**ABSTRACT**

The disulfide-stabilized Fv (dsFv) is a novel form of a variable-region fragment (Fv) of an antibody which is stabilized by an interchain disulfide bond. As a consequence, it is more stable than its Fv analogue. Anti-Tac(dsFv) is derived from anti-Tac(IgG) which specifically binds to the p55 subunit of the interleukin-2 receptor (IL2Rα). The receptor is found in large numbers on activated T cells and many T-cell leukemias. The biodistribution patterns of 125I-anti-Tac(dsFv) and 125I-anti-Tac(IgG) were determined in athymic nude mice bearing two s.c. tumors, one expressing a stably transfected plasmid encoding IL2Rα (ATAC4) and one composed of parental untransfected A431 epidermoid carcinoma cells. Anti-Tac(dsFv), which has a molecular weight of 25,000, was specifically captured by the ATAC4 tumors but not by control A431 tumors. The antigen-specific tumors accumulated >2% of the injected dose/g within 15–45 min after i.v. injection. The level of radioactivity in the ATAC4 tumors was maintained at >1% of the injected dose/g for nearly 6 h, at which time the ATAC4 tumors contained 11-fold more 125I-anti-Tac(dsFv) than did the A431 tumors. Unbound 125I-anti-Tac(dsFv) was rapidly cleared from the blood with apparently biphasic pharmacokinetics (α1 = 10 min; β = ~5.5 h). Initially, the bulk of the 125I-anti-Tac(dsFv) appeared in the kidneys. In contrast, 125I-anti-Tac(dsFv) showed no tumor- or tissue-specific uptake over the 24-h time course of the experiments and remained primarily in the blood stream (blood clearance t1/2 = ~12 h).

This is the first report of the biodistribution of a dsFv fragment. Because of its rapid uptake by the IL2 receptor-bearing tumors, short serum half-life, and increased stability, radiolabeled anti-Tac(dsFv) may be useful for the imaging and therapy of neoplasias expressing the IL2 receptor.

**INTRODUCTION**

Radiolabeled monoclonal antibodies are being actively developed for both the diagnosis and therapy of solid tumors and leukemias (reviewed in Refs. 1–4). Two of the most serious difficulties involved in radioimmunoimaging and radioimmunotherapy are the low specific uptake of the radiolabeled antibody by the tumor and the high level of nonspecific radiation exposure due to its slow plasma clearance. These shortcomings are primarily due to transport problems related to the large size of a monoclonal antibody. Antibodies are large molecules (M, >150,000) that pass through the vascular endothelium into the interstitial space of tumors at a relatively slow rate (5). Therefore, it can take several days to get a substantial amount of antibody delivered to cells in the interior of a tumor mass. Likewise, molecules of this size are not filtered out of the circulation by the kidney (6); therefore, the radiation dose to the whole body and particularly the bone marrow from unbound antibody is undesirably high.

In order to increase the rate of transport of an antibody out of the blood stream, through the interstitial tissue, and into tumors, antibody fragments (e.g., F(ab)_2, Fab, and Fv) have been used (7–13). The smallest of these are Fv fragments. An Fv is an antigen-binding protein composed of only the V_H and V_L from a monoclonal antibody. Fv fragments have been produced in a number of different forms. If the V_H and V_L domains are not covalently linked, then the molecule is known simply as an Fv. If the two domains are covalently connected by a peptide linker into a single polypeptide, then the molecule is called a scFv. Finally, if V_H and V_L are independent gene products but are covalently linked to one another by an interchain disulfide bond, then the molecule is known as a dsFv.

Studies comparing the efficacy of Fv immunotoxins (M, ~62,000) with that of IgG immunotoxins (M, ~200,000) have demonstrated the superiority of the former in irradiating xenograft tumors in athymic nude mice; therapeutic doses required for complete remission were 17-fold lower for an Fv immunotoxin compared to an IgG immunotoxin (3). In cases where factors such as the affinity of the antigen-binding domain, the enzymatic activity of the toxin domain, the cytotoxic activity of the preparation on cultured cells, and the immunoreactivity of the preparation are approximately the same, the increased efficacy can be attributed to better tumor penetration by the Fv immunotoxins, which allows them access to a larger number of tumor cells. Experiments evaluating the ability of radiolabeled antibodies and antibody fragments to penetrate tumors in vivo have demonstrated an inverse relationship between the molecular weight and depth of penetration (14, 15).

Although all three of the Fv analogues should have improved transport properties due to their small size, the dsFvs have distinct advantages. Simple Fvs, being noncovalently associated dimers, are unstable at low protein concentrations because the first-order reaction of dimer dissociation surpasses the second-order reaction of dimer formation. The scFvs are less sensitive to inactivation at low concentrations because the heavy and light variable domains are tethered together by a peptide linker. However, this form of Fv is very susceptible to aggregation; perhaps this is due to a “daisy-chain” formation, which can occur when the V_H and V_L domains dissociate and then reassociate with complementary domains on neighboring dissociated scFvs. The dsFvs and their immunotoxin counterparts are more resistant to denaturation by either elevated temperature or urea than either the simple Fv or the scFv, presumably due to their centrally located interdomain disulfide linkage (16, 17, 31). In addition, in one instance with a scFv made from monoclonal antibody c23, which interacts with the erbB2 oncoprotein product, the dsFv had significantly better binding than the single-chain Fv (18).

We have prepared a dsFv fragment of the anti-Tac antibody (19) by recombinant DNA technology (31). Amino acid residues in conserved framework regions of both the heavy and light variable domains of the anti-Tac antibody were mutated into cysteines at positions compatible with the formation of an interdomain disulfide linkage (i.e., V_H-44gly and V_L-99ser). The mutant subunits form a disulfide-bonded Fv molecule, which binds to the α-subunit of the IL2 receptor (IL2Rα) with an affinity identical to that of humanized anti-Tac IgG and the scFv. This dsFv proved to be substantially more resistant to denaturation by heat or urea treatment than the scFv.

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1 To whom requests for reprints should be addressed, at Laboratory of Molecular Biology, Division of Cancer Biology, Diagnosis and Centers, National Cancer Institute, NIH, 9000 Rockville Pike, Building 37, Room 4E16, Bethesda, MD 20892.

2 The abbreviations used are: V_H, heavy chain variable domain; V_L, light chain variable domain; scFv, single-chain Fv; dsFv, disulfide-stabilized Fv; ID/g, injected dose/g; RI, radiolocalization indices; IL2, interleukin 2.

3 L. Pui and I. Pastan, unpublished data.
We report here the radiolabeling of anti-Tac(dsFv) and anti-Tac(IgG) with $^{125}$I-Bolton-Hunter reagent, followed by determination of the biodistribution of these proteins in athymic nude mice carrying s.c. p55-expressing tumors.

MATERIALS AND METHODS

**Protein Production.** Production of anti-Tac(dsFv) was essentially as described previously (31) with the addition of a final purification with organomercurial affinity gel to remove incompletely oxidized dsFv molecules. Briefly, the VH and VL chains of anti-Tac(dsFv) were expressed in separate Escherichia coli cultures from the plasmids pYR43 and pKW50, respectively (17, 31). The proteins were recovered as cytosolic inclusion bodies from detergent-solubilized cells as described by Buchner et al. (20). Inclusion bodies, solubilized in 6 M guanidine-HCl and reduced with diithioerythritol, were combined in equimolar amounts and refolded by dilution into a glutathione-containing redox-shuffling buffer (16). Refolded anti-Tac(dsFv) was purified by chromatography on Q- and S-sepharose, Mono-S (Pharmacia, Piscataway, NJ), and TSK-3000 (TosoHaas, Philadelphia, PA). The TSK-3000-purified material was mixed with Affi-Gel 501 organomercurial affinity matrix (0.03 ml packed gel/mg protein) to remove any non-disulfide-linked molecules. The IgG antibody used in this study is a humanized form of the anti-Tac monoclonal antibody described by Queen et al. (21).

**Radiolabeling.** Anti-Tac(dsFv) and humanized anti-Tac monoclonal IgG were labeled with mono-iodo-$^{125}$I-Bolton-Hunter reagent (New England Nuclear, Boston, MA) to a specific radioactivity of ~1.4 $\mu$Ci/$\mu$g as described by Bolton and Hunter (22). The labeled proteins were separated from unincorporated radionuclide by size-exclusion chromatography on a PD10 column (Pharmacia Fine Chemicals, Piscataway, NJ).

**SDS-PAGE and Autoradiography.** Labeled proteins were analyzed by electrophoresis through 10–20% gradient polyacrylamide gels (Bio-Rad, Richmond, CA) using the SDS-containing buffer system of Laemmli (23). Samples were pretreated with 5% $\beta$-mercaptoethanol in SDS-PAGE sample buffer at 90°C if reduction was needed. After electrophoresis, the gels were autoradiographed for 12 h.

**Immunoreactivity.** Immunoreactivities of the labeled antibodies were determined by the method of Lindmo et al. (24) on adherent cultures of ATAC4 cells. A trace amount of radiolabeled antibody was incubated with different numbers of cells, ranging from $2 \times 10^4$ to $5 \times 10^5$. The immunoreactivities were ascertained from the Y-intercept of a double reciprocal plot of the fraction of the radioactivity specifically bound versus the cell number. The immunoreactivity for both $^{125}$I-anti-Tac(dsFv) and anti-Tac(IgG) was greater than 90%.

**Cell Lines.** A431, a human epidermoid carcinoma cell line originally obtained from G. Todoro (NIH), was grown in RPMI 1640 supplemented with 5% fetal bovine serum. ATAC4 cells were derived from the A431 cell line by cotransfection with plasmids encoding the $\alpha$ subunit of the IL-2 receptor and a neomycin resistance gene (25). They contain approximately $2 \times 10^9$ subunits per cell. ATAC4 cells were grown in DMEM supplemented with 5% fetal bovine serum and 750 $\mu$g/ml of G-418. Both cell lines were grown at 37°C in 5% CO$_2$ atmosphere.

**Biodistribution Assay.** Athymic female nude mice weighing ~20 g were each inoculated with $3 \times 10^6$ A431 cells (negative control) in the right flank and $3 \times 10^6$ ATAC4 cells in the left flank. After 10–12 days of growth, when tumors were 0.5- to 1.0-cm diameter, the mice received injections via the tail vein with 4 $\mu$g of either humanized anti-Tac IgG or anti-Tac(dsFv) carrying ~5.5 $\mu$Ci of $^{125}$I. At time points between 15 min and 24 h, mice were anesthetized with Metofane (Pitman-Moore, Mundelein, IL), blood samples were taken from the tail, and animals were killed by cervical dislocation. Tumors and organs were removed, rinsed with PBS, weighed, and counted in a Beckman 5500B gamma counter (Beckman Instruments, Fullerton, CA).

**RESULTS**

**Analysis of Labeled Protein.** The anti-Tac(dsFv) used for iodination was prepared to near homogeneity as described in "Materials and Methods" and migrated with a $M_r$ of ~25,000 upon SDS-PAGE (Fig. 1). Analysis of the radioiodinated antibodies by SDS-PAGE followed by autoradiography demonstrated that greater than 95% of the radioactivity was bound to the antibody proteins. The amount of radioactivity in the heavy and light chains of the IgG was directly proportional to the molecular weight of each, indicating that conjugation with Bolton-Hunter reagent did not occur preferentially on either subunit. The VH and VL subunits of the radioiodinated dsFv migrated too close to one another on reducing SDS-PAGE to determine if one of them was preferentially labeled. No loss of immunoreactivity due to labeling was detected for either the IgG or the dsFv. The Y-intercept on the double-reciprocal plot was 0.84 for both antibody forms (Fig. 2).

**Biodistribution.** The biodistribution of $^{125}$I-anti-Tac(dsFv) and $^{125}$I-anti-Tac(IgG) were determined in athymic nude mice each bearing two tumors. One tumor was composed of antigen-positive ATAC-4 cells, and the other was composed of antigen-negative A431 cells. No difference could be detected in growth rate or morphological appearance between the two types of tumors. Radioactivity was determined in tumors, blood, spleen, kidney, liver, stomach, and lung (Fig. 3 and Tables 1 and 2).

Within the first 15 min after injection of $^{125}$I-anti-Tac(dsFv), the ATAC4 tumor accumulated greater than 3% of the ID/g of tissue (Fig. 3). This is approximately equal to the amount of radiolabel in the blood at this time and higher than that found in any other tissue analyzed except for kidney. The ATAC4 tumors maintained a level of $^{125}$I greater than 2% of the ID/g for at least 1.5 h. The uptake of radiolabel in the antigen-positive tumor (ATAC4) was approximately 2-fold higher than that observed for the antigen-negative A431 tumor at 15 min, and by 6 h, this ratio had increased to ~11.

As expected from previous studies, over the first 24 h there was no specific accumulation of $^{125}$I-anti-Tac(IgG) into tumors (Fig. 3B; Table 2). In addition, the level of $^{125}$I in the blood was at least an order of magnitude higher than with the dsFv. Between 15 and 90 min, the blood level remained constant at approximately 32% of the ID/g. The
Table 1. '251-Anti-Tac(dsFv) and '251-Anti-Tac(IgG) Percentage of ID/g and RI.

<table>
<thead>
<tr>
<th>Time</th>
<th>Blood</th>
<th>ATAC4</th>
<th>A431</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Liver</th>
<th>Stomach</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>3.05 (0.65)</td>
<td>3.10 (0.07)</td>
<td>1.55 (0.38)</td>
<td>0.93 (0.20)</td>
<td>121.0 (10.0)</td>
<td>1.64 (0.16)</td>
<td>2.03 (0.28)</td>
<td>1.87 (0.30)</td>
</tr>
<tr>
<td>45 min</td>
<td>3.07 (1.02)</td>
<td>2.03 (0.73)</td>
<td>0.55 (0.16)</td>
<td>0.34 (0.07)</td>
<td>67.7 (9.3)</td>
<td>0.72 (0.15)</td>
<td>0.69 (0.11)</td>
<td>0.72 (0.17)</td>
</tr>
<tr>
<td>90 min</td>
<td>0.78 (0.06)</td>
<td>2.27 (0.42)</td>
<td>0.31 (0.03)</td>
<td>0.28 (0.02)</td>
<td>31.0 (13.3)</td>
<td>0.55 (0.09)</td>
<td>0.75 (0.21)</td>
<td>0.50 (0.10)</td>
</tr>
<tr>
<td>6 h</td>
<td>0.30 (0.05)</td>
<td>2.47 (1.01)</td>
<td>0.97 (0.21)</td>
<td>1.13 (0.03)</td>
<td>2.48 (0.72)</td>
<td>0.71 (0.10)</td>
<td>0.28 (0.09)</td>
<td>0.16 (0.02)</td>
</tr>
<tr>
<td>24 h</td>
<td>0.30 (0.05)</td>
<td>2.47 (1.01)</td>
<td>0.97 (0.21)</td>
<td>1.13 (0.03)</td>
<td>2.48 (0.72)</td>
<td>0.71 (0.10)</td>
<td>0.28 (0.09)</td>
<td>0.16 (0.02)</td>
</tr>
</tbody>
</table>

*Values are % of ID/g tissue (SD); n = 4.

Table 2. '251-Anti-Tac(IgG) Percentage of ID/g and RI.

<table>
<thead>
<tr>
<th>Time</th>
<th>Blood</th>
<th>ATAC4</th>
<th>A431</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Liver</th>
<th>Stomach</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>32.8 (3.3)</td>
<td>1.56 (0.26)</td>
<td>1.42 (0.57)</td>
<td>8.65 (1.66)</td>
<td>7.81 (2.26)</td>
<td>8.58 (2.53)</td>
<td>6.23 (0.23)</td>
<td>7.15 (0.88)</td>
</tr>
<tr>
<td>45 min</td>
<td>32.1 (3.5)</td>
<td>2.62 (0.56)</td>
<td>1.49 (0.51)</td>
<td>8.60 (0.89)</td>
<td>7.26 (1.22)</td>
<td>7.93 (1.49)</td>
<td>2.02 (0.20)</td>
<td>1.40 (0.88)</td>
</tr>
<tr>
<td>90 min</td>
<td>30.2 (3.6)</td>
<td>3.96 (0.63)</td>
<td>2.28 (0.34)</td>
<td>5.33 (0.52)</td>
<td>7.20 (1.39)</td>
<td>5.99 (2.21)</td>
<td>1.92 (0.23)</td>
<td>8.96 (2.09)</td>
</tr>
<tr>
<td>6 h</td>
<td>14.7 (4.4)</td>
<td>5.04 (1.45)</td>
<td>2.96 (0.34)</td>
<td>6.57 (2.57)</td>
<td>4.27 (0.42)</td>
<td>4.76 (1.06)</td>
<td>1.90 (0.19)</td>
<td>4.77 (0.84)</td>
</tr>
<tr>
<td>24 h</td>
<td>8.7 (5.8)</td>
<td>3.78 (2.12)</td>
<td>3.75 (1.47)</td>
<td>5.56 (2.89)</td>
<td>2.45 (0.91)</td>
<td>3.03 (0.40)</td>
<td>1.34 (0.35)</td>
<td>2.22 (1.61)</td>
</tr>
</tbody>
</table>

*Values are % of ID/g tissue (SD); n = 4.
Kidney uptake of the $^{125}$I from the IgG was much different than was seen for the dsFv. The highest kidney level measured was at 90 min (2.5% of the ID). This level decreased over the course of the experiment with approximately monophasic kinetics ($t_{1/2} \approx 14$ h).

**DISCUSSION**

We have studied the biodistribution patterns of $^{125}$I-labeled anti-Tac(dsFv) and humanized antiTac(IgG) in athymic nude mice bearing antigen-positive (ATAC4) and antigen-negative (A431) tumors. The $^{125}$I-antiTac(dsFv) bound quickly and specifically to the antigen-expressing ATAC4 tumor. The level of uptake by the tumors (2–3% of the ID/g tissue) was comparable to that reported for radiolabeled scFvs (12, 26, 27). The antigen-specificity of the binding was demonstrated by the lack of significant uptake of antibody by the homologous, antigen-negative A431 tumor.

Due to the small size of the dsFv ($M_t \approx 25,000$), it is expected to be efficiently filtered from the blood as it passes through the kidney. This was confirmed by the high level of radiiodine in the kidney (>120% of ID/g) at the earliest time point and the correspondingly low level of radiiodine in the blood (3% of ID/g). The kidney does not sequester the radiolabeled protein or the radiiodine but rapidly excretes the bulk of the radiolabel into the urine. The radiiodine in the urine, at all time points tested, is not protein bound, as determined by SDS-PAGE and autoradiography (data not shown). This is to be expected since proteins that are filtered by the kidney are normally degraded quickly by proteases in the proximal tubule cells (6, 28). An additional

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**Fig. 3.** Percentage of ID/g of tissue. The data are derived from mice receiving injections via the tail vein with 4 µg, $5.5$ µCi of (A) $^{125}$I-antiTac(dsFv) or (B) $^{125}$I-antiTac(IgG) at time zero. Each value is the mean from four animals. ☐, blood; ☐, ATAC4; ☐, A431; ☐, spleen; ☐, kidney; ☐, liver; ☐, stomach; ☐, lung.

**Fig. 4.** Blood clearance of radiolabeled antiTac(dsFv) and antiTac(IgG). Whole blood was collected from mice at the specified times after injection with $^{125}$I-antiTac(dsFv) (*) or $^{125}$I-antiTac(IgG) (○). Each value is the mean from four animals.

**Fig. 5.** Tissue: blood ratios were determined based upon the percentage of ID/g at the specified time points. Mice were injected via the tail vein with either (A) $^{125}$I-antiTac(dsFv) or (B) $^{125}$I-antiTac(IgG) as described in the text. Each value is the mean of four animals. ☐, ATAC4; ☐, A431; ☐, spleen; ☐, kidney; ☐, liver; ☐, stomach; ☐, lung.
contribution to the release of radioiodine from the kidney is the abundance of deiodinases in the plasma membrane of renal tubule cells (29, 30).

The large difference in the blood clearance rate between the dsFv and the IgG can be accounted for primarily by the size-dependent differences in renal filtration rate for these proteins. Glomerular filtration by the kidneys has a molecular weight cutoff of approximately 40,000, above which proteins are not efficiently filtered (6, 28). The electrostatic charge on the protein is also a factor (basic proteins being filtered more readily than neutral or acidic ones); the calculated pI of antiTac(dsFv) is ~10. Humanized antiTac(IgG) was used for a control in this study for a number of reasons: (a) IgG antibodies are the most commonly used carriers for site-specific delivery of radioactive compounds to tumors; (b) the humanized form of antiTac(dsFv) has an affinity identical to that of antiTac(dsFv) (31); and (c) the humanized form is more readily available because it can be produced in larger quantities than the original mouse monoclonal antibody.

The rapid clearance of the dsFv from the circulatory system is a distinct advantage compared to the much longer serum half-life of the IgG. For applications when it is necessary to minimize the background level of radioactivity as quickly as possible (e.g., radioimmunomaging and radioimmunotherapy with short-lived radionuclides), this factor may determine the ultimate utility of the method.

In separate experiments, we have investigated the possibility of directly labeling antiTac(dsFv) with 131I using the IodoGen method (Pierce Chemicals, Rockford, IL) which places iodine onto tyrosine residues. It was found that this antibody can be labeled to relatively high specific activity (>7 mCi/mg) while maintaining greater than 80% of its immunoreactivity.4

The characteristics of the antiTac(dsFv) are close to ideal for an agent designed to systemically deliver a radioactive substance or a toxin to tumor cells in vivo. Its low Mr, and cationic charge at neutral pH allow it to be cleared rapidly from the circulation by uptake and degradation in the kidney. Also, due to its low Mr and high affinity, it can pass easily through the capillary endothelium to enter the tumor and accumulate at significant levels in the short period of time required by many therapeutic and diagnostic radioisotopes. We are now beginning to evaluate this antibody for use as a therapeutic agent in mice with tumor xenografts.

4 K. Webber and I. Pastan, unpublished data.

References


Rapid and Specific Uptake of Anti-Tac Disulfide-stabilized Fv by Interleukin-2 Receptor-bearing Tumors

Keith O. Webber, Robert J. Kreitman and Ira Pastan


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