Pretreatment with a Monoclonal Antibody/Interleukin-2 Fusion Protein Directed against DNA Enhances the Delivery of Therapeutic Molecules to Solid Tumors

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Abstract

The efficacy of molecular therapies for human malignancies is limited by inadequate accumulation within solid tumors. Our laboratory has developed a novel approach that uses monoclonal antibodies (MAbs) to direct vasoactive proteins to tumor sites to increase local vascular permeability and, in turn, improve the delivery of therapeutic reagents. Previously, we demonstrated that pretreatment with immunoconjugates containing interleukin-2 (IL-2) enhances specific tumor uptake of radiolabeled MAbs without affecting normal tissues. In the present study, we describe a fusion protein consisting of a chimeric antinuclear antibody and IL-2 (chTNT-3/IL-2) and illustrate its potential for improving the delivery of both MAbs and drugs. The ability of pretreatment with chTNT-3/IL-2 to increase specific tumor uptake of the MAb B72.3 was demonstrated in LS174T colon tumor-bearing mice. Tumor accretion of B72.3 increased nearly 3-fold, with no changes in normal tissues. Abrogation of this effect with N\textsuperscript{G}-methyl-l-arginine, a chemical inhibitor of nitric oxide synthase, suggests that rapid generation of nitric oxide in the tumor is responsible for the enhanced uptake. To demonstrate that pretreatment with chTNT-3/IL-2 can improve the uptake of other clinically relevant MAbs in different tumor models, additional studies were performed in both lung and prostate xenograft models. Pretreatment with the fusion protein increased specific tumor uptake of the MAb NR-LU-10 in A427 lung tumor-bearing mice and enhanced tumor uptake of the MAb CVT-351 in LNCaP prostate tumor-bearing mice, 2.1-fold and 1.7-fold, respectively. Finally, tumor uptake of the radiolabeled thymidine analogue \(^{125}\text{I} \text{UdR}\) also increased \(\sim 3\)-fold after pretreatment, indicating that this approach can be extended to small molecules such as chemotherapeutic drugs. Because TNT-3 recognizes a universal nuclear antigen accessible in degenerating and necrotic cells within all solid tumors, this strategy may be applicable to the majority of human cancers.

Introduction

Physiological barriers to the delivery of therapeutic reagents to solid tumors are a major obstacle to the clinical success of developing molecular therapies (1). For example, the limited clinical responses observed in radioimmunotherapy of solid tumors (2) can be attributed in large part to low tumor localization of radiolabeled MAbs (1). Although xenograft models in nude mice have shown levels of tumor uptake ranging from 1–20\% ID/g, patient studies have demonstrated exceedingly low tumor uptake in the range of 0.01\% ID/g (3). Thus, an extremely small fraction of antibody delivers radionuclide to tumor sites, whereas the majority of the injected dose disperses throughout the body, where it can cause dose-limiting myelosuppression (4). Recognizing that blood flow and vascular permeability are key parameters controlling the egress of therapeutic molecules into tumors (5, 6), our laboratory developed an experimental approach to alter tumor vascular physiology and, in turn, increase the delivery of therapeutic reagents to tumors. This strategy uses MAbs to direct proteins with vasoactive properties to tumor sites to increase local vascular permeability without affecting normal tissues (7). We previously developed immunoconjugates containing cytokines and other vasoactive molecules and examined their ability to increase tumor uptake of radiolabeled MAbs (8). From these studies, it was determined that the immunoconjugates that produced the greatest enhancement of antibody uptake contained IL-2.

IL-2 is a \(M\text{,} 15,000\) protein secreted by activated T cells that supports the proliferation and activation of lymphocytes and other immune cells (9). In clinical studies, IL-2 has shown success in the treatment of several human malignancies, in particular melanoma and renal cell carcinoma (10). It is well established, however, that systemic administration of IL-2 leads to increased permeability of blood vessels in the lungs and other organs leading to a toxic side effect known as the capillary leak syndrome (11–13). In our novel approach, the undesirable prop-

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3 The abbreviations used are: MAb, monoclonal antibody; ID/g, injected dose/g of tissue; IL-2, interleukin 2; muTNT-3, murine TNT-3; chTNT-3, chimeric TNT-3; chTNT-1, chimeric TNT-1; l-NMA, N\textsuperscript{G}-methyl-l-arginine; \(^{125}\text{I} \text{UdR}\), 5-\(^{125}\text{I}\)iodo-2‘-deoxyuridine; NOS, nitric oxide synthase.

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property of IL-2 has been harnessed by using MAbs to target IL-2 to the tumor site. Our laboratory has demonstrated in animal models that administration of immunoconjugates consisting of IL-2 and MAbs directed against various tumor antigens increases local tumor vascular permeability and, in turn, enhances tumor uptake of radiolabeled MAbs (14, 15). The magnitude of enhancement was similar whether the immunoconjugate was directed against tumor-associated cell surface antigens (14), an extracellular matrix protein in the basement membranes of tumor vessels (15), or an intracellular antigen accessible in the necrotic regions of solid tumors (8). For this reason, we chose to develop an antibody/IL-2 fusion protein with specificity for a nuclear antigen that might serve as a universal targeting agent, owing to its ability to target degenerating cells within all solid tumors.

In the present study, we describe a fusion protein consisting of chTNT-3 and IL-2 (chTNT-3/IL-2). Because TNT-3 recognizes DNA in the degrading and necrotic cells within solid tumors, it has the potential to target the majority of human malignancies (16). In this study, we examine the ability of chTNT-3/IL-2 to increase the specific tumor uptake of both MAbs and chemotherapeutic drugs in various tumor xenograft models in the hope that such a strategy may represent a general approach to increase the delivery of therapeutic molecules to solid tumors.

Materials and Methods

Reagents

The plasmid pBC12/HIV/IL-2 containing human IL-2 cDNA (17) was obtained from the American Type Culture Collection (clone 67618; Manassas, VA). The plasmids pEE6hCMV-B and pEE12 were purchased with the Glutamine Synthetase Gene Amplification System from Lonza Biologics (Slough, United Kingdom). Restriction endonucleases, T4 DNA ligase, and other molecular biology reagents were purchased from New England Biolabs (Beverly, MA) or Boehringer Mannheim (Indianapolis, IN). Chloramphenicol, l-lysine, and ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt] were purchased from Sigma Chemical Co. (St. Louis, MO). Iodine-125 and iodine-131 were obtained as sodium iodide in 0.1N sodium hydroxide from DuPont/New England Nuclear (North Billerica, MA). 125IUDR was purchased from Amersham Life Science Inc. (Arlington Heights, IL). BALB/c and athymic nude mice were purchased from Harlan Sprague Dawley (Indianapolis, IN).

Antibodies and Cell Lines

chTNT-3 (IgG1x) was constructed and expressed as described previously (16). chTNT-1 (IgG1x), of which the variable regions of cDNAs were cloned from the murine TNT-1 hybridoma (18), was constructed and expressed in the same manner as chTNT-3. The fusion protein chCLL-1/IL-2, consisting of the chimeric anti-B-cell MAB CLL-1 with human IL-2 at the C-termini of the chimeric heavy chains, was produced as described previously (19). The murine MAB B72.3 (IgG1; Ref. 20), recognizing the tumor-associated glycoprotein TAG-72, was a gift from Celltech Biologicals. The murine MAB NR-LU-10 (IgG2a; Ref. 21), reactive with a Mₐ 40,000 glycoprotein expressed on many epithelial cell carcinomas, was generously provided by Dr. Don Axworthy of NeoRx Corp. (Seattle, WA). The murine MAB CYT-351, also known as 7E11-C5.3 (IgG1; Ref. 22), recognizing a Mₐ 100,000 prostate-specific membrane glycoprotein (23, 24), was generously provided by CYTGEN Corp. (Princeton, NJ). Iodine-125 and iodine-131-labeled MAbs were prepared using a modified chloramine T method, as described previously (25). The N50 murine myeloma cell line was obtained from Lonza Biologicals. The Raji cell line (derived from an African Burkitt’s lymphoma; Refs. 26 and 27), the LS174T human colon adenocarcinoma cell line (28), the A427 human lung adenocarcinoma cell line (29), and the LNCaP human prostatic adenocarcinoma cell line (30) were obtained from the American Type Culture Collection.

Construction of Expression Vectors

The expression vectors were constructed using standard techniques. The expression vector for the chTNT-3 heavy chain, pEE12/chTNT-3 HC (16), was used as the parent vector. This plasmid contains the cDNA sequence for the human-mouse chTNT-3 heavy chain, under the control of the cytomegalovirus major immediate early promoter, and the cDNA sequence for glutamine synthetase, under the control of the SV40 early promoter. To amplify the human IL-2 cDNA from the pBC12/HIV/IL-2 plasmid template, two primers (5’-GTAAAAGGCGCCGCAAGAGGTTGCGGCACCTACTTCAAGTTCTACA-3’ and 5’-TCATGCGCCGCTCAAGTTATGTTAGATGATGCT-3’) were used. The PCR fragment was inserted into the NotI site of pEE12/chTNT-3 HC, resulting in the expression vector 12/chTNT-3/IL-2 encoding a fusion protein consisting of the chTNT-3 heavy chain with human IL-2 at its COOH terminus. The expression vector for the chTNT-3 light chain, pEE6/chTNT-3 LC, was constructed as described previously (16).

Expression and Purification of Antibody Fusion Protein

chTNT-3/IL-2 was expressed in NS0 murine myeloma cells according to the manufacturer’s protocol (Lonza Biologicals). The highest producing clone was incubated in a 10-liter bioreactor, and the fusion protein was purified stepwise from cell culture medium by protein A affinity and ion-exchange chromatography, as described previously (25). Purity of the fusion protein was examined by SDS-PAGE according to the method of Laemmli (31).

Immunosassays

ELISA. Fusion protein-containing supernatants were initially identified by indirect ELISA using microtiter plates coated with single-stranded DNA from calf thymus, as described previously (16). For production rate assays, 10⁶ cells were plated in 1 ml of selective medium and allowed to incubate for 24 h. ELISA was then performed as before. Supernatants were serially diluted and applied to wells of microtiter plates coated with goat antihuman IgG (H+L; CalTag, South San Francisco, CA). Dilutions of a control chimeric MAB were used to generate a standard curve using 4-parameter fit by an automated ELISA reader (Bio-Tek Instruments, Inc., Winoski, VT), from which concentrations of unknowns were estimated. Rates of production were compared to identify the highest producing clones.

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Raji Cell Competition RIA. The immunoreactivity of chTNT-3/IL-2 was also evaluated by a competition RIA for binding to fixed Raji lymphoma cells. For these studies, 2 × 10^6 Raji cells fixed in 2% paraformaldehyde (32) were incubated with 20 ng of 125I-labeled muTNT-3 and serial dilutions of cold chTNT-3, chTNT-3/IL-2, or a MAb recognizing a different nuclear antigen (chTNT-1). The cells and MABs were incubated for 1 h at room temperature with constant mixing. The cells were then washed twice, and the cell pellet-associated radioactivity was measured in a gamma counter. Maximal binding was determined from tubes containing no cold antibodies.

Determination of Avidity. The avidity constant of chTNT-3/IL-2 was determined by a fixed cell RIA using the method of Frankel and Gerhard (33). Raji lymphoma cell suspensions containing 10^6 cells/ml were incubated with 10–110 ng of 125I-labeled chTNT-3/IL-2 in 200 μl of PBS in duplicate for 1 h at room temperature with constant mixing. The cells were then washed three times with PBS containing 1% BSA to remove unbound antibody and counted in a gamma counter. The amount of fusion protein bound was determined by the remaining cell-bound radioactivity (cpm) in each tube and the specific activity (cpm/ng) of the radiolabeled fusion protein. Scatchard analysis was performed to obtain the slope. The equilibrium or avidity constant K_a was calculated by the equation K_a = −(slope/n), where n is the valence of the antibody fusion protein (2 for IgG).

IL-2 Bioassay. Biological activity of the fusion protein was determined by a standard IL-2-dependent T-cell proliferation assay using the murine T cell line CTLL-2. Recombinant IL-2, obtained from Hoffmann La Roche, Inc. (Nutley, NJ), was used as a standard. Briefly, serially diluted samples and standard were incubated with 2 × 10^4 CTLL-2 cells in triplicate for 20 h at 37°C before they were pulsed with [3H]thymidine and harvested 6 h later. Specific activity of the sample was determined by regression of the linear portion of a semi-log graph of cpm versus ng IL-2 for the standard.

Pretreatment Studies. Athymic nude mice, 6 weeks of age, received injections of a 0.2-ml inoculum containing 2 × 10^5 LS174T human colon adenocarcinoma cells s.c. in the left thigh. The tumors were grown for 10–14 days until they reached approximately 1 cm in diameter. In the time-dependence study, groups of mice (n = 4) were administered i.v. injections of 30 μg of chTNT-3/IL-2 at various times before, simultaneously, or subsequently to the i.v. injection of a 0.1-ml inoculum containing 100 μCi/10 μg 125I-labeled B72.3. In the dose-dependence study, groups of mice were administered various doses of chTNT-3/IL-2 2 h before the i.v. injection of 125I-labeled B72.3. As a control, a group of mice received 15 μg of chCLL-1/IL-2. To examine the mechanism of vasopermeability enhancement, a group of mice received 20 mg/kg l-NMA (34), an inhibitor of NO synthase, i.p. 30 min before pretreatment with 15 μg of chTNT-3/IL-2. A control group received l-NMA alone before administration of 125I-labeled B72.3. In the preceding groups, animals were sacrificed by sodium pentobarbital overdose 72 h after injection, and tissues were removed, weighed, and measured in a gamma counter. For each mouse, data were expressed as the percentage of ID/g and tumor:organ ratio (cpm/g tumor:cpm/g organ).

The ability of chTNT-3/IL-2 pretreatment to enhance the specific tumor uptake of radiolabeled MABs was examined in two additional tumor models. Female athymic nude mice, 6 weeks of age, received injections of A427 lung adenocarcinoma cells, and male nude mice (6 weeks of age) received injections of LNCaP prostatic adenocarcinoma cells. The tumors were grown for 10–14 days (A427) or ~8 weeks (LNCaP) until they reached 1 cm in diameter. Groups of mice (n = 7) were then administered i.v. injections of various doses of chTNT-3/IL-2 2 h before the i.v. injection of 125I-labeled MAb. NR-LU-10 was used with the A427 tumor model, and CYT-351 was used with the LNCaP tumor model. Animals were sacrificed 5 days after injection for biodistribution analysis, as described above. To examine whether this pretreatment strategy could be extended to small molecules such as chemotherapeutic drugs, groups of mice (n = 4) received 125I UdR with or without pretreatment with 15 μg of chTNT-3/IL-2. Because of the rapid clearance of this drug, mice were sacrificed 3 h after injection for biodistribution analysis. All data are presented as medians. Significance levels were determined using the Wilcoxon’s rank-sum test.

Results

Construction, Expression, and Purification of chTNT-3/IL-2. A PCR fragment containing the human IL-2 cDNA, preceded by a 7 amino acid linker peptide, was inserted into the NotI site previously appended immediately downstream of the human γ1 terminal codon, producing a TNT-3 VH/human γ1 human IL-2 fusion gene. This resulted in the expression vector 12/chTNT-3 HC/IL-2 encoding a fusion protein consisting of the chTNT-3 heavy chain with human IL-2 at its COOH terminus. This expression vector was cotransfected with the expression vector for the chTNT-3 light chain, pE6/chTNT-3 LC. The fusion protein was expressed in NS0 murine myeloma cells using the Glutamine Synthetase Gene Amplification System (Lanza Biologicals). The highest producing transfectant was scaled up to a 10-liter bioreactor, and the fusion protein was purified stepwise by protein A affinity and ion-exchange chromatography. The production level of chTNT-3/IL-2 was >40 μg/ml. After purification, the chimeric heavy chain fusion protein was intact, as demonstrated by reducing SDS-PAGE. Two bands were resolved for chTNT-3/IL-2 at approximately M, 25,000 and M, 70,000, corresponding to the molecular weights of the immunoglobulin light chain and heavy chain plus cytokine, compared with chTNT-3, the heavy chain of which exhibited an apparent molecular weight of approximately M, 55,000 (Fig. 1).

Immunobiochemical Analysis. The immunoreactivity of chTNT-3/IL-2 was assessed by determining binding to fixed Raji lymphoma cells. In a competition RIA, the fusion protein, chTNT-3, and an isotype-matched control MAb (chTNT-1) were evaluated for their ability to inhibit the binding of 125I-labeled muTNT-3 to Raji cells (Fig. 2). Because it recognizes a different nuclear antigen, chTNT-1 was unable to compete with radiolabeled muTNT-3. chTNT-3/IL-2, however, inhibited binding of 125I-labeled muTNT-3 to a similar extent as chTNT-3. Binding studies were then conducted in which 125I-labeled chTNT-3/IL-2 was incubated with fixed Raji cells, and the bound radioactivity was used to calculate the avidity constant. chTNT-3/IL-2 was found to have a binding constant of...
1.6 \times 10^9 \text{ m}^{-1}, \text{ compared with } 1.4 \times 10^9 \text{ m}^{-1} \text{ for chTNT-3. These studies confirm that chTNT-3/IL-2 maintains the immunoreactivity of chTNT-3 and demonstrate that the cytokine at the COOH terminus of the heavy chain does not interfere with binding to the antigenic target under physiological conditions.}

**IL-2 Bioactivity of chTNT-3/IL-2.** Biological activity of the IL-2 moiety was determined by examining the ability of the fusion protein to support IL-2-dependent T-cell proliferation. A bioassay with the murine T cell line CTLL-2 was performed in which chTNT-3/IL-2 was assayed along with chTNT-3 and a recombinant IL-2 standard. Two molecules of IL-2/antibody were assumed. On a molar basis, the fusion protein displayed roughly 26\% of the activity of the IL-2 standard (data not shown), corresponding to a specific activity of \( 6 \times 10^5 \text{ IU/mg} \text{ chTNT-3/IL-2}. \) As expected, chTNT-3 had no IL-2 activity. chCLL-1/IL-2, which was used as a control for the pretreatment studies, had \( 50\% \) of the activity of the IL-2 standard on a molar basis. This corresponds to a specific activity of \( 8 \times 10^5 \text{ IU/mg} \) (19).

**Pretreatment Studies.** The effect of chTNT-3/IL-2 administration on tumor uptake of the murine MAb B72.3 was evaluated in LS174T colon tumor-bearing nude mice. Biodistribution analyses were performed 72 h after B72.3 injection. To determine the relationship between the timing of treatment and tumor uptake, 30 \( \mu \text{g} \) of chTNT-3/IL-2 were injected i.v. at various times relative to \( ^{125}\text{I}-\text{labeled MAb injection.} \) The highest tumor accretion of B72.3 occurred when chTNT-3/IL-2 was injected 1–3 h before the administration of B72.3. For this reason, a 2-h interval between pretreatment and radiolabeled MAb injection was used for the remainder of the experiments.

The relationship between the dose of fusion protein and tumor uptake of B72.3 was then examined. A dose of 15 \( \mu \text{g} \) resulted in the greatest increase in tumor uptake (Fig. 3B). With higher doses, the magnitude of tumor uptake began to diminish. At the highest dose studied, several normal tissues revealed significantly higher MAb uptake (Table 1). Most importantly, the lung showed a significant increase in uptake (from a median of 0.96\% ID/g with 15 \( \mu \text{g} \) chTNT-3/IL-2 to 1.40\% ID/g with 60 \( \mu \text{g}; P \leq 0.025 \)). This suggests that toxicity to normal tissues can occur with high doses of fusion protein. This inference does not explain the increase in blood levels of B72.3 after the highest dose of fusion protein; the explanation for this increase remains unclear.

Fig. 4 depicts the tissue biodistribution and tumor uptake of \( ^{125}\text{I}-\text{labeled B72.3 under optimal pretreatment conditions. Tumor uptake increased significantly from 4.19\% ID/g (range, 3.64–4.61) to 11.18\% ID/g (range, 10.32–11.70) after pretreatment with chTNT-3/IL-2 (P \leq 0.025 \)}. \) Under these conditions, there was no change in radionuclide MAb uptake in normal tissues, resulting in significantly higher tumor:normal organ ratios (Fig. 4B). On the other hand, pretreatment with the control fusion protein chCLL-1/IL-2 (19), which recognizes B cell malignancies, had no effect on tumor uptake. This demonstrates that tumor localization of the fusion protein is necessary for enhancing specific uptake of radionuclide MAb.

The NOS inhibitor L-NMA was administered before pretreatment with chTNT-3/IL-2 to examine the mechanism of increased tumor vascular permeability. L-NMA abrogated the effect of fusion protein pretreatment on tumor uptake of B72.3 (Fig. 5). The inhibitor alone, however, did not decrease tumor uptake below baseline levels (data not shown). These data strongly suggest that nitric oxide generation is responsible for the enhancement of tumor uptake of MAb.

The effect of chTNT-3/IL-2 administration on specific tumor uptake of the murine MAb NR-LU-10 was then evaluated in A427 lung tumor-bearing nude mice. Various doses of
chTNT-3/IL-2 were administered 2 h before injection of 125I-labeled NR-LU-10. Biodistribution analyses were performed 5 days after NR-LU-10 injection. The tumor uptake of MAb increased with increasing doses of fusion protein (data not shown). Table 2 depicts the biodistribution of NR-LU-10 after pretreatment with 30 μg of chTNT-3/IL-2. Tumor uptake increased significantly from 3.09% ID/g (range, 2.83–3.25) to 6.37% ID/g (range, 5.75–6.71) after pretreatment with chTNT-3/IL-2 (P ≤ 0.001). There was a significant decrease in tissue levels of radiolabeled MAb in many normal organs after fusion protein pretreatment and, consequently, a significant increase in tumor:organ ratios for all normal tissues (Table 2).

Pretreatment with the fusion protein was next evaluated in a human prostatic adenocarcinoma xenograft model. The relationship between dose of chTNT-3/IL-2 and tumor uptake of 125I-labeled CYT-351 was examined in LNCaP prostate tumor-bearing mice. Mice were again sacrificed 5 days after injection for biodistribution. A dose of 30 μg resulted in the greatest increase in tumor uptake (data not shown). As shown in Table 3, tumor uptake increased significantly from 18.59% ID/g (range, 17.29–20.81) to 31.85% ID/g (range, 28.70–34.55) after this pretreatment dose (P ≤ 0.001), with no increase in uptake in normal tissues. On the contrary, there was a significant decrease in the levels of radiolabeled MAb in the majority of normal tissues after pretreatment, again resulting in higher tumor:organ ratios.

Finally, the effect of chTNT-3/IL-2 pretreatment on tumor uptake of 125IUdR was examined, to assess whether this approach could be applied to small molecules such as chemotherapeutic drugs. IUdR was selected as a representative drug because of the availability of a radioiodinated derivative. Again, 15 μg of fusion protein were administered 2 h before injection of 125IUdR in LS174T colon tumor-bearing mice. Because of the short circulation time of this drug, mice were sacrificed 3 h later for biodistribution analysis. Control tumor uptake was 1.44% ID/g (range, 1.28–1.53), increasing significantly to 4.10% ID/g (range, 3.89–4.32) after pretreatment (P ≤ 0.025), representing approximately a 3-fold increase in tumor uptake with no effect on normal tissues (Table 4).

Discussion
In this study, a recombinant fusion protein containing the chimeric MAb TNT-3 and human IL-2 was generated as a universal pretreatment to enhance the delivery of therapeutic molecules to solid tumors. The fusion protein was expressed in mammalian cells using the Glutamine Synthetase Gene Amplification System so that large-scale production can yield sufficient recombinant product for clinical studies (35). Acrylamide gel electrophoresis demonstrated that the chimeric heavy chain fusion protein was intact after purification (Fig. 1). As described previously by our laboratory, for other antibody-cytokine fusion

Table 1 Median percentage ID/g of the MAb B72.3 administered 2 h after pretreatment with the indicated doses of chTNT-3/IL-2 in LS174T human colon adenocarcinoma-bearing nude mice 72 h after injection

<table>
<thead>
<tr>
<th>Organ</th>
<th>15 μg</th>
<th>60 μg</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>1.74</td>
<td>2.78</td>
<td>≤0.025</td>
</tr>
<tr>
<td>Skin</td>
<td>0.74</td>
<td>0.84</td>
<td>≤0.05</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.26</td>
<td>0.41</td>
<td>NS</td>
</tr>
<tr>
<td>Bone</td>
<td>0.23</td>
<td>0.35</td>
<td>≤0.05</td>
</tr>
<tr>
<td>Heart</td>
<td>0.47</td>
<td>0.74</td>
<td>≤0.025</td>
</tr>
<tr>
<td>Lung</td>
<td>0.96</td>
<td>1.40</td>
<td>≤0.025</td>
</tr>
<tr>
<td>Liver</td>
<td>0.52</td>
<td>0.74</td>
<td>≤0.025</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.39</td>
<td>0.53</td>
<td>NS</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.32</td>
<td>0.76</td>
<td>≤0.025</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.75</td>
<td>0.74</td>
<td>NS</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.24</td>
<td>0.43</td>
<td>≤0.025</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.49</td>
<td>0.66</td>
<td>≤0.05</td>
</tr>
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* P* s determined by the Wilcoxon’s rank-sum test.
# NS, not significant.
proteins (19, 36), the IL-2 cDNA was inserted downstream of the terminal codon of the chimeric heavy chain, following a short linker peptide to promote proper folding of the cytokine. The fusion protein retains the immunoreactivity of the parent antibody, as evidenced by competition with 125I-labeled muTNT-3 for binding to fixed Raji Burkitt’s lymphoma cells (Fig. 2). Moreover, chTNT-3/IL-2 maintains the high avidity constant of chTNT-3. The biological activity of the IL-2 moiety was demonstrated by a proliferation assay with a murine IL-2-dependent T cell line.

As our laboratory and others have demonstrated previously, MAb/IL-2 fusion proteins are eliminated rapidly from normal mice (36–38). The therapeutic potential of MAb/IL-2 fusion proteins for eliciting tumor rejection has been demonstrated in animal models (39–41). The rapid clearance of these fusion proteins may prove beneficial in the clinical setting, where potentially injurious exposure of healthy tissues to the high doses of IL-2 (13, 42, 43) necessary to evoke cellular immune responses against solid tumors (10) may be minimized as the antibody concentrates the cytokine at the tumor site (44). It will, of course, be necessary to evaluate the toxicity of MAb/IL-2 fusion proteins in patients because the serum persistence of the fusion protein compared with free recombinant IL-2 (45) may still result in toxicities.

The efficacy of MAb/IL-2 fusion proteins is limited by insufficient accumulation within solid tumors (46). Investigators have shown that IFNs can enhance the expression of tumor-associated antigens leading to increased tumor uptake of MAb s (47, 48). This approach, however, is limited to MAb s directed against tumor antigens that can be up-regulated by such treatment. We have focused our efforts on developing an approach to improve the delivery of antibodies to tumors that might also be applicable to other therapeutic molecules. Our laboratory was the first to use immunoconjugates containing vasoactive cytokines to increase vascular permeability (14). Others have shown that the systemic administration of tumor necrosis factor (49–51), IFN-γ (52), and IL-2 (14, 53) increases tumor uptake of radiolabeled MAb s in mouse models, but as demonstrated in these studies and in those from our laboratory (14), pretreatment with free vasoactive cytokines also results in increased uptake in normal tissues including lung, liver, and spleen. Hence, the targeted delivery of cytokines to tumor sites using MAb s represents a significant advancement of this technology. The fusion protein described in the present study was designed for enhancing tumor uptake of therapeutic molecules in a wide variety of human cancers because TNT-3 recognizes a universal nuclear antigen (16) exposed in the degenerating and necrotic cells present in all solid tumors.

The ability of chTNT-3/IL-2 to increase tumor uptake of both a MAb and a drug (IUdR) was examined in the LS174T colon adenocarcinoma xenograft model. Radiolabeled IUdR has been evaluated in animal tumor models for the diagnosis and therapy of cancers (54) and was used in our studies to determine whether chemotherapeutic drugs can also be enhanced in tumors.
in a specific manner. In this model, increased tumor uptake of 
$^{125}$I-labeled B72.3 after pretreatment with chTNT-3/IL-2 was 
both time- and dose-dependent (Fig. 3). It seems that normal 
tissue toxicity at the highest pretreatment dose administered 
limited the accumulation of B72.3 in the tumor, as levels in 
normal tissues began to increase. Under optimal conditions, 
however, pretreatment with chTNT-3/IL-2 resulted in nearly a 
3-fold increase in tumor accretion of both $^{125}$I-labeled B72.3 
(Fig. 4) and $^{125}$IUdR (Table 4), with no effect on normal tissues. 
These results are similar to those observed with chemically 
produced IL-2 immunoconjugates. The necessity for tumor lo-
calization of IL-2 was evidenced by the absence of an effect 
when mice were pretreated with the control fusion protein 
chCLL-1/IL-2 directed against B-cell malignancies. Further-
more, as illustrated in Fig. 4, the biodistribution of radiolabeled 
B72.3 in normal tissues was similar with or without the control 
and experimental fusion protein pretreatment, indicating that the 
clearance profile of B72.3 was not altered by the administration 
of MAb/IL-2 fusion proteins. In addition to improving the 
delivery of macromolecules, this pretreatment strategy has ex-
citing implications for chemotherapy, as this approach may 
decrease the systemic toxicity of anticancer drugs while produc-
ing higher tumor killing.

The effects of pretreatment with chTNT-3/IL-2 on the 
specific tumor uptake of radiolabeled antibodies were examined 
in two additional tumor models. The MAb NR-LU-10 recog-
nizes a membrane glycoprotein expressed in many carcinomas 
of epithelial origin. The tumor-targeting ability of this MAb has 
been demonstrated previously in colon tumor-bearing mice by 
biodistribution and imaging studies (55). Moreover, clinical 
studies have illustrated the potential of NR-LU-10 both for 
the diagnostic imaging and staging of patients with lung cancer 
(56) and for the radioimmunotherapy of ovarian cancer (57). In 
the present study, the ability of pretreatment with chTNT-3/IL-2 
to enhance the tumor uptake of NR-LU-10 was demonstrated in 
nude mice bearing A427 human lung adenocarcinoma tumors 
(Table 2). The tumor targeting ability of the MAb CYT-351 
reactive with a prostate-specific membrane antigen has been

### Table 2

<table>
<thead>
<tr>
<th>Organ</th>
<th>Percentage of ID/g</th>
<th>Tumor:organ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No pretreatment</td>
<td>Pretreatment</td>
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<tr>
<td>Blood</td>
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<td>1.68</td>
</tr>
<tr>
<td>Skin</td>
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<tr>
<td>Muscle</td>
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<td>0.16</td>
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<tr>
<td>Bone</td>
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<tr>
<td>Heart</td>
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<td>Liver</td>
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<td>Intestine</td>
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<tr>
<td>Kidney</td>
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</tr>
<tr>
<td>Tumor</td>
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<td>6.37</td>
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$^a$ Ps determined by the Wilcoxon’s rank-sum test.

### Table 3

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<th>Percentage of ID/g</th>
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<tr>
<td>Tumor</td>
<td>18.59</td>
<td>31.85</td>
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</table>

$^a$ Ps determined by the Wilcoxon’s rank-sum test.

$^b$ NS, not significant.
permeability. To NOS inhibition, nitric oxide generation can further increase tumors in which baseline vascular permeability is not responsive to NOS inhibition, nitric oxide generation can further increase uptake of radiolabeled MAb after pretreatment with chTNT-3/IL-2 (Fig. 5). It has recently been demonstrated that systemic inhibition of NOS has no effect on vascular permeability of the LS174T xenograft (63). In our study, we, likewise, observed no decrease in tumor uptake of radiolabeled MAb after administration of l-NMA. Hence, it seems that even in those tumors in which baseline vascular permeability is not responsive to NOS inhibition, nitric oxide generation can further increase permeability.

Modifying vascular physiology in the tumor microenvironment represents a strategy with great possibilities for improving drug delivery. Pretreatment with the fusion protein described in this study may be applicable to a wide spectrum of human malignancies because TNT-3 is expected to localize to any tumor that contains degenerating cells and necrosis. Moreover, these studies demonstrate the ability of pretreatment with chTNT-3/IL-2 to increase tumor uptake of both MAbs and small molecules such as chemotherapeutic drugs, which should improve the therapeutic potential of these reagents.

### Acknowledgments

We thank Barbara H. Biela and Myra M. Mizokami for assistance with the animal studies.

### References


### Table 4

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<th>Percentage of ID/g</th>
<th>Tumororgan ratio</th>
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<td>No pretreatment</td>
<td>Pretreatment</td>
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<td>Blood</td>
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<td>1.59</td>
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<tr>
<td>Skin</td>
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<td>1.35</td>
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<tr>
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<td>Tumor</td>
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*Ps determined by the Wilcoxon’s rank-sum test.

### NS, not significant.


Pretreatment with a Monoclonal Antibody/Interleukin-2 Fusion Protein Directed against DNA Enhances the Delivery of Therapeutic Molecules to Solid Tumors


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