Microautoradiographic Analysis of the Normal Organ Distribution of Radioiodinated Single-Chain Fv and Other Immunoglobulin Forms

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ABSTRACT

In previous studies, we have compared the immunoechemical properties, the in vivo pharmacokinetics, and the tumor penetration of a radioiodinated single-chain Fv (sFv) in comparison with other immunoglobulins (Ig) forms (intact IgG, F(ab')2, and Fab') (Cancer Res., 51: 6363-6371, 1991). Biodistribution studies demonstrated a higher percent injected dose/g in the liver and spleen for the intact IgG and F(ab')2. Renal uptake was observed with the Fab' and F(ab')2, whereas the sFv demonstrated no specific localization in either of these organs. The 125I-labeled sFv also demonstrated a more even distribution throughout the tumor xenografts as compared to the other Ig forms (Cancer Res., 52: 3402-3408, 1992). Subsequent studies utilizing the sFv conjugated with a radiometal (121I) demonstrated that the sFv was being metabolized by the kidney, and a significantly higher percent injected dose/g was obtained with a 121I-labeled sFv as compared to a 125I-labeled sFv (Cancer Res., 52: 6413-6417, 1992). These previous studies indicated the potential utility of radioiodinated sFv and other Ig fragments for use in radiodiagnostic procedures. In the present study, the distribution in normal tissues of the 4 Ig forms of monoclonal antibody (MAb) CC49, which is directed against a pancarcinoma antigen (tumor-associated glycoprotein-72-2), 125I-labeled sFv, Fab', F(ab')2, and IgG of MAb CC49 were administered to athymic mice either bearing or not bearing the tumor-associated glycoprotein-72 positive human colon carcinoma xenograft (LS-174T). At various intervals following the i.v. injection of the Ig forms, the liver, spleen, kidneys, and lungs were removed for autoradiographic analyses. Dramatic differences were observed in the kidney; the IgG was found only in the renal vasculature, whereas the Fab', F(ab')2, and sFv showed a high density of grains in the cortical tubules. In the liver, the IgG and F(ab')2 were found in association with hepatocytes, Kupffer cells, and in the sinusoids; the Fab' and sFv were primarily associated with the Kupffer cells. In the spleen, the sFv forms localized to the marginal zones surrounding the lymphoid follicles. No specific accumulation of grains for any of the Ig forms was observed in the lung. In each of the tissues, the clearance rates were related to the size of the Ig form. The localization in the liver and spleen was determined to be antibody-mediated. No specific localization was observed when 125I-labeled BL-3, an isotype-matched control MAb, was injected into tumor-bearing mice, or, when the 125I-labeled CC49 IgG was administered to non-tumor-bearing mice. By defining the interactions of a MAb and its modified forms not only with tumor lesions but also with normal organs and tissues, more rational protocols for uses of a MAb and its various forms, including sFv's, can be designed for diagnostic and therapeutic applications.

INTRODUCTION

When using radiolabeled MAbs in diagnostic or therapeutic applications, the concern of toxicity to normal organs is ever present. Intact Ig remains in the circulation for several days, which increases the potential of bone marrow toxicity due to therapeutic doses, or extends diagnostic procedures while the signal-to-noise ratio decreases to acceptable levels. Intact Ig's have also demonstrated a reduced ability to rapidly penetrate tumor lesions as compared to small Ig forms (1, 2). Furthermore, hepatic accretion is often observed in the liver when intact IgG is used, especially when conjugated with radiometals. Smaller Ig forms have exhibited more rapid clearance from the plasma compartment and greater tumor penetration (2). Although hepatic uptake is often reduced when fragments are used, renal uptake has been observed instead (3-6). Such unwanted accumulation in normal organs: (a) reduces the amount of radiolabeled MAb available for tumor targeting; (b) makes the detection of tumors in some normal organs difficult; and (c) increases the rad dosage received by the normal organs. The design and rational use of MAb in the clinical setting mandates an understanding of how the MAb conjugate is taken up not only in the tumor lesion, but also in normal tissues. There are a number of factors that can affect accretion of MAbs in normal tissues. These include the form of the MAb (i.e., intact Ig, fragments, sFv), vascular permeability of the tissue, antigen expression and distribution, as well as the biochemical properties of the antigen (e.g., secreted or membrane-associated).

The very rapid clearance of the genetically engineered sFv's makes these molecules very appealing for diagnostic and some therapeutic purposes (5, 6). In the present study, autoradiographic techniques were utilized to assess the accretion of the radioiodinated forms of MAb CC49 (intact IgG, F(ab')2, Fab', and sFv) by normal tissues. MAb CC49 (7) is a murine IgG1 that reacts with a pancarcinoma antigen, TAG-72 (8). CC49 reacts with adenocarcinomas of the colon, gastrointestinal tract, and pancreas, as well as carcinomas of the ovary, breast, prostate, and lung (non-small cell) (9). Radiiodinated MAb CC49 has been shown to localize human tumor xenografts in athymic mice (10) and has shown efficacy in reducing or abrogating tumor growth (11, 12). In addition, in several clinical trials, 131I-labeled CC49 IgG and 125I-labeled CC49 IgG have demonstrated targeting of tumor lesions in carcinoma patients (13), via γ-scanning, the use of an intraoperative hand-held probe (14), and direct biopsy analysis.

In previous studies, the relative affinities, in vitro binding properties, in vivo pharmacokinetics, and biodistribution (%ID/g in tumor and normal tissues) of the MAb CC49 Ig forms were compared (6). Microdistribution of tumor penetration was also compared (2). It was found that the relative affinity constants of the bivalent forms (IgG and F(ab')2) were 7- to 10-fold greater than those for the monovalent forms (Fab' and sFv).Pharmacokinetic studies in mice and rhesus monkeys revealed that the radiiodinated sFv had a T1/2 of <4 min. In biodistribution studies, the %ID/g of the monovalent forms were appreciably lower than the bivalent forms, however, the radiolocalization indices for the sFv was similar to, or greater than, the IgG, F(ab')2, or Fab' (6). At 24 h, the %ID/g in the tumor were 17.5, 19.2, 3.7, and 1.4 for the intact IgG, F(ab')2, Fab', and sFv, respectively. The %ID/g for the normal organs were 7.7, 11.1, 0.9, and 0.2 in the liver, 5.2, 3.5, 0.2, and 0.2 in the spleen; and 3.4, 2.2, 4.4, and 0.1 in the kidneys. Tumor penetration was assessed by quantitative autoradiographic analysis using athymic mice bearing a human colon carcinoma xenograft (LS-174T) (2). It was found that the sFv distributed more even throughout the tumor xenografts as compared to the intact IgG and F(ab')2. Subsequent studies utilizing the sFv conjugated with a radiometal (121I) demonstrated that the sFv was being metabolized by the kidney, and a significantly higher percent injected dose/g was obtained with a 121I-labeled sFv as compared to a 125I-labeled sFv (Cancer Res., 52: 6413-6417, 1992). These previous studies indicated the potential utility of radioiodinated sFv and other Ig fragments for use in radiodiagnostic procedures. In the present study, the distribution in normal tissues of the 4 Ig forms of monoclonal antibody (MAb) CC49, which is directed against a pancarcinoma antigen (tumor-associated glycoprotein-72) (121I-labeled sFv, Fab', F(ab')2, and IgG of MAb CC49 were administered to athymic mice either bearing or not bearing the tumor-associated glycoprotein-72 positive human colon carcinoma xenograft (LS-174T). At various intervals following the i.v. injection of the Ig forms, the liver, spleen, kidneys, and lungs were removed for autoradiographic analyses. Dramatic differences were observed in the kidney; the IgG was found only in the renal vasculature, whereas the Fab', F(ab')2, and sFv showed a high density of grains in the cortical tubules. In the liver, the IgG and F(ab')2 were found in association with hepatocytes, Kupffer cells, and in the sinusoids; the Fab' and sFv were primarily associated with the Kupffer cells. In the spleen, the sFv forms localized to the marginal zones surrounding the lymphoid follicles. No specific accumulation of grains for any of the Ig forms was observed in the lung. In each of the tissues, the clearance rates were related to the size of the Ig form. The localization in the liver and spleen was determined to be antibody-mediated. No specific localization was observed when 125I-labeled BL-3, an isotype-matched control MAb, was injected into tumor-bearing mice, or, when the 125I-labeled CC49 IgG was administered to non-tumor-bearing mice. By defining the interactions of a MAb and its modified forms not only with tumor lesions but also with normal organs and tissues, more rational protocols for uses of a MAb and its various forms, including sFv's, can be designed for diagnostic and therapeutic applications.

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2 The abbreviations used are: MAb, monoclonal antibody; Ig, immunoglobulin; sFv, single-chain Fv; BSM, bovine submaxillary mucin; %ID/g, percent injected dose/g; TAG-72, tumor-associated glycoprotein-72; IC, immune complex.

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throughout the tumor, whereas the IgG remained more concentrated in the region of or adjacent to the capillaries. The 125I-labeled CC49 Fab' F(ab')2 exhibited an intermediate penetration to the sFv and IgG that was size related. The sFv attained maximum penetration of the tumor xenograft at 30 min, whereas the intact IgG required 48 to 96 h to reach an equivalent level. A subsequent study revealed that the apparent absence of sFV accretion in the kidney was probably due to dehalogenation. When “dual-label” studies (15) were performed comparing 177Lu- and 125I-labeled CC49 sFv, significant differences were found in the metabolic patterns. The 177Lu-labeled sFv showed a higher %ID/g in the liver and spleen, and a very dramatic increase in the kidney; it appeared that the sFv was being metabolized.

In light of these findings, radiiodinated sFv’s and fragments of MAbs may be more useful than when labeled with a heavy metal. Because these molecules have such a rapid clearance from the plasma compartment, they may be more advantageous in diagnostic applications such as radioimmunoguided therapy or immunoscintigraphy. Both of these procedures would be greatly abbreviated by the use of sFv’s. Immunotherapy with radioiodinated sFv’s or fragments in multiple dosing protocols should also not be precluded. For this reason, the following study was designed to compare the distribution of radiiodinated Ig forms of MAb CC49 in normal tissues. Microautoradiographic techniques were employed to define the mechanism by which radiolabeled MAbs are sequestered in normal organs. This study also addresses the influence of antigen on the distribution of a MAb and its various forms.

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 described previously (7). CC49 IgG was purified from ascitic fluid as reported previously (10). The MAb was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the protein concentration was determined by the method of Lowry et al. (16). As a control IgG, BL-3 (IgG1, an isotype-matched control) was used.

Fab' and F(ab')2 fragments were generated by the digestion of purified CC49 IgG using pepsin as detailed elsewhere (17).

The CC49/212 sFv gene was constructed by combining the Vι subdomain sequence and a complementary DNA copy of the Vι subdomain sequence via a linker sequence designated linker 212, and expressed in Escherichia coli as described previously (5). CC49 sFv protein expression has been detailed elsewhere (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 described previously (18).

Radioiodination and Radioimmunoassay. MAb CC49, IgG1, F(ab')2, Fab', sFv, antifluorescein sFv, and control MAB BL-3 IgG were labeled with Na125I using Iodo-Gen (19) as described previously (10). The iodination protocol yielded specific activities of 11.9 μCi/μg for the CC49 IgG and 9.2-10.8 μCi/μg for the CC49 fragments and Fv, with up to 30-50% incorporation of the Na125I. The specific activities of the antifluorescein sFv and BL-3 were 8.0 μCi/μg and 5.2 μCi/μg, respectively. The immunoreactivity of the 125I-labeled CC49 Ig forms was assessed using BSM-coated beads in a column format as described previously (15). The preparation of the beads has been detailed elsewhere (8). Briefly, BSM (2 mg protein:1 ml packed gel) was coupled to Reacti-Gel HW65F (Pierce, Rockford, IL). To perform the radioimmunoassay, the BSM-beads (250 μl packed volume) were placed in a column and washed (3 X 1 ml) with 1% bovine serum albumin in phosphate-buffered saline. The 125I-labeled Ig form was applied onto the column (23 nCi in 50 μl) and counted in a γ-scintillation counter. The column was washed with 1% bovine serum albumin in phosphate-buffered saline (3 X 1 ml), the eluate applied immediately to a second column prepared as above, and washed. The radioactivity remaining on each column was measured, and the percent bound to the columns was calculated. The percent bound for each of the 125I-labeled Ig forms was greater than 90%.

Tumor Model. Six-week-old female athymic mice (nu/nu), obtained from Charles River, Inc. (Wilmington, MA), were injected s.c. in the scapular region with 1 X 10^6 LS-174T human colon carcinoma cells (20) in 0.2 ml. Animals were utilized for studies when the tumors measured approximately 1 cm in maximal diameter. The 125I-labeled CC49 forms, or control Ig forms, were injected i.v. At various times, animals were killed by exsanguination (2-3 mice/group). The liver, spleen, kidney, and lung were excised, fixed in 10% buffered formalin, and counted in a γ-scintillation counter. The cpm/mg for the normal organs was determined. The formalin-fixed tumor tissues were paraffin-embedded and processed for autoradiography. Non-tumor-bearing athymic mice were used as a control.

Microautoradiography. Microautoradiographic analysis was performed examining the distribution of the 125I-labeled CC49 IgG, fragments, sFv, antifluorescein sFv, and BL-3 in the normal organs in LS-174T tumor-bearing athymic mice. Details of the microautoradiographic procedure have been published previously (2, 21). Briefly, 5-μm tumor sections were deparaffinized with xylene, hydrated, and then dipped in Ilford K.5 (Ilford Limited, Mobberley, Cheshire, United Kingdom) photographic emulsion maintained at 50°C. After air drying, the slides were stored at ~70°C in light-proof boxes for 7 days. The slides were then developed in Kodak D-19 developer for 6 min, fixed in Kodak Fixer and counterstained with hematoxylin, dried, and mounted. Each section was evaluated for the presence of silver grains, indicative of MAb localization. The sections were scored + for positive to +++ for a very heavy density, ± for scattered grains, and − for no evident grains as compared to background grains.

RESULTS AND DISCUSSION

In the present study, a detailed evaluation of the microdistribution of the 4 Ig forms of 125I-labeled CC49 was conducted in athymic mice bearing the human colon carcinoma xenograft, LS-174T, in an attempt to determine their localization to normal organs. Autoradiographic analysis was performed on tissue sections of the liver, kidney, spleen, and lung from tumor- and non-tumor-bearing mice following i.v. injection with 125I-labeled CC49 IgG (Table 1). In the livers of LS-174T tumor-bearing mice, grains were associated with the Kupffer cells. The grains remained evident even at 72 h after injection (Fig. 1B). In contrast, in the non-tumor-bearing mice, there are qualitatively fewer grains localized in the Kupffer cells (Fig. 1D). In the spleens of the tumor-bearing mice, an accumulation of grains was observed surrounding the lymphoid follicles (Fig. 1: A, 48 h; and C, 72 h), whereas only a background number of grains are detectable in the non-tumor-bearing mice (Fig. 1E). When 125I-labeled BL-3, an isotype matched control, was administered to tumor-bearing mice, the microdistribution pattern was similar to that of the non-tumor-bearing mice (data not shown). The absence of any specific localization by BL-3 in the liver and the spleen suggests that the uptake in these organs is not a Fc receptor-mediated event. This is also supported by the distribution patterns of the fragments and sFv, discussed below. This is in contrast to the findings of Jones et al. (22), in which the F(ab')2 fragment of MAb B6.2 did not accumulate to the same degree as the intact IgG in the liver. This particular study used non-tumor-bearing mice. Our results suggest that the microdistribution observed in the liver and the spleen is specific and is due to the presence of antigen (TAG-72). The possibilities for the antigen-mediated uptake

| Table 1 | Comparison of localization of 125I-labeled CC49 IgG between LS-174T-bearing and non-tumor-bearing mice at 72 h after administration* |
|---|---|---|---|---|
| Liver | Hepatocytes | Kupffer cells | Spleen | Marginal zone |
| LS-174T-bearing mice | + | + | + | + |
| Non-tumor-bearing mice | + | + | ± | − |

* Athymic mice bearing LS-174T tumor xenografts and non-tumor-bearing mice were given injections of 125I-labeled CC49 IgG (112 μCi). The mice were killed and the liver and spleens were processed for autoradiography at 72 h.

b For scoring, + for positive to +++ for a very heavy density, ± for scattered grains, and − for no evident grains above background.
NORMAL ORGAN DISTRIBUTION OF SINGLE-CHAIN Fv

Fig. 1. Autoradiographs of normal organs from tumor- and non-tumor-bearing athymic mice following i.v. injection with 125I-labeled CC49 IgG. In the tumor-bearing mice, grains are accumulated in the marginal zone of the spleen at 48 h after administration (A, arrows), in the Kupffer cells of the liver (B, arrows), and in the marginal zone of the spleen (C, arrows) at 72 h. In contrast, in the non-tumor-bearing mice, there are fewer grains localized in the Kupffer cells in the liver (D) and in the spleen (E). × 400.

of CC49 IgG include the following: (a) Antigen is shed from the tumor into the circulation and is taken up and processed by macrophages in the liver and spleen. The CC49 epitope is then presented by the macrophage and is recognized by MAb CC49. (b) MAb CC49 forms complexes with circulating shed antigen that are then taken up by the liver and spleen. (c) CC49-TAG-72 complexes form at the tumor, and the complexes are then shed with subsequent uptake in the liver and spleen. To confirm the presence of TAG-72 in these tissues, the normal organs of athymic mice, tumor and non-tumor-bearing, were stained with biotinylated CC49 using immunohistochemical techniques (data not shown). The mice did not receive a prior administration of MAb CC49. The study revealed positive staining of the Kupffer cells in the liver and in the marginal zones of the spleens from the LS-174T tumor-bearing athymic mice only. The pattern observed was identical to that visualized by the autoradiography. This finding thus supports the hypothesis that circulating TAG-72 is taken up by the macrophages in the livers and spleens of tumor-bearing mice. It is possible that the high accumulation of 125I-labeled CC49 IgG (13, 23) that has been observed in the spleens of some colon carcinoma patients could be due to antigen-antibody complexes depositing in the spleen or antigen-positive areas in the spleen, which MAb CC49 is recognizing.

In earlier studies (2, 6), comparisons were made on the biodistribution and tumor penetrance of the 4 iodinated Ig forms of MAb CC49. Appreciable differences were observed between intact IgG, Fab', F(ab')2, and the sFv, i.e., high renal uptake of the Fab and
NORMAL ORGAN DISTRIBUTION OF SINGLE-CHAIN Fv

F(ab')2 fragments, which is consistent with other investigations (24-31). It was of interest to determine the microdistribution of the fragments, sFv, and, as discussed, the intact IgG in normal organs. The microdistribution of the 4 CC49 Ig forms was assessed in the kidney, liver, and spleen. The distribution within the kidney showed the most dramatic differences among the Ig forms (Fig. 2; Table 2). The CC49 IgG showed only a few grains associated in the capillaries of the glomeruli and peritubular vasculature (Fig. 2A). The number of grains is greatly decreased by 72 h. No accumulation was observed in the cytoplasm of the cortical tubules, suggesting that the IgG does not filtrate at the glomerulus. A major accumulation was observed in the cortical tubules of the kidney 6 h after injection of 125I-labeled F(ab')2 (Fig. 2B, arrows). This finding indicates that the F(ab')2 does filtrate at the glomerulus and is reabsorbed into the cytoplasm of the cortical tubules. This was also observed by Jönsson et al. (24). The grain density in this case is reduced at 24 h, and in essence is cleared by 48 h. This clearance is slightly faster than what was reported by Pervez et al. (27) for a F(ab')2. The difference may be due to the presence of circulating antigen and IC formation that occurs upon giving mice or patients injections of MAb CC49. For the Fab', a similar but more rapidly occurring pattern is discerned. Extensive accumulation was observed in the cortical tubules of the kidney by 30 min. The accumulation of grains due to the Fab' was greater than that seen for the F(ab')2 (Fig. 2C, arrows), even at 6 h, however, its clearance is faster than that of the F(ab')2. The grain density reaches background level 24 h after injection of the 125I-labeled Fab. The CC49 sFv shows a heavy accumulation in cortical tubules by 30 min (Fig. 3), which resembles the Fab' pattern. The density of grains, however, is less than the Fab' and the grains are cleared by 6 h (Fig. 2D), in contrast to the 24 h required for the Fab'. The results suggest that the clearance of the CC49 sFv through the kidney, as with the fragments, appears to be the major route of elimination, and that its clearance is much faster than the fragments.

In the liver, grains of the IgG are distributed diffusely at 6 h (Table 3). Some grains are observed in the sinusoids, due to circulation in the vascular compartment. Grains are also detectable associated with hepatocytes and Kupffer cells, however, the denser accumulation of grains was observed with the Kupffer cells. Autoradiographic findings demonstrated that the number of grains in the liver decreased by 96 h. At 7 days, most of the IgG circulating in liver is cleared from the sinusoids, whereas some grains remain associated with the Kupffer cells. The CC49 F(ab')2 localized in the sinusoids, hepatocytes, and Kupffer cells at 6 h after administration. Fewer grains are observed in the sinusoids, as compared with the IgG, probably due to its rapid plasma clearance. At 24 h, the grain density in the sinusoids and hepatocytes was reduced, whereas grains are still associated with the Kupffer cells at this timepoint. Thereafter, the grains show a gradual

Table 2. Localization of 125I-labeled CC49 forms in the kidney at 6 h after injection

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cortical tubules</th>
<th>Glomerulus</th>
<th>Peritubular</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC49 IgG</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CC49 F(ab')2</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CC49 Fab'</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CC49 sFv</td>
<td>±</td>
<td>-</td>
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</tr>
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</table>

a Athymic mice bearing the LS-174T tumor xenograft were given injections of equivalent µCi (112 µCi) amounts of the radioiodinated CC49 Ig forms. The mice were killed and the kidneys were processed for autoradiography at 6 h.

b See Table 1 for details.

Fig. 2. Microdistribution of 125I-labeled CC49 forms in the kidney of LS-174T tumor-bearing athymic mice. Athymic mice bearing LS-174T tumors were administered 112 µCi of 125I-labeled CC49 IgG (A), F(ab')2 (B), Fab' (C), and sFv (D). The mice were sacrificed at 6 h and the kidney processed for autoradiography. The CC49 F(ab')2 (B) and Fab' (C) grains accumulated in the cortical tubules of the kidney (arrows) x 400.
clearing from the liver between 48 and 96 h, with only a few grains remaining at the Kupffer cells at 96 h. It was found that the Fab' cleared more rapidly than the F(ab')₂. The Fab' did not demonstrate any association with hepatocytes or the sinusoids. Most of the grains are cleared from the liver, and some grains remain in Kupffer cells by 24 h. For the CC49 sFv, some grains are localized in Kupffer cells, and a few are observed in the sinusoids of the liver. The majority of the grains are completely cleared from the liver by 6 h. The localization and pharmacokinetics pattern of the intact IgG and F(ab')₂ in the liver are consistent with several studies in which it was found that the hepatocyte plays an important role in the clearance of IC (32–34). Since the liver is the major site of metastatic spread for most carcinomas, unwanted accumulation in the liver makes the detection of tumors at this site more difficult. In a previ-

Table 3 Localization of ¹²⁵I-labeled CC49 forms in the liver at 6 h after injection

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Hepatocytes</th>
<th>Kupffer cells</th>
<th>Sinusoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC49 IgG</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CC49 F(ab')₂</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CC49 Fab'</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CC49 sFv</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Athymic mice bearing the LS-174T tumor xenograft were given injections of equivalent μCi (112 μCi) amounts of the radioiodinated CC49 Ig forms. The mice were killed and the livers were processed for autoradiography at 6 h.

b See Table 1 for details.
ous study (15), $^{177}$Lu-labeled CC49 sFv showed a greater uptake in the liver and spleen and a much higher uptake in the kidney, as compared to $^{125}$I-labeled sFv. The radiometal-labeled sFv showed a very different metabolic pattern than the iodinated sFv, which is most likely due to retention of the metal by organs metabolizing the sFv, whereas the radioiodine is rapidly removed and excreted. In fact, the autoradiographic findings reported here showed almost no localization for $^{125}$I-labeled CC49 sFv at 6 h after injection. It thus appears that radioiodine-labeled sFv may have advantages over intact immunoglobulins and fragments in certain applications.

In the spleen, CC49 IgG showed an accumulation of grains surrounding the lymphoid follicle in the marginal zone at 6 to 96 h (Table 4; Fig. 1A). Only a few grains remain in the spleen at 7 days after injection of the IgG. Fewer grains are localized in the spleen for the F(ab')$_2$ than for the IgG, and most are cleared by 72 h. The Fab' shows an accumulation around the lymphoid follicles 30 min after injection, but is cleared by 6 h. The sFv behaved in a manner similar to that of the Fab'. The marginal zone of the spleen contains a subset of macrophages that is postulated to be the more differentiated population of macrophages in the spleen. The finding of silver grains in this region of the spleen supports the hypothesis that antigen is either being presented by the macrophages, or IC are being deposited and metabolized.

No specific accumulation was observed for the 4 forms of CC49 IgG in the lung. The grains appear localized in the vasculature in the lung. The only apparent difference among the Ig forms is the rate of clearance in the lung, i.e., the sFv clears from the lung faster than the Fab', F(ab')$_2$, and the intact IgG. It is believed that the microdistribution patterns observed in the autoradiographs, as well as the density of the grains, of the normal organs are representative of in vivo events. Since formalin fixation of tissues results in the cross-linking of neighboring proteins, the radiolabeled Ig forms would not be removed during processing of the tissues for autoradiography.

If one looks at the difference between the distribution patterns of CC49 and the control IgG, BL-3, CC49 IgG localizes at the Kupffer cells in the liver and areas outlining the lymphoid follicles of the spleen. CC49 also demonstrates a longer retention time in these tissues. As shown in Fig. 1, the distribution and residence time of the CC49 IgG are probably due to antigen-antibody interactions. Such is not the case for the control IgG. BL-3 represents the metabolic pattern of normal IgG, for example, fewer grains are observed in the Kupffer cells, and its clearance is faster than that of the CC49 IgG. In the spleen, no specific localization was observed with BL-3.

To emphasize the rapid kinetics of the CC49 sFv, the early timepoint (30 min after administration) is illustrated in Table 5 and Fig. 3, along with the control sFv. When CC49 sFv is compared with a control sFv (anti-fluorescein sFv), the same kinds of differences between the 2 Ig forms were observed in both the liver (Fig. 3, A and D) and the spleen (Fig. 3, C and F). The CC49 sFv grains accumulated in the Kupffer cells of the liver and in the marginal zone of the spleen, the antigen-positive areas of these tissues, whereas no specific localization was observed with the control sFv. Extensive accumulation was observed in the cortical tubules of the kidney (Fig. 3, B and E) at 30 min after injection. A major elimination route for the 2 sFv's appears to be the kidney (Fig. 3, B and E).

Among the problems that may be associated with using radiolabeled MAbs for immunodiagnosis and immunotherapy is the accretion of radionuclides by normal organs. This is most apparent in the liver when intact IgG is used (32, 33). Although liver uptake can be reduced when fragments are used (3), kidney uptake of fragments takes place (24–31). Such unwanted accumulation in normal organs reduces the amount of radiolabeled MAb available for tumor targeting and makes the detection of tumors in the normal organs difficult. In a previous study (15), $^{177}$Lu-labeled CC49 sFv showed a greater uptake in liver and spleen and a much higher uptake in kidney, as compared to $^{125}$I-labeled sFv (19). The radiometal-chelated sFv showed a very different metabolic pattern than the radioiodinated sFv. The metal is being retained by the tissues metabolizing the sFv, whereas iodinated sFv is rapidly dehalogenated and excreted. In fact, the autoradiographic findings reported here showed that almost no localization in normal organs was observed for the $^{125}$I-labeled CC49 sFv at 6 h after injection. The time course of an $^{111}$In- and $^{125}$I-labeled MAb accretion by rat liver has been reported by Sands (33). At early timepoints (up to 1 min), the radiolabels show equivalent uptake by the liver. However, the %ID/g of the radiolabels begin to diverge at 5 min. The radiometal remains trapped as the MAb is metabolized, whereas the radioiodine is excreted. It thus appears that radioiodine-labeled Ig forms may have advantages over metal-conjugated immunoglobulins and fragments in cancer detection and therapy. Due to their rapid clearance and ability to penetrate tumor lesions (2, 6), sFv molecules have further advantages over intact IgG and fragments.

The rationale of this study was to gain an understanding of the mechanism of normal organ uptake of radioiodinated MAbs. The results of the experiments reported here show that at the early time points, grains are distributed in both hepatocytes and Kupffer cells, but at the later time points, the predominant cell type responsible for the uptake of radiolabeled MAbs by the liver appeared to be the nonparenchymal cells rather than parenchymal cells.

Many papers have reported on the localization of antibodies in the liver using several methods (22, 32–39). One approach has involved injecting animals with radiolabeled MAb, harvesting the livers at various timepoints, fractionating the cells, and identifying the cell types associated with radioactivity. Some reports demonstrated that the accretion of radiolabeled MAbs is approximately equal for both hepatocytes and nonparenchymal cells (22, 37) when the values are adjusted for the proportion of each cell population. In contrast, other investigators have demonstrated that intact Ig was associated with the parenchymal cells than nonparenchymal cells (32, 34). However, it was not clear whether or not the hepatocytes and Kupffer cells were identified and quantitated.

### Table 4 Localization of $^{125}$I-labeled CC49 forms in the spleen at 6 h after injection

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Marginal zone</th>
<th>Intravascular</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC49 IgG</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>CC49 F(ab')$_2$</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>CC49 Fab'</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>CC49 sFv</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

* a: Athymic mice bearing the LS-174T tumor xenograft were given injections of equivalent μCi (112 μCi) amounts of the radioiodinated CC49 Ig forms. The mice were killed and the spleens were processed for autoradiography at 6 h.

* b: See Table 1 for details.

### Table 5 Kinetics of CC49 sFv and control sFv localizing in normal organs

<table>
<thead>
<tr>
<th>Organs</th>
<th>10 min</th>
<th>30 min</th>
<th>CC49 sFv, 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Hepatocytes +</td>
<td>Hepatocytes +</td>
<td>Hepatocytes +</td>
</tr>
<tr>
<td></td>
<td>Sinusoids +</td>
<td>Sinusoids +</td>
<td>Sinusoids +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kupffer cells ++</td>
</tr>
<tr>
<td>Spleen</td>
<td>Intravascular +</td>
<td>Intravascular +</td>
<td>Intravascular +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Marginal zone +</td>
</tr>
<tr>
<td>Kidney</td>
<td>Cortical tubules +++</td>
<td>Cortical tubules ++</td>
<td>Cortical tubules ++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>Intravascular +</td>
<td>Intravascular +</td>
<td>Intravascular +</td>
</tr>
</tbody>
</table>

* a: Athymic mice bearing the LS-174T tumor xenograft were given injections of equivalent μCi (112 μCi) amounts of the radioiodinated CC49 Ig forms. The mice were killed and the livers, spleens, kidneys, and lungs were processed for autoradiography at the time points indicated.

* b: See Table 1 for details.
In the report by Beatty et al. (34), it was demonstrated that much of the liver uptake correlated with the formation of antigen-antibody complexes. Skogh et al. (40) correlated an increased liver accretion via Kupffer cells with increased levels of IC. Interestingly, sinusoidal endothelial cells were also observed to sequester IC. Beatty et al. (34) and others (37) have correlated IC formation with carcinoembryonic antigen levels, which result in an increased uptake of the radiolabeled MAB in the liver and the spleen. On the other hand, Perala-Heape et al. (35) did not observe any influence of circulating antigen (prostatic acid phosphatase) levels on liver uptake of a $^{111}$In-labeled F(ab')$_2$. However, IC formation was not demonstrated in this study.

The most popular theory for the mechanism of liver uptake is that of a Fc receptor-mediated interaction. The Fc receptor for IgG in the liver exists not only on hepatocytes, but also on Kupffer cells and endothelial cells (41). The evidence in support of this theory is that liver uptake is reduced when the Fc portion of a MAB is removed. Removal of the Fc fragment, however, also affects the rate of blood clearance of MAB, through the reduction in size, and therefore should influence subsequent liver uptake. One possible explanation for the reduced liver uptake of the fragments is that the more rapid clearance from blood reduces the available fragments for liver accumulation. The results reported here showed that CC49 fragments and sFv localized in Kupffer cells, as does CC49 IgG, but the grains in the sinusoids are cleared more rapidly for the fragments and sFv than IgG.

In this instance, the localization of the CC49 Ig forms appears to be antigen-mediated (the control Ig did not demonstrate any localization). Other mechanisms of IC clearance include complement- and carbohydrate-mediated interactions. Several studies have shown that complexes activate complement and will bind to complement type 3 receptors on erythrocytes. The IC is dissociated from the erythrocyte as it transverses the liver (40, 42, 43) and/or the spleen (44). This does not appear to be the primary mechanism of clearance for soluble IC. Carbohydrate receptors appear to function through recognition of the antigen. Rifai et al. (45) have demonstrated the specific uptake of galactose-rich IC by parenchymal cells, whereas mannose-rich IC are predominantly sequestered by nonparenchymal cells. The biochemical nature of the antigen, as well as the size of the complex, appear to determine the fate of the IC and the mechanism of clearance.

Clearance through the kidney of the antibody fragments and sFv seems to be the major route of elimination for these molecules. Excessive accumulation of $^{125}$I-labeled CC49 Fab' and F(ab')$_2$ was observed in the cortical tubules. The results of this study indicate that the fragments filtrate at the glomerulus and are reabsorbed by cortical tubules. The rapid decrease of radioactivity is most likely due to dehalogeneration and excretion of the radioiodine from the kidney. The localization of radioiodinated CC49 sFv in normal kidney was also readily apparent at 0.5 h postinjection. Most of the $^{125}$I-labeled sFv is completely cleared from the kidney by 6 h, indicating that active metabolism is taking place. To test this hypothesis, dual-label studies were performed (15) comparing $^{177}$Lu- and $^{125}$I-labeled CC49 sFv in mice bearing LS-174T xenografts. The %ID/g of kidney for $^{177}$Lu-labeled CC49 sFv was much higher than that for $^{125}$I-labeled CC49 sFv at all timepoints.

According to human renal physiology, there is no hindrance to the movement of molecules with $M_r < 7.000$, and essentially total hindrance to molecules with $M_r > 60,000$. For molecules between these extremes, the filterability progressively decreases. It should be noted that not only does size affect filtration at kidney, but also electric charge is a critical variable in determining penetration by proteins. Even though CC49 F(ab')$_2$ has a $M_r$ of 100 kD, our results indicate that it is filtered at the glomerulus and reabsorbed by the cortical tubules. Since MAB fragments are an artifact of the laboratory, it is difficult to predict their behavior in vivo. It is possible that the F(ab')$_2$ might be cleaved into Fab' fragments in vivo, which is then an appropriate size that can be filtered by the glomerulus. Motta-Hennessy et al. (36) analyzed the radioactivity of tissue extracts of athymic mice given injections of $^{111}$In-labeled F(ab')$_2$ by high performance liquid chromatography and concluded that the labeled fragment was processed in the liver and resulted in the formation of Fab' fragments. Immunolocalization studies using fragments of MABs have resulted in a higher uptake in the kidney (26). The Fab' or F(ab')$_2$ fragments of several anti-melanoma MABs have also demonstrated renal uptake, either in animal models (24, 25) or in patients (4, 30, 31), by autoradiographic or scintigraphic techniques.

The results reported here can offer further evidence for the potential utility of radioiodinated CC49 sFv for tumor detection and therapy, since the $^{125}$I-labeled CC49 sFv is not retained in normal organs after 6 h. The enhanced target localization, as reported previously, with reduced accumulation in normal organs may permit a more sensitive diagnostic application of CC49 sFv and other anti-tumor sFv's in future clinical studies.

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References


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Microautoradiographic Analysis of the Normal Organ Distribution of Radioiodinated Single-Chain Fv and Other Immunoglobulin Forms

Takashi Yokota, Diane E. Milenic, Marc Whitlow, et al.