Rapid Tumor Penetration of a Single-Chain Fv and Comparison with Other Immunoglobulin Forms

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ABSTRACT

Single-chain antigen-binding proteins, or sFvs, represent potentially unique molecules for targeted delivery of drugs, toxins, or radionuclides to a tumor site. In previous studies (Cancer Res., 51: 6363-6371, 1991) using a human colon carcinoma xenograft model, it was demonstrated that the sFv has an extremely rapid plasma and whole body clearance, as compared to intact IgG or Ig fragments. One potential consequence of the rapid sFv pharmacokinetic properties was the reduced percentage of injected dose/g of the radiolabeled sFv found in the tumor throughout a range of time points. The present study was designed to define the tumor penetration properties of a radiolabeled sFv in comparison with other Ig forms. 125I-labeled sFv, Fab', F(ab')2, and IgG forms of monoclonal antibody CC49, directed against the human pancarcinoma antigen TAG-72, were used to target the LS-174T human colon carcinoma xenograft in athymic mice. At various time points after systemic Ig administration, quantitative autoradiographic analyses of surgically removed tumors were used to define the rate and degree of penetration of the various Ig forms. These studies revealed that most of the intact IgG delivered to the tumor was concentrated in the region of or immediately adjacent to vessels, while the sFv was more evenly distributed throughout the tumor mass. The distributions of the Fab' and F(ab')2 fragments showed intermediate penetration in a size-related manner. The sFv demonstrated maximum tumor penetration at 0.5 h postinjection, while the intact IgG reached an equivalent degree of penetration at 48 h postinjection. These studies thus reveal a greater degree of uptake throughout the tumor for the sFv than would be expected by gross analyses of percentage injected dose/g and demonstrate an extremely rapid tumor penetration of the sFv. These studies should aid in the rational design of potential applications of drug-, toxin-, and radionuclide-conjugated sFvs in cancer therapy.

INTRODUCTION

One potential advantage for the therapeutic or diagnostic use of a sFv2 molecule (1-6) is the possibility that, due to its relatively small size (M, 27,000 versus M, 50,000 to 150,000 for other immunoglobulin forms), it may penetrate tumors more rapidly and evenly. Previous qualitative autoradiographic studies by several groups have shown, in animal models, that intact IgG accumulates in the perivascular regions of the tumor (7-13). A more diffuse distribution of intact IgG was not observed until after 24 h, but even at that time, the distribution of the radiolabeled MAb was still heterogeneous. A more uniform and greater penetration of tumor by intact IgG can be attained by increasing the amount of MAb being injected (7, 14, 15). Kennel et al. (7) observed that doses ranging from 2 to 113 µg did not alter the perivascular microdistribution of a MAb at 48 h; when 500 µg was administered, a more diffuse distribution resulted.

Autoradiographic analyses of tumors have also been performed with patient biopsy specimens following the administration of radiolabeled MAb (16, 17). Distribution patterns ranged from perivascular to diffuse throughout the tumor lesion. A reduction in the size of the immunoglobulin molecule has also been proposed as a means of increasing tumor penetration by MAb. Kennel and his coworkers (7) investigated the ability of a F(ab')2 fragment to penetrate tumors. They found that the fragment gave a microdistribution pattern similar to that of the intact IgG at 48 h. The major difference between the fragment and the intact IgG was that the fragment appeared to clear from the tumor more quickly than the intact IgG.

In the studies reported here, we have used quantitative autoradiography to define tumor penetration of four Ig forms, using the intact IgG, F(ab')2, Fab', and sFv of MAb CC49. MAb CC49 is a murine IgG second-generation antibody to B72.3 and reacts with the human pancarcinoma antigen TAG-72 (18). CC49 was developed by immunizing mice with purified TAG-72 (18) and has been shown to have a relative Ks of 16.18 x 10^-5 M^-1. CC49 reacts with tumor cells in lesions from colorectal, ovarian, mammary, gastric, non-small cell lung, and pancreatic cancer (19). Radiolabeled CC49 has also been demonstrated to localize human tumor xenographs in athymic mice (18) and has been effective in reducing and eliminating tumor growth (20, 21). Furthermore, preliminary studies of two clinical trials using 131I-CC49 IgG indicate that CC49 demonstrates efficient targeting of colorectal carcinoma lesions (22, 23). In a previous study (6), we have generated and compared all four Ig forms of MAb CC49 (intact IgG, F(ab')2, Fab', and sFv) as to their physical properties, relative affinities, in vivo pharmacokinetics, biodistribution to tumor and normal tissues, and tumor:normal tissue ratios. In those studies (6), the relative Ks of the dimeric intact IgG and F(ab')2 were shown to be approximately 7-10-fold higher than those of the monomeric Fab' and sFv. Pharmacokinetic studies using radiolabeled CC49 IgG, F(ab')2, Fab', and sFv in mice revealed that the sFv had an extremely rapid plasma clearance (T1/2α = 3.7 min), along with a very rapid whole body clearance. Pharmacokinetic studies in rhesus monkeys also demonstrated the CC49 sFv to have a rapid clearance from the plasma compartment (T1/2α = 3.9 min). In biodistribution studies, using the LS-174T human colon carcinoma xenograft in athymic mice, the %ID/g in the tumor at 24 h for monomeric CC49 sFv (1.7%) and Fab' (3.7%) were much lower than for the dimeric intact IgG (27.2%) and Fab' (19.2%). However, tumor:normal tissue ratios (radiolocalization indices) for the sFv were equal to or greater than those for the other Ig forms. At 6 h, sFv tumor:blood radiolocalization indices were 16:1 and reached 94:1 at 72 h. Both 131I-F(ab')2, and 131I-Fab' demonstrated high kidney uptake, whereas no kidney uptake was observed with 131I-sFv. Thus, the tumor:normal tissue ratios of the CC49 sFv form were comparable to those of the intact IgG, although at different time intervals, but the %ID/g in tumor of the sFv was approximately 8-10-fold lower than intact IgG.

There are several questions that remain unanswered in com-
paring the use of intact IgG versus sFv for in vivo diagnostic or therapeutic applications. These include: (a) Does the sFv penetrate tumors more rapidly than intact IgG? (b) Does sFv penetrate tumors more evenly than intact IgG? and (c) Is the higher %ID/g seen in tumors for IgG versus sFv due to more MAb in the intravascular or perivascular regions of the tumor or due to more IgG diffused throughout the tumor? Answers to these questions are necessary in the rational design of more efficient therapeutics of solid tumors and are the subject of this investigation.

MATERIALS AND METHODS

Monoclonal Antibody, Fragments, and sFv. MAb CC49, a murine IgG1, was developed by the immunization of mice with purified TAG-72 as previously described (18). Ascitic fluid containing MAb CC49 was generated by the i.p. inoculation of approximately 1 x 10^6 hybridoma cells into BALB/c mice that were previously primed with pristane. The ascitic fluid was harvested and clarified at 10,000 x g for 10 min before storage at -20°C. CC49 IgG was purified from ascitic fluid by ammonium sulfate precipitation followed by ion-exchange chromatography (DE52) as previously described (24). The fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and pooled appropriately. The protein concentration was determined by the method of Lowry et al. (25). As a control IgG BL-3 (IgG1, an isotype-matched control) was used.

Fab' fragments were generated by the digestion of purified CC49 IgG using pepsin as detailed elsewhere (26). Briefly, CC49 IgG was incubated with 10 mM dithiothreitol followed by 22 mM iodoacetamide to block the thiol groups. The IgG was then digested with 2% pepsin (protein:protein) at 37°C for 16 h. The Fab' fragments were then dialyzed in 5 mM sodium phosphate (pH 7.5) and further purified using ion-exchange chromatography (DE52). F(ab')2, fragments were prepared in the same manner, but without dithiothreitol or iodoacetamide treatment.

The CC49/212 sFv gene was constructed by combining the V1 region sequence and a complementary DNA copy of the V1 region sequence via a linker sequence, designated linker 212, and expressed in Escherichia coli as previously described (5). CC49 sFv protein expression was induced by a 42°C temperature shock for 1 h as detailed elsewhere (6). Cells were lysed by homogenization in 300 mM NaCl in 50 mM Tris-HCl with 1 mM disodium EDTA (pH 8.0) at 4°C (Manton-Gaulin cell homogenizer; Gaulin, Everett, MA). Following the homogenization, the material was centrifuged, and the resulting cell pellet was resuspended in 1 mM NaCl in 50 mM Tris-HCl with 1 mM disodium EDTA (pH 8.0) at 4°C and centrifuged again.

The cell pellet was resuspended in 6 mM guanidine-HCl, stirred at room temperature for 1 h, and centrifuged. The CC49/212 sFv was refolded by slow dilution (1:10) into 50 mM KCl in 50 mM Tris-HCl with 10 mM CaCl2 (pH 8.0) at 6°C. After resting for 21 h at 6°C, the solution was filtered and concentrated, and the buffer was exchanged using a Pellicon tangential flow apparatus (Millipore, Bedford, MA). The refolded CC49/212 sFv was purified using a Poly CATA cation exchange column (Poly LC, Columbia, MD) (21.5 x 250 mm), using a 50-min linear gradient of 1 mM calcium acetate in 40 mM 3-(N-morpholino)propanesulfonic acid (pH 6.3) and 5 mM calcium acetate in 40 mM 3-(N-morpholino)propanesulfonic acid (pH 7.5). The material in the peak fractions was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing and was found to be a homogeneous entity, as previously reported (6).

As a control sFv, sFv 4-4-20 derived from an antifluorescein antibody was used; it contained the same 212 linker as the CC49 sFv and was constructed, expressed, and purified as previously described (27).

Radiolabeling. MAb CC49 IgG, F(ab')2, Fab', and sFv, antifluorescein sFv, and BL-3 in the LS-174T tumor xenografts. Details of the microautoradiographic procedure have been published previously (30). Briefly, 5-μm tumor sections were deparaffinized with xylene, hydrated, and then dipped in Ilford K5 photographic emulsion (Ilford, Ltd., Mobberley, Cheshire, England) maintained at 50°C. After air drying, the slides were stored at -70°C in light-proof boxes. In the studies comparing the four Ig forms of CC49, the exposure time was 6 days. In subsequent experiments, the cpm/mg was determined, and the exposure time ranged from 5 to 7 days. The slides were then developed in Kodak D-19 developer for 6 min, fixed in Kodak Fixer, counterstained with hematoxylin, dried, and mounted.

In order to quantitate the data, sections of tumor photomicrographs were analyzed for grain density as a function of distance from the vessel margin (7). From five to five photographs from each time point were used in each analysis. Each photograph contained one or two blood vessels. Three distinct regions around each blood vessel were utilized to quantitate silver grains. One hundred-μm2 areas were counted at radiating 10-μm intervals from the vessel margin. The first 100-μm2 area radiating from the vessel was scored as <10 μm, since it was impossible to score the numerous grains inside the vessel and adjacent to the vessel wall. Thus, for the <10 μm area, only those grains that were distant from the vessel wall were counted. For all other areas, all grains were counted. Each data value is an average of 12-16 areas analyzed.

RESULTS

Previous studies (6) have shown that when equal μg and μCi amounts of 125I-CC49 IgG forms were administered to mice bearing the LS-174T human colon carcinoma xenograft, the %ID/g in the tumor for intact IgG, F(ab')2, Fab', and sFv at 6 h was 10.2, 20.8, 6.2, and 2.6, respectively, while at 24 h the %ID/g was 27.2, 19.2, 3.7, and 1.7, respectively. On the other hand, the %ID/g in the blood was 17.5, 6.2, 0.9, and 0.3 at 6 h and 10.7, 1.0, 0.1, and <0.1 at 24 h for the IgG, F(ab')2, Fab', and sFv, respectively. In an attempt to define and compare the penetration of each of the Ig forms within the tumor mass, 112 μCi of 125I-labeled Fab' and sFv and 15 μCi 125I-IgG and F(ab')2 were administered to athymic mice bearing the LS-174T xenograft tumor. These injected doses corresponded to 3.4, 6.8, 9.6, and 19 μg of IgG, F(ab')2, Fab', and sFv, respectively, which...
concentrated within 40 µm (approximately 2 cell diameters) of the vessel margins in the LS-174T xenograft. The intact IgG is illustrated in the photomicrographs of tumor sections following autoradiography (Fig. 1). At 6 h postadministration, the sFv demonstrates a diffuse pattern of distribution throughout the tumor section (Fig. 1D). In contrast, the intact IgG (Fig. 1A) is restricted to perivascular or intravascular areas. The F(ab)\(_2\) and Fab' fragments gave distribution patterns intermediate to those of the intact IgG and sFv. The F(ab)\(_2\) showed a more diffuse distribution than the IgG, while the Fab' showed a still greater penetration of the tumor (Fig. 1, B and C, respectively).

Grain density was quantitated as a function of tumor depth (the distance from the vessel margin) (Fig. 2) and further illustrates the degree of tumor penetration by each of the Ig forms. Temporal differences between the Ig forms are also apparent in Fig. 2. As shown (Fig. 2A), 6 h after injection of the intact IgG, most of the silver grains are found to be concentrated within 40 µm (approximately 2 cell diameters) of the vessel margins in the LS-174T xenograft. The intact IgG does not exhibit further penetration into the tumor until 48–96 h postinjection (Fig. 2, C–E).

The F(ab)\(_2\) shows a greater penetration of tumor, reflected in both the density of grains and the depth of penetration, than that of the IgG, at the earlier time points (Fig. 2, A and B). After 24 h, however, there is a decrease in both of these parameters. The more diffuse pattern of distribution by the Fab' observed at 6 h in Fig. 1 is also reflected in the quantitative analysis. In fact, the quantitative analysis demonstrated that the Fab' showed its greatest penetration of tumor at 6 h (Fig. 2A). This was followed by a decrease in the penetration and density of grains, with a significant reduction at 72 and 96 h (Fig. 2, D and E). A maximal distance of 70 µm from the vessel margin was achieved by the Fab', and by 96 h very few grains were observed near the vessel, suggesting that the \(^{125}\text{I}-\text{Fab}'\) is being cleared from the tumor xenograft. Of the Ig forms, the sFv demonstrated the greatest penetration of the LS-174T xenograft. A distance of 100 µm (the maximum distance measured) was attained by the \(^{125}\text{I}-\text{sFv}\) at 6 h postinjection. At subsequent time points, the density of the sFv grains steadily decreased, but the sFv appeared to persist in the tumor longer than either the F(ab)\(_2\) or Fab' fragment.

The distribution patterns of each of the Ig forms are perhaps more apparent when the total number of silver grains is compared. Most of the intact IgG was found to be restricted to 40 µm from the vessel margins (Fig. 3A) and did not exhibit an increase in the total number of grains or further penetration until 48 to 96 h postinjection. There was no appreciable difference between 72 and 96 h. The F(ab)\(_2\) demonstrated a more diffuse distribution (Fig. 3B), which is reflected in a greater number of grains found distal to the vessel margins. The total number of grains plateaued at 24 h, after which the number of grains decreased. In contrast to the other Ig forms, the total number of grains was low, even at the 24-h time point, when the peak accumulation of grains was observed. The Fab' showed a greater penetration (70 µm) of the tumor at 6 h. The total number of grains steadily declined, and by 96 h very few grains were evident in the tumor. The sFv shows a dramatic accumulation in the total number of grains at 6 h postinjection (Fig. 3D). The total number of grains was maximal when compared to the other Ig forms, in addition to being the most evenly distributed, at 50–80 µm from the vessel margins. This even distribution of the \(^{125}\text{I}-\text{sFv}\) was maintained over the 96-h period of the study, even though the total number of grains steadily decreased.

As illustrated in Fig. 1, a more diffuse distribution is observed after administration with the F(ab)\(_2\) and Fab' fragments than...
TUMOR PENETRATION OF A SINGLE-CHAIN Fv

Fig. 3. Quantitative analysis of the total number of grains as a function of distance from blood vessels. Concentric areas were counted at 10 μm intervals from blood vessel. A, CC49 IgG; B, F(ab')2; C, Fab'; D, sFv. Distances from the vessel margin are: <10 μm (D); 10–20 μm (S); 20–30 μm (O); 30–40 μm (S); 40–50 μm (O); 50–60 μm (O); 60–70 μm (O); 70–80 μm (O).

Table 1 Comparison of total number of grains for each of the CC49 Ig forms

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>IgG</th>
<th>F(ab')2</th>
<th>Fab'</th>
<th>sFv</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>191</td>
<td>459</td>
<td>1131</td>
<td>1953</td>
</tr>
<tr>
<td>24</td>
<td>211</td>
<td>480</td>
<td>831</td>
<td>1546</td>
</tr>
<tr>
<td>48</td>
<td>455</td>
<td>268</td>
<td>726</td>
<td>1004</td>
</tr>
<tr>
<td>72</td>
<td>915</td>
<td>344</td>
<td>304</td>
<td>826</td>
</tr>
<tr>
<td>96</td>
<td>933</td>
<td>205</td>
<td>85</td>
<td>592</td>
</tr>
<tr>
<td>Total</td>
<td>2705</td>
<td>1756</td>
<td>3077</td>
<td>5921</td>
</tr>
</tbody>
</table>

* The total number of grains was determined for an area with an 80-μm radius from a blood vessel. The values represent an average of 12–16 areas.

Quantitative analysis supports the observation that the sFv achieves maximal penetration by 30 min (Fig. 5A). There is a subsequent decrease in the density of the grains by 1 h; however, there is no further decrease at the 3- or 6-h time points. In points were chosen since, in the previous experiments, at 6 h the sFv exhibited the greatest penetration and density in the tumor.

The ability of the CC49 sFv and the intact IgG to localize and penetrate tumors is illustrated in Fig. 4. The 125I-sFv is observed penetrating the tumor as early as 10 min post MAb injection (Fig. 4A). The greatest penetration of grains is observed at 30 min, with a corresponding diffuse distribution throughout the tumor. In contrast, the intact IgG exhibits only a few grains penetrating the tumor at 6 h (Fig. 4E), while the remainder accumulates in the vascular compartment. By 24 and 48 h, the intact IgG has disseminated from the vascular lumen (Fig. 4, F and G) and by 96 h demonstrates a more diffuse distribution.

Quantitative analysis supports the observation that the sFv achieves maximal penetration by 30 min (Fig. 5A). There is a subsequent decrease in the density of the grains by 1 h; however, there is no further decrease at the 3- or 6-h time points. In

Table 1 presents the total number of grains, in the area within a radius of 80 μm from vessels, for each of the MAb CC49 Ig forms. The sFv demonstrated the highest number of grains, which occurred at 6–48 h. The F(ab')2 and Fab' fragments also demonstrated a high accumulation of grains at the early time points; however, neither of these two fragments reached the same level as that of the sFv molecule. In contrast, the IgG did not reach a maximal accumulation until 72–96 h. Furthermore, the sFv reached maximal levels that were 2-fold greater than those of the IgG. The totals listed at the bottom of Table 1 are for comparative purposes only.

Experiments were then conducted to compare the penetration of CC49 sFv and IgG in the LS-174T xenograft when equivalent μg and μCi amounts were injected per mouse. To determine the optimal time of penetration of the tumor by the CC49 sFv, tumors were excised at 10 min, 30 min, 1 h, 3 h, and 6 h postinjection and processed for autoradiography. These time

Fig. 4. Microdistribution of 125I-labeled CC49 sFv and 125I-labeled IgG in LS-174T tumor xenografts. Athymic mice bearing LS-174T tumors were administered 112 μCi of 125I-labeled CC49 sFv (A–D) and 125I-labeled CC49 IgG (E–H). For the CC49 sFv, the time points are: A, 10 min; B, 30 min; C, 1 h; and D, 6 h. For the CC49 IgG, the time points are: E, 6 h; F, 24 h; G, 48 h; and H, 96 h. × 400.
CC49 sFv localization and penetration of the LS-174T tumor, previous observations. To demonstrate the specificity of the remains at a plateau for the remainder of the time. The IgG, tumor sections was compared to that of the CC49 IgG (Table between 48 and 96 h.

When the radiolabeled CC49 sFv or Fab’ forms were injected into athymic mice bearing the LS-174T tumor xenograft is greater for the intact IgG or Fab’, F(ab’), and intact IgG (Fig. 2). When the total number of grains of the CC49 sFv within the tumor sections was compared to that of the CC49 IgG (Table 2), the sFv reaches the apex at 30 min, decreases at 1 h, and remains at a plateau for the remainder of the time. The IgG, however, peaks and plateaus at 48–96 h. This is consistent with previous observations. To demonstrate the specificity of the CC49 sFv localization and penetration of the LS-174T tumor, the control antifluorescin sFv, and control IgG (BL-3) were utilized in these studies. As depicted in Table 2 and Fig. 7, very few grains were detected in the tumor.

When the total number of grains observed that actually penetrated into the tumor was higher for the sFv than for the Fab’ (Table 1). Likewise, the F(ab’), demonstrated a greater number of grains than the IgG. Of the four Ig forms, the intact IgG resulted in the lowest number of total grains at early time points. Subsequently, after determining the optimal time of penetration of the intact IgG and sFv, the sFv and IgG were then compared at equivalent µg and µCi amounts. Since very low levels of the Ig forms are being administered in these studies, we do not believe that the actual relative amounts (µCi or µM) of IgG or sFv administered are crucial in this model system. The level of antigen is well in excess of the amount of antibody injected and should not have an affect on the tumor targeting of the antibody. Higher doses of antibody (>250 µg) could affect the %ID/g (24). Microscopic examination of sections from the intact 125I-IgG-injected group showed that while the penetration of grains in the tumors increased progressively from 6 h to 96 h, very few grains were observed in the deeper parts of the tumors at the early time points. In contrast, grains extended beyond the perivascular area in the sFv-treated LS-174T tumors by 30 min postinjection. Grains were still detectable at 72 h in deeper regions (80–100 µm) of the tumors. When the total number of grains was compared, it was found that the sFv at 10 min to 6 h did not differ greatly from the intact IgG at 6 to 96 h (Table 2). Interestingly, the total numbers of grains are at similar levels in the tumors increased progressively from 6 h to 96 h, very few grains were observed in the deeper parts of the tumors at the early time points. In contrast, grains extended beyond the perivascular area in the sFv-treated LS-174T tumors by 30 min postinjection. Grains were still detectable at 72 h in deeper regions (80–100 µm) of the tumors. When the total number of grains was compared, it was found that the sFv at 10 min to 6 h did not differ greatly from the intact IgG at 6 to 96 h (Table 2). Interestingly, the total numbers of grains are at similar levels of grains counted in 100 µm² areas at 10-µm intervals from the blood vessels. For the sFv (A), time points are: 10 min (O), 30 min (¢), 1 h (△), 3 h (○), and 6 h (□). For the IgG (B), time points are: 6 h (O), 24 h (△), 48 h (○), 72 h (□), and 96 h (□). contrast, the IgG requires 48–96 h to achieve a comparable density of grains and degree of penetration (Fig. 5B). An analysis of the total number of grains (Fig. 6) further illustrates the contrast between the sFv and intact IgG. The sFv attains a maximal total number of grains by 30 min, while the IgG reaches a similar level of total grains in the tumor xenograft between 48 and 96 h.

When the total number of grains of the CC49 sFv within the tumor sections was compared to that of the CC49 IgG (Table 2), the sFv reaches the apex at 30 min, decreases at 1 h, and remains at a plateau for the remainder of the time. The IgG, however, peaks and plateaus at 48–96 h. This is consistent with previous observations. To demonstrate the specificity of the CC49 sFv localization and penetration of the LS-174T tumor, the control antifluorescin sFv, and control IgG (BL-3) were utilized in these studies. As depicted in Table 2 and Fig. 7, very few grains were detected in the tumor.

**DISCUSSION**

We have shown that gross accumulation (%ID/g) of MAb CC49 in the LS-174T tumor xenograft is greater for the intact IgG than for the F(ab’), Fab’, and sFv. For example, the %ID/g in the tumor is 27.2, 19.2, 3.7, and 1.7 for the IgG, F(ab’), Fab’, and sFv, respectively, at 24 h. A different picture emerges, however, when one examines the microdistribution and the degree of penetration by each of the Ig forms. The sFv penetrates more deeply into portions of the tumor distal from the blood vessels than do the Fab’, F(ab’), and intact IgG (Fig. 2). When the radiolabeled CC49 sFv or Fab’ forms were injected at a µCi dose that was 8 times greater than the intact IgG or F(ab’), to ensure delivery of equal amounts to the tumors, the total number of grains observed that actually penetrated into the tumor was higher for the sFv than for the Fab’ (Table 1). Likewise, the F(ab’), demonstrated a greater number of grains than the IgG. Of the four Ig forms, the intact IgG resulted in the lowest number of total grains at early time points. Subsequently, after determining the optimal time of penetration of the intact IgG and sFv, the sFv and IgG were then compared at equivalent µg and µCi amounts. Since very low levels of the Ig forms are being administered in these studies, we do not believe that the actual relative amounts (µCi or µM) of IgG or sFv administered are crucial in this model system. The level of antigen is well in excess of the amount of antibody injected and should not have an affect on the tumor targeting of the antibody. Higher doses of antibody (>250 µg) could affect the %ID/g (24). Microscopic examination of sections from the intact 125I-IgG-injected group showed that while the penetration of grains in the tumors increased progressively from 6 h to 96 h, very few grains were observed in the deeper parts of the tumors at the early time points. In contrast, grains extended beyond the perivascular area in the sFv-treated LS-174T tumors by 30 min postinjection. Grains were still detectable at 72 h in deeper regions (80–100 µm) of the tumors. When the total number of grains was compared, it was found that the sFv at 10 min to 6 h did not differ greatly from the intact IgG at 6 to 96 h (Table 2). Interestingly, the total numbers of grains are at similar levels

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**Table 2** Comparison of the total number of grains following administration of sFv and IgG

<table>
<thead>
<tr>
<th>Time (Hr)</th>
<th>Total no. of grains</th>
<th>Control IgG</th>
<th>CC49 sFv</th>
<th>Control sFv</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1036</td>
<td>6</td>
<td>10.17</td>
<td>0.17</td>
</tr>
<tr>
<td>15</td>
<td>1629</td>
<td>24</td>
<td>0.5</td>
<td>2584</td>
</tr>
<tr>
<td>48</td>
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<td>48</td>
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<td>1935</td>
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<td>1580</td>
</tr>
<tr>
<td>96</td>
<td>2554</td>
<td>96</td>
<td>6</td>
<td>1861</td>
</tr>
</tbody>
</table>

*The total number of grains was determined for an area with an 80-µm radius from a blood vessel. The values represent an average of 12–16 areas.*
at 24 h after administration (Tables 1 and 2), even though there is a significant difference between the %ID/g of the IgG (23.2%) and sFv (2%) in the tumor. The discrepancy between the total number of grains and the %ID/g is most likely due to the uneven distribution of the intact IgG restricted to the intravascular space, whereas the sFv penetrated much more deeply into portions of the tumors distal from the blood vessels. It is important to again point out that quantitation of grains was begun at 10 μm from the vessel margins, since the grains in the intravascular space are too dense. These results indicate that more sFv is effectively delivered throughout the tumor mass than would have been estimated based on the %ID/g in the tumor.

Experiments reported here were conducted comparing equivalent amounts of all four Ig forms delivered to the tumor, as well as equivalent doses of sFv and intact IgG. It was found that each form behaved in a distinct manner in relationship to size, in terms of penetration, as analyzed by grain density and total number of grains present in the tumor. These data are consistent with aspects of the hypothesis by Jain and Baxter (31, 32), in which it is proposed that the capillary endothelium is a major barrier to injected MAb. They have shown mathematical models for the transport of fluid and macromolecules in a tumor. Since the intact IgG localized in the tumor has penetrated the capillary wall, but does not move very far into the tumor, the uneven distribution of the intact IgG in the tumor cannot be due solely to the poor penetration of the capillary wall. Shed antigen most likely represents a major barrier to the effective penetration of tumor by MAbs. If tumor cells shed antigen into the stromal compartment, MAbs may be intercepted before they reach the parenchymal compartment of the tumor. Some other barriers may exist in the tumor tissue which may restrict the penetration of the intact IgG to distal areas from the blood vessels. These include antibody affinity, concentration and antibody size, and vascular permeability and intratumor pressure (33, 34). The present study suggests that the barrier is size dependent, since the affinity is equivalent for CC49 Fab' and CC49 sFv and equivalent for IgG and F(ab')2.

To address the effects of these other parameters, several systems need to be further evaluated, including effect of affinity. For example, the intact CC49 IgG and F(ab')2 used in this study displayed relative Kd's of approximately 4 x 10^8 M^-1, while those for the sFv and Fab' were 8-fold and 7.4-fold lower, respectively. To address the effects of Kd's on tumor penetration, it may be necessary to conduct studies using MAbs with a wide range of Kd's, varying from approximately 10^7 to 10^13 M^-1. It will also be necessary to conduct these studies using fragments or other forms of these MAbs.

There are a number of reasons why the CC49 sFv may prove potentially useful for clinical applications in the management of cancer. First, penetration by the sFv is rapid and homogeneous. The sFv penetrates as a massive wave, radiating out from the blood vessels, and reaches the deeper areas of the tumor within 30 min. In the case of drugs and toxins, the uniform distribution suggests a higher probability of success for delivery by sFv. In addition, the rapid clearance of the sFv from the blood pool may reduce unwanted toxicity to normal tissues. Radiolabeled intact IgG remains in the circulation for several days with potential manifestations in bone marrow toxicity. Rapid elimination from blood could be overcome by repeated or continuous injection of high doses of sFv to increase the dosage delivered to tumor lesions. Therefore, multiple administrations resulting in higher concentrations of sFv with a diffuse and more uniform penetration into tumor tissue could result in a more efficient therapeutic effect.

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Rapid Tumor Penetration of a Single-Chain Fv and Comparison with Other Immunoglobulin Forms

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