Increased Antitumor Effect of Immunoconjugates and Tumor Necrosis Factor in Vivo

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ABSTRACT

The potential of specifically targeting antineoplastic drugs and toxins to tumors with the use of monoclonal antibodies (MoAbs) reactive with tumor-associated antigens is currently being examined. N-Acetyl-melphalan-MoAb (N-AcMEL-MoAb) conjugates have previously been shown to have greater antitumor activity than N-AcMEL, melphalan, or MoAb alone against both subcutaneous and ascites murine thymomas in mice. Although this conjugate is also a highly selective tumor inhibitor in vitro, it may not reach all the tumor cells in a high concentration, and consequently larger tumors (≥0.4 cm²) cannot be eradicated. This conjugate is representative of many drug-MoAb conjugates in that they are unable to gain adequate access to the tumor site to exert their cytotoxic effect. To potentiate the antitumor effect of the N-AcMEL-MoAb conjugate, studies were undertaken to analyze its action in combination with recombinant human tumor necrosis factor α (rTNF-α), a monokine, capable of causing acute necrosis of syngeneic tumor transplants in mice. Treatment of mice with murine thymomas (0.4 to 0.6 cm² in size) demonstrated that 30% of the tumors in mice receiving conjugate and rTNF-α partially or completely regressed, while no regressions were observed in the tumors of mice receiving N-AcMEL-anti-Ly-2.1 conjugate or rTNF-α alone. This and other experiments indicated that the antitumor effect and tumor localization of N-AcMEL-MoAb conjugates can be enhanced in vivo by rTNF-α, thereby enabling successful eradication of larger established subcutaneous murine tumors.

INTRODUCTION

There are many problems associated with the use of immunoconjugates, such as the complexity of coupling relatively hydrophobic and bifunctional drugs to antibody (1), the potency (2) and nonspecific toxicity (3) of drug/toxin-MoAb conjugates, and the heterogeneity of tumor cells (4, 5), which have all been addressed. Successful treatment of subcutaneous tumors by intratumor immunotoxin therapy (6) suggests that the relative inaccessibility of solid tumor to drug/toxin-MoAb conjugates is a major limitation of the more widely applicable i.v. route of administration. One method of partially overcoming this problem is to use vasoactive agents. By their selective action on normal blood vessels, vasoactive drugs can alter the tumor/normal tissue perfusion ratio, thereby enhancing the access of drug-MoAb conjugates to tumors and increasing the effectiveness of tumor therapy (7). However, it is clear from these studies that other methods have to be found to get more antibody out of the circulation and into the tumor. It is apparent that antibodies produced in response to bacterial infection require a local inflammatory response (with vasodilation and increased permeability of vessels) to permit the antibodies to reach their target, and on the basis of these observations, we have used TNF-α, a monokine capable of inducing hemorrhagic necrosis and a subsequent inflammatory response in tumors, to increase the potential antitumor effect and tumor access of immunoconjugates.

In partially purified form, TNF-α is cytostatic or cytotoxic for a variety of tumor cells in vitro (8) while having a number of noncytotoxic effects on normal cells (9). Recently, rTNF-α protein (10) has confirmed the selective cytotoxic effects in vitro on tumor cells previously observed with the natural TNF-α. TNF-α binds to specific receptors on the membrane of target cells (11) and is subsequently internalized and degraded (12), although the presence or absence of TNF receptors, however, is not correlated with sensitivity or resistance to the cytolytic effects of TNF-α (13). The overall spectrum of the antitumor activity of TNF-α is not yet understood, and in addition to a direct cytostatic or cytotoxic effect on tumor cells, TNF-α is also capable of eliciting an antitumor effect by a direct cytotoxic effect on capillary endothelial cells, thereby leading to vascular damage and subsequent hemorrhagic tumor necrosis (14).

Studies analyzing the effect of TNF-α in combination with other lymphokines (murine γ-interferon) and immunochemotherapy in an in vivo model system are also a prerequisite for exploring the full potential of TNF-α given that TNF-α has a very narrow therapeutic range. We now present evidence that the antitumor effect of N-AcMEL-MoAb conjugates can be enhanced by recombinant TNF-α, enabling improved therapy of larger established murine thymomas.

MATERIALS AND METHODS

Tumor Growth. The E3 clonal variant of the murine thymoma ITT(1)75NS (1) was maintained in vitro in DME, supplemented with 10% heat-inactivated newborn calf serum (Flow Laboratories, Sydney, Australia), 2 mM glutamine (Commonwealth Serum Laboratories, Melbourne, Australia), 100 IU of penicillin/ml (Commonwealth Serum Laboratories), and 100 μg/ml of streptomycin (Gibco Laboratories, Melbourne). For in vivo experiments E3 was maintained by serial passage in the ascites form in C57Bl/6 x BALB/c F1 (hereafter called B6CF1) mice; cells from the ascites fluid were washed and centrifuged (400 x g, 5 min) twice in DME and PBS (pH 7.3), resuspended in PBS, and injected s.c. into mice. Tumor cells were injected s.c. into the abdominal wall and were allowed to develop into palpable tumors before commencing treatment. Mice were then subjected to a series of i.p. and i.v. treatments, and the size of the tumors was measured daily with a caliper square measuring along the perpendicular axes of the tumors; the data were recorded as the mean tumor size [product of two diameters ± SE (<5%)]. Experimental groups of 10 to 20 mice, all of the same sex and age, were used while a number of parameters were recorded during the course of experiment for each group, including: (a) the number of deaths due to rTNF-α toxicity (determined by histopathology); (b) %T/C tumor size = the mean tumor size of a treated group of mice relative to the mean tumor size of PBS-treated mice on Day 20; (c) the proportion of tumors which remained completely regressed 100 days after tumor inoculation; and (d) the proportion of partial (>50% reduction in original tumor size) and complete tumor regressions combined.

Mice. B6CF1 mice were produced in the Department of Pathology, University of Melbourne.

MoAb. The MoAb used in this study was anti-Ly-2.1, a murine MoAb reactive with the murine Ly-2.1 specificity (IgG2a) (15). This MoAb was isolated from ascitic fluid by precipitation with 40% am-
monium sulfate, dissolution in PBS, and dialysis with the same buffer. This crude preparation was adsorbed onto Protein A-Sepharose (Phar- macia, Inc.), washed extensively in PBS (pH 7.3), and eluted with 0.2 M glycine-HCl (pH 2.8). Following neutralization, MoAb was dialyzed against PBS, checked for >95% purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, aliquoted, and stored at ~80°C.

Preparation of N-AcMEL-MoAb. Preparation and quantitation of N-AcMEL and N-AcMEL-MoAb conjugate have previously been described (1). An active ester of N-AcMEL was coupled to MoAb for 1 h at room temperature, free drug and other unreacted starting materials were removed by centrifugation and gel filtration [Sephadex G-25 (PD-10; Pharmacia)], and the amount of drug incorporated was determined by absorbance spectrophotometry at 258 nm after subtracting protein contribution estimated by Bradford analysis. N-AcMEL-anti-Ly-2.1 conjugates used in tumor growth experiments were composed of 20 to 25 molecules of N-AcMEL per molecule of MoAb.

Human rTNF-α and rIFN-γ. Both human rTNF-α (6 × 10^7 units/mg) (purity, >99%; ≤1.0 ng of endotoxin/mg) and mouse rIFN-γ (1 to 2 × 10^7 units/mg) were generously supplied by Boehringer Ingleheim. Both biologicals were diluted in PBS and stored in single-dose aliquots until required.

Radioiodination of rTNF-α. rTNF-α was labeled with 125I using Iodogen (Pierce Chemical Co., Rockford, IL) (11), the labeled protein was separated from the unbound 125I using a PD-10 column (Pharmacia) equilibrated with PBS, and the iodinated material eluted in the void volume (2 ml) was divided into aliquots before storing at ~80°C. This procedure did not result in any obvious loss of biological activity.

In Vitro Activity. Two different assays were performed to assess the activity of N-AcMEL-anti-Ly-2.1 and both rTNF-α and rIFN-γ against the E3 cell line. (a) For N-AcMEL-anti-Ly-2.1, by measuring the inhibition in [3H]thymidine incorporation into E3 cells after 24-h exposure to N-AcMEL-MoAb conjugates, the 50% inhibitory dose of N-AcMEL-anti-Ly-2.1 has previously been demonstrated to be 5.0 to 8.0 × 10^−6 M (1). (b) For rTNF-α and rIFN-γ, the sensitivity of the E3 cell line to rTNF-α and rIFN-γ was tested by assessing cell viability. Five hundred µl of E3 cells (4 to 5 × 10^6/ml) were added to a flat-bottomed plate and incubated for 4 h at 37°C. rTNF-α, rIFN-γ, or both together (125 µl) were added to the cells using triplicate wells/sample; control wells received 125 µl of PBS. Cells were left at 37°C in a 7% CO2 atmosphere for 72 h, and then 25 µl of each sample was removed and added to an equal volume of trypan blue solution. The number of viable cells was counted in a Neubauer hemocytometer, and the result was expressed as a percentage reduction in the viability relative to the control samples. The standard error for any given sample was generated by multiple determinations and did not exceed 5% for any point. Parallel analysis of the activity of rTNF-α was also performed using an inhibition of [3H]thymidine incorporation assay after 72-h exposure.

In Vitro Binding of 125I-labeled rTNF-α to E3 Cells. Fifty-µl samples of E3 cells (1 to 2 × 10^6 cells/ml) were added to a 96-well round-bottomed PVC microtiter plate containing various concentrations of unlabeled rTNF-α (≤1000-fold excess) and 50 µl of 125I-rTNF-α [500,000 cpm (2.9 × 10^6 cpm/µg)]. After an incubation of 4 h at 37°C, the cells were washed 4 times in DME containing 10% newborn calf serum, and the cell-bound radioactivity was determined in a gamma counter. The resulting data were evaluated by Scatchard analysis (16), and the number of TNF receptors per cell calculated given those data was corrected for nonspecific binding in the presence of a 1000-fold excess of unlabeled rTNF-α. Results are the average of quadruplicate determinations ± standard error of the mean. Since these studies were carried out at 37°C, it is possible that the measured binding parameters were influenced by cellular metabolic processes such as receptor biosynthesis, internalization, or recycling.

Biodistribution of 125I-Anti-Ly-2.1. B6CF1 mice bearing the subcutaneous E3 tumor (0.2 to 0.5 cm^2) were used to compare the distribution of 125I-anti-Ly-2.1 in the presence or absence of therapeutic levels of rTNF-α (5 µg). Groups of 4 mice were sacrificed 24 h after the injection of labeled anti-Ly-2.1, and the biodistribution of 125I-anti-Ly-2.1 was determined by counting the radioactivity of blood, heart, spleen, liver, kidneys, and tumor. The distribution of isotope was reported as a localization [percentage of radioactivity (cpm)/g of tissue]. All mice received 1.5 × 10^6 cpm of 125I activity with 60 µg of unlabeled MoAb by tail vein injection 24 h after the i.p. administration of rTNF-α or PBS. It should be noted that rTNF-α injected 24 h earlier did not enhance the localization of a nonreactive 125I-250-30.6 (reactive with colon carcinoma) conjugate (tumor/blood ratio = 0.16 ± 0.01). Results were tabulated as the mean ± standard error.

Biodistribution of 125I-rTNF-α. Studies were conducted to determine the biodistribution of 125I-rTNF-α in selected organs and tissues of B6CF1 mice with or without subcutaneous E3 tumors (0.2 to 0.5 cm^2). Groups of 4 mice were sacrificed 6 and 24 h after the i.p. injection of labeled rTNF-α (2 × 10^6 cpm/5 µg/mouse), and the uptake of 125I-rTNF-α was determined by counting the radioactivity of blood, heart, spleen, liver, kidneys, brain, and tumor. The distribution of isotope was recorded as a localization [percentage of radioactivity (cpm)/g of tissue].

RESULTS

In Vivo Antitumor Activity. The effective therapeutic use of rTNF-α is limited by its toxicity (17, 18); thus an initial study was designed to elucidate a suitable nontoxic schedule of rTNF-α treatment in conjunction with a constant dose (24 µg) of N-AcMEL-anti-Ly-2.1 conjugate, and the tumor growth of the (Ly-2.1+) E3 (1) thymoma and the histopathology of the major organs of B6CF1 mice were monitored.

N-AcMEL-anti-Ly-2.1, MEL, and anti-Ly-2.1 MoAb were administered i.v. on Days 6 (tumor size, 0.25 cm²), 9, and 14 with or without rTNF-α i.p. on Days 7, 8, and 13 after tumor inoculation at doses described in Fig. 1. Tumor growth (Fig. 1) indicated that both N-AcMEL-anti-Ly-2.1 conjugate and rTNF-α had significant antitumor effect when used alone. The mean tumor size of the drug-antibody conjugate-treated mice (Fig. 1F) was 24% that of control-treated mice on Day 20 (mean PBS tumor size = 2.66 cm²) after tumor inoculation. In addition, a substantial reduction in tumor growth was achieved by the injection (3 times, 5 µg) of rTNF-α (32% of control mean tumor size) (Fig. 1B). However, there was a rapid decline in efficacy at lower doses (3 times, 1 µg) (66% of control mean tumor size) (Fig. 1A) and the occurrence of liver and kidney toxicity at higher doses (3 times, 10 µg) of rTNF-α (20% died) (Fig. 1C). By contrast the combination of conjugate and rTNF-
α caused a more effective reduction in tumor growth than either one of the two agents used alone. For example 15 μg (3 times, 5 μg) of rTNF-α totally eradicated 10% of tumors (Fig. 1B), but in combination with 24 μg of N-AcMEL-anti-Ly-2.1 conjugate (where 20% were eradicated), 50% (5 of 10) of the tumors were eradicated (Fig. 1F). Similar synergistic effects were observed in groups receiving (3 times, 1 μg, or 3 times, 10 μg) rTNF-α and 24 μg of the immunoonjugate (Fig. 1E). It should also be noted that rTNF-α and a mixture of MEL and anti-Ly-2.1 MoAb (Fig. 1E) was more efficacious than MEL (Fig. 1C) and anti-Ly-2.1 alone (Fig. 1D); we noted that the antitumor effect of TNF has previously been demonstrated to be enhanced by chemotherapeutic agents in vivo (19).

Having established a nontoxic single therapeutic dose of rTNF-α (5 to 10 μg) it was necessary to further test the antitumor effect of varying doses of N-AcMEL-anti-Ly-2.1 conjugate using larger groups of mice. In addition, to attempt to increase the antitumor effect of rTNF-α, some groups received exogenous murine rIFN-γ (18). rTNF-α and rIFN-γ were administered i.p. on Days 6 (tumor size = 0.3 cm²), 7, 10, 13, and 16 after tumor inoculation with or without N-AcMEL-anti-Ly-2.1 i.v. on Days 6, 8, and 10. The mean PBS tumor size at Day 20 was 3.30 cm². rIFN-γ marginally increased the efficacy of the combined treatment with immunoonjugate and rTNF-α (Fig. 2). Mice receiving rIFN-γ in combination with 12 μg of conjugate and 25 μg of rTNF-α (Fig. 2G) had a smaller mean tumor size and greater percentage of regressions (90%) than mice receiving 12 μg of conjugate and 25 μg of rTNF-α (80%) (Fig. 2E). Importantly, 35% of tumors could be completely eradicated in mice receiving the highest dose of rTNF-α and N-AcMEL-anti-Ly-2.1 conjugate (Fig. 2F). By reducing the immunoonjugate dose from 24 μg (Fig. 2F) to 12 μg (3 times, 4 μg) (Fig. 2E), the antitumor effect in combination with 25 μg of rTNF-α was markedly reduced. However, the effect was superior to that of conjugate (Fig. 2C) or rTNF-α alone (Fig. 2A). Between 80 and 90% of tumors in mice receiving conjugate and rTNF-α (Fig. 2, E and F) demonstrated partial or complete regression of the tumors compared with 40% for N-AcMEL anti-Ly-2.1 conjugate-treated mice (Fig. 2D).

Given that effective conjugate treatment is limited to small subcutaneous tumors (1), the antitumor efficacy of combination therapy was determined against larger tumors (Fig. 3). Treatments were not commenced until tumors were 0.4 to 0.6 cm² in size, and groups of 10 B6CF1 mice were then given one of the following: (a) PBS; (b) a total of 45 μg of rTNF-α i.p. over Days 9, 11, 14, 17, and 21 after tumor inoculation; (c) a total of 26 μg of N-AcMEL-anti-Ly-2.1 i.v. over Days 8, 10, 14, and 15; or (d) a combination of b and c. It was apparent that N-AcMEL-anti-Ly-2.1 conjugate or rTNF-α alone inhibited tumor growth (by approximately 50%). However, neither treatment enabled partial or complete regression of tumor mass (Fig. 3). By contrast, combined treatment with immunoonjugate and rTNF-α was effective in eradicating 10% of tumors, and another 20% demonstrated a partial regression in tumor size, while the mean tumor size of this group was 15% that of PBS-treated mice (Day 22). In addition, at the completion of combined treatment, remaining viable tumors continued to grow at a rate markedly slower than those of the other treated groups. Histological examination of tumors, treated with a combination of conjugate and rTNF-α, which initially regressed and then regrew, suggested that peripherally located viable tumor may be responsible for recurrence of tumor growth (data not shown).

The experiments performed above clearly indicated an enhanced in vivo antitumor effect when N-AcMEL-MoAb conjugate and rTNF-α were administered in combination. The antitumor activity of rTNF-α is not yet completely understood, and therefore the mechanism underlying its enhancement of N-AcMEL-MoAb antitumor activity was unclear. It was initially necessary to determine whether rTNF-α could exhibit a direct cytotoxic effect on E3 tumor cells in vivo.

In Vitro Activity of rTNF-α. The cytotoxicity of rTNF-α and rIFN-γ was tested on E3 target cells after 72-h exposure (Fig. 4). The E3 cell line was inhibited 70 to 80% at doses of >100 units/ml by both rTNF-α and rIFN-γ, while resistant to doses <1 unit/ml, with resistance being defined as less than 30% inhibition of control growth. In contrast to IFN-γ, TNF-α showed little species specificity, and previous comparisons between murine and human recombinant TNF-α performed in vitro have shown a similar degree of lysis of human and murine tumor cell lines (20). The effects of simultaneously combining various concentrations of human rTNF-α and murine rIFN-γ were also evaluated (Fig. 4), and no synergistic cytotoxic effect...
was observed. For some cell lines, however, it has been demonstrated that synergy with IFN-γ is more pronounced when the in vitro efficacy of rTNF-α was measured by enhanced antitumor effect in vivo. Similar results were obtained when the in vitro efficacy of rTNF-α was measured by the inhibition in [3H]thymidine incorporation into E3 cells after 72-h incubation at 37°C with rTNF-α (20). This may explain the in vivo antitumor activity of N-AcMEL-MoAb conjugate by increasing its uptake into tumor tissue.

Biodistribution studies of 125I-rTNF-α in B6CF1 mice with subcutaneous E3 tumors (Table 2) demonstrated that, after 6 h, levels of 125I, as defined by the localization index, were higher in the tumor than in the heart, lungs, brain, spleen, and liver, but lower at 24 h than the liver, spleen, and kidneys. Kidney also had the highest concentration of 125I after 6 h, probably due to the short serum half-life of TNF (approximately 30 min) (22). Given the relatively minor localization of 125I-rTNF-α to the E3 tumor and the large central areas of hemorrhagic necrosis macroscopically observed in rTNF-α-treated E3 tumors (data not shown), the bulk of the rTNF-α antitumor effect may be related to vascular phenomena resultant from TNF-α administration.

**DISCUSSION**

MoAbs which react specifically with antigens preferentially associated with tumor cells are potentially useful for targeting antineoplastic drugs. These may improve the therapeutic efficacy of drugs by increasing the localization of drug in tumors and by minimizing toxic effects of drugs which represent a major limitation to conventional chemotherapy. Antibody conjugates with plant toxins and cytotoxic drugs have been produced with adequate antibody and drug activity (1, 2, 23), and many of these have demonstrated therapeutic effects against ascites tumors and small s.c. implanted murine and human tumors. If drug-MoAb conjugates have a role in the therapy of metastatic deposits near to or distant from the primary lesion, then successful treatment requires that the immunoconjugate localizes effectively and penetrates regions of the tumor which enable its progressive growth. However, the experimental immunochemotherapy of larger tumor appears to be limited by tumor burden and the inaccessibility of some tumor cells to immunoconjugate treatment (1, 7). Indeed, the absence of truly defined "tumor-specific targets" and the poor tumor localization properties of currently available MoAb reagents (24) may limit the effectiveness of immunoconjugate treatment of large solid tumors. In this study the use of rTNF-α to enhance the antitumor activity of N-AcMEL-MoAb conjugate against large solid tumors highlights the possible use of combined treatment modalities.

**Table 1 Biodistribution of 125I-MoAb in the presence of rTNF-α**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>125I-Anti-Ly-2.1</th>
<th>125I-Anti-Ly-2.1 and rTNF-α</th>
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<tr>
<td>Blood</td>
<td>18.31 ± 0.61</td>
<td>11.90 ± 0.30</td>
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<tr>
<td>Tumor</td>
<td>14.87 ± 0.90</td>
<td>19.30 ± 0.68</td>
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<tr>
<td>Liver</td>
<td>2.12 ± 0.28</td>
<td>1.70 ± 0.12</td>
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<tr>
<td>Spleen</td>
<td>3.40 ± 0.21</td>
<td>2.18 ± 0.12</td>
</tr>
<tr>
<td>Heart</td>
<td>3.35 ± 0.26</td>
<td>1.83 ± 0.06</td>
</tr>
<tr>
<td>Kidneys</td>
<td>3.23 ± 0.43</td>
<td>1.90 ± 0.04</td>
</tr>
<tr>
<td>Tumor blood</td>
<td>0.81 ± 0.03</td>
<td>1.63 ± 0.02</td>
</tr>
<tr>
<td>Liver blood</td>
<td>0.11 ± 0.01</td>
<td>0.15 ± 0.07</td>
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</tbody>
</table>

* Mean ± SE.

**Table 2 Biodistribution of 125I-rTNF-α**

<table>
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<tr>
<th>Tissue</th>
<th>Localization (% of radioactivity/g of tissue)</th>
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</thead>
<tbody>
<tr>
<td>Blood</td>
<td>2.10 ± 0.14</td>
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<tr>
<td>Tumor</td>
<td>1.54 ± 0.12</td>
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<tr>
<td>Liver</td>
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<td>Brain</td>
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<tr>
<td>Lung</td>
<td>1.45 ± 0.06</td>
</tr>
</tbody>
</table>

* Mean ± SE.
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REFERENCES


15. Fiers, W. In vivo anti-tumor activity of recombinant human and murine TNF, alone and in combination with murine IFN-γ, on a syngenic murine melano-


21. Fiers, W. In vivo antitumor activity of recombinant human and murine TNF, alone and in combination with murine IFN-γ, on a syngenic murine melano-


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