Cytokine Targeting in Tumors Using a Bispecific Antibody Directed against Carcinoembryonic Antigen and Tumor Necrosis Factor α

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ABSTRACT

The use of tumor necrosis factor α (TNFα) in cancer therapy is limited by its short circulatory half-life and its severe systemic side effects. To overcome these limitations, we evaluated the capability of a bispecific antibody (BAb) directed against carcinoembryonic antigen (CEA) and human TNFα to target this cytokine in tumors. A BAb was constructed by coupling the Fab’ fragments from an anti-CEA monoclonal antibody (MAB) to the Fab’ fragments from an anti-TNFα MAb via a stable thioether linkage. The double specificity of the BAb for CEA and TNFα was demonstrated using a BIAcoretwo-step analysis. The affinity constants of the BAb for CEA immobilized on a sensor chip and for soluble TNFα added to the CEA-BAb complex were as high as those of the parental MAbs (1.7 x 10^9 M^-1 and 6.6 x 10^8 M^-1, respectively). The radiolabeled 125I-labeled BAb retained high immunoreactivity with both CEA and TNFα immobilized on a solid phase. In nude mice xenografted with the human colorectal carcinoma T380, the 125I-labeled BAb showed a tumor localization and biodistribution comparable to that of 125I-labeled anti-CEA parental Fab, with 25–30% of the injected dose (ID)/g tumor at 24 h and 20% ID/g tumor at 48 h. To target TNFα to the tumor, a two-step i.v. injection protocol was used first, in which a variable dose of 125I-labeled BAb was injected, followed 24 or 48 h later by a constant dose of 125I-labeled TNFα (1 μg). Mice pretreated with 3 μg of BAb and sacrificed 2, 4, 6, or 8 h after the injection of TNFα showed a 1.5- to 2-fold increased concentration of 125I-labeled TNFα in the tumor as compared to control mice, which received TNFα alone. With a higher dose of BAb (25 μg), mice showed a better targeting of TNFα with a 3.2-fold increased concentration of 125I-labeled TNFα in the tumor: 9.3% versus 2.9% ID/g tumor in control mice 6 h after TNFα injection. In a one-step injection protocol using a premixed BAb-TNFα preparation, similar results were obtained 6 h postinjection (3.5-fold increased TNFα tumor concentration). A longer retention time of TNFα was observed leading to an 8.1-fold increased concentration of TNFα in the tumor 14 h postinjection (4.4 versus 0.5% ID/g tumor for BAb-treated and control mice, respectively). These results showed that our BAb is able, first, to localize in a human colon carcinoma and, then, to immunoabsorb the i.v.-injected TNFα, leading to its increased concentration at the tumor site.

INTRODUCTION

Since the discovery of the MAβ3 technique by Köhler and Milstein (1), MAbs directed against tumor cells have been used for the identification and in some cases for the destruction of these cells (2). To make MAbs more efficient in the destruction of tumors, several forms of immunoconjugates have been designed by coupling drugs, toxins, or radioisotopes to MAb. One of the limitations of these immunoconjugates is the direct covalent link between the therapeutic molecule and the MAb. The biodistribution of the conjugate compared with that of the original MAb can be modified, and the efficacy of the therapeutic molecule on the target-tumor cells can be altered because of lower affinity for its receptor, for example, and to inhibition of its internalization in the form of a conjugate. To overcome these problems, a new approach was developed based on BAbs in which one arm of the antibody is directed against a tumor-associated antigen and the other arm against a therapeutic molecule (3–5), a radiolabeled peptide, and/or a chelate (6). This approach presents at least two advantages in the case of a therapeutic molecule: (a) the BAb, which is nontoxic by itself, can be injected initially in relatively large amounts, and then, when the BAb is already concentrated in the tumor, the therapeutic molecule is injected and can reach the tumor more selectively and rapidly; and (b) the therapeutic molecule is not covalently linked to the antibody and can easily “jump” onto its natural receptor present at the surface of the targeted cells or in neighboring cells leading to a broader biological effect.

The use of cytokines as adjuvants for different forms of cancer therapy represents one of the most promising areas of applied cancer research (7–11). Among these cytokines, TNFα has been shown in different animal models to have a potent antitumor activity (7–9, 12, 13). In different clinical trials of cancer therapy by systemic injection of TNFα, the results have been disappointing mainly because patients were found to have significantly lower maximum tolerated dose (about 10 μg/kg; Refs. 14 and 15) as compared with mice (400 μg/kg; Refs. 9 and 13). Studies involving regional (11, 16, 17) or intratumoral (18) injection of TNFα have demonstrated its potential for cancer therapy but only if a high enough therapeutic concentration of TNFα in the tumor with a nontoxic systemic concentration was obtained.

We report here the preparation, characterization, and preliminary in vivo evaluation of the first BAb directed against both the CEA, which is one of the best target antigens for in vivo tumor localization of MAbs (19, 20), and the TNFα cytokine. Using nude mice bearing human colon carcinoma xenografts, we have demonstrated the capability of the BAb to localize in a CEA-expressing tumor and concentrate TNFα in this tumor.

MATERIALS AND METHODS

MAbs and Fab(ab′)2 Fragments. Anti-CEA MAb 35A7 is specific for CEA and does not bind to cross-reacting antigens or to granulocytes (19). MAb 35A7 has been used for treatment of a CEA-producing human colon carcinoma transplanted into nude mice (2) and for radiodiagnostic purposes in patients (20). MAb 35A7 (IgG1) was purified from mouse hybridoma ascites fluid by ammonium sulfate precipitation (45% saturation at 4°C) followed by ion-exchange chromatography on DEAE 52 cellulose (Whatman, Balston, UK) using 0.03 M phosphate buffer (pH 8.0). MAb trifluoroacetic acid (TFA) was kindly provided by Dr. M. Brochhaus (Hoffmann-La Roche AG, Basel, Switzerland). In control experiments, normal mouse IgG1 purified from the mouse myeloma P-3X65 was used as irrelevant IgG1 (21). Fab(ab′)2 fragments were prepared by digestion with pepsin.
Co., St. Louis, MO) at a 3:100 (w/w) ratio of pepsin/ IgG and incubated at 37°C for 22 h with MAB 35A7 or 4 h in the case of tnfl8. The reaction was stopped by adding 2.5 M Tris (pH 8.0), and the F(ab')2 fragments were separated from the digest mixture by gel filtration on a Superdex 200 column (Pharmacia, Uppsala, Sweden). Each purified F(ab')2 fragment gave a band on SDS-PAGE (polyacrylamide, 7.5%) containing more than 95% of the deposited proteins.

**Preparation of BAb.** Heterodimeric F(ab')2 molecules containing two different mouse Fab' fragments were constructed as described by Glennie et al. (22) with modifications. Briefly, F(ab')2 fragments of the two MAb's in 20 mM HEPES buffer (pH 7.0), containing 150 mM NaCl and 10 mM EDTA were reduced by addition of 10 mM cysteamine (Fluka, Buchs, Switzerland) for 1 h at 37°C. The Fab' fragments were isolated immediately by G25-PD10 column chromatography (Pharmacia) equilibrated with 50 mM acetate, 0.5 mM EDTA (pH 5.3). The Fab' fragments from MAB 35A7 were mixed with a large excess (200-fold) of bifunctional cross-linker N,N'-p-phenylene-dimaleimide (Sigma Chemical Co.) diluted in dimethylformamide for 1 h at room temperature. The final concentration of dimethylformamide did not exceed 20%. This Fab' derivative was purified by G25-PD10 column chromatography with 50 mM acetate and 0.5 mM EDTA (pH 5.3). It was then added immediately to the Fab' fragment of tnfl8 with a 1:1 (mol/mol) ratio and concentrated to approximately 8–10 mg/ml. After incubation overnight at 4°C, the pH was adjusted to 8.0 with 20% of a 0.5 M HEPES buffer, and then the fragments were reduced with cysteamine at a final concentration of 10 mM for 1 h at 37°C. The resulting sulfhydryl groups were blocked by N-ethylmaleimide (Sigma Chemical Co.) at a final concentration of 30 mM for 20 min at room temperature. Finally, the BAb was separated from other products and residual reactants by passage through a Superdex 200 column (Pharmacia) gel filtration column equilibrated with 0.1 M Tris-HC1 and 0.15 M NaCI (pH 7.0). The degree of purification achieved was determined by SDS-PAGE 8.5% under reducing conditions with 2-mercaptoethanol and under nonreducing conditions. A standard protein mixture (Sigma Chemical Co.) for SDS-PAGE gel electrophoresis was used to determine molecular weights. Under the experimental conditions, molecular weight markers are applicable only between 29,000 and 97,000.

**BIAcore™ Analysis.** The binding affinities of antibodies to CEA and TNFα were measured using plasmon surface resonance with BIAcore (Pharmacia Biosensor, Uppsala, Sweden) as described by Griffiths et al. (23). The different antigens were covalently immobilized on separate sensor chips through their amine groups. The antigens were diluted in 10 mM acetate buffer at 25 μg/ml (pH 3.8) for CEA and 40 μg/ml (pH 4.2) for TNFα. To analyze the binding of soluble MAb's, MAB 35A7, tnfl8, 35A7 F(ab')2, tnfl8 F(ab')2, or CEA were injected at a concentration of 10 μg/ml in PBS. The injection was performed at a flow rate of 5 μl/min, and regeneration of the sensor chip was performed using 15 μl of 100 mM HCl. For determination of binding activities to both CEA and TNFα, BAb at 10 μg/ml in PBS was injected onto the CEA- or TNFα-coated sensor chip and followed when equilibrium was reached by a pulse of TNFα at 20 μg/ml or CEA at 95 μg/ml, respectively, as described by Holliger et al. (24).

The kinetic parameters of the binding reaction were determined using BIAevaluation software (Pharmacia Biosensor). The dissociation rate (off-rate) constant K_d was determined by iterative fitting of the experimental and theoretical curves. The association rate (on-rate) constant K_a was determined by iterative fitting of experimental and theoretical curves for each concentration and by plotting the slopes ks of dR/dt = f(R) versus the concentration values [the slope of ks = f(C) gives the value of k_assoc], where R = BIAcore™ response in RU, t = time, and C = concentration of analyte.

**Radiolodination.** Batches of 10–40 μg of 35A7 F(ab')2, tnfl8 F(ab')2, BAb, and human recombinant TNFα (gift of Dr. W. Lesslauer, Hoffmann-La Roche, Basel, Switzerland) were labeled with 100–200 μCi (1 Ci = 37 Gbq) of 125I or 131I, kindly provided by CisBio International (CisBio International, Gif-sur-Yvette, France) by the iodogen method (Pierce Chemical Co., Rockford, IL), yielding a specific activity of 1–4 μCi/μg for 35A7 F(ab')2, tnfl8 F(ab')2, BAb, and 10–20 μCi/μg for human recombinant TNFα.

**Immunoreactivity of Radiolabeled F(ab')2, BAb, and TNFα.** Immunoreactivity of radiolabeled BAb or 35A7 F(ab')2 for CEA was determined in vitro in a direct binding assay. About 20 pmol of 125I-labeled BAb or 131I-labeled 35A7 F(ab')2 were incubated for 16 h at 25°C with 3 μg of purified CEA chemically coupled to Sepharose-CNBr (Pharmacia). The percentage of binding was determined by measuring the radioactivity bound to the CEA. Immunoreactivity of radiolabeled BAb or 131I-labeled tnfl8 F(ab')2 was incubated for 16 h at 25°C with TNFα-coated polystyrene beads. After washing, the percentage of binding was determined by measuring the radioactivity bound to the beads. Immunoreactivity of radiolabeled TNFα was determined on tnfl8 MAb chemically coupled to Sepharose-CNBr. About 10 ng of 125I-labeled TNFα were incubated for 16 h at 25°C 3 μg of tnfl8 coupled to Sepharose. The percentage of binding was determined by measuring the radioactivity bound to tnfl8 MAb. The double specificity of the BAb for CEA and TNFα was determined in a one-step sandwich binding assay, in which various amounts of BAb (500, 250, 125, 60, and 30 ng) were incubated with 5 ng of 125I-labeled TNFα and 3 μg of CEA coupled to Sepharose for 16 h at 25°C. Percentage binding was determined by measuring the radioactivity bound to the CEA. In all experiments, nonspecific binding was determined by similar incubation with an irrelevant protein coupled to Sepharose.

**Human Tumor Xenografted Model.** The human colon carcinoma T380 (25) was serially transplanted s.c. into the right flank of 7–9-week-old female Swiss nu/nu mice (Iffa Credo, l'Arbresle, France). After 2 weeks, the mice were grouped according to tumor size by measuring tumor diameters (3 dimensions). Tumor volume was calculated by the formula vol = r1 x r2 x r3 x 4/3π (r = radius). Lugol iodine solution (5%) was added to the drinking water (0.2 ml/300 ml) 2 days before injection of the iodine-labeled proteins.

**Biodistribution of Radiolabeled Antibodies.** Nude mice bearing human colon carcinoma T380 were injected i.v. with a mixture of 125I-labeled BAb (3 μCi, 3 μg) and 131I-labeled 35A7 F(ab')2 (2 μCi, 3 μg) or with a mixture of 125I-labeled BAb (3 μCi, 3 μg) and 131I-labeled tnfl8 F(ab')2 (2 μCi, 3 μg). The total amount of fragments from each injected antibody was adjusted by adding unlabeled F(ab')2 fragments. Three groups of three mice were killed 6, 24, or 48 h postinjection. The radioactivity in all tissues was quantified by dual-channel γ scintillation counting of both iodine isotopes. The results were expressed as the % ID/g of tissue. As above, all data were corrected for physical decay of the isotope and for overlap of 131I into the 125I channel.

**Biodistribution of TNFα in Nude Mice.** Mice bearing T380 were injected i.v. with 2 μCi of 125I-labeled TNFα and a total amount of 500 ng of TNFα adjusted by adding unlabeled protein. Nine groups of mice (with different numbers of mice per group, results being the average of different experiments) were killed 10, 20, or 40 min, or 2, 4, 6, 8, 14, or 24 h after injection. The radioactivity in all tissues was quantified by γ scintillation counting of iodine isotope. The results were expressed as the % ID/g of tissue. As above, all data were corrected for physical decay of the isotope.

**Two-Step Injection Protocol.** Mice bearing T380 were injected i.v. with 125I-labeled BAb (3 μCi, 3 μg and 3 μCi, 25 μg) and 125I-labeled TNFα (2 μCi, 1 μg). Control mice were injected with 131I-labeled TNFα (2 μCi, 1 μg) alone. The total amount of each injected protein was adjusted by adding unlabeled proteins. Mice were then sacrificed 2, 4, 6, or 8 h after the TNFα injection, and the radioactivity in all tissues was quantified by γ scintillation counting of both iodine isotopes. The results were expressed as the % ID/g of tissue. As above, all data were corrected for physical decay of the isotope.

**One-Step Injection Protocol.** Mice bearing T380 received one injection i.v. with a mixture of 125I-labeled BAb (3 μCi, 25 μg) and 131I-labeled TNFα (2 μCi, 1 μg) preincubuted for 16 h at 4°C. Control mice were injected with 131I-labeled TNFα (2 μCi, 1 μg) alone. The total amount of each injected protein was adjusted by adding unlabeled proteins. Mice were then sacrificed 6 or 14 h after the injection of the mixture or TNFα alone. The radioactivity in all tissues was quantified by γ scintillation counting of both iodine isotopes. The results were expressed as the % ID/g of tissue. As above, all data were corrected for physical decay of the isotope.

**Statistical Analysis.** Results for TNFα tumor uptake in the two-step injection protocol (see Fig. 4) were evaluated by a two-way ANOVA after a square root transformation of the data.

**RESULTS**

**Characterization of the BAb.** BAb was purified by gel filtration on a Superdex 200 column with a retention time comparable to that of F(ab')2 fragments. The BAb fractions were pooled and were analyzed
on SDS-PAGE in comparison with F(ab')2 fragments of anti-CEA 35A7 and anti-TNFα tnfl8 MAb (Fig. 1). Under nonreducing conditions (Fig. 1A), the BAB showed one band with an apparent molecular size of 100 kd very similar and intermediary between those of the 35A7 F(ab')2 and tnfl8 F(ab')2. After reduction with 2-mercaptoethanol (Fig. 1B), the BAB showed a 50-kd band due to the dimer of pepsin-treated heavy chain covalently linked by the noncleavable thioether bond and two smaller bands of 25 and 28 kd, corresponding to the light chains of MAb tnfl8 and MAb 35A7, respectively.

Kinetic binding constants of BAB for CEA and TNFα were determined using BiAcore and compared with intact parental 35A7 and tnfl8 MABs and their F(ab')2 fragments. This technique allows real-time analysis of biospecific interactions without the need for labeling. As shown in Table 1, the affinity constant of BAB for CEA immobilized on the sensor chip compared favorably with that of 35A7 MAb and its 35A7 F(ab')2 fragments (1.7 × 10⁹, 9.7 × 10⁸, and 2.2 × 10⁸ M⁻¹, respectively). The affinity constant of BAB for the TNFα-bearing sensor chip was also very close to that of tnfl8 MAb and its tnfl8 F(ab')2 fragments (9.4 × 10⁹, 1.8 × 10⁸, and 2.5 × 10⁸ M⁻¹, respectively). The double specificity of the BAB for both CEA and TNFα was investigated using BiAcore in a two-step assay to simulate the future in vivo use (Table 1). BAB bound on the CEA sensor chip showed an affinity constant for soluble TNFα comparable to that of BAB for TNFα immobilized on the sensor chip (6.6 × 10⁸ and 9.4 × 10⁸ M⁻¹, respectively). Similar results were obtained in the reverse experiment (binding of soluble CEA to BAB bound to the TNFα-sensor chip).

These results demonstrate the double specificity of our anti-CEA/anti-TNFα BAB. The conservation of high-affinity constants of both arms for their respective antigens allowed an efficient immunoadsorption of soluble TNFα on CEA-bearing surfaces.

Before every in vivo experiment, radiolabeled BAB and parental F(ab')2 fragments were evaluated in direct binding assays. The percentage binding of ¹²⁵I-labeled BAB on CEA immobilized on Sepharose was 51.1 ± 11.1% (n = 14) as compared with 75.0 ± 3.0% (n = 3) for ¹²⁵I-labeled 35A7 F(ab')2 fragments. On TNFα-coated polystyrene beads, the binding of ¹²⁵I-labeled BAB and ¹²⁵I-labeled tnfl8 F(ab')2 was 10.1 ± 3.0% (n = 12) and 37.6 ± 5.0% (n = 4), respectively. All these tests were performed in parallel and showed that radiolabeled BAB gave about 80% of the binding of radiolabeled 35A7 F(ab')2 on CEA-Sepharose and 35% of the binding of radiolabeled tnfl8 F(ab')2 on TNFα beads. The double specificity of the BAB was confirmed in a sandwich binding assay, in which 125 ng of BAB bound to 3 μg of CEA linked to Sepharose were able to retain 35% of the 5 ng of the ¹²⁵I-labeled TNFα added. In control experiments under the same conditions, only 2% of the ¹²⁵I-labeled TNFα were bound to an irrelevant protein linked to Sepharose.

Biodistribution of Radiolabeled BAB and Parental F(ab')2 Fragments. BAB was compared in vivo with the parental F(ab')2 fragments in nude mice bearing human colon carcinoma T380 xenografts. For each comparison, three groups of three mice bearing size-matched T380 received an i.v. coinjection of ¹²⁵I-labeled BAB and ¹³¹I-labeled tnfl8 F(ab')2 or ¹²⁵I-labeled BAB and ¹³¹I-labeled 35A7 F(ab')2, and were dissected 6, 24, and 48 h later. The time-dependent biodistribution study of ¹²⁵I-labeled BAB versus ¹³¹I-labeled tnfl8 F(ab')2 (Fig. 2) showed a tumor localization of 23.2 ± 4.1% ID/g for the BAB as early as 6 h postinjection. At 24 h postinjection, the same % ID/g tumor was observed, but the ratios of the concentration in the tumor to that in nontumor tissues were increased, giving values ranging from 10 to 45 for most normal organs except blood, which gave a value of 6.3 ± 0.9. These ratios reached their highest values at 48 h postinjection, ranging from 13.3 ± 4.5 for blood to 107.0 ± 20.6 for muscle. The coincjected ¹³¹I-labeled tnfl8 F(ab')2 showed no specific localization in any organ at any time confirming that tumor localization of the BAB was due to its single anti-CEA arm, which retained high reactivity in vivo.

When ¹²⁵I-labeled BAB was coincjected with ¹³¹I-labeled 35A7 F(ab')2, BAB showed a slightly better tumor localization than the 35A7 F(ab')2 fragments at any time after injection despite the monovalence of the anti-CEA arm. In normal tissues, BAB concentration was also slightly higher than that of F(ab')2 fragments. The BAB coincjected with 35A7 F(ab')2 gave a slightly lower tumor uptake than the same BAB coincjected with tnfl8 F(ab')2, e.g., 18.9 ± 7.4 versus 23.3 ± 2.3% ID/g at 24 h postinjection). This could be due to a competition between the two anti-CEA antibodies directed against the same CEA epitope.

Kinetic of Biodistribution of Radiolabeled TNFα. The time-dependent biodistribution of radiolabeled TNFα was measured in

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**Table 1. Affinity constants of different soluble proteins for a CEA or TNFα sensor chip**

<table>
<thead>
<tr>
<th>Soluble proteins</th>
<th>CEA</th>
<th>TNFα</th>
</tr>
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<tbody>
<tr>
<td>BAB</td>
<td>1.7 × 10⁹</td>
<td>9.4 × 10⁸</td>
</tr>
<tr>
<td>TNFα on BAB</td>
<td>6.6 × 10⁹</td>
<td>ND</td>
</tr>
<tr>
<td>CEA on BAB</td>
<td>ND</td>
<td>2.5 × 10⁸</td>
</tr>
<tr>
<td>35A7</td>
<td>9.7 × 10⁸</td>
<td>ND</td>
</tr>
<tr>
<td>35A7 F(ab')₂</td>
<td>2.2 × 10⁹</td>
<td>No fixation</td>
</tr>
<tr>
<td>tnfl8 F(ab')₂</td>
<td>ND</td>
<td>1.8 × 10⁹</td>
</tr>
<tr>
<td>TNFα</td>
<td>No fixation</td>
<td>ND</td>
</tr>
<tr>
<td>TNFα on BAB</td>
<td>ND</td>
<td>No fixation</td>
</tr>
</tbody>
</table>

*The Ka (kₐ/kₐ₋) values (M⁻¹) for the interaction of different soluble proteins with CEA or TNFα immobilized on different sensor chips were determined using BiAcore and BlAevaluation (Pharmacia). For the determination of the Ka values of CEA or TNFα on BAB, BAB was first exposed to a TNFα- or CEA-bearing sensor chip, and when equilibrium was reached, CEA or TNFα was then injected. ND, not done.

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**Fig. 1.** Gel electrophoretic analysis of BAB. Parental MABs anti-CEA 35A7, anti-TNFα tnfl8, and corresponding F(ab')₂ fragments were compared with BAB on an 8.5% SDS-PAGE gel under nonreducing (A) and reducing (B) conditions. Under the experimental conditions, molecular weight markers are applicable only between 29,000 and 97,000. Results are shown after Coomassie blue staining.
nude mice bearing T380 tumors from 10 min to 24 h after i.v. injection (Fig. 3). The results showed a very rapid clearance from the circulation by kidney elimination (34.7 ± 4.4% ID was recovered per g kidney 10 min after injection).

The tumor uptake of radiolabeled TNFa increased from 10 min to 2 h postinjection (2.6 ± 0.2, 3.5 ± 0.4, 5.4 ± 0.6, and 6.3 ± 1.1% ID/g tumor at 10, 20, 40 min, and 2 h, respectively) and then decreased (5.2 ± 1.1, 3.6 ± 0.8, 2.3 ± 0.3, 0.9 ± 0.3, and 0.3 ± 0.1% ID/g tumor at 4, 6, 8, 14, and 24 h, respectively). There was no preferential accumulation of radiolabeled TNFa in any organ; the high radioactivity observed in stomach (not shown) is due to accumulation of free iodine as demonstrated by Palladino et al. (26), who compared the biodistribution of 125I-labeled recombinant human TNFa versus [3H] recombinant human TNFa.

The blood disappearance curves of 125I-labeled TNFa fitted a biexponential model with values of 9 min, 55 s and 5 h, 17 min for the α and β half-lives, respectively.

Targeting of TNFa in the Tumor by the BAb. Before in vivo experiments, immunoreactivity of radiolabeled TNFa was evaluated in direct binding assays. The percentage of binding of 125I-labeled TNFa on fn18 coupled to Sepharose was 79.8 ± 1.2%. In control experiments under the same conditions, only 2.8 ± 1.0% of the 125I-labeled TNFa was bound to an irrelevant protein linked to Sepharose.

The ability of BAb to target TNFa in the CEA-expressing tumor T380 was evaluated by different protocols including various schedules of injection and doses. In the first series of experiments, we used a two-step injection protocol. From BAb distribution studies (Fig. 2),
a delay of 24 h was chosen between BAb and TNFα injections. At that time, the BAb is clearly localized in the tumor. The blood concentration of BAb is not too high (about 4% ID/g) but is sufficient to trap any excess of i.v.-injected TNFα. Mice were preinjected with 3 µg of 125I-labeled BAb 24 h before the injection of 1 µg of 131I-labeled TNFα. Mice sacrificed 2, 4, 6, and 8 h after TNFα injection showed a tumor uptake of BAb similar to that obtained previously with a % ID/g of tumor ranging from 30.97 ± 3.34 to 24.47 ± 6.02 for the nude mice dissected 26 h (24 + 2 h) to 32 h (24 + 8 h) postinjection, respectively. At all times, the BAb-injected mice showed a higher tumor uptake of TNFα and higher ratios of tumor:normal tissue concentration than control mice, which received only TNFα. Analysis of the results showed that the cumulative increase of TNFα tumor uptake in BAb-treated mice, as compared with controls, was quantitatively important. For instance, as shown in Fig. 4, the area under the curve of the BAb-treated tumors showed that between 2 and 8 h after TNFα injection, the BAb-treated mice retained 81.7% more TNFα in the tumors than the controls. The highest difference of TNFα tumor concentration between BAb-treated mice and control mice was obtained 6 h after TNFα injection: tumor uptake of TNFα was 2-fold greater in the BAb preinjected mice compared with control mice (7.20 ± 0.84 versus 3.61 ± 0.83% ID/g tumor, respectively; Fig. 5A).

Tumor:normal tissue ratios of TNFα concentration were also increased in BAb-preinjected mice with values ranging from 1.70 ± 0.25 for blood to 9.45 ± 2.04 for muscle as compared with 1.25 ± 0.38 and 6.62 ± 2.84 for the same organs in control mice that received only TNFα.

In the second series of experiments, we used this delay at 6 h between TNFα injection and sacrifice to analyze the effect of BAb dose. A BAb dose of 2.5 µg was compared to the previous dose of 3 µg. With this higher dose, 2 delays of BAb pretreatment were studied at 24 and 48 h. The 48-h delay was tested with this BAb dose because the circulating BAb concentration was only about 2% ID/g (Fig. 2), at 24 and 48 h. The 48-h delay was tested with this BAb dose because the circulating BAb concentration was only about 2% ID/g (Fig. 2), which represents about 1 µg of circulating BAb per mouse. Mice that received 1 µg of 131I-labeled TNFα 24 h after BAb injection of 25 µg and that were sacrificed 6 h after injection of TNFα showed a 3.1-fold tumor uptake of TNFα as compared to control mice, which received only TNFα (9.28 ± 1.06 versus 2.96 ± 0.62% ID/g tumor, respectively; Fig. 5B). As compared with previous mice injected with 3 µg of BAb and the same protocol, mice injected with this higher dose of BAb showed increased tumor:normal tissue ratios of TNF concentration with values ranging from 2.89 ± 0.58 for blood to 12.5 ± 2.92 for muscle. A BAb pretreatment period of 48 h resulted in higher tumor:normal tissue ratios (from 3.37 ± 0.68 for blood to 14.41 ± 3.07 for muscle) with a similar tumor uptake of TNFα (9.59 ± 0.66% ID/g of tumor).

In a third series of experiments, to avoid any problem of TNFα toxicity, a one-step injection protocol was used with a premixed BAb-TNFα preparation. BAb and TNFα were mixed at a molar ratio of 12.5:1 (25 µg BAb and 1 µg TNFα) and incubated for 16 h at 4°C. Mice were sacrificed 6 h postinjection for comparison with previous series or 14 h postinjection to analyze the effect of BAb pretreatment at the time at which 76% of free TNFα is eliminated from the mouse in our model (Fig. 3). The TNFα tumor uptake enhancement, as compared to mice receiving TNFα only, was 3.25 (7.15 ± 1.41 versus 2.2 ± 0.8% ID/g) and 8.15 (4.4 ± 0.8 versus 0.54 ± 0.2% ID/g) for the 6- and 14-h period, respectively (Fig. 6). With this one-step protocol, TNFα was stabilized by the BAb. The recovered TNFα was 35.6% versus 31.1% for the 6-h period and 8.0% versus 4.8% for the 14-h period in BAb-treated and control mice, respectively. The calculation of the area under the curve of the % ID TNFα/g tumor between 6 and 14 h for mice injected with the mixture showed a 319% increase as compared to control mice injected with TNFα alone.

**DISCUSSION**

Different attempts have been made to target cytokines in tumors in animal models as well as in humans. Some authors have used intratumoral or locoregional injection of TNFα (16, 18). Others have coupled interleukins to MAbs (27—29) or designed fusion proteins containing antitumor scFv linked to cytokines (30, 31), while, in the field of gene therapy, genes of different cytokines have been transfected into tumor cells (32, 33). We describe here, for the first time to our knowledge, the use of a BAb to target a cytokine to a tumor. As a model, we prepared and characterized a BAb reacting with CEA and TNFα. CEA is one of the best target antigens for in vivo tumor localization of MAbs (20), and TNFα has been shown not only to induce necrosis in tumors (16) but also to induce tumorvascular hemorrhage (34), to augment the lethal effects of radiation against certain tumor cell lines (35—38), and to increase the permeability of the tumor vessels before inducing their disruption (39—41). We demonstrated (a) that our anti-TNFα/anti-CEA BAb can localize with a high degree of specificity into human colon carcinoma xenografted in nude mice, and (b) that the BAb can help to target to the tumor radiolabeled TNFα either injected 24 or 48 h after the BAb or coinjected with the BAb.

**Fig. 4.** Comparison of the kinetics of TNFα tumor uptake in nude mice bearing a T380 xenograft treated or not with BAb. Control mice (C) and mice pretreated (24 h before) with 3 µg of BAb (O) received an i.v. injection of 131I-labeled TNFα and were dissected 2, 4, 6, or 8 h later. Results are expressed as the mean of percentages of ID of TNFα/g tumor with one SD (bars) for seven or eight mice. The gray area represents an 81.7% increase of tumor TNFα uptake in BAb-treated mice as compared with control mice. A two-way ANOVA concluded to a significant effect of BAb pretreatment (P < 0.001).
because they may be valid only for anti-CEA MAb 35A7, which is known to localize very efficiently in tumor, even as Fab fragments (19, 20), and to be very stable upon chemical modification (44, 45).

The BAb affinity constants were determined using BlAcore technology. We demonstrated that the kinetic binding constants of the BAt, against CEA and TNFα were not significantly different from those of the parental F(ab')2 fragments: about $1.7 \times 10^9 \text{ M}^{-1}$ for CEA and $9.4 \times 10^8 \text{ M}^{-1}$ for TNFα. In a two-step BlAcore experiment such as that used by Holliger et al. (24) for the determination of the bispecificity of their “diabodies,” TNFα was bound to a CEA-bearing sensor chip incubated previously with BAb. This binding of TNFα to immobilized CEA via the BAb not only confirms its bispecificity but also represents an in vitro precursor experiment for the TNFα tumor targeting strategy that we want to develop in vivo.

As a first step for the in vivo evaluation of the anti-CEA/anti-TNFα BAb, we used as target the human colorectal carcinoma T380 grafted in nude mice. An extensive time-dependent biodistribution study of $^{125}$I-labeled TNFα allowed us to determine the behavior of free TNFα in this model. The short blood half-lives of $^{125}$I-labeled TNFα ($t_{1/2} = 9 \text{ min}$, $55 \text{ s}$; $t_{1/2} = 5 \text{ h}$, $17 \text{ min}$) were in good agreement with those reported by different authors (9, 26). Radiolabeled TNFα

Using anti-CEA 35A7 and anti-TNFα tmf18 F(ab')2 fragments, BAb was prepared by the bis-maleimide linker method according to Glennie et al. (22). Preparation of BAb by chemical coupling of Fab' fragments was preferred, as a first approach, to the quadroma (42) or genetic engineering technologies (24), because the chemical coupling is relatively easy and allows the production of BAb in amounts compatible with in vivo evaluation (about 1 mg of BAb can be produced for 2.5 mg of F(ab')2 fragments from each of the two parental MAbs). Among the various coupling procedures, the bis-maleimide linker method provides a bispecific molecule insensitive to 2-mercaptoethanol reduction in vitro and stable in vivo. Quadri et al. (43) used this procedure to stabilize antiferritin F(ab')2 fragments and demonstrated a 2.5-fold higher tumor uptake as compared with unmodified F(ab')2 in nude mice bearing hepatoma tumor xenografts. Interestingly, our BAb, which represents a monovalent anti-CEA molecule, localized as well as the divalent F(ab')2 fragments from the parental anti-CEA MAb 35A7 in a human colon carcinoma grafted in nude mice. This observation suggests strongly that divalent antibodies or F(ab')2 fragments use only one arm for their localization in CEA-expressing tumors. These results, however, cannot be generalized.
diffused rapidly into the human colon carcinoma T380 grafted in nude mice followed by a rapid decrease of % ID/g tissue. The tumor was the only tissue, with skin to a lesser extent, which showed an increase of TNFα concentration from 10 min to 2 h postinjection, with values of 2.65 ± 0.20 and 6.34 ± 1.17% ID/g tumor, respectively. This tumor uptake of TNFα can be explained by the relatively high blood volume, blood flow, and vascular permeability, which we have demonstrated in this human colon carcinoma xenograft (39). It is known that these vascular parameters are essential for tumor penetration of circulating ligand (46). An alternative explanation of the relatively high tumor localization of TNFα in control mice is the possible expression of the TNFα-receptor p55 or p75, which can retain the cytokine in the tumor microenvironment, or the presence of the TNFα-receptor p55 on endothelial cells in tumor vessels of murine origin. It is known that murine p55 receptor can bind human TNFα (47). The expression of the TNFα receptor on tumor cells or in tumor vessels may represent an advantage for the induction of the biological effect of TNFα, but, in the present evaluation of tumor targeting of TNFα, its presence may increase the baseline value of free TNFα tumor uptake.

The efficiency of BAb to target TNFα in a CEA-expressing tumor was demonstrated in different protocols. In the two-step protocol, the tumor targeting of TNFα was demonstrated even with a dose of only 3 μg of BAb (BAb:TNFα molar ratio, 1.5), because we obtained 7.20% ID TNFα/g tumor versus 3.61% ID TNFα/g tumor in the control mice. Pretreatment of mice with 25 μg of BAb (BAb:TNFα molar ratio, 12.5) allowed a better targeting of TNFα in the tumor with up to 9.3% versus 2.9% ID TNFα/g tumor in control mice and higher tumor:normal tissue ratios ranging from 2.89 ± 0.58 for blood to 12.50 ± 2.92 for muscle. In all these experiments, whole-body retention of TNFα was slightly increased in BAb-treated animals as compared to control mice, suggesting that when injected into BAb-pretreated mice, TNFα is at least partially bound to circulating BAb. As shown by Tracey et al. (48) and Ashkenazi et al. (49), anti-TNFα antibodies or TNF receptor immunoadhesins have been shown to reduce TNFα systemic toxicity. To avoid any TNFα toxicity, BAb was also evaluated in a one-step protocol using a premixed BAb-TNFα injection (BAb:TNFα molar ratio, 12.5). BAb was also efficient to target TNFα in the CEA-expressing tumor, with this protocol giving a 3.25 and 8.15 TNFα tumor uptake enhancement as compared to control mice, 6 h and 14 h postinjection of the mixture, respectively. Taken together, these data demonstrate the targeting of TNFα in a CEA-expressing human tumor xenograft in nude mice, suggesting that such a BAb could be used to increase selectively the antitumor effect of a given amount of injected TNFα with relatively reduced systemic toxicity.

Different authors have tried to use antibodies to target TNFα. Hoogenboom et al. prepared a fusion protein consisting of an anti-transferrin receptor Fab fragment linked to TNFα (31). These authors showed conservation of TNFα and MAb activities on target cells in vitro. However, to our knowledge, no results have been published concerning the in vivo use of this fusion protein. Rosenblum et al. (29) chemically coupled TNF to a MAb directed against melanoma cells (ZME-018). They first published an in vitro evaluation showing cytotoxic activity of the conjugate on A375-M melanoma cells. In a recent paper, the same group studied the pharmacokinetics of a ZME-TNF conjugate and showed, in a single therapeutic experiment in nude mice bearing A375-M tumors, that injection of the immunoconjugate induced growth inhibition of the tumors as compared with mice treated with TNF alone or with saline (50). However, the biodistribution results were only given in tissue: blood ratios 72 h postinjection, precluding calculation of the dose of ZME-TNF targeted in the tumor. Khawli et al. (28) prepared seven vasoactive immunonoconjugates with the murine MAb TNT-1, which binds necrotic regions of tumors. The authors showed an increased tumor uptake of TNT-1 Fab(ab’)2 in mice pretreated with a TNT-1/TNFα conjugate as compared with control mice pretreated with intact TNT-1. The increase was significant, 4.00 versus 1.28% ID/g tumor; however, the authors did not analyze the tumor uptake of the TNT-1/TNFα conjugate itself, precluding the calculation of the dose of TNFα targeted in the tumor by the conjugate.

In the present study, we demonstrated for the first time that an anti-CEA/anti-TNFα BAb can facilitate the targeting of TNFα in a CEA-expressing tumor. Up to now, all our experiments have been performed in the model of nude mice bearing human tumor xenografts, because we were only addressing the mechanistic aspect of cytokine targeting. The next step will be the analysis of the biological effect of the targeted TNFα from the vasoactive (39–41), the inflammatory, and the antitumor point of view (16). However, there is a need for an immunocompetent animal in which all the effects of the targeted cytokines can be analyzed. Such an animal model is being developed in our laboratory with CEA-transgenic mice transplanted with syngenic CEA-expressing tumor and will be used in future experiments.


Cytokine Targeting in Tumors Using a Bispecific Antibody Directed against Carcinoembryonic Antigen and Tumor Necrosis Factor α

Bruno Robert, Jean-Pierre Mach, Jean-Claude Mani, et al.


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