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Original Paper

Repeating the Avidin "Chase" Markedly Improved the Biodistribution of Radiolabelled Biotinylated Antibodies and Promoted the Excretion of Additional Background Radioactivity

H. Kobayashi, H. Sakahara, K. Endo, Z-s. Yao, S. Toyama and J. Konishi

Immunoscintigraphy using radiolabelled biotinylated monoclonal antibodies followed by infusion of avidin as a "chase" has been recently reported to improve the biodistribution for both immunoscintigraphy and radioimmunotherapy. In this study the circulating protein-bound and avidin-binding fractions of radiolabelled biotinylated antibodies were determined serially after injection of an avidin "chase", and the effect of repeating the avidin chase was also studied. Nude mice bearing KT005 human osteogenic sarcoma were injected with radiolabelled biotinylated antitumour monoclonal antibody (OST7). After injection of an avidin chase, the protein-bound and avidin-binding fractions in plasma were determined serially using the trichloroacetate method and avidin-Sepharose gel. The biodistribution of radiolabelled biotinylated OST7 was compared after single and double avidin chases with no chase. At 6 h after the first avidin chase in mice injected with radioiodinated and technetium-labelled biotinylated OST7, 67.7% and 67.8%, respectively, of the plasma radioactivity was available for binding to avidin and was cleared from the circulation. Reinjection of avidin decreased the plasma radioactivity and improved the biodistribution of the radiolabelled biotinylated antibodies. Repeating the avidin chase markedly improved the biodistribution of the radioiodine-labelled biotinylated antibody when compared with the use of a single avidin chase. This new method for radioimmunotherapy is sure to protect the critical organs from radiation injury without decreasing the therapeutic effect.

Key words: monoclonal antibody, avidin, biotin, chase, radioimmunotherapy
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INTRODUCTION

DIAGNOSTIC EXAMINATION and radiotherapy using radiolabelled monoclonal antibodies have been performed by many investigators [1–4]. The major problem with such procedures is the relatively low tumour-to-normal tissue radioactivity ratio and the small dose of radioactivity which consequently reaches the target tumour. Many attempts to improve the biodistribution of radiolabelled monoclonal antibodies have been reported, including the use of antibody fragments [5, 6], the subsequent injection of a second antibody [7], and pretargeting methods using either a bifunctional antibody or an avidin–biotin system [8–13]. We and other investigators have already reported the effectiveness of using a radiolabelled biotinylated antibody with an avidin "chase" for immunoscintigraphy and radioimmunotherapy [14–16]. Avidin chase can concentrate the radiolabelled biotinylated antibody into the liver and spleen immediately after injection. The radionuclides rapidly detach from the antibody and are excreted into the urine and faeces. The radioactivity accumulated in the tumour would be frozen during the period chasing the background activity [14]. In this study we additionally investigated the serial changes of circulating protein-bound and avidin-binding antibody fractions in mice after the injection of radiolabelled biotinylated antibodies and a subsequent avidin chase, and also evaluated the effect of repeating the avidin chase.

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MATERIALS AND METHODS

Cells

KT005 human osteosarcoma cells [17] were grown in RPMI 1640 medium (Nissui, Tokyo, Japan) containing 10% fetal calf

serum (GIBCO Laboratories, Grand Island, New York, U.S.A.) and 0.03% L-glutamine at 37°C in 5% CO_2. Subconfluent cells were removed from the culture dishes using calcium- and magnesium-free phosphate-buffered saline (PBS) containing 0.02% EDTA to preserve their antigenicity.

Monoclonal antibodies

The OST7 antibody (IgG1) was raised against a human osteogenic sarcoma [18], and it has been shown to react with human osteogenic sarcoma cells with the antigen being an alkaline phosphatase-related substance [19, 20]. The antibody was purified from the ascites of hybridoma-bearing mice using Protein A column chromatography (Bio-Rad, Richmond, California, U.S.A.). Monoclonal antibody 56C (IgG1), which recognises human chorionic gonadotropin, was used as the isotype-matched control antibody [21].

Biotinylation of monoclonal antibodies

The monoclonal antibodies were biotinylated as described previously [14]. Two to three sulphosuccinimidyl-6-(biotinamido) hexanoate (NHS-L-biotin) complexes were conjugated to each antibody, and more than 90% of the antibodies retained both antigen-binding and avidin-binding activity, as determined by the 2-(4'-hydroxyazobenzene) benzoic acid method of Green [22] and a binding assay with avidin–Sepharose gel (Pierce Chemical Co., Rockford, Illinois, U.S.A.)., respectively [14, 16].

Radiolabelling and quality control

Unconjugated and biotinylated monoclonal antibodies were radioiodinated using the chloramine-T method [23, 24]. Purified monoclonal antibodies (40 μg) in 0.3 M phosphate buffer (pH 7.5) and iodine-125 [^{125}I] (11.1 MBq) (DuPont, Billerica, Massachusetts, U.S.A.) were mixed with 3.0 μg of chloramine-T (Nakarai Chemicals, Kyoto, Japan) dissolved in 0.3 M phosphate buffer. After being allowed to react for 5 min, the radiolabelled antibody was separated from free iodine by PD-10 gel chromatography. The specific activity of the ^{125}I-labelled antibody was approximately 222 MBq/mg.

Unconjugated and biotinylated monoclonal antibodies were labelled with ^{99m}Tc by the direct method [5, 25, 26]. A monoclonal antibody solution (2.5 mg/ml) in 0.05 M PBS was incubated with 2-mercaptoethanol at room temperature for 30 min at a molar ratio of 1 : 1000 and the reduced antibody was purified by PD-10 gel chromatography. Immediately afterwards, 100 μg of the reduced antibody was mixed with 5 μl of the solution from a hydroxymethylene diphosphonate (HMDP) bone-scanning kit (Nihon Mediphysics, Nishinomiya, Japan) reconstituted with 5 ml of 0.9% sodium chloride for injection and 14.8 MBq of pertechnetate eluted from a ^{99}Mo/^{99m}Tc generator (Daiichi Radioisotope Laboratories, Tokyo, Japan).

Radiolabelled antibodies were analysed by size exclusion high-performance liquid chromatography on a TSKG3000SW column (Tosoh Co., Tokyo, Japan) and cellulose acetate electrophoresis. More than 95% of the radioactivity was associated with the IgG fraction, and no high molecular weight species (indicating the presence of antibody aggregates) were observed, as well as no free ^{125}I, free ^{99m}Tc, or free ^{99m}Tc-HMDP (data not shown).

Cell binding assay

The ^{125}I- and ^{99m}Tc-labelled monoclonal antibodies (3–5 ng/100 μl) were incubated with increasing concentrations of KT005 (10^4–5×10^6/100 μl) in 5.7 × 46 mm microcentrifuge tubes for 1 h at 4°C. After centrifugation at 10 000 ***g***, the supernatant was aspirated and the tubes were cut. The radioactivity bound to the cells was counted in an auto-well gamma counter. Specific binding to the cells was calculated by subtracting the nonspecific binding of ^{125}I-labelled control biotinylated 56C from the binding of radiolabelled biotinylated and unbiotinylated OST7. The binding of 56C to OST7 was less than 3% of the added radioactivity. The immunoreactive fraction of the radiolabelled antibodies was determined as the maximum binding percentage to the cell antigen by the modified method of Lindmo and associates [14, 17, 27, 28].

Biodistribution and pharmacokinetic studies

For *in vivo* studies of the radiolabelled monoclonal antibodies, 5×10^6 KT005 cells were inoculated subcutaneously into female BALB/c-nu/nu mice. After 12 days the tumours grew to approximately 200 mg in weight. Potassium iodide solution was administered to the mice from 1 day before the injection of radioiodinated antibodies to inhibit the uptake of released radioiodine by the thyroid.

Experiment 1. (*Blood clearance study*). To analyse the changes in protein-bound and avidin-binding radioactivity in nude mice which received the radiolabelled biotinylated antibody followed by an avidin chase, nude mice bearing KT005 tumours were injected with 30 μg of avidin at 6 h (Groups 1 and 3) or 24 h (Group 2) as well as 6 and 11.5 h (Groups 1 and 3) or 24 and 29.5 h (Group 2) after injection of the ^{125}I- (Groups 1 and 2) and ^{99m}Tc-labelled (Group 3) biotinylated antibodies (Figure 1a). A plasma sample (200 μl) was collected from each group of mice by cardiac puncture at 6, 6.5, 8, and 12 h (Groups 1 and 3) or 24, 24.5, 26 and 30 h (Group 2) after injection of the biotinylated antibody and its radioactivity was counted. Then 100 μl of plasma were added to 1 ml of 10% trichloroacetate and incubated for 5 min at room temperature. After centrifugation at 1000 ***g*** for 1 min, the radioactivity of the supernatant was defined as that of the free radionuclide and the radioactivity of the pellet was defined as that of protein-bound radionuclide. Another 100 μl of plasma were mixed with approximately 0.5 ml of avidin–Sepharose gel (Pierce Chemical Co., Rockford, Illinois, U.S.A.), and then the gel was washed with 0.05 M PBS. The radioactive fraction of the gel and the eluent were counted and the proportion of the radioactive biotinylated antibodies which could bind avidin was determined. Between three and four were tested in each group.

Experiment 2. (*Biodistribution study*). To examine the effect of repeated avidin chases, nude mice bearing KT005 tumours were injected into the tail vein with 37 kBq/10 μg of ^{125}I- (Groups A and B) and ^{99m}Tc-labelled (Group C) biotinylated OST7, and then the mice were intravenously injected with 30 μg of avidin at 6 (Groups A-1, C-1) or 24 h (Group B-1) or with 30 μg of avidin at 6 and 11.5 h (Groups A-2, C-2) or at 24 and 29.5 h (Group B-2). Control groups of mice did not receive any avidin chase (Group A-0, B-0, C-0) (Figure 1b). Each group of mice was killed, the organs and tumours were removed and weighed, and then the radioactivity was counted at 12 h (Groups A, C) or 30 h (Group B) after injection of the radiolabelled biotinylated antibody. Four to eight mice were tested in each group. Both the percentage of the injected dose per gram (%ID/g) of tissue and the tumour-to-normal tissue ratios were determined.

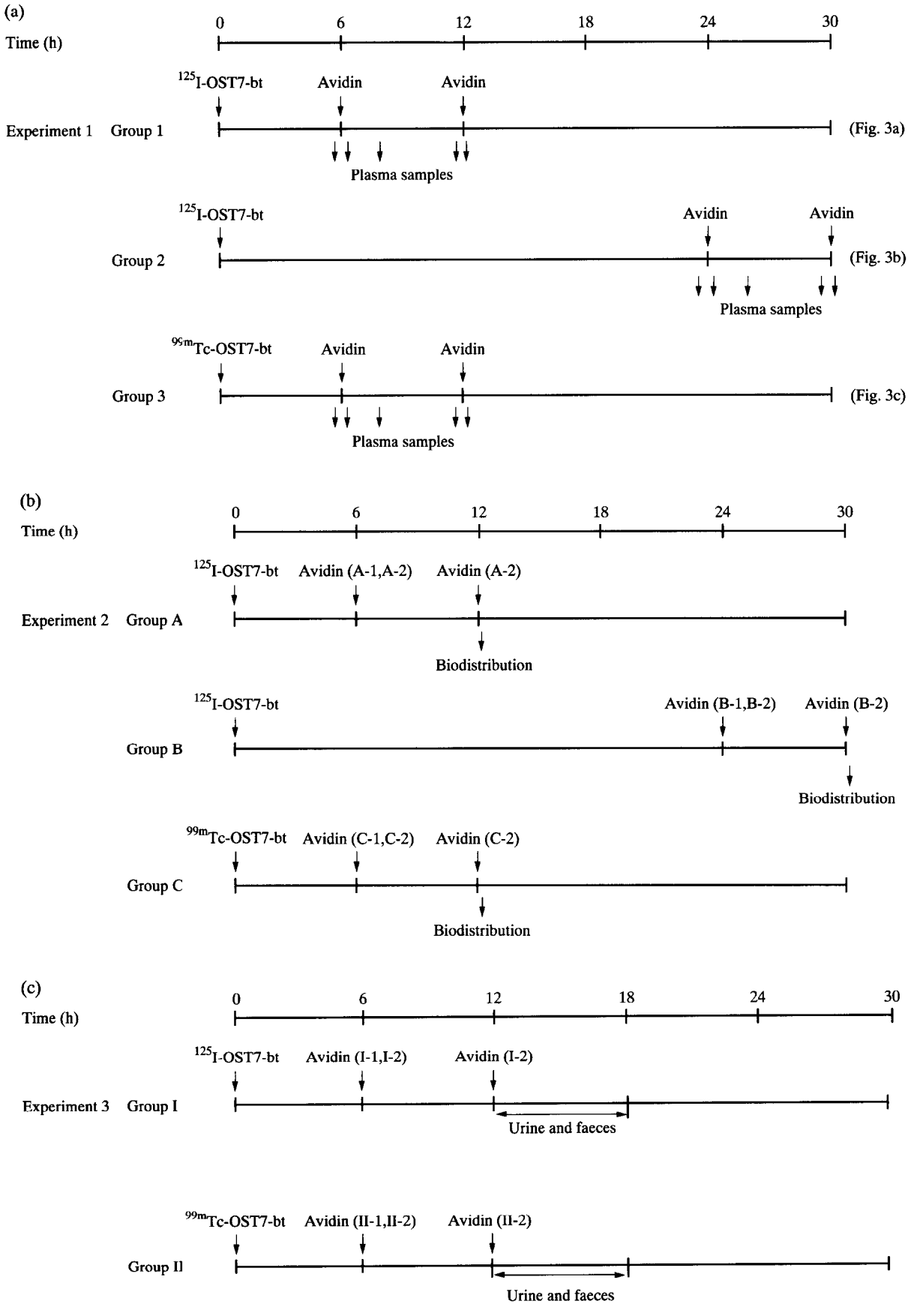

Figure 1. Protocols of the experiments using the mice bearing KT005. (Experiment 1:(a), Experiment 2:(b), Experiment 3:(c)).

Experiment 3. (*Excretion study*). Two groups of mice were placed in metabolic cages and the cumulative ^{125}I (Group I) and ^{99m}Tc (Group II) excretion in the urine and faeces was determined from 12 to 18 h after the intravenous injection of 10 μg of ^{125}I- and ^{99m}Tc-labelled biotinylated antibody. Both groups of mice were given 30 μg of avidin in 150 μl of 0.05 M PBS 6 h after injection of the antibody, following which one group of mice was given 30 μg of avidin in 150 μl of 0.05 M PBS again, and the other control group was given 150 μl of 0.05 M PBS alone, 11.5 h after injection of the antibody (Figure 1c). Three mice were tested in each group and the experiment was performed three times.

All procedures involving animals were carried out in accordance with the regulations for animal welfare in Japan.

RESULTS

The procedures, such as radiolabelling and biotinylation, did not reduce the immunoreactivity of OST7 (Figure 2). Avidin injection quickly decreased the circulating blood radioactivity. With both the radioiodine- and technetium-labelled biotinylated antibodies, over 60% (6.0/8.1, 4.8/7.9) of the plasma radioactivity was localised in the protein-bound fraction unavailable for binding to avidin 30 min after injection of the chase, but this decreased to below 20% within 2 h after avidin injection. However, the protein-bound fraction of the total radioactivity available for binding with avidin increased to 68% (10.7/16.0, 7.0/10.4, 5.0/7.4) 6 h after injection of the chase. This fraction of the blood radioactivity could subsequently be recleared from the circulation by repeating the avidin chase (Table 1, Figure 3). In agreement with the aforementioned results, the biodistribution study showed that reinjection of the avidin chase decreased the blood radioactivity and increased antibody accumulation in the liver and spleen (Table 2). The tumour-to-blood and tumour-to-lung radioactivity ratios increased with time of injected avidin chase (Figure 4). However, the tumour-to-liver and tumour-to-spleen radioactivity ratios decreased after the second chase, because the values of %ID/g in these organs increased immediately after avidin injection. Normally liver and spleen radioactivity is excreted rapidly, so that these ratios would increase over the value without reinjection of avidin within a few hours after reinjection [14]. Urinary and/or faecal excretion also increased to approximately twice that after a single avidin chase (Table 3).

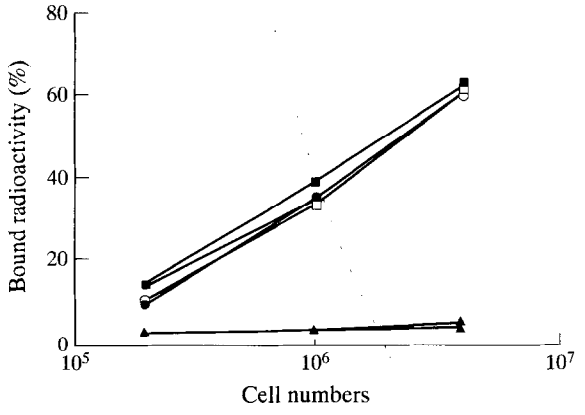

Figure 2. Binding of ^{125}I-labelled unbiotinylated OST7 (○), ^{99m}Tc-labelled unbiotinylated OST7 (□), ^{125}I-labelled biotinylated OST7 (●), ^{99m}Tc-labelled biotinylated OST7(■), ^{125}I-labelled control biotinylated 56C (△) and ^{125}I-labelled control biotinylated 56C (▲) to KT005 cells. The percentage of bound radioactivity is plotted against the number of cells.

DISCUSSION

The results of these experiments indicate that nearly all the circulating biotinylated antibodies were bound to avidin within 30 min after injection of the avidin chase, and that the complexes were rapidly trapped in the liver and spleen. However, biotinylated antibodies available for binding to avidin gradually increased again from 2 h after avidin injection and the proportion of circulating antibodies in the blood, which could bind to avidin, reached 68% at 6 h after the chase. This may have occurred because antibodies trapped in the tissue were not avidinylated after the chase and re-entered the blood as the level of circulating antibody decreased. However, the blood radioactivity at 6 h after avidin injection, which was higher than that at 30 min after injection, could be decreased to a level below that after the first chase by repeating the avidin chase. From the results of the biodistribution study, if the first avidin chase is injected too early to accumulate the biotinylated antibody in the target tumour after the injection of radiolabelled biotinylated antibody, the radioactivity in the target tumour may be lower than that without avidin chase, because the avidin chase rapidly clears the circulating antibody and inhibits the additional accumulation of the antibodies in the tumour. Therefore, the first avidin chase should be injected 24 h or more after the radiolabelled biotinylated antibody injection in mice, when the antibodies have fully accumulated in the target tumour.

When a radioiodinated biotinylated antibody was used, radioiodine was detached from the antibody by the reticuloendothelial system or the hepatocytes in the liver and spleen and provided a major contribution to the slightly increased blood radioactivity 2 h after avidin injection. The protein-bound radioactivity present in the blood 2 h after avidin injection was less than that at 30 min after avidin injection. When a ^{99m}Tc labelled biotinylated antibody was used in the previous and present studies [14], the radioactivity in the blood 2 h after injection showed the lowest value at all time points examined. Although the changes of blood radioactivity after avidin chase were similar to those of the protein-bound fraction when the radioiodine-labelled biotinylated antibody was used, free technetium-99m did not appear in the blood or was not detached from the antibody in the liver and spleen, so a difference in blood radioactivity between the radioiodinated and technetium-99m labelled antibodies was seen 2 h after avidin injection. The different behaviours between the iodine- and technetium-labelled antibodies, as seen in blood clearance studies, could be caused by the instability of the technetium-labelled antibody. Although the direct labelling methods reported by Schwarz and colleagues and Mather and Ellison were very easy and useful [25, 26], the technetium-labelled antibody was relatively unstable *in vivo* [5]. So the technetium-labelled biotinylated antibody, detected by this method, re-entering into the circulation would be less than the iodine-labelled biotinylated antibody.

Immunoscintigraphy and radioimmunotherapy using radiolabelled biotinylated antibodies and an avidin chase would seem to be easy to perform clinically. For immunoscintigraphy, the reinjection of avidin before obtaining delayed images more than 6 h after the first avidin injection would improve contrast and decrease radiation dose to the patient, although the tumour-to-liver and tumour-to-spleen radioactivity ratios may be temporarily increased. For radioimmunotherapy using ^{131}I-labelled monoclonal antibodies, although the exact dosimetry of the cumulative radiation to all the organs on the study using our method would be very difficult because of complicated

Table 1. Plasma radioactivity fractions after injection of ^{125}I-labelled biotinylated OST7 followed by the injection of avidin chase (mean ± S.D.)

Time of first avidin injection	^{125}I-labelled biotinylated OST7										^{99m}Tc-labelled biotinylated OST7				
	6 h					24 h					6 h				
Time of second avidin injection	ND	ND	ND	ND	11.5 h	ND	ND	ND	ND	29.5 h	ND	ND	ND	ND	11.5 h
Time after antibody injection (h)	6	6.5	8	12	12	24	24.5	26	30	30	6	6.5	8	12	12
Total plasma	66.8 ± 4.5†	6.5 ± 1.1	11.4 ± 1.5	16.0 ± 1.4	8.6 ± 0.7	33.0 ± 2.6	8.1 ± 0.9	9.5 ± 1.7	10.4 ± 2.3	5.4 ± 0.4	60.4 ± 6.5	7.9 ± 0.8	6.4 ± 0.4	7.4 ± 1.3	3.3 ± 0.6
Free radionuclide	1.5 ± 0.3	1.8 ± 0.2	4.8 ± 1.0	2.3 ± 0.3	3.2 ± 0.6	1.6 ± 0.2	0.9 ± 0.2	3.1 ± 0.4	1.1 ± 0.2	1.4 ± 0.1	1.0 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.3 ± 0.0	0.4 ± 0.1
Protein-bound, avidin-binding	62.0 ± 5.5	1.7 ± 0.2	3.7 ± 0.5	10.7 ± 0.8	1.4 ± 0.4	31.1 ± 4.6	1.2 ± 0.1	4.1 ± 0.3	7.0 ± 0.5	0.5 ± 0.1	58.7 ± 5.3	2.7 ± 0.5	3.6 ± 0.3	5.0 ± 0.8	0.5 ± 0.1
Protein-bound, unavailable to bind avidin	3.3 ± 0.4	3.0 ± 0.4	2.9 ± 0.2	3.0 ± 0.6	4.0 ± 0.5	0.3 ± 0.0	6.0 ± 1.5	2.3 ± 0.3	2.3 ± 0.4	3.5 ± 0.4	0.7 ± 0.3	4.8 ± 0.3	2.2 ± 0.3	2.1 ± 0.2	2.4 ± 0.5

ND, not done; † Percentage of injected dose per gram plasma.

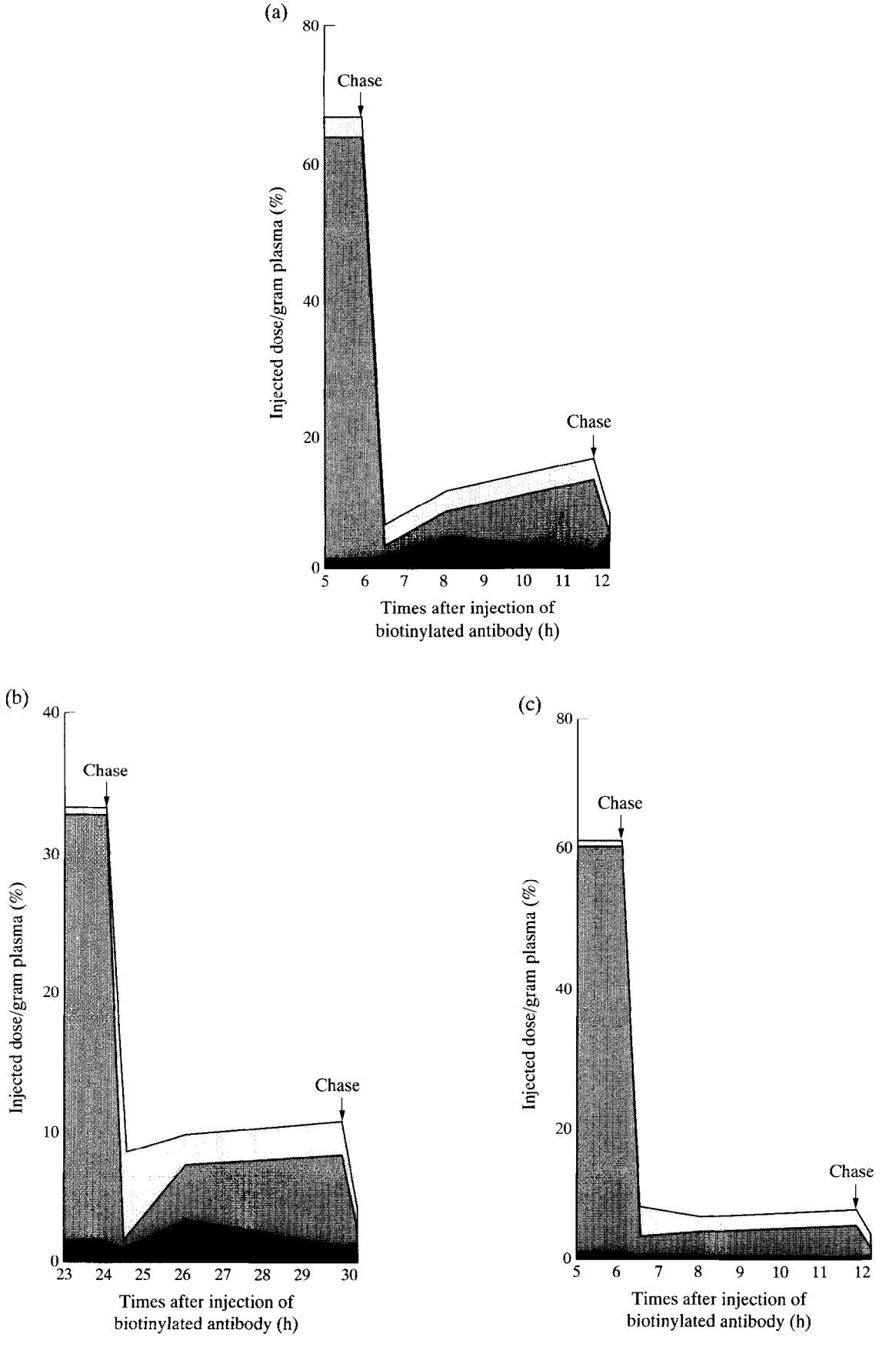

Figure 3. Plasma radioactivity fractions after injection of 10 μg of ^{125}I-labelled biotinylated OST7 (a) Group 1; (b) Group 2; or (c) ^{99m}Tc-labelled biotinylated OST7: Group 3 is shown. Free nucleotide, (■); protein-bound radioactivity available for binding, (▨); and radioactivity unavailable for binding to avidin is shown in the light grey square (□).

Table 2. Biodistribution of 10 μg of biotinylated OST7 after one or two chases with 30 μg of avidin in mice bearing KT005 tumours (mean ± S.D.)

Time after antibody	^{125}I-biotinylated OST7						^{99m}Tc-biotinylated OST7		
	12 h (n = 5)* (Group A)			30 h (n = 6) (Group B)			12 h (n = 5) (Group C)		
Time of avidin injection	None (A-0)	6 h (A-1)	6, 11.5 h (A-2)	None (B-0)	24 h (B-1)	24, 29.5 h (B-2)	None (C-0)	6 h (C-1)	6, 11.5 h (C-2)
Blood	18.72 ± 0.87†	5.04 ± 0.34	2.63 ± 0.38	11.66 ± 1.76	4.32 ± 0.32	2.74 ± 0.17	17.34 ± 1.40	3.03 ± 0.55	1.11 ± 0.13
Liver	5.27 ± 0.37	5.40 ± 0.41	8.86 ± 2.29	2.84 ± 0.50	2.30 ± 0.67	4.60 ± 0.81	6.47 ± 0.36	22.61 ± 3.47	31.50 ± 5.99
Kidney	5.77 ± 0.48	2.97 ± 0.39	3.04 ± 0.53	3.04 ± 0.62	1.57 ± 0.29	1.59 ± 0.11	9.15 ± 0.58	9.39 ± 0.91	8.90 ± 1.03
Intestine	1.89 ± 0.31	1.28 ± 0.14	1.50 ± 0.14	0.79 ± 0.18	0.53 ± 0.09	0.67 ± 0.25	2.76 ± 0.68	5.05 ± 1.06	5.60 ± 0.81
Stomach	2.49 ± 0.93	5.29 ± 1.38	8.02 ± 1.94	0.47 ± 0.06	1.26 ± 0.56	0.92 ± 0.21	1.37 ± 0.43	0.83 ± 0.18	1.28 ± 0.29
Spleen	3.90 ± 0.47	9.25 ± 2.71	11.26 ± 1.65	1.89 ± 0.49	1.89 ± 0.74	2.71 ± 0.35	3.93 ± 0.50	17.70 ± 6.01	20.96 ± 3.17
Lung	8.60 ± 1.05	3.58 ± 0.59	3.01 ± 0.46	4.82 ± 0.68	2.17 ± 0.21	1.98 ± 0.13	7.31 ± 0.70	3.80 ± 0.53	3.30 ± 0.63
Muscle	1.05 ± 0.17	0.85 ± 0.17	0.79 ± 0.07	0.97 ± 0.24	0.72 ± 0.19	0.68 ± 0.08	0.80 ± 0.13	0.52 ± 0.08	0.44 ± 0.07
Bone	1.95 ± 0.39	1.34 ± 0.18	1.24 ± 0.31	1.01 ± 0.15	0.73 ± 0.15	0.74 ± 0.11	1.55 ± 0.31	1.30 ± 0.48	1.06 ± 0.38
Tumour	22.42 ± 2.30	14.71 ± 3.09	14.55 ± 0.61	19.47 ± 6.04	17.69 ± 1.44	17.76 ± 4.01	25.12 ± 2.25	16.05 ± 1.75	17.05 ± 1.94

*Numbers in parentheses are the number of animals; † Percentage of the injected dose per gram of tissue.

Table 3. Excretion percentages of the injected radiolabelled biotinylated antibodies in the 6 h period after avidin chase (n = 3 × 3 times)

Collection period for urine and faeces	^{125}I-labelled biotinylated OST7 12–18 h		^{99m}Tc-labelled biotinylated OST7 12–18 h	
Time from antibody to avidin	6 h (I-1)	6, 11.5 h (I-2)	6 h (II-1)	6, 11.5 h (II-2)
Urinary excretion	5.66–6.25*	11.15–13.37	4.32–4.71	7.59–8.14
Faecal excretion	0.08–0.10	0.17–0.20	1.34–1.46	3.18–3.30

* Percentage of injected radioactivity.

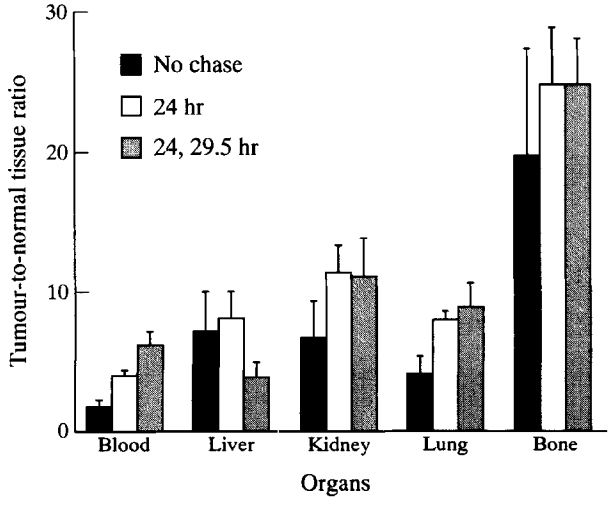

Figure 4. Tumour-to-normal tissue radioactivity ratios in mice (Group B) at 30 h after the injection of ^{125}I-labelled biotinylated OST7 and a subsequent single injection (24 h) or double injection (24 and 29.5 h) of 30 μg of avidin.

behaviours of the radionuclide, the reinjection of avidin at appropriate times after the first chase would decrease the cumulative radiation dose to all the organs, especially the bone marrow and lungs, which are the most critical organs for radiotoxicity [4]. However, it would not decrease the tumour dose of radioactivity. This method would protect non-target organs from unnecessary irradiation and would thus allow an increase of the initial dose injected into the patient and a stronger effect on the target tumour.

In conclusion, immunoscintigraphy and radioimmunotherapy using radioiodinated biotinylated antibodies and a repeated avidin chase may be more valuable than the use of conventional radiolabelled monoclonal antibodies or radiolabelled biotinylated antibodies with a single avidin chase.

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