo [5×10^5 cells (Group II)], or tumor cells admixed with corresponding numbers of 3T3-IL-12 (Group III). Lower doses of BL-6 tumor cells (1×10^4 or 5×10^3 cells) were also inoculated in the same manner with 3T3-Neo or 3T3-IL-12 (5×10^3 or 5×10^3 cells, respectively) to test the effect of IL-12 on at lower tumor inoculation. To determine the influence of various quantities of local IL-12 secretion on tumor progression, 1×10^4 BL-6 cells were mixed with 1×10^5 3T3 cells consisting of graded percentages of 3T3-IL-12 (0, 0.1, 1, 10, 100%) and 3T3-Neo, and injected into mice. Tumor emergence was examined as noted previously. The effect of local IL-2 expression was also examined using the same system. One $\times 10^4$ BL-6 cells were mixed with 1×10^5 3T3-Neo (Group I) or 3T3-IL-2 (Group II), inoculated i.d. into C57BL/6 mice, and examined for tumor emergence in a blinded fashion.

IL-12 Testing in an Immunization Model. One $\times 10^6$ irradiated BL-6 tumor cells (10,000 rads) were mixed with nonirradiated 5×10^5 NIH3T3 cells consisting of graded percentages of 3T3-Neo/3T3-IL-12 using 0, 1, or 100% 3T3-IL-12 in Groups I, II, and III, respectively. Cell mixtures were injected i.d. in the shaved area of the left flank on day 0 and day 7. On day 14, 1×10^5 or 1×10^4 nonirradiated BL-6 cells were injected i.d. in the shaved area of the right flank of immunized mice. Tumor emergence was examined as noted previously.

Cell Proliferation Assay. To determine the direct effect of mIL-12 on BL-6 tumor proliferation, 5×10^5 BL-6 cells were split into T-75 flask containing complete media with or without 250 units/ml of murine IL-12. Forty-eight h later, the number of total cells in each flask was enumerated.

Histological Analysis. Tumor cells (1×10^6 cells/mouse)were inoculated with or without irradiation as in the other experiments, and tumors were harvested 8 days after tumor challenge. One-half of the tumors was immediately frozen and embedded in OCT compound. Serial 5-μm sections were made from these samples using a cryostat and underwent hematoxylin and eosin staining and immunofluorescent staining using antibodies to the T-cell receptor, CD4, CD8 (all from Becton Dickinson, Mountain View, CA), or collagen type III (Biodesign, Kennebunkport, ME). The rest of the samples were fixed in 2.5% glutaraldehyde, dehydrated through alcohols, embedded, and prepared for the electron microscopy using standard procedures.

Statistical Analysis

The percentage of mice without tumor in each group was calculated and compared to the other groups using a Student t test. The differences were considered statistically significant when P was <0.05.

RESULTS

IL-12 mRNA and Bioactive Protein Are Expressed in Transfected Fibroblasts. To generate 3T3 fibroblast cells secreting IL-12, vectors expressing p35 (BL-pSV35) and p40 (BL-pSV40), were co-transfected with a neomycin phosphotransferase expression plasmid (JC125-Neo). Following transfection, selection in G418, and expansion of G418-resistant colonies, the supernatant obtained from each colony was tested for IL-12 secretion using a T-cell growth factor biological assay. Seven of 48 colonies were found to produce bioactive IL-12. Among these colonies, colony 8 was chosen for the use in murine studies because of its much higher expression (240 units/10^6 cells/48 h) compared with the others. This activity, measured in a PHA-blast assay, was confirmed to be murine IL-12 and bioactive in a 4-day PHA blast assay performed on protein captured by solid phase anti-murine p40 monoclonal antibody (termed antibody capture bioassay; data not shown). This IL-12-expressing clone (3T3-IL-12) was used in all subsequent studies, and the 3T3-IL-12 supernatant was harvested at the time of *in vivo* use in every experiment to confirm the expression of IL-12. Reverse transcription-PCR of transfected cells demonstrated the expression of both the murine p35 and p40 chains in 3T3-IL-12 but not in the parental NIH3T3 cells, where expression of neither chain was detected (Fig. 1). The growth rates of the 3T3-IL-12 and 3T3-Neo were not significantly different in culture (data not shown).

IL-12 Does Not Directly Alter Tumor Growth *in Vitro*. To determine 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 mIL-12 were $1.7 \pm 0.1 \times 10^6$ and $1.6 \pm 0.2 \times 10^6$, 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

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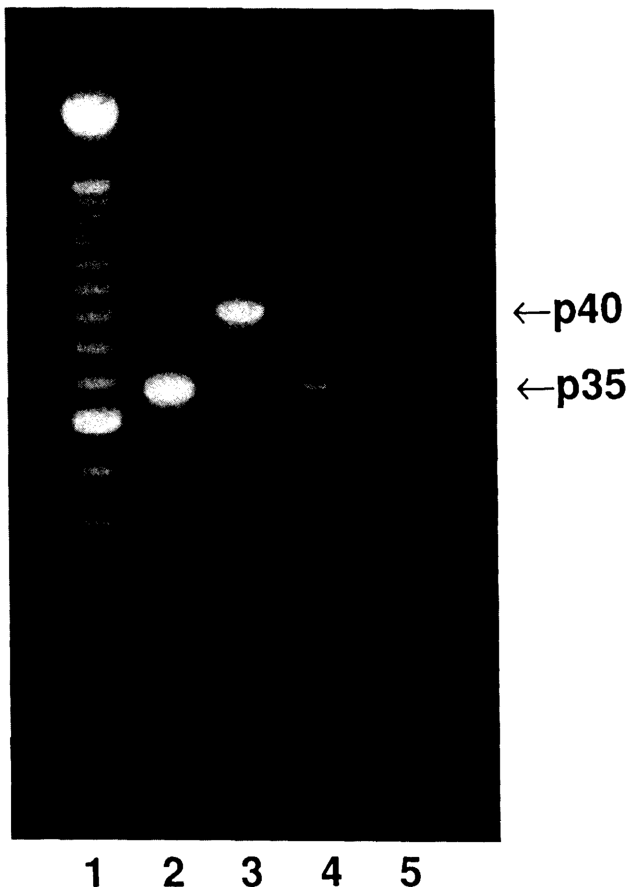

Fig. 1. Expression of IL-12 m-RNA in fibroblasts transfected with both p35 and p40 genes. Reverse transcription-PCR was performed for mRNA harvested from NIH3T3 cells (clone 8) transfected with both IL-12 genes. *Lane 1*, DNA size marker (123-base pair DNA ladder; GIBCO BRL, Gaithersburg, MD); *Lanes 2* and *3*, control DNA plasmids (BL-pSV35 and 40), *Lanes 4* and *5*, samples amplified for p35 and p40, respectively.

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, 1×10^6 tumor cells were inoculated with 3T3-IL-12 or 3T3-Neo (Fig. 2*A*). 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 5×10^3 and 1×10^4 tumor cells and repeated experiments using 1×10^6 tumor cells. Within all experiments, the days of tumor emergence in Group II (3T3-Neo) were not significantly later than those in Group I (tumor only).

Varying numbers of 3T3-IL-12 were inoculated with BL-6 (Fig. 2*B*).In this particular experiment, the 3T3-IL-12 after 10 passages, expressing 100 units/10^6 cells/48 h in culture, was used. One $\times 10^4$ BL-6 cells were inoculated with 1×10^5 3T3 cells (3T3-Neo + 3T3-IL-12) containing varying percentages of 3T3-IL-12. Animals inoculated with BL-6 cells and 100% of 3T3-IL-12 showed a statistically significant delay in tumor emergence (P = 0.016). Animals with 10% or less 3T3-IL-12 showed only a marginal delay. The animals which received 3T3-IL-12 in the site contralateral to the tumor inoculum also showed a delay of tumor emergence which failed to reach significance compared with the mixed inoculum (P = 0.099). To compare the effect of IL-12 with IL-2 on tumor growth in this particular model, 3T3-IL-2 was also evaluated. 3T3-IL-2 used in this experiment produced 100 units/10^6 cells/48 h in a T-cell growth factor bioassay. No significant delay was observed (P = 0.337) (data not shown).

Emergence of Detectable Tumor Following Challenge with Nonirradiated 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 1×10^5 BL-6 tumor cells (Fig. 3*A*), the date of emergence of palpable tumors in Group II (1% 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 1×10^4 BL-6 challenge (Fig. 3*B*), the date of emergence of palpable tumors in Group II (1% 3T3-IL-12) was significantly 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. 4*A*, *arrowheads*). Tumor cells inoculated with high amounts of 3T3-IL-12 showed reduced tumor volume and necrosis associated with encapsulation (Fig. 4*B*, *arrowheads*). Along with this capsule of palisading fibroblasts, macrophages were observed in low frequencies surrounding the tumor at day 8 post-tumor inoculation (Fig. 4*C*) and these cells were confirmed to be inflammatory cells including macrophages by electron microscopy (Fig. 4*D*). 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. 4*E*). 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. 4*F*) or 1% of 3T3-IL-12 (Fig. 4*G*) displayed some $CD4^+$ tumor infiltration lymphocytes, but the tumor inoculum mixed with 100% 3T3-IL-12 (Fig. 4*H*) 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

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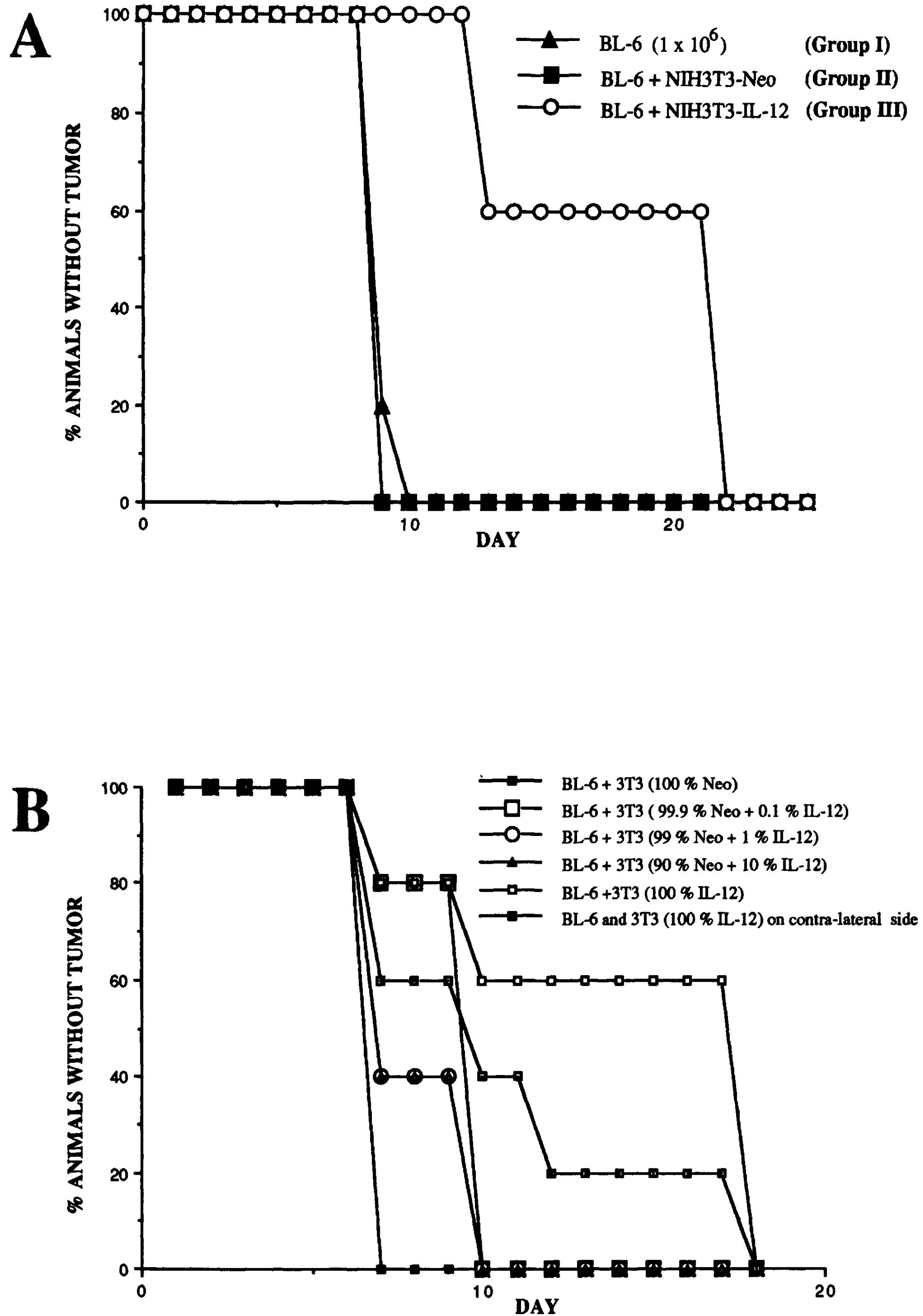

Fig. 2. Delivery of IL-12 by fibroblasts significantly delays tumor establishment. Emergence of detectable tumors after inoculation of BL-6 (BL-6, 1×10^6; NIH3T3-Neo or IL-12, 5×10^5 cells/animal). Each experiment was repeated twice with similar results. *A*, emergence of palpable tumors after inoculation 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 1×10^4 and 1×10^5 (cells/animal) in every group [$P = 0.016$; BL-6 + 3T3 (100% 3T3-IL-12) *versus* BL-6 + 3T3 (100% 3T3-Neo)].

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except the encapsulation. This fibrous capsulation was also observed in the tumor inoculum without irradiation (data not shown).

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 T_H1 (*i.e.*, IL-2) and T_H2 (*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 targetted 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

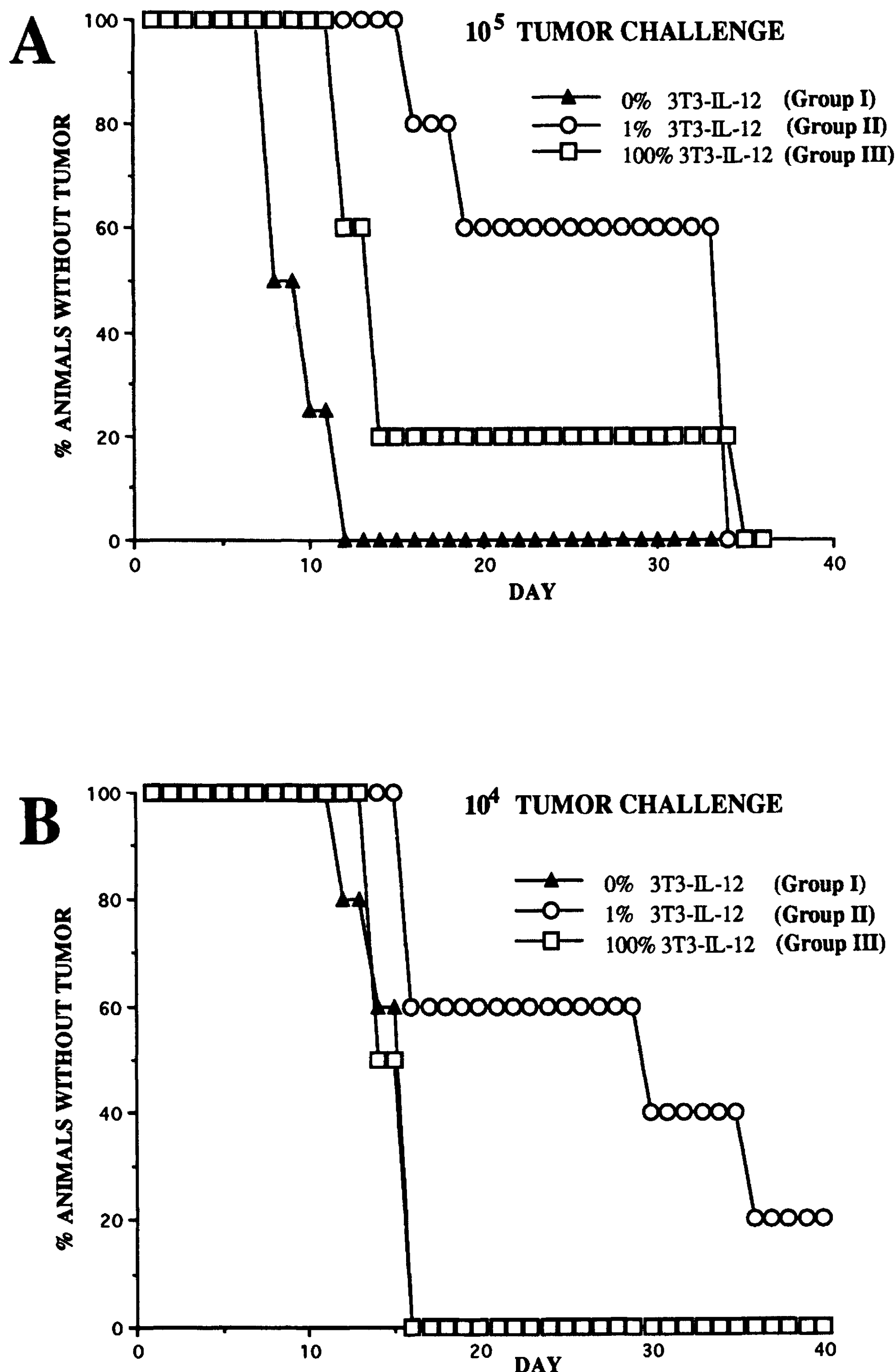

Fig. 3. Dose-dependent effect of IL-12 delivery on establishment and immune reactivity to tumor. Emergence of palpable tumors at the tumor challenge site after the immunization using irradiated BL-6 mixed with 3T3-IL-12. One $\times$ 10^6 irradiated BL-6 tumors (10,000 rads) were mixed with nonirradiated 5 $\times$ 10^5 NIH3T3 cells consisting of graded percentages of 3T3-IL-12 (colony 8; 0, 1, 100% for Group I, II, and III, respectively) and 3T3-Neo, and inoculated i.d. in the shaved area of the left flank on days 0 and 7. On day 14, 1 $\times$ 10^5 (*A*) or 1 $\times$ 10^4 (*B*) of nonirradiated BL-6 cells were inoculated i.d. in the shaved area of the right flank of immunized mice. Tumor emergence was examined as noted previously. (*A*) In 1 $\times$ 10^5 BL-6 challenge, the date of emergence of palpable tumors in Group II (1% 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 marginally delayed compared with that in Group I (control) but not significantly (P = 0.164). (*B*) In 1 $\times$ 10^4 BL-6 challenge, the date of emergence of palpable tumors in Group II (1% 3T3-IL-12) was significantly delayed compared with that in Group I (control) (P = 0.027), but that in Group III (100% 3T3-IL-12) was not delayed compared with that in Group I (control) significantly (P = 0.853).

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).[4] It has been demonstrated that systemic application of IL-12 can suppress and

[4] C. L. Nastala, H. Edington, W. Storkus, T. G. McKinney, H. Tahara, M. J. Brunda, M. K. Gately, R. Schreiber, and M. T. Lotze. Recombinant interleukin-12 administration induces tumor regression in association with interferon-gamma and nitric oxide production, submitted for publication.

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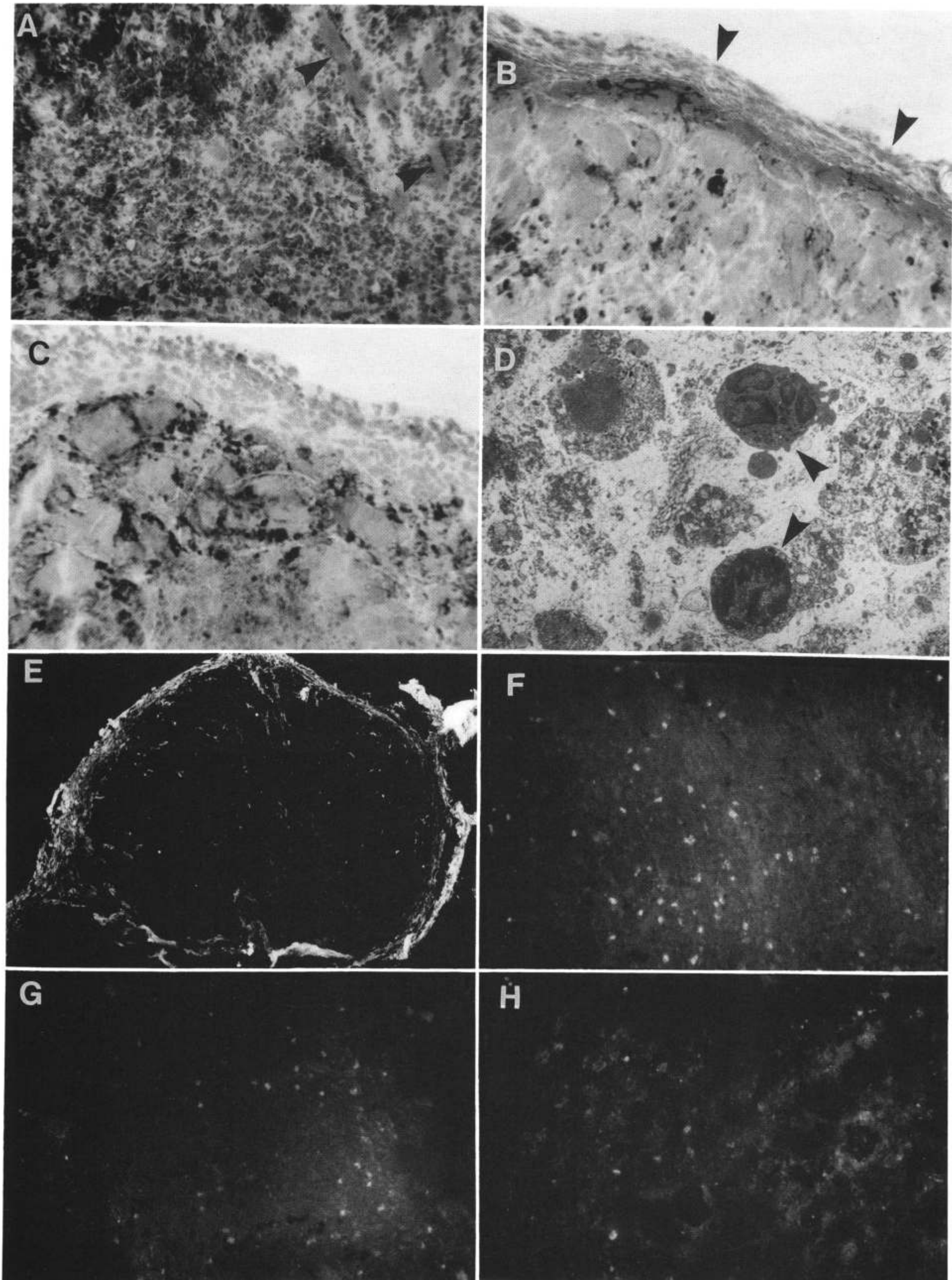

Fig. 4. Prominent capsule formation around tumors is associated with local IL-12 delivery. Histological examination of the tumor inocula mixed with 3T3-Neo or 3T3-IL-12 at 8 days after the inoculation. H & E staining of BL-6 tumor with 3T3-Neo (*A*) showed invasion into the muscle layer (*arrowhead*) 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).

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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 1×10^6 TS/A cells (H-2^d, kindly provided by Dr. Guido Forni, Turin, Italy) were inoculated in the allogeneic host, C57BL/6-*H-2*^{b}), 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 transfected with GM-CSF, IL-4, or IL-6 (25). Moreover, BL-6 cells are so poorly immunogenic and invasive (14) that they can grow even in a fully allogeneic mouse strain when 5×10^5 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.[5] 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)[4,6] 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.[4] 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.[4] Thus, it appears that systemic IL-12 treatment initially suppresses tumor growth rather than inducing tumor-specific immunomediated 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.[4] 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_H1 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 perilymphatically 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.*[6] 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 IFN-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.

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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,[7] 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.

ACKNOWLEDGMENTS

We thank Dr. Maurice K. Gately (Hoffmann-La Roche) for performing antibody captured IL-12 PHA blast assay and critical reading of the manuscript; Drs. Tim Anderson and Michael J. Brunda (Hoffmann-La Roche), Chet L. Nastala (University of Pittsburgh), and Drs. Jordan S. Orange and Christine A. Biron (Brown University) for valuable information; and Steven S. Hurd (University of Pittsburgh) for excellent technical assistance.

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[7] Manuscript in preparation.

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