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ORIGINAL ARTICLE

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Antitumor effects of the combination immunotherapy with interleukin-12 and tumor necrosis factor α in mice

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Abstract There is strong evidence that antitumor activity of interleukin-12 (IL-12) in vivo is mediated, in part, through interferon (IFNγ) produced by IL-12-stimulated natural killer and T cells. Since IFNγ and tumor necrosis factor α (TNFα) have been reported to synergize in antitumor effects in a number of models, we decided to examine whether the combined treatment with recombinant mouse IL-12 and recombinant human TNFα would produce similar effects. The efficacy of the combined IL-12/TNFα immunotherapy was evaluated in three tumor models in mice: B16F10 melanoma, Lewis lung (LL/2) carcinoma and L1 sarcoma. Intratumoral daily injections of 1 μg IL-12 in combination with 5 μg TNFα into B16F10-melanoma-bearing mice resulted in a significant retardation of the tumor growth as compared with that in controls and in mice treated with either cytokine alone. Similar effects were obtained using 0.1 μg IL-12 and 5 μg TNFα in LL/2 carcinoma and L1 sarcoma models. Antitumor activity against L1 sarcoma was still preserved when TNFα at a low dose (1 μg) was combined with 0.1 μg IL-12 and applied for a prolonged time. Potentiation of antitumor effects, which was observed in IL-12/TNFα-based immunotherapy, could result from at least three different mechanisms, partly related to stimulation of IFNγ and TNFα production in treated mice: (a) direct cytostatic/cytotoxic effects on tumor cells, (b) induction of antitumor activity of macrophages, and (c) inhibition of blood vessel formation in the tumor. Our studies demonstrate that combination tumor immunotherapy with IL-12 and TNFα may be more effective than single-cytokine treatment, and suggest possible mechanisms by which IL-12 and TNFα may exert potentiated therapeutic effects against locally growing tumors.

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Introduction

Interleukin-12 (IL-12), a heterodimeric cytokine of p35 and p40 subunits, was discovered as a natural-killer(NK)-stimulatory factor [15] and also as a cytotoxic lymphocyte maturation factor [29]. IL-12 is produced mainly by monocytes/macrophages and mediates a broad range of biological effects. In particular, IL-12 enhances NK- and T-cell-mediated cytotoxicity [15, 10] and stimulates proliferation of these cells [9], promotes differentiation of Th1 clones [19] and may induce secretion of interferon γ (IFNγ) from both resting and activated NK cells and T lymphocytes [15, 4]. IL-12 has also been demonstrated to play a role in intrathymic T cell development [12] as well as in the maturation of bone marrow cells [1].

When administered in vivo, IL-12 has been shown to exert antitumor activity against a number of murine tumors resulting in the inhibition of tumor growth as well as the reduction of the number of experimental metastases [3, 21, 36]. Combining IL-12 with other agents may enhance its antitumor efficacy and indicate the directions to pursue in clinical trials. Several attempts have already been undertaken to enhance the antitumor activity of IL-12. For example, IL-12 administered in combination with IL-2 displayed greater antitumor activity than that observed with either IL-12 or IL-2 alone [35]. Synergy in the induction of tumor immunity has also been observed between IL-12 and B7-1 [7]. Recently, IL-12 has been found to be an effective antitumor agent in established murine tumors when combined with macrophage-colony-stimulating factor and radiation treatment [31].

Since IFNγ seems to be required for optimal antitumor effects of IL-12 [3, 21], and given the evidence that IFNγ and TNFα synergize in their antitumor activity [2, 16, 30], we decided to examine whether the antitumor effects of

IL-12 could be strengthened by administration of this cytokine together with TNFα in established murine tumors.

Materials and methods

Mice

BALB/c and (C57BL/6×DBA/2)F1 mice, hereafter called B6D2F1, 8–12 weeks of age, were used in the experiments. Breeding pairs were obtained from the Inbred Mice Breeding Center of the Institute of Immunology and Experimental Medicine, Wrocław, Poland. Mice were bred in a local animal facility and were kept in conventional conditions with full access to food and water during experiments.

Tumors

The following tumor cell lines were used: B16F10 melanoma (obtained from Dr. M. Kubin, Wistar Institute, Philadelphia, Pa.), Lewis lung carcinoma (LL/2) and L1 sarcoma (from Dr. P. Janik, Institute of Oncology, Warsaw, Poland). Tumor cells were cultured in Dulbecco's modified Eagle medium (Gibco BRL, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum, antibiotics, 2-mercaptoethanol (50 μM) and L-glutamine (2 mM) (all from Gibco BRL). Cells were maintained in 25-cm^2 tissue-culture flasks (Nunc, Roskilde, Denmark) at 37 °C in a humidified atmosphere of 5% CO_2 and were passaged two or three times weekly using trypsin/EDTA (Gibco BRL) pretreatment.

Cytokines

Recombinant mouse IL-12 (specific activity 4.6×10^6 U/mg protein) was a generous gift from the Genetics Institute, Cambridge, Mass. Recombinant human TNFα was kindly provided by Knoll AG/BASF, Ludwigshafen, Germany (specific activity 5×10^7 U/mg protein), and from Dr. W. Stec, Department of Bioorganic Chemistry, Center for Molecular and Macromolecular Studies, Łódź Poland (specific activity 4.3×10^7 U/mg protein). TNFα from both sources was found to have comparable biological efficacy when tested in vitro and in vivo. Cytokines were diluted with 0.1% bovine serum albumin (BSA; Sigma Chemicals, St. Louis, Mo.) in phosphate-buffered saline (PBS; Gibco BRL) for in vivo experiments. Murine recombinant IFNγ (specific activity 1×10^7 U/mg protein) was purchased from Genzyme Corporation, Cambridge, Mass.

Determination of endogenous IFNγ and TNFα in mouse serum

Serum levels of IFNγ and TNFα were measured following a 7-day intertumoral (i.t.) treatment of tumor-bearing mice with recombinant mouse IL-12 (0.1 μg, daily) and/or recombinant human TNFα (5 μg, daily). Mice were bled (under ether anesthesia) 4 h after the last injection of cytokines, and the levels of endogenous IFNγ and TNFα in serum samples were measured using enzyme-linked immunosorbent assay (ELISA) kits purchased from Genzyme Corp. (Factor-test-X for mouse TNFα, Intertest-γ for mouse IFNγ). In preliminary testing, a Factor-test-X kit was found specifically to detect mouse TNFα and no crossreactivity was observed with the recombinant human TNFα that was used for the treatment of the mice.

MTT assay

The direct cytostatic/cytotoxic effect of IL-12, TNFα and IFNγ on B16F10 melanoma cells was tested in a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [16]. Briefly, tumor cells were dispensed in a 96-well flat-bottomed microtiter plate (Nunc) at a concentration of 2.5×10^3 cells in 100 μl/well. Plates were incubated overnight, and then serial dilutions of the tested cytokines, alone or in combination, were added in quadruplicate to a final volume of 200 μl. After an incubation period of 68 h, 25 μl MTT (Sigma Chemicals) solution was added to each well. The plates were centrifuged 4 h later and 200 μl supernatant was carefully removed from each well and replaced with 200 μl acid dimethylsulfoxide. After solubilization of formazan crystals, the plates were read on an ELISA reader (Organon Technika, Reader 510), using a 540-nm filter. The cytostatic/cytotoxic effect was expressed as relative viability (percentage of control) and was calculated as follows:

$$\text{Relative viability } (\%) = \frac{\text{experimental absorbance} - \text{background absorbance}}{\text{absorbance of untreated controls} - \text{background absorbance}} \times 100$$

Assay for macrophage cytostatic/cytotoxic activity and production of nitric oxide (NO) by macrophages

B6D2F1 mice were treated i.p. with IL-12 (0.1 μg, daily) and TNFα (5 μg, daily), alone or in combination, for 3 days. One day after the last injection, peritoneal exudate cells were collected by lavage of the peritoneal cavity with chilled Dulbecco′s PBS (Gibco BRL) containing 10 U/ml heparin. After centrifugation, cells were plated in 92 × 21-mm plastic petri dishes (Nunc) in culture medium, incubated for 1 h at 37 °C in 5% CO_2, and washed gently three times with warm RPMI-1640 medium to remove non-adherent cells. Adherent cells with the morphological characteristics of macrophages were harvested by forced rinsing with cold Dulbecco′s PBS (without calcium and magnesium), using a 50-ml syringe and 20-gauge needle and, in addition, any remaining cells were scraped by a rubber policeman. Following one washing, macrophages were resuspended in culture medium, counted and dispensed into wells of a 96-well flat-bottomed microtiter plate at a concentration of either 10^5 or 5×10^4 cells in 0.1 ml/well. Then, 5×10^3 B16F10 melanoma cells in 0.1ml culture medium were added to each well (within 2 h), giving the E:T ratio 20:1 or 10:1 respectively. Cultures were incubated for 3 days. The cytocidal/cytostatic effect of macrophages on melanoma cells was tested in a standard MTT assay, where cells were incubated with MTT for the last 4 h of a 3-day culture. The antitumor effect of macrophages from IL-12- and/or TNFα-treated mice, expressed as the relative viability of tumor cells (percentage of control cultures) was calculated according to the formula: relative viability (%) = $[(A_{exp} - A_{min})/(A_{max} - A_{min})] \times 100$, where: A_{exp} = absorbance in experimental macrophage cultures with B16F10 melanoma cells after subtracting absorbance of macrophages alone, A_{min} = background absorbance, A_{max} = absorbance in control macrophage cultures with B16F10 melanoma cells after subtracting the absorbance of macrophages alone. The means and standard errors of the mean were determined for quadruplicate samples.

NO production by macrophages from mice treated with IL-12 and/or TNFα was assessed by nitrite accumulation in 18-h culture supernatants using a colorimetric microtiter assay with Griss reagent. Briefly, samples of 100 μl were incubated with equal parts of 50 μl 1% sulfanilamide dihydrochloride (Sigma) in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylendiamine (Sigma) in distilled water at room temperature for 15 min. The absorbance was determined with an ELISA reader using a 540-nm filter. Concentrations of nitrite were determined from a linear standard curve generated from 1.25–100 μM sodium nitrite in culture medium. The means and standard deviations were determined for triplicate samples.

Angiogenesis assay

The tumor-induced angiogenesis assay was used as described previously [18]. Briefly, female BALB/c mice were immunosuppressed by X-irradiation (6 Gy) and, 1 day later, were anesthetized with chloral hydrate and shaved on both flanks. To induce angiogenesis, each mouse was given tumor-cell suspension intradermally (3×10^5 B16F10 melanoma cells in 0.1 saline with trypan blue for better visualization of the injection site) and, after 3 days, the mice were killed and the inner surface of their skin was assessed according to Sidky and Auerbach [28] for the number of newly formed blood vessels. The effects of IL-12 and/or TNFα on angiogenic response was assessed blindly 1 day following three cytokine injections (0.1 μg and/or 5 μg respectively, daily, on days 0, 1, and 2).

In vivo experiments

B16F10 melanoma, LL/2 carcinoma or L1 sarcoma cells, prepared as described elsewhere [17], were injected into the footpad of the right hind limb at the dose of 1×10^6 cells (B16F10 melanoma in B6D2F1 mice and L1 sarcoma in BALB/c mice) or 5×10^5 cells (LL/2 carcinoma in B6D2F1 mice) in 20 µl medium. Mice were treated intratumorally with IL-12 and/or TNFα according to protocols that are presented in the legends of tables and figures showing the results of individual experiments, starting from day 7 after tumor cell inoculation. Cytokines were given at daily doses of 1 µg or 0.1 µg (IL-12) and 5 µg or 1 µg (TNFα) in 20 µl 0.1% BSA/PBS. Each injection of cytokines was paralleled by i.t. injections of 0.1% BSA/PBS into mice from the control groups. Local tumor growth was determined by measuring footpad diameter (anterior-posterior dimension) with calipers every 2 days, starting from day 7 following inoculation of tumor cells. Tumor size was calculated according to the formula: tumor diameter = (diameter of footpad with growing tumor) – (diameter of non-treated contralateral footpad). Independently, in all experiments, two-dimensional measurements of the footpad (anterior-posterior + side-to-side measure) were performed to evaluate the tumor growth. No substantial difference between the one-dimensional and two-dimensional methods of evaluation of the tumor size was found.

Statistical analysis

Differences between samples in tests in vitro and differences in tumor diameter in experiments in vivo were analyzed for significance by Student's *t*-test (two-tailed). To determine the difference in mouse survival between experimental groups, the Mann-Whitney *U*-test was used.

Results

Antitumor effects of IL-12 and TNFα against B16F10 melanoma, LL/2 carcinoma, and L1 sarcoma

To evaluate the effects of IL-12 and TNFα on B16F10 melanoma, LL/2 carcinoma, and L1 sarcoma, mice were injected with tumor cells on day 0 and treated i.t. daily with IL-12 and/or TNFα, starting on day 7 after tumor cell inoculation. As shown in Fig. 1A, administration of IL-12 at a daily dose of 1 µg for 7 days induced significant retardation of the B16F10 melanoma growth ($P<0.05$, from day 17). TNFα was ineffective in this treatment schedule. The most potent antitumor effect was observed when melanoma-bearing mice were treated with both TNFα and IL-12. This double-cytokine treatment produced a significant delay of tumor progression, not only in comparison with controls and TNFα-treated mice (from day 17, $P<0.01$ and $P<0.05$ respectively) but also when compared with mice treated with IL-12 alone (from day 23, $P<0.05$). The delay in tumor growth in mice treated with TNFα + IL-12 resulted in nonsignificant prolongation of mouse survival (the median survival time in this group was 62 days, in comparison with 46 days in IL-12-treated mice, 39 days in TNFα-treated animals, and 42 days in diluent-injected controls). The combined treatment with IL-12 and TNFα was also effective in LL/2 carcinoma and L1 sarcoma (Figs. 1B, 2) despite the lower IL-12 doses that were used in these models. The reduction of the IL-12 dose was necessary to avoid toxic effects. Beginning on day 13, significantly smaller tumors were observed in LL/2-carcinoma-bearing mice injected with IL-12 and TNFα together (0.1 µg and 5 µg respectively, for 6 days) in comparison with controls or the IL-12-treated group ($P=0.01$ and $P<0.05$ respectively) (Fig. 1B). Under the same experimental conditions, IL-12 was completely ineffective, and TNFα produced a statistically significant retardation of tumor growth in the last two measurements (on days 25 and 27, $P<0.01$).

In comparison with B6D2F1 mice injected with B16F10 melanoma or LL/2 carcinoma, BALB/c mice bearing L1

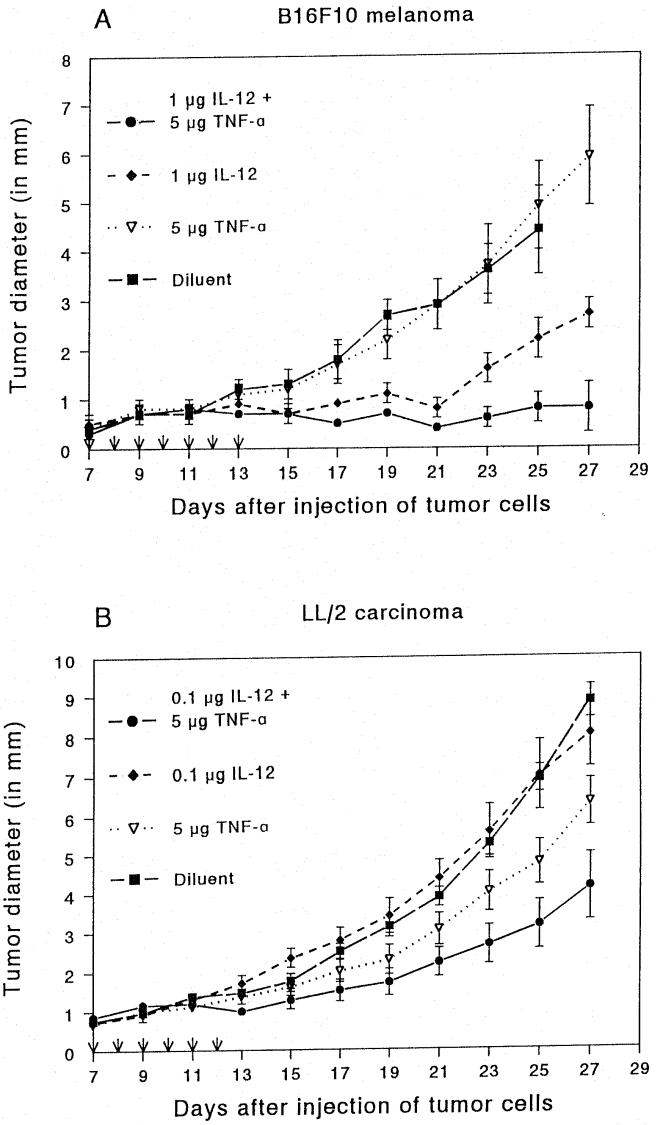

Fig. 1A, B Effect of treatment with interleukin-12 (*IL-12*) and/or tumor necrosis factor α (*TNFα*) on growth of B16F10 melanoma and Lewis lung (LL/2) carcinoma. **A** Mice were inoculated with 10^6 melanoma cells into the footpad of the right hind limb and then treated intratumorally with 1 µg IL-12 and/or 5 µg TNFα per day, starting on day 7 after tumor inoculation for 7 consecutive days (*arrows*). **B** Mice were inoculated with 5×10^5 LL/2 carcinoma cells into the footpad of the right hind limb and then treated intratumorally with 0.1 µg IL-12 and/or 5 µg TNFα per day for 6 consecutive days, starting on day 7 after tumor inoculation (*arrows*). Groups consisted of four to six mice. Data are expressed as means ± SE

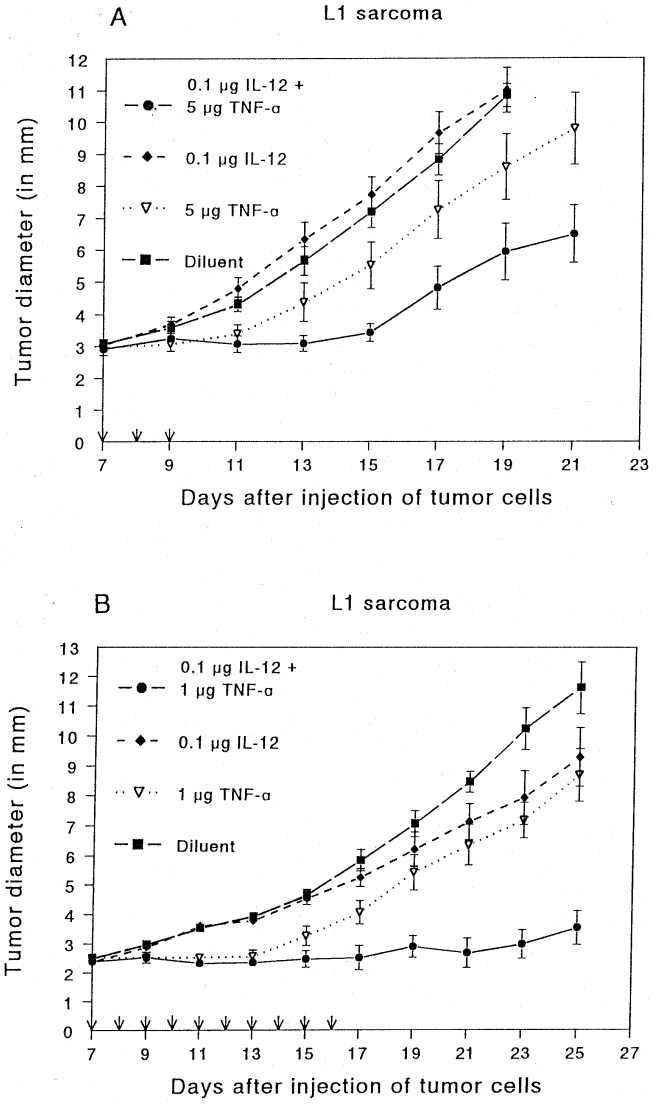

Fig. 2A, B Effect of treatment with IL-12 and/or TNFα on growth of L1 sarcoma. Mice were inoculated with 1×10^6 L1 sarcoma cells into the footpad of the right hind limb and treated intratumorally with 0.1 μg IL-12 and/or 5 μg TNFα per day for 3 consecutive days (**A**) or with 0.1 μg IL-12 and/or 1 μg TNFα per day for 10 consecutive days (**B**), starting on day 7 after tumor inoculation (*arrows*). Groups consisted of five or six mice. Data are expressed as means ± SE

sarcoma were found to be very susceptible to the action of IL-12 + TNFα (and also to TNFα alone). One of six mice treated with both cytokines died on day 10 after tumor inoculation, so a 3-day treatment schedule was adopted. We observed a similar high susceptibility of BALB/c mice in our earlier studies on the effect of TNFα and actinomycin D [17]. As shown in Fig. 2A, the response pattern of L1 sarcoma to the treatment with IL-12 and/or TNFα was quite similar to that observed in LL/2 carcinoma. Significant retardation of tumor growth was demonstrated in mice treated with both cytokines ($P<0.01$, as compared with IL-12- or diluent-treated mice from day 11, and $P<0.05$, as compared with mice injected with TNFα alone on day 15),

Table 1 Serum levels of endogenous interferon γ (*IFNγ*) and tumor necrosis factor α (*TNFα*) following treatment with recombinant mouse interleukin-12 (*IL-12*) and recombinant human TNFα. Mice were treated intratumorally for 1 week with IL-12 (0.1 μg, daily) and/or TNFα (5 μg, daily), beginning on day 7 after tumor inoculation. Mice were bled 4 h after the last injection and levels of endogenous IFNγ and TNFα were measured by enzyme-linked immunosorbent assay, as described in Materials and methods. Data represent mean ± SE serum levels from four or five mice per group

Treatment	IFNγ level (pg/ml)	TNFα level (pg/ml)
IL-12	7811±1804*	51±23**
TNFα	0±0	12±1
IL-12 + TNFα	1978±513	56±8***
Diluent	0±0	5±4

* $P<0.001$ as compared with the group receiving IL-12 + TNFα
** $P<0.01$ as compared with diluent-treated mice
*** $P<0.001$ as compared with diluent-treated mice

while administration of TNFα produced a minor antitumor effect ($P<0.05$, as compared with controls on day 11 only) and IL-12 treatment was completely ineffective. To determine if a decrease of TNFα dose could reduce toxicity in BALB/c mice, 1 μg instead of 5 μg TNFα was used with 0.1 μg IL-12 in mice inoculated with L1 sarcoma. As seen in Fig. 2B, application of a lower dose of TNFα allowed prolongation of cytokine treatment (10 days) while potentiation of the antitumor effect of TNFα by IL-12 was still preserved [$P<0.001$, from day 11, when compared with controls or IL-12-treated mice, and $P<0.05$ on day 17 (and $P<0.01$ from day 19), when compared with mice treated with TNFα]. What is more important, no life-threatening toxicity was found in this treatment schedule and, in one mouse in the group treated with both cytokines, complete tumor regression was observed. In both the LL/2 carcinoma and L1 sarcoma models, treatment of mice with IL-12 in combination with TNFα resulted in slightly longer survivals in comparison with those observed in other groups. However, the differences did not reach statistical significance (data not shown).

Serum levels of endogenous IFNγ and TNFα

Since one of the properties of IL-12 in vivo is its ability to induce the secretion of IFNγ [21], we decided to measure serum level of this cytokine, as well as the level of endogenously produced TNFα, shortly after the 7-day course of IL-12/TNFα treatment in tumor-bearing mice. As shown in Table 1, high levels of IFNγ were detected in sera of mice treated with 0.1 μg IL-12 (mean 7811 pg/ml, $P<0.001$ in comparison with controls). Surprisingly, combination therapy with 0.1 μg IL-12 and 5 μg TNFα induced a much lower level of IFNγ in the serum (mean 1978 pg/ml, $P<0.001$ as compared with the IL-12-treated group or controls). Treatment with IL-12, alone or in combination with TNFα, also led to elevation of the endogenous TNFα level, but this increase was not so dramatic as that of IFNγ (51 pg/ml TNFα was detected in the serum of IL-12-treated

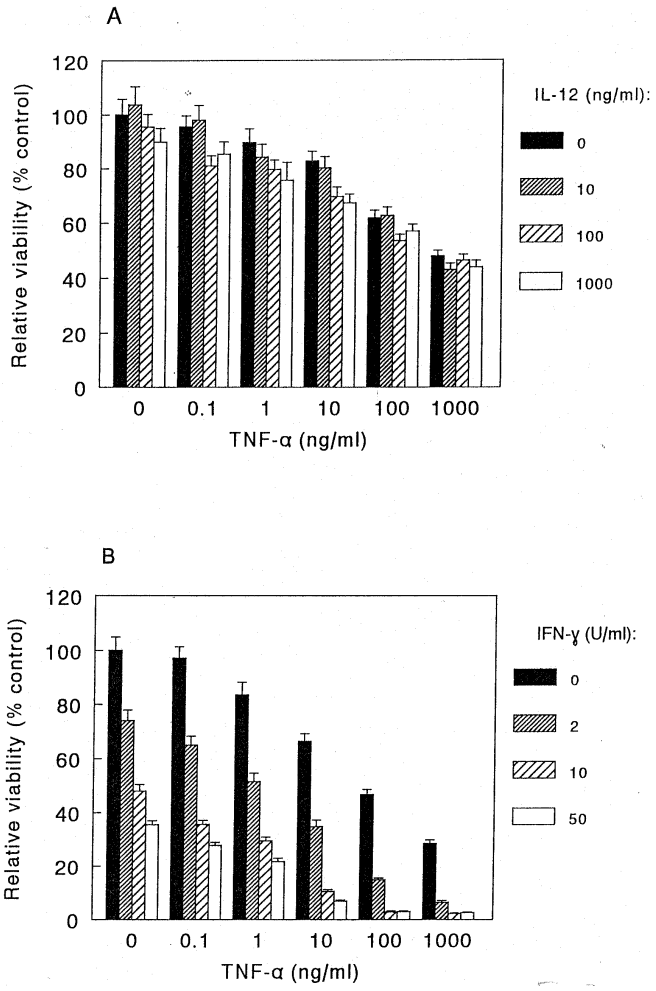

Fig. 3A, B Effects of IL-12 and TNFα, or interferon γ (IFNγ) and TNFα on B16F10 melanoma cells in vitro. One-day-old monolayers of melanoma cells were exposed for 72 h to various concentrations of IL-12 and TNFα (**A**) or IFNγ and TNF α (**B**). Cytostatic/cytotoxic effects of cytokines, expressed as relative viability (percentage of control), were tested in a standard MTT assay (see Materials and methods). Data are expressed as means ± SE

mice and 56 pg/ml TNFα in mice treated with both IL-12 and TNFα, $P<0.01$ and $P<0.001$ respectively in comparison with controls).

The combination of IL-12 and TNFα in vitro, in contrast to the IFNγ and TNFα combination, does not seem to result in potentiation of the cytostatic/cytotoxic effect on B16F10 melanoma cells

To determine whether the direct antitumor effect of IL-12 could contribute to inhibition of the tumor growth following treatment with IL-12 + TNFα in vivo, B16F10 melanoma cells were incubated in vitro with IL-12 and/or TNFα for 3 days and the viability of cells was tested in a MTT assay. As shown in Fig. 3A, TNFα exerted a dose-dependent cytostatic/cytotoxic effect in this experiment. Adding IL-12 to melanoma cell cultures seemed to reduce the viability of

Table 2 Antitumor activity of peritoneal macrophages from IL-12 and/or TNFα-treated mice against B16F10 melanoma cells. Mice were treated i.p. with IL-12 (0.1 μg, daily) and/or TNFα (5 μg, daily) for 3 days (three or four mice per group). Peritoneal macrophages, obtained as described in Materials and methods, were dispensed into wells of a 96-well flat-bottomed microtiter plate at a concentration of either 1×10^5 or 5×10^4 cells/well. Then, 5×10^3 B16F10 melanoma cells were added to each well within 2 h, giving the E:T ratio 20:1 or 10:1 respectively. After a 3-day incubation, cytostatic/cytotoxic effects of macrophages on melanoma cells, expressed as the relative viability of tumor cells (% of control cultures), were tested in a standard MTT assay (see Materials and methods)

Source of macrophages	Viability of B16F10 melanoma cells in cultures with macrophages, mean ± SE (% of control cultures)	
	E:T = 20:1	E:T = 10:1
Mice treated with:		
IL-12	51±2	55±2
TNFα	118±7	128±3
IL-12 + TNFα	38±2*	58±4
Diluent	100±4	100±1

* Significantly different in comparison with each other group ($P<0.001$), at the 20:1 ratio

melanoma cells, at least in cultures in which TNFα was combined with the highest concentration of IL-12 (1000 ng/ml). However, only in three IL-12/TNFα combinations (for TNFα concentrations 0.1, 10 and 100 ng/ml) was the difference statistically significant ($P<0.001$, $P<0.001$, and $P<0.05$ respectively, as compared with cultures with no IL-12). In contrast, strong cytostatic/cytotoxic effects against melanoma cells were observed when TNFα was combined with IFNγ (Fig. 3B). In cultures with the highest concentrations of TNFα and IFNγ almost no viable tumor cells were detected. Therefore, it seems highly probable that the tumor-growth-inhibitory effect observed when IL-12 was injected intratumorally together with TNFα was due to the IL-12-induced stimulation of IFNγ production rather than any direct effect of IL-12 itself.

Augmented antitumor activity of macrophages from mice treated with IL-12 and TNFα

As shown in Table 1, treatment with IL-12 induced a high level of IFNγ in serum. Since, in many experimental settings in vitro, IFNγ has been demonstrated to synergize with TNFα in stimulation of macrophages for tumoricidal activity [5, 14, 16], we decided to examine whether the combined treatment of mice with IL-12 and TNFα could produce a similar effect in vivo. To study this problem, mice were treated i.p. with IL-12 and/or TNFα for 3 days, and the antitumor effects of ex vivo peritoneal macrophages was tested against B16F10 cells in a 3-day MTT assay. The data presented in Table 2 show that, indeed, the mechanisms considered above could contribute to the antitumor effects of IL-12/TNFα-based therapy and could explain the higher antitumor efficacy of double-cytokine treatment in comparison with single-agent therapy. Ex vivo macro-

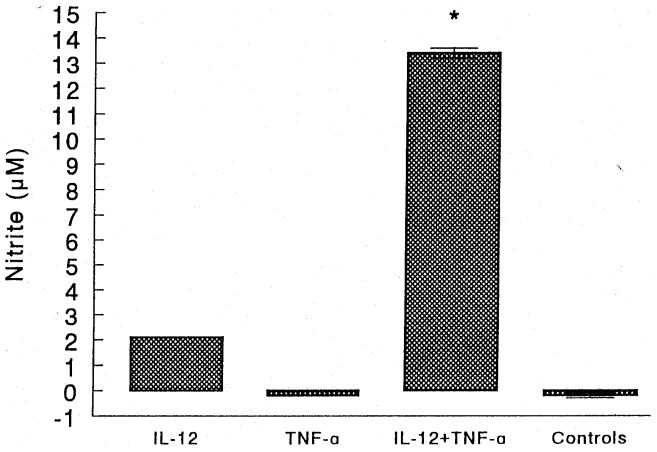

Fig. 4 Nitric oxide (NO) production by macrophages from mice treated with IL-12 and/or TNFα. Peritoneal macrophages from mice treated with 0.1 μg IL-12 and/or 5 μg TNFα for 3 days were cultured for 18 h and NO production was then assessed by nitrite accumulation in culture supernatants as described in Materials and methods. Data are expressed as means ± SE. *$P<0.001$ as compared with other groups

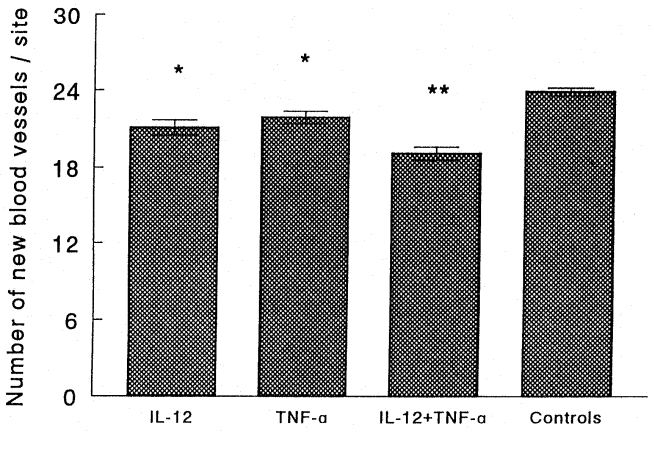

Fig. 5 Effects of IL-12 and TNFα on tumor-cell-induced angiogenesis. X-irradiated BALB/c mice were injected intradermally with 3×10^5 B16F10 melanoma cells and treated inraperitoneally for 3 consecutive days with 0.1 μg IL-12 and/or 5 μg TNFα per day, starting on the day of tumor cell inoculation. Angiogenic response was evaluated blindly 1 day following the last cytokine injection. Data are expressed as mean number ± SE of newly formed blood vessels at a single injection site. Each group consisted of four mice injected with B16F10 cells into three or four sites. *$P<0.001$ as compared with controls, **$P<0.01$ as compared with TNFα-treated mice and $P<0.001$ as compared with controls and mice treated with IL-12

phages from mice treated with both cytokines together were able to inhibit proliferation of B16F10 melanoma cells in cultures more effectively than those from either IL-12- or TNFα-treated mice ($P<0.001$, at the E:T ratio of 20:1). This effect, at least in part, was mediated by nitric oxide (Fig. 4).

Antiangiogenic effects of IL-12 and TNFα

In studies by Voest et al. [34], IL-12 has been proven to inhibit fibroblast-growth-factor-induced corneal neovascularization. To determine whether IL-12, alone or in combination with TNFα, could induce a similar effect in the tumor, we used the tumor-cell-induced angiogenesis model, which was effective in previous studies on retinoids and IFNα [18]. As shown in Fig. 5, short-term (3 days) intraperitoneal administration of either 0.1 μg IL-12 or 5 μg TNFα resulted in significant inhibition of blood vessel formation at the site of B16F10 cell injection (a mean of 21.1 and 21.9 blood vessels in IL-12- and TNFα-treated mice respectively, in comparison with 23.9 blood vessels in diluent-injected controls, $P<0.001$). The combined treatment with IL-12 and TNFα was more effective in inhibiting the formation of new blood vessels than was treatment with either cytokine alone (a mean of 19.1 blood vessels, $P<0.001$ as compared with TNFα-treated and $P<0.01$ as compared with IL-12-treated mice) - altogether a 20% decrease in the number of newly formed vessels was observed in comparison with controls ($P<0.001$).

Discussion

In the present study, the combination immunotherapy with IL-12 and TNFα resulted in potentiation of antitumor effects in three different tumor models in mice, both sensitive and non-sensitive to antitumor activity of TNFα (Figs. 1, 2). TNFα did not by itself show any antitumor activity against B16F10 melanoma at a daily dose of 5 μg but it significantly strengthened the antitumor effects of 1 μg IL-12 when used in a 7-day treatment schedule (Fig. 1A). In Lewis lung carcinoma and L1 sarcoma models, minor antitumor activity displayed by TNFα was potentiated when the same dose of TNFα (5 μg) was applied with a lower dose of IL-12 (0.1 μg) (Figs. 1B, 2A), and also, in the case of L1 sarcoma, when 1 μg TNFα was combined with 0.1 μg IL-12 (Fig. 2B). Retardation of the tumor growth was not associated with significant prolongation of mouse survival in our treatment schelule (one cycle of daily injections). However, it is highly probable that changing the therapeutic protocol (e.g. adminstration of cytokines in several cycles) would improve the overall results and could bring about statistically significant differences in mouse survival between groups.

Augmentation of the antitumor effect by IL-12 and TNFα has not been reported to date. The only study dealing with combined use of IL-12 and TNFα examined the in vitro effect of these and other cytokines on splenocytes from severe combined immunodeficiency (SCID) mice: an increase in the IFNγ level was found in splenocyte cultures incubated with IL-12 and TNFα [32]. Since IFNγ seems to play an important role in the development of the antitumor effects of IL-12 [21] and since IFNγ has been demonstrated to synergize in the antitumor effect with TNFα [2, 30], it could be assumed that the beneficial antitumor effect of the IL-12 + TNFα-based therapy could result, at least in part, from the joint action and cooperation of TNFα and IFNγ. In

an attempt to shed more light on the participation of endogenously produced IFNγ (and also TNFα) in antitumor effects observed in our study, we measured serum levels of these cytokines in mice treated with IL-12 and/or TNFα. Treatment with IL-12 induced high serum levels of IFNγ (Table 1), confirming observations of other authors who demonstrated stimulation of IFNγ production in vivo by IL-12 [21, 11]. Unexpectedly, in mice treated with IL-12 in combination with TNFα, IFNγ levels were significantly lower, suggesting that antitumor effects observed in mice treated with both cytokines could be mediated, at least in part, by IFNγ-independent mechanisms. The possibility cannot be excluded that lower IFNγ levels in mice treated with IL-12 and TNFα, in comparison with those in mice injected with IL-12 alone, could result from exhaustion of NK cells [11] (and maybe other cells) responsible for IFNγ production. Rapid consumption of IFNγ in the course of IL-12 and TNFα therapy can also be considered, as up-regulation of IFNγ receptors has been observed on monocytic cells following treatment with TNFα [23].

Apart from stimulating the IFNγ production, IL-12, alone or in combination with TNFα, induced increased production of endogenous TNFα in our system (Table 1). Stimulation of TNFα synthesis by IL-12 has also been observed by other investigators [21]. Taking into account high-level production of IFNγ induced by IL-12 treatment (Table 1) it could be supposed that some of the augmented antitumor activity observed in tumor-bearing mice treated with IL-12 in combination with TNFα could result from well-established synergistic antitumor effects of IFNγ and TNFα [2, 16, 30]. This synergy in vivo was also demonstrated in our previous study and could result from both direct and indirect antitumor activity of TNFα and IFNγ. In the present study we confirmed potentiation of cytostatic/cytotoxic activity of TNFα and IFNγ on B16F10 melanoma cells in vitro (Fig. 3B). According to earlier reports, synergistic cytostatic/cytotoxic effects of IFNγ and TNFα against various tumor cells was probably due to reciprocal regulation of receptor expression exerted by these cytokines [23, 33]. Additive rather than synergistic interactions between TNFα and IFNγ are probably responsible for melanoma cell killing in our experiments, as the net cytostatic/cytotoxic effect against B16F10 cells in cultures with both cytokines is the sum of effects of each agent used alone (Fig. 3B).

As far as indirect antitumor effects of IFNγ and TNFα are concerned, these cytokines could synergize in inducing tumoricidal activity of macrophages in vitro [5, 14], which was also demonstrated in our previous report on a MmB16 melanoma in mice [16]. In the present study, we examined antitumor activity of ex vivo macrophages isolated from mice treated with IL-12 and/or with TNFα. As shown in Table 2, macrophages from mice treated with IL-12 + TNFα exerted significantly greater antitumor activity against melanoma B16F10 cells and produced more NO (Fig. 4) as compared with macrophages isolated from any other group. Since infiltration by macrophages is a common feature of melanoma [13], and since tumors regressing after local IL-12 delivery contained an increased macrophage infiltrate [36], macrophage tumoricidal activity could participate in potentiating antitumor effects observed in our in vivo study in mice treated with IL-12 and TNFα.

It has long been known that vascular endothelial cells in tumor blood vessels constitute one of the targets of TNFα activity. TNF exhibits cytostatic and cytotoxic effects on endothelial cells [24], inhibits their migration [20], and induces procoagulant activity in these cells, adding thrombus formation in tumor blood vessels and hemorrhagic necrosis of the tumor to the mechanisms responsible for antitumor effects of this cytokine [26]. IL-12 has only recently been shown to exhibit angiostatic activity [25]. IFNγ-induced C-X-C chemokine, known as interferon-inducible protein 10 (IP-10), has been implicated in the angiostatic effects of IL-12 and IFNγ. In our experiments, both IL-12 and TNFα exerted mild but significant angiostatic activity and this activity was significantly increased in mice subjected to combined IL-12 + TNFα treatment (Fig. 5). Therefore, the possibility cannot be excluded that the antiangiogenic effect could play a role in a strengthened antitumor activity of our IL-12 + TNFα experimental protocol and could especially be expressed in the long-term treatment schedule (Fig. 2B).

Since induction of IFNγ production is important [21] but not sufficient [3] to explain all antitumor effects of IL-12, other mechanisms, besides those discussed above, could participate in potentiating the antitumor activity exerted by IL-12/TNFα immunotherapy. IL-12 exhibits pleiotropic immunological activities, which include promotion of the development of Th1 clones and enhancement of NK- and T-cell-mediated cytotoxicity [10, 15, 19]. Similarly, TNFα mediates a broad range of immunoregulatory effects and, apart from influencing macrophage function [5], facilitates the generation of cytotoxic cells and enhances the expression of MHC antigens (for review see [27]). As far as NK cells are concerned, they seem to contribute significantly to the antitumor effect of IL-12/TNFα-based therapy only at the beginning of the treatment. Similarily to Gately et al. [11], we observed a sharp increase of NK activity (against YAC-1 cells) in cultures of splenocytes from mice treated with a single dose of IL-12, alone or in combination with TNFα. Prolonged daily administration of IL-12 caused a decline in NK activity (data not presented). A specific T cell response may also play a role in the antitumor effects in IL-12/TNFα-treated mice since the induction of antitumor immunity by mouse tumor cells transfected with the IL-12 gene has recently been reported [22] and $CD4^+$ and $CD8^+$ T lymphocytes seemed to be necessary for optimal antitumor activity following in vivo treatment with IL-12 [21]. However, further studies should be performed to test this hypothesis.

One of the strategies to broaden the therapeutic margin of cytokines in cancer treatment is to use them together or in combination with chemotherapeutics. For example, TNFα with IFNγ (and melphalan) have been successfully applied in the treatment of locally growing tumors in an isolated-limb perfusion model [8]. Quite recently, it has been reported that, in IL-12-based immunotherapy, the amount of this cytokine at the tumor site was critical for

the antitumor effects [6]. These observations make IL-12/TNFα combination immunotherapy a potential therapeutic approach that could be useful in a locoregional treatment of neoplasia, which is supported by the results of our present studies in a murine model.

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