

Effect of Immune Complex Formation on the Distribution of a Novel Antibody to the Ovarian Tumor Antigen CA125

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ABSTRACT:

3A5 is a novel antibody that binds repeated epitopes within CA125, an ovarian tumor antigen that is shed into the circulation. Binding to shed antigen may limit the effectiveness of therapeutic antibodies because of unproductive immune complex (IC) formation and/or altered antibody distribution. To evaluate this possibility, we characterized the impact of shed CA125 on the in vivo distribution of 3A5. In vitro, 3A5 and CA125 were found to form ICs in a concentration-dependent manner. This phenomenon was then evaluated in vivo using quantitative whole-body autoradiography to assess the tissue distribution of ¹²⁵I-3A5 in an orthotopic OVCAR-3 tumor mouse model at different stages of tumor burden. Low doses of 3A5 (75 μg/kg) and pathophysiological levels of shed CA125 led to the formation of ICs in vivo that were rapidly distrib-

uted to the liver. Under these conditions, increased clearance of 3A5 from normal tissues was observed in mice bearing CA125-expressing tumors. Of importance, despite IC formation, 3A5 uptake by tumors was sustained over time. At a therapeutically relevant dose of 3A5 (3.5 mg/kg), IC formation was undetectable and distribution to normal tissues followed that of blood. In contrast, increased levels of radioactivity were observed in the tumors. These data demonstrate that CA125 and 3A5 do form ICs in vivo and that the liver is involved in their uptake. However, at therapeutic doses of 3A5 and clinically relevant CA125 levels, IC formation consumes only a minor fraction of 3A5, and tumor targeting seems to be unaffected.

Introduction

Ovarian cancer is the fifth most common cause of cancer death in women. Each year, ovarian cancer is diagnosed in approximately 20,000 American women, and approximately 15,000 die of the disease (American Cancer Society, 2007). Current treatment options consist of surgery followed by chemotherapy and occasionally radiation therapy, but prognosis remains poor because of the high metastatic potential of this disease (Piver et al., 1991) and long-term risk of tumor recurrence after surgery. To improve therapeutic outcome, monoclonal antibodies specific for ovarian tumor antigens are being developed (Schultes et al., 1998; Noujaim et al., 2001; Ehlen et al., 2005).

One such ovarian-specific tumor antigen is MUC16, a member of the mucin family of receptors (O'Brien et al., 2001; Yin and Lloyd, 2001), which is overexpressed on the membrane of epithelial ovarian cancer cells. The extracellular domain of MUC16, called CA125, is shed from the tumor cell surface (Beck et al., 1998). For many years,

the presence of shed CA125 in the serum has proven useful as a marker not only for the presence of ovarian cancer but also for response to therapy and disease recurrence (Bast et al., 1983, 1998; Verheijen et al., 1999; Berek et al., 2008). Although the expression pattern of MUC16 has made this protein a potentially important target for antibody therapy (Harris, 2004; Nicodemus and Berek, 2005), there is concern that shed CA125 may induce immune complex (IC) formation with therapeutic antibodies and prevent their effectiveness.

In this study, we describe a novel CA125-specific antibody, named 3A5, which recognizes not just a single epitope but rather a repeated epitope within this tumor antigen. 3A5 was previously found to bind more sites per cell than an antibody that binds to a single epitope (Chen et al., 2007). This property is hypothesized to enhance the therapeutic effectiveness of 3A5 and explains why 3A5 is currently a component of an antibody-drug conjugate strategy (Trail et al., 2003; Polakis, 2005; Wu and Senter, 2005; Chen et al., 2007; Junutula et al., 2008). This strategy consists of combining the targeting power of 3A5 with the chemotherapeutic effect of a cytotoxic drug, demonstrating potent antitumor activity in the OVCAR-3 ovarian tumor model (Chen et al., 2007; Junutula et al., 2008). Because of the multivalence of 3A5, IC formation may have a greater impact on tumor targeting and/or in vivo distribution of this antibody in comparison with that on

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ABBREVIATIONS: IC, immune-complex; luc, luciferase-expressing; QWBA, quantitative whole-body autoradiography; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; SEC-HPLC, size exclusion high-performance liquid chromatography; %ID/g, percentage of injected dose normalized to a gram of tissue; ELISA, enzyme-linked immunosorbent assay.

antibodies binding with a lower stoichiometry. To address this potential problem, we performed studies to determine the conditions and kinetics by which IC formation between 3A5 and CA125 occurs in vitro, to explore the tissue distribution of preformed 3A5-CA125 IC, and to characterize the in vivo impact of circulating CA125 on 3A5 distribution to target tissues at early and late stages of the disease, using an orthotopic ovarian cancer animal tumor model.

In this model, immune-compromised mice were injected with OVCAR-3 cells, a human ovarian carcinoma cell line, directly into the peritoneal cavity. This model recapitulates ovarian cancer in human patients in aspects such as extrapelvic extension, ascites fluid accumulation, and at later stages dissemination within the peritoneal organs. In addition, this mouse model also resembles the human disease in its heterogeneity in the levels of shed CA125 antigen in the serum and ascites (Chen et al., 2007). Thus, this tumor model is thought to be a suitable representation of ovarian cancer in humans, providing clinically relevant information on the antitumor activity of the potential therapeutic agent.

Unlike with subcutaneous xenograft models, a major challenge with this model is the quantitative assessment of drug distribution and progression of the tumor because of its localization within the organs in the peritoneum. Hence, in this study, in vivo bioluminescence imaging of luciferase-expressing OVCAR-3 (OVCAR-3/luc) tumor cells was used to allow the in vivo characterization of tumor burden, and quantitative whole-body autoradiography (QWBA) was used to assess drug distribution, overcoming difficulties associated with the traditional tissue dissection.

In these studies, we found that the interaction of 3A5 with CA125 results in the formation of ICs in vitro. ICs were also formed in vivo in the OVCAR-3 tumor model, in which the liver was identified as the major organ responsible for its uptake and clearance. However, IC formation did not affect tumor targeting.

Materials and Methods

Reagents and Cell Lines. The anti-MUC16 antibody (3A5) was described previously (Chen et al., 2007). The CA125 human ovarian cancer antigen (lot no. L1040309M) was purchased from United States Biological (Swampscott, MA). Genentech, Inc. (South San Francisco, CA)-engineered recombinant humanized IgG1 specific for glycoprotein D (anti-gD) was used as an isotype control for 3A5. ^{125}I was obtained as sodium iodide in 10^{-5} N sodium hydroxide from PerkinElmer Life and Analytical Sciences (Waltham, MA), and 75 μg of 3A5 was labeled with 1 mCi of ^{125}I using the Iodogen method (Pierce Chemical, Rockford, IL). The pooled ^{125}I -3A5 was >97% trichloroacetic acid (TCA) precipitable. The resulting specific activity was 9.50 $\mu\text{Ci}/\mu\text{g}$. OVCAR-3/luc cells were described previously (Chen et al., 2007).

In Vitro Studies. Different concentrations of purified CA125 human cancer antigen and the ^{125}I -radiolabeled 3A5 were incubated in phosphate-buffered saline (PBS) for various times at 37°C to characterize the kinetics of IC formation over time. The following variables were evaluated for effects on IC formation: 1) CA125 concentration (ranging from 0 to 2000 U/ml); 2) ^{125}I -3A5 concentration (ranging from 0.005 to 1.0 $\mu\text{g}/\text{ml}$); and 3) incubation time (ranging from 0 to 24 h). Size exclusion-high performance liquid chromatography (SEC-HPLC) was used to characterize IC formation. The samples were resolved by a BioSep 4000 column (Phenomenex, Torrance, CA) using PBS as the mobile phase. Quantitation was based on the peak area under the concentration-time curve, divided by the sum of all peak areas under the concentration-time curve $\times 100$.

In Vivo Studies. In all studies, mice were weighed, tagged, and housed in cages equipped with a coated wire floor to prevent animal contact with feces and urine. To prevent ^{125}I sequestration by the thyroid, animals in both groups received an intraperitoneal injection of 100 μl of 30 mg/ml sodium iodide 1 and 24 h before dosing with the radiolabeled dosing material. All dosing materials were prepared at Genentech, Inc., the night before use, and dose volumes (100 μl) were adjusted with the vehicle PBS. Mice were distributed into groups and dosed as described under *Results*. All animals were euthanized

by intravenous injection of pentobarbital through the tail vein at specified time points. All studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). Data are presented as mean \pm S.D. Statistical analysis was performed using a two-tailed *t* test; $p \leq 0.05$ was considered significant.

Tissue Distribution of Preformed ICs. Thirty normal female CD1 mice (Charles River Laboratories, Inc., Wilmington, MA), 6 to 8 weeks old and weighing between 17.6 and 20.9 g, were used to determine whether in vitro preformed ICs affected the distribution and clearance of ^{125}I -3A5 in a normal mouse model. Preformed ^{125}I -3A5-CA125 IC was prepared in vitro by incubating purified CA125 (2500 U/ml) with ^{125}I -3A5 (1 $\mu\text{g}/\text{ml}$) for 1 h at 37°C. Each mouse was given a single intravenous bolus injection of ^{125}I -3A5-CA125 IC or ^{125}I -3A5 alone (control group). The dose of radioactivity in both groups was 800 $\mu\text{Ci}/\text{kg}$. At terminal time points (0.25, 1, 6, 24, and 120 h, $n = 3/\text{time point}$), blood (200 μl) was collected via cardiac puncture under general anesthesia into lithium heparin Microtainers (BD Biosciences, San Jose, CA) and kept at 4°C. Plasma was separated from whole blood by centrifugation. Tissue distribution in this model was assessed by tissue dissection of major organs of distribution. The tissues harvested from each mouse were heart, kidneys, liver, lungs, spleen, duodenum, and large intestines (intestinal contents were rinsed out with ice-cold PBS). All organs were rinsed with PBS and blotted dry, weighed, and frozen at -70°C until total radioactivity counting. Counts per minute in dissected tissues, plasma, and dosing solution were determined using a gamma counter (Wallac 1470; PerkinElmer Life and Analytical Sciences). The counts per minute value was then used to calculate the percentage of injected dose normalized to a gram of tissue (%ID/g) or milliliter of fluid by dividing injected counts per minute with counts per minute per gram of tissue $\times 100$. Plasma TCA precipitation was used to measure antibody-bound radioactivity in circulation. In brief, the precipitated radioactivity present in 20 μl of plasma was prepared by adding 200 μl of ice-cold 1% bovine serum albumin in PBS (pH 7.2) to each plasma sample and vortex mixing, followed by adding 500 μl of ice-cold 20% TCA and vortex mixing again. After 30 min of incubation on wet ice, the samples were centrifuged at 14,000 rpm for 5 min. The supernatant was aspirated, and the radioactivity in the pellet of each sample was quantified for 1 min using the gamma counter. The radioactivity before and after TCA precipitation ($\times 100$) was used to determine the percentage of antibody-bound radioactivity for each plasma sample.

Impact of Circulating CA125 on 3A5 Tissue Distribution. Fifty C.B-17 ICR SCID female mice (Charles River Laboratories), weighing between 20 and 25 g were used to assess in vivo IC formation and distribution of 3A5 in mice bearing tumors that shed CA125 antigen into circulation. Mice were inoculated intraperitoneally with OVCAR-3/luc tumor cells, and tumor burden was determined by bioluminescence as described in Chen et al. (2007). Inoculated mice were observed at ventral view by bioluminescence on the day of cell inoculation to determine cell distribution and then once weekly thereafter. Mice with similar bioluminescence signals 2 weeks after cell inoculation were separated into two groups: 1) early stage of tumor burden, at approximately 35 days after inoculation, characterized as little ascites development and tumors within the peritoneal organs and 2) late stage tumor burden, at approximately 75 days after inoculation, characterized as established ascites and solid tumors within the peritoneal organs. Animals were weighed throughout the experiment and monitored closely for signs of distress (including distended abdomen, pale skin, and lethargy). If signs of distress or a loss of >20% of original body weight was observed, the mice were euthanized.

The serum CA125 levels from tumor-bearing mice were measured before 3A5 dosing using a CA125 ELISA kit (Alpha Diagnostic International, San Antonio, TX) according to the protocol supplied by the manufacturer. Mice at an early stage of tumor burden were given a single intravenous bolus injection via tail vein of 75 $\mu\text{g}/\text{kg}$ ^{125}I -3A5, equivalent to 600 $\mu\text{Ci}/\text{kg}$ radioactivity. Mice at a late stage of tumor burden study were given a single intravenous bolus injection of 75 $\mu\text{g}/\text{kg}$ of either ^{125}I -3A5 or ^{125}I -anti-gD (isotype control antibody) in addition to unlabeled antibody to complete a total dose of 3.5 mg/kg. Terminal time points were 0.25, 8, 24, and 48 h postdose ($n = 3/\text{time point}$ for tumor-bearing mice and $n = 2/\text{time point}$ for nontumor-bearing mice) for the early stage tumor burden study and 0.25, 8, 24, 48, and 120 h postdose for the late-stage tumor burden study when 3A5 was used and 0.25, 24, and 120 h postdose for the control isotype antibody ($n = 3/\text{time point}$). Blood was

collected at terminal time points via retro-orbital bleeding under isoflurane anesthesia (150 μ l into lithium heparin Microtainers) for plasma separation. Plasma was kept at -70°C until analysis by SEC-HPLC and TCA precipitation as described previously. After euthanasia, animals were secured on a foam board and immersed in an alcohol bath at -70°C until the bodies were completely frozen. Tissue distribution of ^{125}I -3A5 in the OVCAR-3/luc tumor-bearing mice was assessed by quantitative QWBA using the whole-body cryosectioning technique developed by Ullberg and Larsson (1981). QWBA allowed for the tissue distribution assessment of the test antibodies, avoiding the challenge of tissue dissection where tumors grow within the normal organs. Animals were embedded in 4% carboxymethylcellulose and stored at -70°C until sectioning. Sagittal sections of 20- μm thickness were obtained using a cryostat microtome (CM3600; Leica, Wetzlar, Germany) at -20°C . The sections were collected at three levels of interest in the sagittal plane, and all major tissues, organs, and fluids were included in these levels. Sections were lyophilized and mounted on clear tape (Filmolux 610 soft polyvinyl chloride glossy sheets, 6 \times 8 inches; Neschen AG, Bückeberg, Germany). The whole-body cryosections were covered with Mylar film (Fralock, Canoga Park, CA) and exposed overnight at room temperature to phosphor-imaging plates (YBIP 2025MS; Fuji Film Medical Systems, Inc., Stamford, CT) along with ^{125}I calibration standards. These were prepared from normal whole blood spiked with known concentrations of ^{125}I , ranging from 0.01 to 15 $\mu\text{Ci}/\text{ml}$. After the exposure time, the phosphor-imaging plates were scanned using a Fuji Film BAS-5000 scanner (Fuji Film Medical Systems, Inc.) to obtain digital images of the radioactivity in each section. The MicroComputer Imaging Device (MCID Analysis, version 7; Imaging Research Inc., ON, Canada) was used to quantify the concentrations of radioactivity in the calibration standards and tissues of whole-body sections, as described by Potchoiba et al. (1995) for ^{14}C xenobiotics. The quantitation method described here was demonstrated to be as accurate and precise as the values obtained from gamma or liquid scintillation counter methods (Potchoiba et al., 1995, 1998; Busch et al., 2000; Steinke et al., 2000). The lower limit of quantification was determined by the lower detected concentration in the calibration standards (0.01 $\mu\text{Ci}/\text{ml}$). The concentration of radioactivity from tissues was extrapolated from each standard curve as microcuries per gram of tissue, assuming that 1 g of tissue weight was equivalent to 1 ml and converted to a percentage of the injected dose. The ^{125}I -associated antibodies distributed equally to ascites and solid tumors; therefore, both compartments were added together during the quantitation and are reported as ascites-tumor values.

Results

IC Formation In Vitro. To determine the in vitro conditions under which CA125 and ^{125}I -3A5 formed ICs, various concentrations of 3A5 were incubated with clinically relevant concentrations of CA125 (Bast et al., 1983). Thus, as represented in Fig. 1A, CA125 at a fixed concentration of 1000 U/ml was incubated for 24 h at 37°C , according to Haisma et al. (1987), with increasing concentrations of ^{125}I -3A5 (0.1, 0.5, and 1 $\mu\text{g}/\text{ml}$). After incubation, samples were analyzed by SEC-HPLC to determine the extent of IC formation. At a fixed CA125 concentration of 1000 U/ml, the addition of 0.1 $\mu\text{g}/\text{ml}$ ^{125}I -3A5 resulted in approximately 40% of the antibody forming IC (Fig. 1A). Because the antibody concentration was increased relative to CA125, the proportion of IC formation relative to the total antibody decreased (Fig. 1A). Likewise, when the ^{125}I -3A5 concentration was fixed at 0.1 $\mu\text{g}/\text{ml}$ in the presence of various concentrations of CA125, IC formation was concentration-dependent such that the percentage of free 3A5 decreased with increasing CA125 antigen (Fig. 1B). In these studies, a CA125 concentration of 1000 U/ml with 0.1 $\mu\text{g}/\text{ml}$ 3A5 resulted in 40 to 60% of the antibody forming ICs by 24 h (Fig. 1, A and B). Therefore, this concentration of CA125 and 3A5 was used to determine the kinetics of IC formation in vitro over 0, 0.5, 4, and 24 h. The formation of ICs was rapid, with 80% of the total IC formation occurring within the first 0.5 h (Fig. 1C). These data indicate that the proportion of IC formed between 3A5 and CA125 is dependent on the relative concentrations of the antigen, and antibody. IC formation is

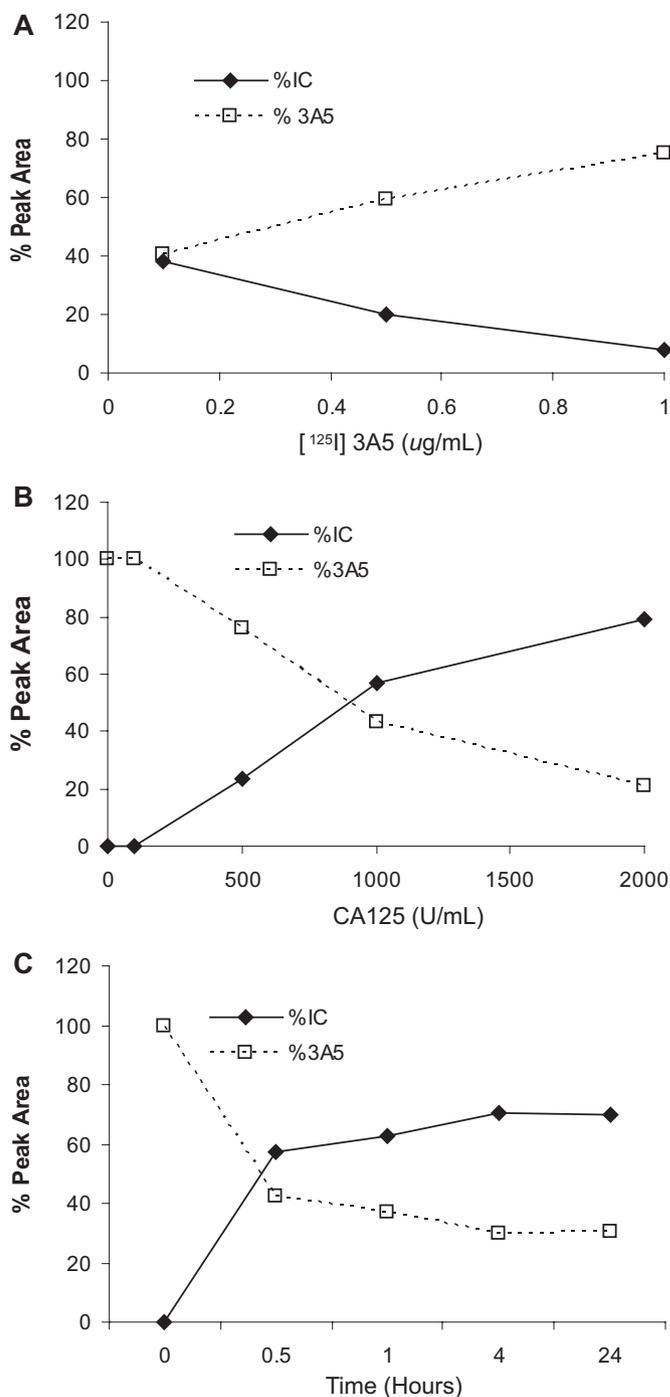


FIG. 1. In vitro formation of 3A5 and CA125 immune complex. A, increasing concentrations of ^{125}I -3A5 with a fixed purified CA125 concentration of 1000 U/ml. B, increasing concentrations of purified CA125 with a fixed ^{125}I -3A5 concentration of 0.1 $\mu\text{g}/\text{ml}$. The y-axis represents the percent peak area. The x-axis represents the different concentrations of either antibody or antigen, respectively. C, percentage of IC formation between 0.1 $\mu\text{g}/\text{ml}$ ^{125}I -3A5 + 1000 U/ml purified CA125 over a 24-h incubation time period at 37°C . Data are presented as percent IC formation (%IC) and percent free 3A5 (%3A5) of two replicates per time point per condition.

enhanced when the concentration of CA125 is greater than that of 3A5.

Tissue Distribution and Clearance of ICs in Normal Mice. To determine whether IC formation altered the kinetics of tissue distribution and clearance of ^{125}I -3A5 in a normal mouse model, ^{125}I -3A5-CA125 IC was preformed in vitro before administration in mice. In an effort to more closely mimic the level of IC formation that may be

observed in the clinic, ICs were generated by incubating 2500 U/ml purified CA125 with 1 $\mu\text{g/ml}$ ^{125}I -3A5, which resulted in a dose of 5560 U/kg and 2 $\mu\text{g/kg}$ CA125 and ^{125}I -3A5, respectively. SEC-HPLC confirmed that 10% of the 3A5 was present as ICs (Fig. 2A, left panel), as predicted from in vitro data (Fig. 1B). No IC was present in the control group dosing material (Fig. 2A, right panel). At 0.25 h after dosing with ^{125}I -3A5-CA125 IC or ^{125}I -3A5, the SEC-HPLC profile of plasma showed no detectable IC peak, whereas ^{125}I -3A5 was present in plasma of mice dosed with ^{125}I -3A5 (Fig. 2B, left and right panels, respectively), suggesting that circulating 3A5-CA125 IC is cleared more rapidly than uncomplexed 3A5.

The impact of IC formation on the tissue distribution of 3A5 was determined using tissue dissection for quantitation of total radioactivity. There was no substantial difference in the tissue distribution of

^{125}I -3A5-CA125 IC or ^{125}I -3A5, with exception of the liver (Fig. 2C, left and right panels, respectively). Uptake of radioactivity at 0.25 h postdose was significantly higher ($*p < 0.05$) in the livers from mice dosed with preformed IC compared with that of mice dosed with ^{125}I -3A5 alone. These data suggest a role for the liver in the rapid uptake of IC. Overall, in both groups the highest %ID/g was observed in the blood and highly perfused organs (spleen > lungs > liver > kidney > heart), indicating equilibration between the blood and other normal tissues, resulting in a parallel decrease with blood concentrations (Fig. 2C).

Impact of Circulating CA125 on 3A5 Tissue Distribution. Early stage of ascites and tumor development. To explore whether tumors that shed CA125 would result in IC formation and alteration of the tissue distribution of 3A5, OVCAR-3/luc tumor cells were inoculated

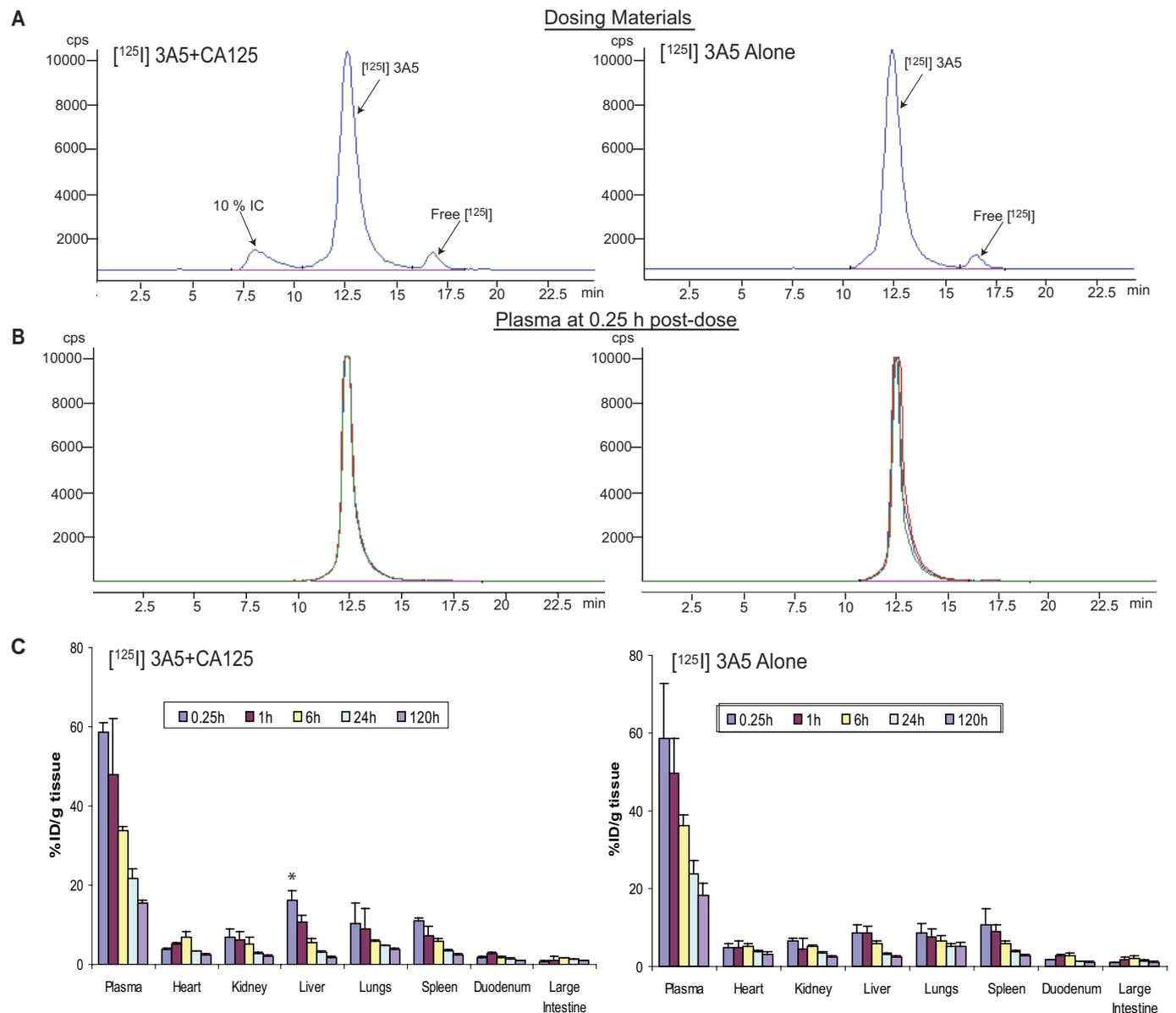


FIG. 2. Preformed 3A5 ICs are rapidly cleared by the liver in mice. SEC-HPLC chromatograms of the dosing solution and plasma samples from normal mice at 0.25 h postdose with either the preformed IC (A, left panel) or ^{125}I -3A5 alone (A, right panel). B, left panel, plasma profiles from normal mice after an intravenous dose of the preformed IC showing IC clearance from the circulation at 0.25 h postdose. B, right panel, plasma profile from normal mice dosed with the antibody alone at 0.25 h postdose showing a profile similar to that of the dosing solution. Overlapped profiles from three independent animals in each group at 0.25 h sampling time are shown. C, graphs represent %ID/g in normal mice after a single intravenous bolus injection of ^{125}I -3A5 + CA125 (left panel) or ^{125}I -3A5 alone (right panel). Tissue distribution was determined by the tissue dissection method at 0.25, 1, 6, 24, or 120 h postdose. Data are expressed as the mean \pm S.D. values of three animals per time point in each group. Statistically significant liver uptake was observed in mice dosed with the preformed IC compared with liver from mice dosed with ^{125}I -3A5 alone ($*, p \leq 0.05$).

into mice (Chen et al., 2007). A model at an early stage of ascites development and tumor burden, characterized by bioluminescence, was used to investigate the interaction of the antibody with the circulating shed antigen with minimal interference of tumor burden. Circulating levels of CA125 ranged from 61 to 102 U/ml, as determined by ELISA. Based on the conditions that resulted in IC formation *in vitro*, it was predicted that circulating CA125 levels needed to be greater relative to the 3A5 concentrations to facilitate IC formation. Therefore, a dose of ^{125}I -3A5 (75 $\mu\text{g}/\text{kg}$) to mice with an early stage of tumor burden was expected to result in approximately 10% IC formation. In parallel, 75 $\mu\text{g}/\text{kg}$ ^{125}I -3A5 was administered to nontumor-bearing mice (without CA125).

In tumor-bearing mice, 8% of 3A5-related radioactivity was present as IC in plasma at 0.25 h postdose (Fig. 3A). At 8 h postdose, the products of IC degradation were detected in plasma, as evidenced by the presence of a lower molecular weight peak in SEC-HPLC. By 24 and 48 h postdose, plasma radioactivity was diminished to below the level of detection. In contrast, plasma from nontumor-bearing mice showed no evidence of IC formation at any time point, and radioactivity clearance was slower than that of tumor-bearing mice (Fig. 3B).

To further characterize the *in vivo* catabolism of ^{125}I -3A5 in plasma over the time course of the study, TCA precipitation was performed on plasma samples from tumor-bearing and nontumor-bearing mice collected at terminal time points. In both groups, ^{125}I -3A5 was approximately 100% precipitable at all time points (Fig. 3, C and D), with the exception of the 8-h postdose samples from tumor-bearing

mice, in which the percentage of precipitable radioactivity declined to 75%. These data are consistent with the degradation products observed by SEC-HPLC at 8 h postdose in these mice (Fig. 3A).

To assess the tissue distribution of ^{125}I -3A5 in tumor-bearing and nontumor-bearing mice, QWBA was used. Autoradiograms from tumor and nontumor-bearing mice are shown in Fig. 4, A and C, respectively; data are represented as %ID/g in Fig. 4, B and D, respectively. Of importance, at 0.25 h postdose, radioactivity in the liver of tumor-bearing mice was 2-fold higher than that in nontumor-bearing mice (31.6 + 1.57 versus 15.21 + 2.74% ID/g, respectively). These data confirm the role of the liver in the uptake of ICs. At the remaining time points, levels of radioactivity in the liver decreased in parallel with those in the blood. These data together suggest that a fraction of the low dose of 3A5 forms IC by 0.25 h, and it is taken by the liver the remaining free 3A5 rapidly declines from blood. Thus, even if more CA125 becomes available in circulation, the rapid clearance of antibody in blood may impair the formation of new ICs over time. Consistent with previous evidence of degradation products at 8 h postdose in tumor-bearing mice, high radioactivity levels were observed in the intestines and stomach. This is indicative of the route of elimination of ^{125}I degradation products and of the known role of the stomach in the absorption of free ^{125}I , respectively (Regoeczi, 1987). Ascites-tumor uptake of ^{125}I -3A5 increased until 8 h postdose and remained unchanged over the 48-h time course of the study (Fig. 4B, inset).

In nontumor-bearing mice, radioactivity was largely distributed in the blood. After equilibration with highly perfused organs (spleen >

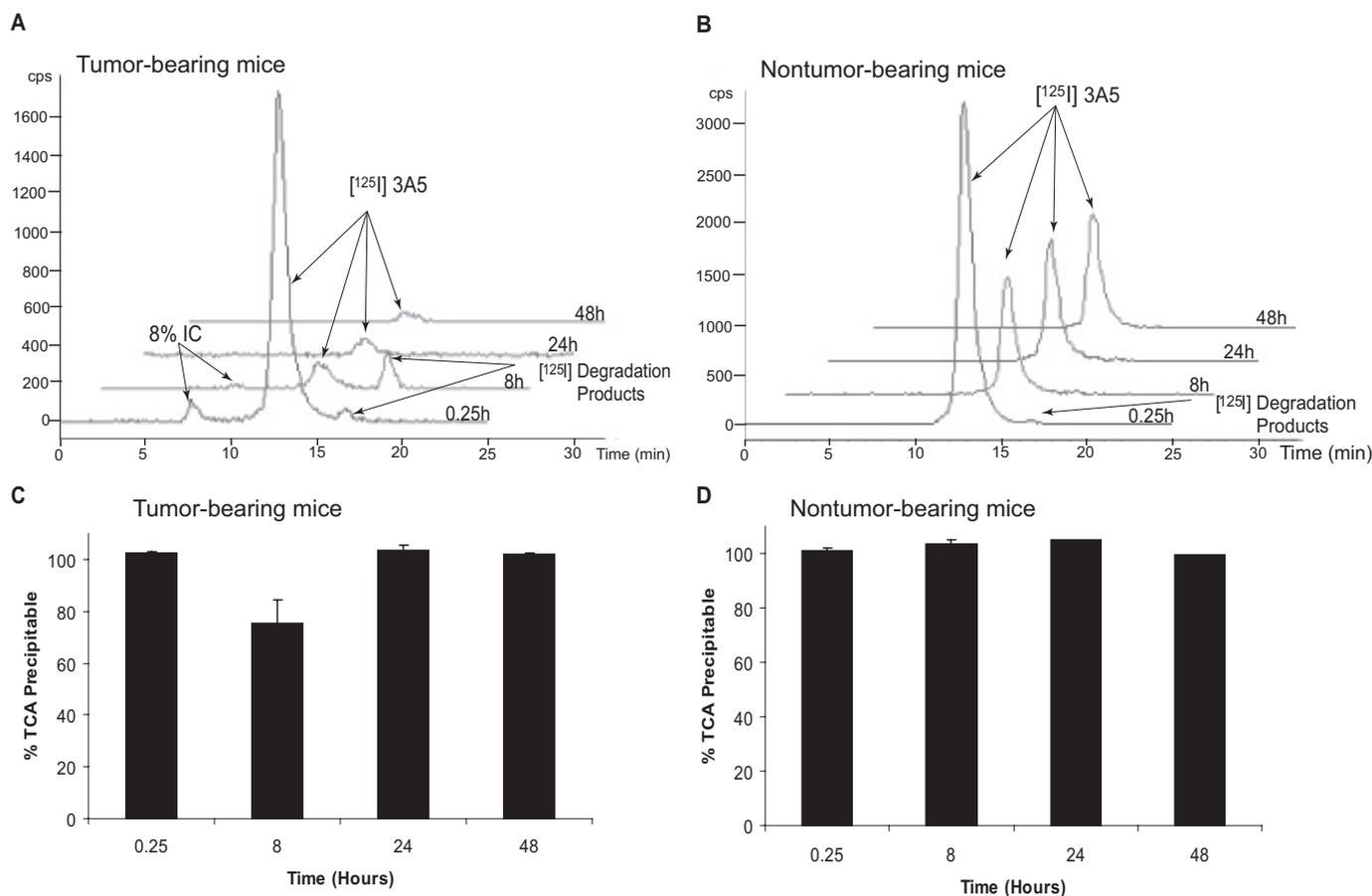


FIG. 3. 3A5 forms ICs in OVCAR-3/luc tumor-bearing mice. SEC-HPLC profiles of representative plasma samples collected at indicated time points after a single intravenous dose of 75 $\mu\text{g}/\text{kg}$ ^{125}I -3A5 into OVCAR-3/luc tumor-bearing mice (A) with circulating levels of CA125 or nontumor-bearing mice (B). The TCA-precipitable fraction of ^{125}I -3A5 in plasma after intravenous administration into OVCAR-3/luc tumor-bearing mice (C) with circulating levels of CA125 and nonbearing-tumor mice (D). Data are expressed as the mean \pm S.D. values of three animals per time point in each group, with the exception of nontumor-bearing mice at 8 and 24 h with $n = 2$. Intestines Cont., intestines contents.

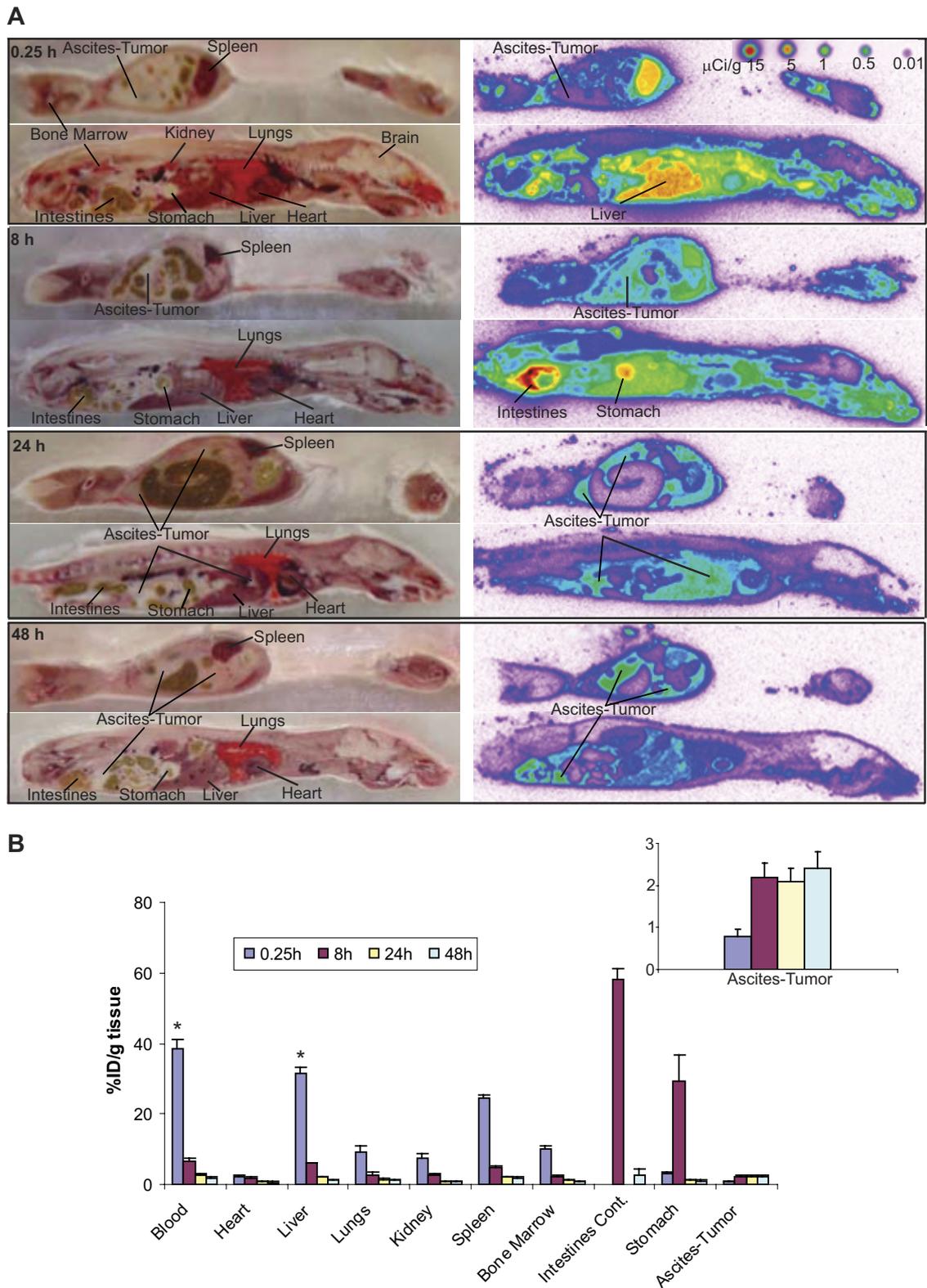


FIG. 4. 3A5 distributes to ascites-tumor in OVCAR-3/luc tumor-bearing mice despite IC formation. Representative whole-body sagittal sections of a mouse at different time points after ^{125}I -3A5 administration. Anterior and posterior sectioning levels depict ascites-tumor and major organs of distribution, respectively. Left panel, digital pictures of the sections for anatomical localization purposes. Right panel, autoradiograms with the distribution of ^{125}I -3A5 after an intravenous dose of $75 \mu\text{g}/\text{kg}$ into OVCAR-3/luc tumor-bearing mice (A) at an early stage of tumor burden and nontumor-bearing mice (C). Tissue distribution was determined by QWBA at 0.25, 8, 24, and 48 h postdose. The standard curve is represented as a pseudo-color heat map, inserted above the autoradiograms. Graphs represent %ID/g after a single intravenous injection of $75 \mu\text{g}/\text{kg}$ ^{125}I -3A5 into OVCAR-3/luc tumor-bearing mice (B) (inset displays 3A5 uptake by tumor on a smaller scale) and nontumor-bearing mice (D). Data are expressed as the mean \pm S.D. values of three animals per time point, with the exception of nontumor-bearing mice at 8 and 24 h where $n = 2$. Significant differences in the extent of ^{125}I -3A5 distribution to the liver and blood were observed in the tumor versus nontumor-bearing mice (*, $p \leq 0.05$). Intestines Cont., intestines contents.

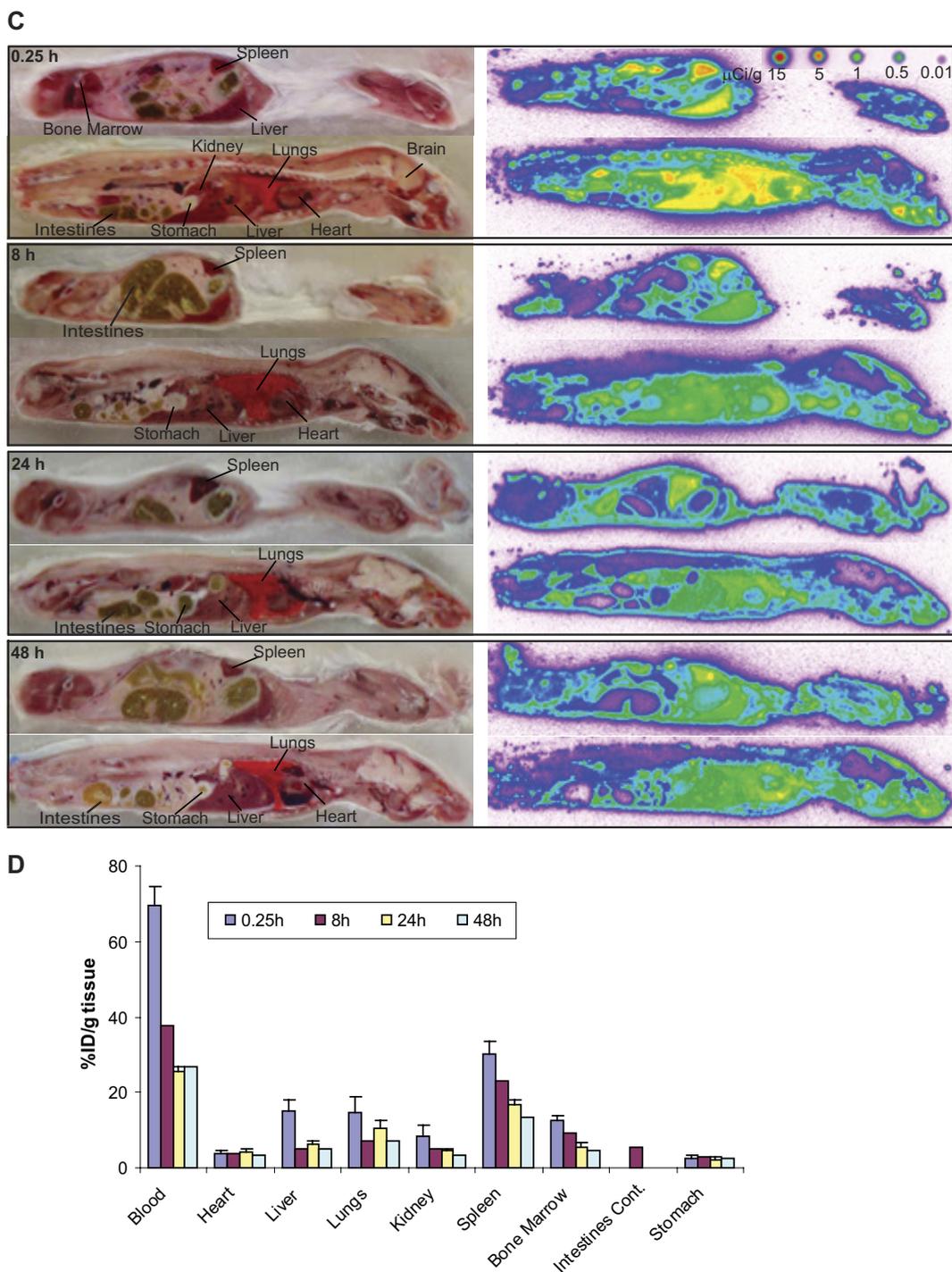


FIG. 4—Continued.

lungs > liver > kidney = bone marrow > heart), levels in the blood and tissues remained relatively sustained over the 24 h postdose (Fig. 4, C and D).

Late-stage ascites and tumor development. The distribution of ^{125}I -3A5 was characterized in normal versus tumor tissues in a more clinically relevant model: mice presenting with circulating CA125 levels consistent with those observed in patients with ovarian cancer (Bast et al., 1983) and with "late-stage" tumors. At this stage of tumor burden (75 days after inoculation) circulating CA125 levels in mice ranged from 69 to 318 U/ml. Higher levels of CA125 were not

achievable without compromising the animal's health. In this study a therapeutically relevant dose of 3A5 (^{125}I -3A5 + unlabeled 3A5 to complete a total dose of 3.5 mg/kg), as determined by tumor efficacy studies of a 3A5 antibody-drug conjugate (Chen et al., 2007), was administered to mice bearing OVCAR-3/luc tumors. After the intravenous bolus administration neither IC formation nor degradation products were detected in plasma at any time point as determined by SEC-HPLC and TCA precipitation (Fig. 5, A and inset, respectively). QWBA assessment of ^{125}I -3A5 tissue distribution in tumor-bearing mice is depicted in Fig. 5, B and C, with data represented as %ID/g in Fig. 5C. These

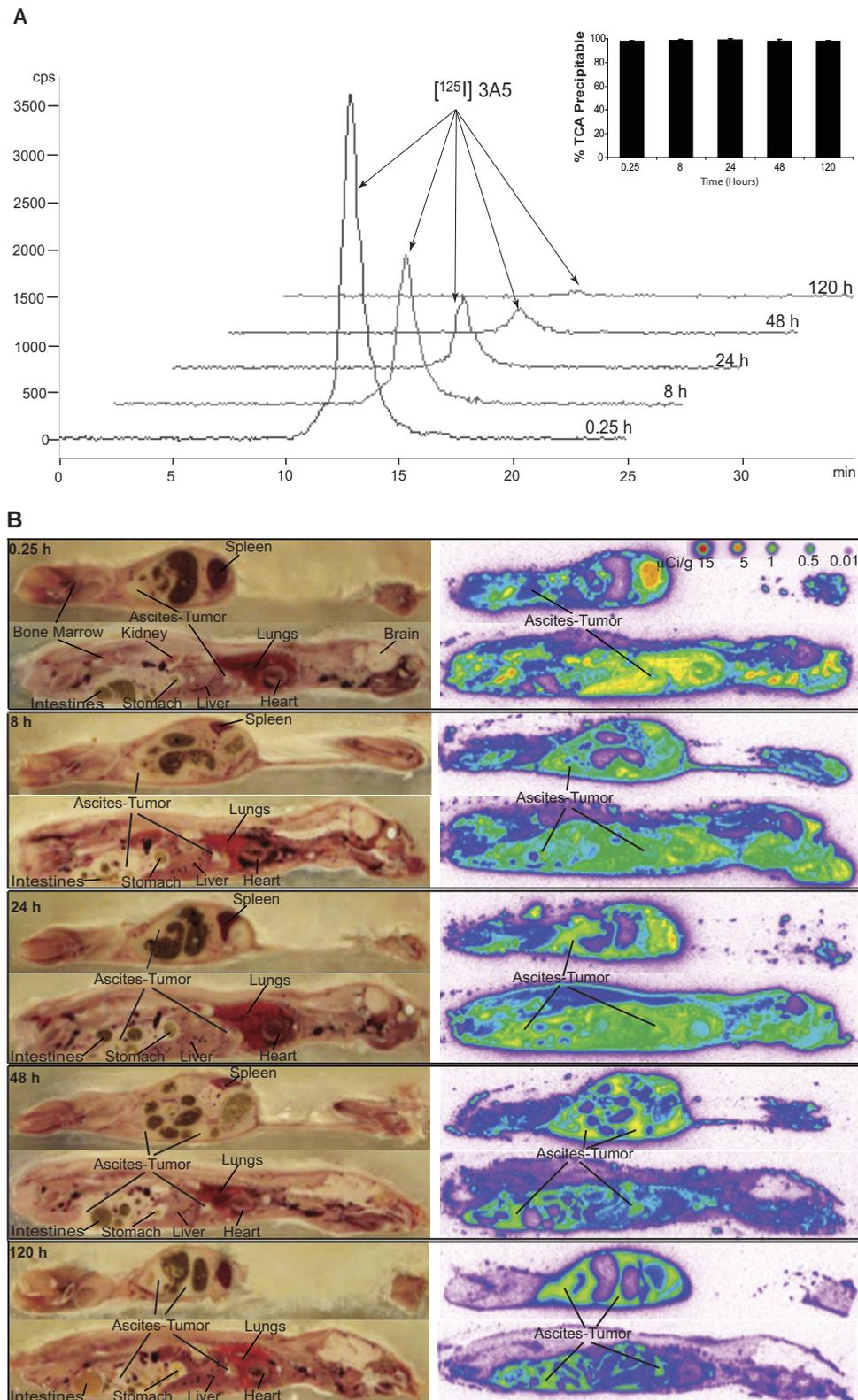


FIG. 5—Continued.

experiments revealed high radioactivity levels in the blood and highly perfused organs. Radioactivity levels in these normal tissues decreased in parallel with blood radioactivity. In contrast, the %ID/g in ascites-tumor increased by 8 h postdose and remained relatively unchanged over the remaining time course of the study (Fig. 5, B and C).

To determine the specificity of 3A5 uptake by ascites-tumor, the

distribution of 3A5 distribution was compared with that of an isotype control antibody in tumor-bearing mice. Mice bearing OVCAR-3/luc tumors at a late stage of the disease were dosed with ^{125}I -anti-gD (isotype control antibody) + unlabeled anti-gD for a total dose of 3.5 mg/kg. Tissue/blood ratios, a measure of tissue-specific uptake, were calculated from mice dosed with isotype control or 3A5 antibody (Fig. 6,

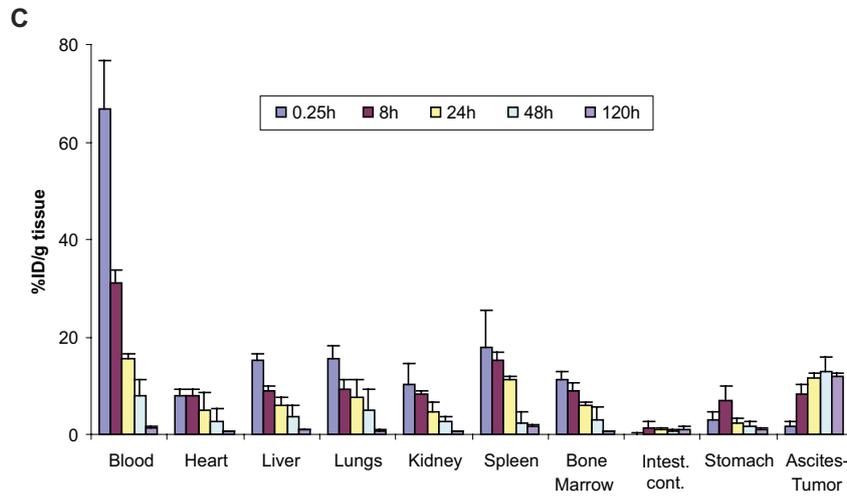


FIG. 5. Immune complex is not detected at a therapeutically relevant dose of 3A5. A, plasma SEC-HPLC profile from OVCAR-3/luc tumor-bearing mice with circulating levels of CA125 after a single intravenous dose of 3.5 mg/kg (¹²⁵I-3A5 + unlabeled 3A5). Inset displays the percentage of antibody that was TCA-precipitable from plasma samples over time. Data are expressed as the mean ± S.D. of three animals per time point. B, representative whole-body sagittal sections of OVCAR-3/luc tumor-bearing mice at different time points postdose. Anterior and posterior sectioning levels depict ascites-tumor and major organs of distribution, respectively. Left panel, digital pictures of the sections for anatomical localization purposes. Right panel, autoradiograms with the distribution of 3.5 mg/kg (¹²⁵I-3A5 + unlabeled 3A5) at 0.25, 8, 24, 48, and 120 h. The standard curve is represented as a pseudo-color heat map, inserted above the autoradiograms. Tissue distribution was assessed by QWBA. C, graph represents %ID/g after a single intravenous dose of 3.5 mg/kg (¹²⁵I-3A5 + unlabeled 3A5) into OVCAR-3/luc tumor-bearing mice at a late stage of tumor burden with measurable levels of CA125. Data are expressed as the mean ± S.D. values of three animals per time point. Intest. cont., intestinal contents.

A and B, respectively). A ratio >1, indicative of the specific uptake of an antibody by a tissue, was only observed at 120 h in the ascites-tumor (8.85 ± 1.04) of mice dosed with 3A5 (Fig. 6B), whereas ascites-tumor from the control antibody revealed ratios <1 (Fig. 6A). All normal tissues from 3A5 and control antibody had ratios <1 at all time points (Fig. 6).

Discussion

In recent years, therapeutic antibodies have been used to specifically target tumor cells based on their surface expression of unique antigens. In some cases, the antigen is shed from the cell surface into the circulation where the antibody may form ICs with the circulating

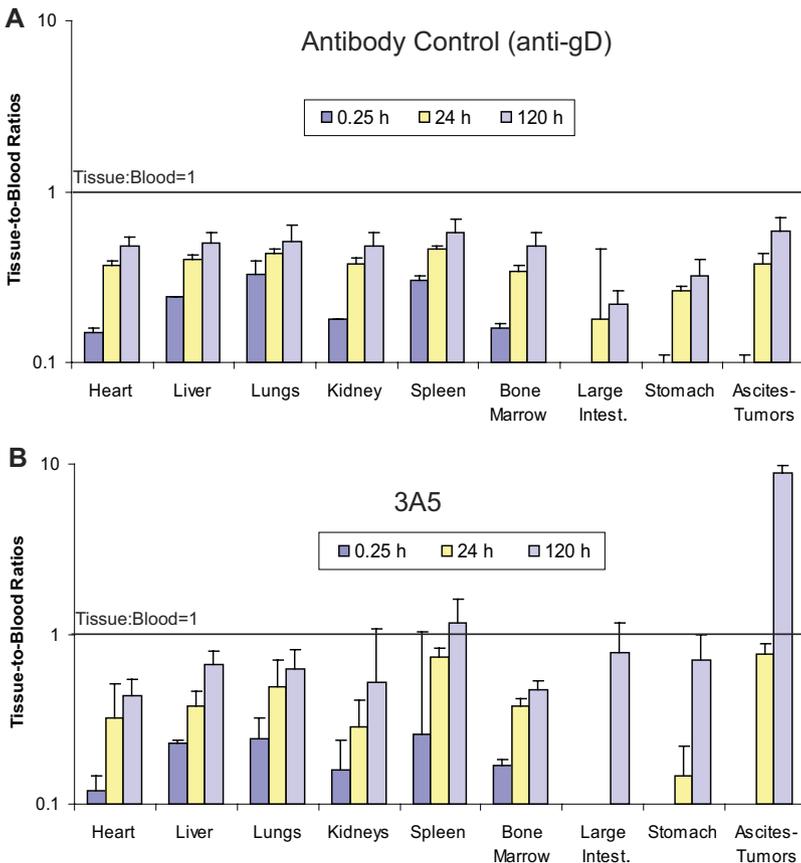


FIG. 6. Tumor/blood ratio of 3A5 or control antibody after a single intravenous dose of 3.5 mg/kg ¹²⁵I-3A5 + unlabeled 3A5 or ¹²⁵I-anti-gD + unlabeled anti-gD into OVCAR-3/luc tumor-bearing mice at a late stage of tumor burden at 0.25, 24, and 120 h postdose. Data are expressed as the mean ± S.D. values of three animals per time point. Large Intest., large intestine.

antigen, potentially affecting therapeutic efficacy by preventing it from reaching the tumor and/or altering the antibody kinetics of distribution and clearance (Bruno et al., 2005). A number of ovarian cancer preclinical and clinical studies have addressed the potential risks of shed antigen (Hagan et al., 1985; Haisma et al., 1987; Pimm et al., 1989; Kobayashi et al., 1993; Pimm, 1995; Sakahara et al., 1996; Davies et al., 1997; McQuarrie et al., 1997; Prinssen et al., 1998; Maeda et al., 2004). In patients with ovarian cancer, IC formation was detected upon delivery of an anti-CA125 antibody. However, the effect of circulating CA125 antigen did not appear to compromise pharmacokinetics of the antibody when it was used in excess of the circulating antigen (Sakahara et al., 1996; McQuarrie et al., 1997).

Here we studied 3A5, a novel antibody (Chen et al., 2007), that binds to a repeated epitope on CA125 and is currently a component of an antibody-drug conjugate strategy for the treatment of epithelial ovarian cancer (Chen et al., 2007). Because of the avidity of 3A5 to CA125, IC formation could affect delivery of the antibody to the tumors and induce unwanted toxicities in normal tissues. To address this issue, we performed a series of studies to assess IC formation in vitro and in vivo, as well as to characterize the impact of 3A5-CA125 interaction on the kinetics of 3A5 distribution.

We demonstrated that the in vitro interaction between ^{125}I -3A5 and CA125 can result in IC formation. The proportions of ICs are dependent on the relative concentrations of antibody and antigen. Increased concentrations of CA125 and a low concentration of 3A5 resulted in a proportional increase in IC formation (Fig. 1). Of importance, when the concentration of 3A5 was increased while that of the antigen was held constant, the proportion of IC was low relative to total 3A5. The in vitro kinetics of IC formation was rapid, in line with that reported by Haisma et al. (1987). IC formation between 3A5 and circulating CA125 was consistently detected in vivo in tumor-bearing mice only at low concentrations of the antibody relative to the antigen, which was determined by ELISA. When mice presenting with clinically relevant CA125 levels (69–318 U/ml) (Bast et al., 1983) were given a therapeutic dose of 3A5 (3.5 mg/kg) (Chen et al., 2007), no IC was detected. Taken together, these data suggest that IC formation at therapeutic 3A5 concentrations seems to consume a small fraction of the total 3A5. Furthermore, whether ICs were administered preformed to normal mice or formed in vivo in tumor-bearing mice, they were rapidly cleared from circulation, with the liver demonstrating a major role in the uptake and degradation. Consistent with liver catabolism, there was evidence of IC degradation products in the plasma, the contents of the intestines, and the stomach, an organ known to absorb free ^{125}I -(Regoeczi, 1987). Aside from the liver, we observed no overall difference in tissue kinetics in normal mice dosed with either 3A5-CA125 IC preformed in vitro or 3A5 alone (Fig. 2C). However, when IC was formed in vivo by administering a low dose of 3A5 (75 $\mu\text{g}/\text{kg}$) to tumor-bearing mice, faster clearance of the antibody was observed in normal tissues of these mice compared with 3A5 dosed to normal mice (Fig. 4, A and B). This effect was not as pronounced when tumor-bearing mice received a therapeutically relevant dose of 3A5 (3.5 mg/kg) (Fig. 5, B and C).

An important finding in these studies was that 3A5 distribution to the tumor was achieved despite IC formation. At the therapeutic dose of 3A5, persistent uptake of antibody in ascites-tumors was observed, with no IC detected and no uptake or accumulation in normal tissues. Specificity of 3A5 for ascites-tumors was demonstrated by the high tissue/blood ratios, whereas the isotype control antibody and normal tissue ratios revealed blood-related distribution only (Fig. 6). These data indicate that despite the binding of 3A5 to repeated epitopes expressed within CA125, IC formation did not affect tumor targeting.

The data herein describe the circumstances under which ICs form in

vitro and in vivo. In conclusion, the interaction between 3A5 and CA125 resulted in the formation of ICs that were rapidly distributed from circulation to the liver. At a therapeutically relevant dose, however, the proportion of IC relative to total 3A5 appeared to be minor, with a consequent greater distribution of uncomplexed 3A5 to the tumor. As a point of consideration, it remains possible that as the concentration of 3A5 decreases in vivo due to normal clearance, conditions may arise that result in a proportional increase in IC formation, a phenomenon that should be monitored clinically.

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