Effect of Tumor Necrosis Factor on the Antitumor Efficacy and Toxicity of Aminopterin-Monoclonal Antibody Conjugates: Parameters for Optimization of Therapy

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ABSTRACT

Immunoconjugates of monoclonal antibodies with drugs, isotopes, or toxins are currently being investigated for their therapeutic effect on tumors. However, all have problems of access of the immunoconjugate to the tumor, particularly with solid tumors. To address this problem, we have used aminopterin-monoclonal antibody (AMN-mAb) conjugates combined with murine tumor necrosis factor (mTNF-α), which is known to have specific effects on tumor vascularization. In a murine model, well-established tumors (measuring 1.0–1.4 cm in diameter) were either totally eradicated or considerably reduced in size with combined therapy—a greater effect than with either mTNF-α or AMN-mAb used alone. The mechanisms involved in the improved antitumor effect were investigated using in vitro assays, autoradiography, and biodistribution experiments. mTNF-α was found both to increase the cytotoxic activity of the conjugate in vitro and to increase in vivo tumor localization of mAb up to 5-fold. The timing of mTNF-α administration was crucial to effects on tumor localization; mTNF-α given with mAb caused the greatest increase in localization and mTNF-α given well before mAb decreased localization. mTNF-α also reduced the toxicity to mice of AMN-mAb depending on the timing of injection. These results indicate that mTNF-α has a useful role in potentiating immunoconjugate therapy but shows the need for careful planning of the dose regimen.

INTRODUCTION

Immunoconjugates of isotope, drug, and toxin are being investigated for their beneficial effects in the treatment of human cancer, and preclinical studies with these substances have shown promise—so much so that a number of clinical trials are now in progress (1–3). However, what is clear from the preclinical studies is that tumors in favorable sites can be totally eradicated by immunoconjugates, but in other less accessible sites tumors may shrink but may not be eradicated. Thus, tumors growing in ascites fluid (and therefore readily accessible to immunoconjugates) can be eradicated (4), and when toxic whole ricin-antibody conjugates are injected directly into solid s.c. tumors, the tumors can also be totally eradicated (5). However, these 2 models, while giving impressive results leading to the cure of cancers, are unrealistic because human tumors rarely present in such accessible forms. The most realistic experimental model involves i.v. treatment of solid tumors growing s.c. or in other sites in animals. Unfortunately, under these circumstances the results with immunoconjugates are far less impressive. There are a number of reasons for this but foremost is the access of the immunoconjugate to the tumor. Antibodies do not actively seek out tumor cells, rather, in the process of circulation and diffusion some of the immunoconjugate will bind to the tumor. With this in mind, we have examined a number of modes of treating tumors where the accessibility of antibodies will be increased by injection of a potent immunoconjugate of AMN-mAb2 and demonstrate that mTNF-α has beneficial effects. A problem with some immunoconjugates is their toxic side effects and in this study we investigate the effects of mTNF-α on the toxicity of AMN-mAb. Careful timing of doses was performed in conjunction with biodistribution studies, and it was noted that the benefits of mTNF-α are dependent on the timing of administration.

MATERIALS AND METHODS

Tumor Growth. The Ly-2.1+ E3 clonal variant of the murine thymoma ITT(1)75NS (7) was maintained in vitro in Dulbecco’s modified Eagle’s medium, supplemented with 10% heat-inactivated newborn calf serum (Flow Laboratories, Sydney, Australia), 2 mM glutamine (Commonwealth Serum Laboratories, Melbourne, Australia), 100 IU penicillin/ml (Commonwealth Serum Laboratories), and 100 µg/ml streptomycin (GibcoLabs, Melbourne, Australia). For in vivo experiments, E3 was maintained by serial passage in the ascites form in C57BL/6 × BALB/c F1 (hereafter called B6CF1) mice produced in the Department of Pathology, University of Melbourne; cells from the ascites fluid were washed and centrifuged (400 × g, 5 min) twice in Dulbecco’s modified Eagle’s medium and PBS (pH 7.3), resuspended in PBS, and 10 × 10⁶ cells injected s.c. into the abdominal wall of mice. Mice were subjected to a series of i.v. or i.p. treatments, and the size of the tumors was monitored with a caliper square measuring along 2 perpendicular axes of the tumors; the data were recorded as the mean tumor size (product of 2 diameters) ± SE. Experimental groups of 8–10 mice of the same sex and age were used.

mAbs. The mAb used in this study was anti-Ly-2.1, a murine IgG2a mAb reactive with the murine Ly-2.1 specificity (8). This mAb was isolated from ascites fluid by adsorption onto Protein-A-Sepharose (Pharmacia Inc., Uppsala, Sweden), washing with PBS and elution with citrate buffer (pH 4.5). mAbs were concentrated by precipitation with 50% ammonium sulfate, resuspended and dialyzed in PBS, and stored at −70°C. mAbs were labeled with 121I using Enzymobead radioiodination reagent (Bio-Rad, Richmond, CA).

Preparation of AMN-mAb. An active ester of AMN was prepared using a method described previously (9). Briefly, AMN (Sigma, St. Louis, MO) was dissolved in DMSO at 4.39 mg/100 µl, and N-hydroxysuccinimide (2.3 mg/50 µl DMSO) and N,N-dicyclohexylcarbodiimide (2.06 mg/50 µl DMSO) were added. The mixture was incubated at room temperature for 4 h and then stored at 4°C, protected from light. The active ester (15–30 fold excess) was added to mAb (3–5 mg/ml) and the mixture incubated for 1 h at room temperature. The reaction mixture was centrifuged to remove precipitated protein, and unbound drug was removed by gel filtration (PD-10 column; Pharmacia Inc.). The protein concentration was determined by the Bradford dye-binding assay (10) and the amount of AMN bound to the mAb was estimated by absorbance spectrophotometry at 370 nm (ε = 8.13 × 10³ M⁻¹ cm⁻¹). AMN-mAb conjugates used in tumor growth experiments contained 4–6 molecules of AMN per molecule of mAb.

In Vitro Activity. Cytotoxicity of mTNF-α (recombinant mTNF-α)

1 The abbreviations used are: AMN, aminopterin; DMSO, dimethyl sulfoxide; mAb, monoclonal antibody; mTNF-α, murine tumor necrosis factor; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; Eo, molar absorptivity constant.

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RESULTS

In Vitro Effects of mTNF-α in Combination with AMN-mAb Conjugate. The in vitro effects of mTNF-α in the presence of AMN-mAb on the growth of E3 cells were assessed by combining mTNF-α with AMN-mAb, incubating with E3 cells for 24 h, and measuring the cytotoxicity of various combinations (Fig. 1). The inhibitory effect obtained by either agent used alone was enhanced when mTNF-α and AMN-mAb were used together. First, conjugate containing AMN in doses greater than $6 \times 10^{-7}$ M completely inhibited cell proliferation as did concentrations of mTNF-α greater than 1000 units/ml. However, when using $10^{-7}$ M AMN, which caused a 30% inhibition of deoxyuridine uptake alone, significant additional inhibitory effects on the tumor cells were noted with the addition of increasing amounts of mTNF-α. When 10 units/ml of mTNF-α (which causes 2% inhibition alone) was combined with this dose of mTNF-α, the inhibition of [3H]deoxyuridine uptake was increased to 50%; increasing doses of mTNF-α caused further increases in inhibition. Thus, mTNF-α and AMN-mAb displayed additive cytotoxic effects.

Effect of mTNF-α on the Biodistribution of Radiolabeled mAbs. To optimize the therapeutic effects of mTNF-α with immunonjugates, localization studies were performed to determine the most appropriate times for dosage. Radiolabeled mAb was injected into mice that had received mTNF-α at various times and doses, and the distribution of the mAb was ascertained (Fig. 2). The simultaneous administration of mTNF-α and labeled mAb (group A) resulted in a 4.7-fold increase in the tumor:blood ratio of mAb and 4.8-fold more radioactivity in the tumor than when mAb had been injected alone. This increase was similar whether 1 or 2 $\mu$g of mTNF-α was injected (groups A and B). When mTNF-α was injected 4 h before mAb (group C), a lesser increase in the tumor:blood ratio (1.5-fold) was obtained. Furthermore, the tumor:blood ratio was reduced by half when mTNF-α was injected both 1 and 2 days before mAb (group D), and this reduction was only partially abrogated by an additional simultaneous injection of mTNF-α with the mAb (group E). Thus, the greatest increase in tumor localization of radiolabeled mAb occurred when mTNF-α was injected simultaneously with the mAb; previous injections of mTNF-α significantly reduced tumor localization of mAb.

The increase in tumor localization when mTNF-α was injected simultaneously with mAb corresponded with a significant decrease in the radioactivity in all normal organs measured except stomach, intestine, and muscle (Fig. 3); this decrease was not seen when mTNF-α was injected 24 and 48 h prior to mAb.

Thus, mTNF-α increased or decreased the localization of radiolabeled mAb to the tumor depending on the timing of administration. This suggests that, if the effects of mTNF-α on [125I]mAb correspond to similar effects on the localization of AMN-mAb, the dose schedule is a critical factor in optimizing therapeutic results. In addition, an increase in the amount of mAb taken up by the tumor coincided with a reduction in localization of mAb to normal organs, and this could cause a

![Fig. 1. Cytotoxicity of mTNF-α and AMN-mAb on E3 cells. [3H]Deoxyuridine uptake of E3 cells after incubation with mTNF-α and AMN-mAb at a concentration of 0 M (●), 1 $\times$ 10^{-7} M (●), 6 $\times$ 10^{-7} M (●), and 2.5 $\times$ 10^{-6} M (●). Uptake is expressed as the percentage of [3H]deoxyuridine uptake by untreated E3 cells.](Image)

![Fig. 2. Localization of mAb in mice after mTNF-α treatment. Tumor uptake of [125I]mAb in mice treated with (A) 1 $\mu$g of mTNF-α simultaneously with mAb, (B) 2 $\mu$g of mTNF-α simultaneously with mAb, (C) 1 $\mu$g of mTNF-α 4 h before mAb, (D) 1 $\mu$g of mTNF-α 24 and 48 h before mAb, and (E) 1 $\mu$g of mTNF-α 24 and 48 h before and simultaneously with mAb. Tumor:blood ratio (●) and percentage of injected dose in the tumor (●) were measured 24 h after [125I]mAb administration and expressed as a percentage of the uptake in saline-treated control mice (——).](Image)
reduction in the toxicity of AMN-mAb conjugates when used in conjunction with mTNF-α.

** Autoradiography of Tumors Treated with Radiolabeled mAb and mTNF-α.** The intensity and distribution of 125I-labeled mAb within the tumor was investigated by autoradiography of tumors. Tumor-bearing mice were given injections of radiolabeled mAb and mTNF-α in the same dose schedules as in the localization experiments, and tumors were excised 24 h later and autoradiographed. Control mice receiving radiolabeled mAb showed a diffuse accumulation of silver granules throughout the tumor (Fig. 4A). Mice treated with mTNF-α simultaneously with mAb showed an obvious increase in the number of granules in the tumor (Fig. 4B), and mice given mTNF-α 4 h before mAb showed a lesser increase (Fig. 4C). This increase was not distributed evenly throughout the tumor but was found in patches, possibly associated with vessels. Mice given previous daily injections of mTNF-α showed a lower density of granules in the tumor than did control mice (Fig. 4D). Although a more subjective analysis, these results support the data provided by the biodistribution experiments described above and suggest that mTNF-α affects the localization of mAb to the tumor in different ways, depending on the timing of administration.

**Effect of mTNF-α on the Therapeutic Activity of AMN-mAb Conjugates in Tumor-Bearing Mice.** Three separate studies were performed to assess the effect of mTNF-α on immunoconjugate efficacy in vivo. In the first experiment, mice with relatively large tumors (1.2–1.4 cm²) were used to investigate the effect of combining mTNF-α and AMN-mAb treatment in 2 different doses with 2 different schedules of administration. Mice received mTNF-α i.v. in doses of 0.75 µg on days 8, 10, 12, and 14 after tumor injection and a total of 27 or 54 µg of AMN was given i.v. as immunoconjugate over the same days. A total dose of 54 µg of AMN was chosen, because this is approximately the 20% lethal dose for AMN-mAb, and so the effects of mTNF on the toxicity of the conjugate could also be investigated. Mice were treated with: (a) PBS; (b) mTNF-α; (c) AMN-mAb (27 µg); (d) AMN-mAb (54 µg); (e) mTNF-α simultaneously with AMN-mAb (27 µg); (f) mTNF-α simultaneously with AMN-mAb (54 µg); and (g) mTNF-α 4 h before AMN-mAb (27 µg). The first point to note is that these relatively large and established tumors (1.2–1.4 cm²) were resistant to therapy either with AMN-mAb alone (even at toxic levels) or with mTNF-α alone (Fig. 5). However, when mTNF-α and AMN-mAb were used together, a significant growth inhibition was achieved; by day 18 the mean tumor size of mice treated with mTNF-α simultaneously with 27 µg of AMN-mAb (group E) was 60% of the mean tumor size of the saline-treated control mice (group A). Combined mTNF-α and immunoconjugate treatment (groups E, F, and G) resulted in similar inhibition with the 2 doses of AMN-mAb and the 2 treatment schedules. It is also important to note that mTNF-α did not substantially alter the toxicity of the immunoconjugates in mice receiving 54 µg of AMN-mAb, because 2 of 9 mice (22%) treated with 54 µg of AMN-mAb died of toxicity and 1 of 9 mice (11%) treated with 54 µg of AMN-mAb and mTNF-α died of treatment-related toxicity. mTNF-α alone had no toxic effects.

In the second study, mice with slightly smaller tumors (1.0–1.2 cm) were treated i.v. with AMN-mAb and mTNF-α at 1 µg doses. Groups of mice received: (a) PBS; (b) mTNF-α; (c) AMN-mAb; (d) mTNF-α 4 h before AMN-mAb; and (e) mTNF-α simultaneously with AMN-mAb. Mice received 1 µg of mTNF-α on each of days 7, 9, 11, and 13 and a total of 50 µg of AMN as conjugate over the same days. Some antitumor effect was obtained with either AMN-mAb or mTNF-α when each was used alone, but the results were significantly improved when the agents were used together (Fig. 6). On day 18, for mice treated with either mTNF-α or AMN-mAb (groups B and C), mean tumor sizes were 87% and 62% of control tumor size, respectively. However, when treated with both AMN-mAb and mTNF-α, tumors were either 11% (AMN-mAb and mTNF-α at the same time, group E) or 15% (AMN-mAb given 4 h after mTNF-α, group D) of the size of control tumors. Some tumors disappeared altogether and by day 20, 2 of the 9 mice (22%) treated with mTNF-α 4 h before AMN-mAb (group D), and 6 of the 10 mice (60%) treated with mTNF-α simultaneously with AMN-mAb (group E) had no detectable tumor. Mice with tumors that had regressed were observed and tumors reappeared either at the primary site or elsewhere. Evidence of tumor growth was found in the lymph nodes, kidneys, liver, and lungs (data not shown). Again, there was no significant difference in toxicity between mice treated with AMN-mAb alone or with mTNF-α because 22% and 20%, respectively, of mice died of toxicity. mTNF-α had no toxic effects.

To maximize the effects of mTNF-α on immunoconjugate therapy, the authors suggest combining mTNF-α with AMN-mAb at optimal doses and schedules to achieve optimal efficacy while minimizing toxicity.
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Fig. 4. Distribution of mAb in tumors. Silver granules indicate accumulation of 125I-mAb in tumors of mice treated with (A) 125I-mAb, (B) mTNF-α simultaneously with 125I-mAb, (C) mTNF-α 4 h before 125I-mAb, or (D) mTNF-α 24 and 48 h before 125I-mAb.

Localization, a third experiment was performed in which mice were given injections of AMN-mAb and mTNF-α 4 days apart (Fig. 7). Mice received: (a) PBS; (b) AMN-mAb; (c) mTNF-α; and (d) AMN-mAb and mTNF-α, and treatment commenced when tumors were 0.4 cm². mTNF-α was given i.v. in doses of 0.5 and 0.75 μg on days 4 and 8 and a total of 90 μg of AMN conjugated to mAb was injected i.p. on the same days. mTNF-α was injected immediately prior to AMN-mAb. By day 14, saline-treated control mice (group A), had a mean tumor size of 4.7 cm². mTNF-α administration alone had little effect on tumor growth because on day 14, mTNF-α-treated mice (group C) had a mean tumor size of 3.5 cm² (76% of control size). AMN-mAb alone (group B) caused a significant tumor inhibition (1.27 cm² or 27.3% of control size by day 14), and AMN-mAb and mTNF-α administration (group D) inhibited tumor growth to a similar extent (1.0 cm² or 21.5% of control size on day 14). Two of the 8 mice (25%) treated with AMN-mAb died of toxicity and all showed symptoms of toxicity (piloerection and inactivity). However, mice receiving AMN-mAb in conjunction with TNF showed no signs of toxicity. Although the protocol used in this experiment is not optimal in terms of
antitumor effect, it demonstrates that mTNF-α can reduce the toxicity of AMN-mAb.

DISCUSSION

One of the major limitations in immunoconjugate therapy is access of relatively large molecules into poorly vascularized tumors (11, 12); improving the blood flow to tumors could be a means of increasing immunoconjugate access and therefore efficacy. TNF-α is known to have selective effects on tumor vasculature and is a suitable candidate for combination therapy with immunoconjugates; a number of studies have been reported in which TNF-α is used to advantage in combination with other drugs (13, 14). TNF-α has been shown to have specific cytotoxic effects on some tumor cells and to cause a diverse range of other effects \( \textit{in vitro} \) and \( \textit{in vivo} \) (15, 16). A more complete knowledge of the effects of TNF, especially with regard to the localization and therapeutic activity of drug-mAb conjugates, is necessary if it is to be used effectively with immunoconjugates in anticancer therapy. Preliminary investigations have shown that human TNF-α can be used to increase both the tumor localization and the antitumor effect of \( N \)-acetylmalphalan-mAb conjugates (6). This report describes the use of AMN-mAb in investigating some aspects of the effects of mTNF-α on immunoconjugate activity in a murine tumor model and in evaluating the potential role of TNF in clinical immunochemotherapy.

The studies reported in this article show that mTNF-α in combination with AMN-mAb has an additive effect \( \textit{in vitro} \) in which the cytotoxicity of AMN-mAb is increased with the addition of increasing concentrations of mTNF-α. In previous work with TNF-α and immunoconjugates (6), TNF-α was injected 4 h before the conjugate or on different days. In the current study, investigations to optimize the dose schedule and improve therapeutic results were performed. Reports on the effect of TNF-α on tumor vasculature (17, 18) suggest that increased vessel wall permeability occurs almost immediately after administration of TNF-α, and that break- down of tumor blood vessels and blood stasis commences within 4 h of administration. These effects on tumor vasculature alter the localization of mAb to the tumor, although other effects (possibly such as an increase in antigen expression) may also influence the distribution of mAb. Thus TNF-α is likely to be of benefit to immunoconjugates soon after administration but not if given well before immunoconjugate administration, because vessels could become occluded. Experiments investigating the localization of radiolabeled mAb after administration of mTNF-α at various times showed greatest tumor localization of mAb when mTNF-α was injected simultaneously with mAb. The experiments also showed a decrease in the tumor:blood ratio of radiolabeled mAb when mTNF-α was injected 24 and 48 h before administration of the mAb. These results indicate that the dose schedule is an important factor in producing optimum results with combination therapy and that perhaps multiple, frequent dose regimes with mTNF-α may be less effective than single dose regimes because of the destructive effects of mTNF-α on tumor blood vessels.

The increase in tumor localization of radiolabeled mAb injected simultaneously with mTNF-α coincided with a significant decrease in the amount of mAb detected in most normal organs. Only the stomach had increased localization with mTNF-α, and this may be a measure of free \(^{125}\)I rather than radiolabeled mAb (19). Such decreases in the amount of immunoconjugate in normal organs are important because this may reduce the toxicity and allow higher doses to be given. This is supported by the toxicity observed in the third therapy experiment; mice receiving AMN-mAb at a 25% lethal dose showed no signs of toxicity when mTNF-α was administered with the conjugate. In the first and second therapy experiments, previous injections of mTNF-α may have prevented the decrease in localization to normal organs, and this may explain why there was no significant reduction of toxicity with mTNF-α. Because the beneficial effects of mTNF-α on the localization of mAb (both in the tumor and in normal organs) were reduced with previous injections of mTNF-α, optimization of combined drug therapy may require a drug-mAb conjugate that can be administered less frequently without reduction in antitumor effects.

The first experiment investigating the therapeutic effect of mTNF-α with immunoconjugates was performed on mice with larger tumors, of a size not previously reported to be responsive to systemic immunochemotherapy. Mice were treated with AMN-mAb at a 20% lethal dose to investigate toxicity as well as antitumor effect; neither mTNF-α nor AMN-mAb had any effect on tumor growth when used alone, but when both agents were used a substantial reduction in tumor growth occurred. The improvement in tumor uptake when mTNF-α was given simultaneously with AMN-mAb (indicated by the biodistribution experiments) did not result in significantly better antitumor effects than when mTNF-α was given 4 h before AMN-mAb. A possible reason for the lack of a significant difference in therapeutic effect between the 2 dose schedules for combined therapy is that multiple doses of mTNF-α were given. This is supported by the biodistribution data, which showed that mTNF-α given 1 and 2 days before mAb reduced tumor access. Another reason may be that the tumor was saturated with mAb and this is supported by the fact that 54 \( \mu \)g of AMN gave no better therapeutic effect than 27 \( \mu \)g (approximately 2 mg mAb). This suggests that optimal results may require the use of a potent immunoconjugate that can be used in smaller, less frequent doses.

In the second therapy experiment, there was a slight antitu- mor effect with mTNF-α or AMN-mAb used alone, which was again substantially improved when the agents were used together. All mice treated with both mTNF-α and AMN-mAb showed partial or total tumor regression and the mean survival time was significantly increased. Regrowth of tumors occurred in every case, sometimes more than 20 days after the original tumor had disappeared. In 7 of the 9 mice with complete tumor regression, secondary tumors appeared in the liver, kidneys, lymph nodes, and lungs as well as or instead of in the primary
tumor site. Metastases have not been observed in this tumor model before (even when mice were kept alive for an equivalent time after tumor injection by intratumoral ricin injections), suggesting that the metastasis was influenced by treatment. Although this is a radical suggestion, the effects of TNF are so broad that such effects cannot be discounted. TNF may cause vascular or immunological changes that promote passage of the tumor cells away from the primary tumor site, or TNF could help select for a metastatic variant of E3. If TNF has caused metastasis, this has important implications in the clinical use of TNF (currently at Phase I–II stage of testing) that should be investigated.

It was clear in these studies that combined AMN-mAb and mTNF-α treatments caused significantly greater inhibition of tumor growth than treatment with AMN-mAb or mTNF-α alone. This improvement could be due to a number of factors; increased localization of immunoconjugate to the tumor caused by effects of mTNF-α on tumor vasculature or antigen expression; additive cytotoxic effects of mTNF-α and AMN-mAb; or other, less direct effects of mTNF-α such as those on the host immune system. The biodistribution studies clearly implicate improved localization, but this does not exclude other factors.

This study has demonstrated that dramatic improvements in immunoconjugate therapy can be obtained with mTNF-α, because large tumors that are not responsive even to toxic levels of AMN-mAb or mTNF-α can be significantly inhibited, and has pointed to a number of factors that should be considered when planning immunochemotherapy regimes using mTNF-α. It seems probable that the beneficial effects seen in the above therapy experiments can be further improved using the knowledge about mTNF-α obtained to date; regimes should involve single or perhaps more widely spaced dose schedules, more toxic molecules attached to less mAb may increase the advantage obtained with mTNF-α, toxic levels may be controlled better with an awareness of the factors involved in the distribution of the drug, and the use of immunoconjugates of drugs known to synergize with mTNF-α may improve antitumor effects. This study provides encouraging evidence for the potential success of combining TNF and immunoconjugates in the clinical treatment of cancer and indicates the value of further detailed investigations in this area.

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