Decreased Renal Accumulation of Biotinylated Chimeric Monoclonal Antibody-Neocarzinostatin Conjugate after Administration of Avidin

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Murine monoclonal antibodies (mAbs) such as A7 administered to humans induce a human anti-mouse antibody response. Moreover, because Fab fragments of mAbs are able to penetrate target tumors easily, they may be more suitable than intact mAb to be carriers of anticancer agents such as neocarzinostatin (NCS), which are rapidly inactivated in the blood. To address these problems, chimeric A7 Fab fragment-NCS conjugate (chA7Fab-NCS) was produced. However, large amounts of ^{125}I-labeled chA7Fab-NCS accumulate in the kidney and can lead to renal dysfunction. To decrease renal accumulation of chA7Fab-NCS, chA7Fab was biotinylated and administered with a subsequent injection of avidin. Human pancreatic carcinoma-bearing nude mice were injected with ^{125}I-labeled biotinylated chA7Fab-NCS with or without subsequent administration of avidin. The accumulation of ^{125}I-labeled biotinylated chA7Fab-NCS in tissue samples was measured at appropriate time intervals. ^{125}I-labeled biotinylated chA7Fab-NCS was cleared more rapidly from the blood and the kidney with the administration of avidin than without it. There was no difference between tumor accumulation in these groups. The tumor/blood ratio of radioactivity of ^{125}I-labeled biotinylated chA7Fab-NCS was significantly higher with subsequent administration of avidin than without avidin. The administration of biotinylated chA7Fab-NCS followed by avidin may enhance safety and permit the administration of larger doses of NCS without the subsequent development of renal failure. A larger amount of ^{125}I-labeled biotinylated chA7Fab-NCS was retained in the liver and spleen with the subsequent administration of avidin than without avidin.

Key words: Pancreatic cancer — Chimeric antibody — Biotinylated antibody — Avidin-biotin complex

The development of hybridoma technology offers the possibility of an effective means of delivering chemotherapy to cancer cells. A number of mAbs have been linked to a variety of antitumor drugs and cytotoxins in attempts to increase the effectiveness of chemotherapy.[1, 2] We generated the mAb A7, and covalently conjugated it to the antitumor antibiotic NCS (A7-NCS).[3] The conjugate A7-NCS has been used clinically to treat patients with colorectal and pancreatic carcinomas.[4] However, murine mAbs such as A7, when administered to humans, induce a HAMA response[5–7] that may reduce the tumor localization of the mAb and lead to an anaphylactic reaction. In general, because Fab fragments of mAbs are

able to penetrate target tumors easily, they may be more suitable than intact mAbs as carriers of anticancer agents, such as NCS, which are rapidly inactivated in the blood. To solve these problems, chA7Fab has been produced using recombinant DNA techniques as a new carrier of NCS.[8] In an experiment using pancreatic cancer-bearing nude mice, ^{125}I-labeled chA7Fab-NCS accumulated in tumors earlier than ^{125}I-labeled A7-NCS, and significantly larger amounts of ^{125}I-labeled chA7Fab-NCS had accumulated in the tumors 1 h after injection, a time when NCS is still active.[9, 10] However, a relatively large amount of ^{125}I-labeled chA7Fab-NCS accumulated in the kidney, as previously observed with chA7Fab.[10] Because renal dysfunction is a major side effect in chemotherapy with NCS in humans, renal accumulation of the chA7Fab-NCS conjugate needs to be minimized.

Avidin has a high binding affinity for biotin, a 244 D vitamin found in low concentrations in tissues and in blood.[11] The bond formation is completed within 15 min, and once formed, is extremely stable.[12] The strong affinity of the avidin-biotin system has drawn the attention of several researchers working on background reduction in imaging applications of mAbs.[13–15] The avidin-biotin

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system provides an extremely rapid increase in the contrast between blood and target tissue. In the present study, the biotinylated chA7Fab-NCS conjugate was administered to nude mice with pancreatic cancer. Thirty min later, a time when NCS is still active, avidin was injected and the kinetics of accumulation of biotinylated chA7Fab-NCS in various tissues were examined. Accumulation with avidin injection was compared to accumulation without avidin injection.

MATERIALS AND METHODS

Cell line and tumor xenografts The pancreatic carcinoma cell line HPC-YS[16] was established from a ductal cell adenocarcinoma of the human pancreas and was obtained from Dr. N. Yamaguchi (Research Institute of Neurology and Geriatrics, Kyoto Prefectural University of Medicine). Cultured HPC-YS cells were maintained in RPMI 1640 media supplemented with 10% FBS (Flow Laboratories, Inc., Rockville, MD) and harvested after a brief treatment with EDTA, washed in PBS and resuspended in PBS. Approximately 5×10^6 viable cells were injected subcutaneously into the left flank of athymic 8-week-old male nude mice (BALB/C, *nu/nu*) (SLC Co., Shizuoka). Tumor masses were detected 7 days after inoculation in all mice injected with HPC-YS cells.

Preparation of chA7Fab-NCS ChA7Fab was produced using recombinant DNA techniques as previously described.[10] The NCS conjugation procedures have been described by Yamaguchi *et al.*[8] Briefly, chA7Fab and NCS were mixed separately with SPDP, and chA7Fab conjugated to PDP (chA7Fab-PDP) and NCS-PDP were collected. NCS-PDP was thiolated with DTT, then chA7Fab-PDP was combined with NCS-SH, and the mixture was loaded onto a Q-Sepharose column. Elution was conducted using a linear gradient from 0 to 200 mM NaCl in 0.02 M Tris-HCl buffer. The conjugation ratio used was 1 mol of NCS per mol of chA7Fab, i.e., 4.5 mg of chA7Fab was bound to 1 mg of NCS.

Preparation of radiolabeled chA7Fab-NCS Radiolabeling of chA7Fab-NCS with ^{125}I (IMS 30, Amersham Japan, Ltd., Tokyo) was performed using the chloramine-T method.[17] Iodinized chA7Fab-NCS was separated from excess reactants by gel filtration on a Sephadex G-25 column. ChA7Fab-NCS was labeled with ^{125}I to a specific activity of 1.0 μCi/μg.

Biotinylation of ^{125}I-labeled chA7Fab-NCS Three hundred micrograms of NHS-Biotin (Pierce 23225, Rockford, IL) in dimethyl sulfoxide was added to 200 μg of ^{125}I-labeled chA7Fab-NCS in 50 mM sodium bicarbonate buffer, pH 8.5. Following incubation on ice for two hours, the preparations were incubated for 30 min at room temperature. Biotinylated ^{125}I-labeled chA7Fab-NCS was separated from unreacted biotin by gel filtra-

tion on a Sephadex G-25 column. The biotinylation ratio used was 2 mol of biotin per mol of chA7Fab-NCS.

Binding activity of biotinylated chA7Fab-NCS to HPC-YS cells The binding activity of biotinylated chA7Fab-NCS was measured by using a competitive radioimmunoassay with HPC-YS cells. Aliquots of HPC-YS cells (5×10^5) were incubated with ^{125}I-labeled chA7Fab-NCS (1×10^5 cpm) in the presence of 4-fold serially diluted chA7Fab-NCS or biotinylated chA7Fab-NCS in PBS at 37°C for 60 min. The concentration of chA7Fab-NCS or biotinylated chA7Fab-NCS ranged from $(1/4)^9 \times 10^{-5}$ to 1.0×10^{-5} mol/liter. After incubation, the cell pellets were subjected to γ-scintillation counting, and the percent inhibition was calculated as compared to the control.

Kinetics of ^{125}I-labeled chA7Fab-NCS accumulation after administration of avidin The kinetics of ^{125}I-labeled biotinylated chA7Fab-NCS accumulation after the administration of avidin was investigated in athymic nude mice bearing HPC-YS tumors. Fourteen days after tumor cell inoculation, 32 mice were divided into two groups. Sixteen mice from each group were injected with 0.7 μCi of ^{125}I-labeled biotinylated chA7Fab-NCS in 100 μl of PBS containing chA7Fab-NCS (chA7Fab, 2.25 mg/kg; NCS, 500 μg/kg). Each of the mice was injected intravenously with 30 μg of streptavidin (Sigma, St. Louis, MO) in 100 μl of 50 mM HEPES in 5% mannitol buffer, pH 7.4 or the same solution without streptavidin 30 min after the injection of ^{125}I-labeled biotinylated chA7Fab-NCS. The mice were killed 0.5, 6, 12, or 24 h after the injection of avidin. The tumors, blood, and normal organs (heart, lung, liver, spleen, pancreas, colon, and kidney) were weighed. The radioactivity in each tissue was then measured using a γ-scintillation counter (Auto-Gamma 5000, Packard, Meriden, CT). The results from the different tissues were expressed as cpm/g and compared with each other. To compare the kinetics of ^{125}I-labeled biotinylated chA7Fab accumulation in each group, the results were presented as %ID/g. Student's t test was used to check for statistically significant differences between groups. To compare the specific localization of the probes in the tumor to that in the blood, the ratio of radioactivity in the tumor to that in the blood was calculated.

In a separate experiment, we examined the kinetics of ^{125}I-labeled biotinylated chA7Fab-NCS accumulation in the blood, kidney, liver, and spleen at 5 min intervals for 30 min after avidin administration. The results are presented as %ID/g of each tissue.

RESULTS

Binding activity of biotinylated chA7Fab-NCS to HPC-YS cells The binding of biotinylated chA7Fab-NCS to
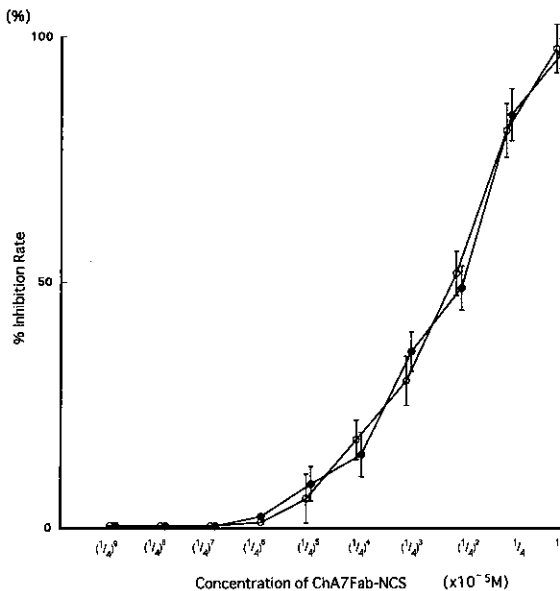
(%)

Fig. 1. The binding activity of biotinylated chA7Fab-NCS to HPC-YS cells was compared to that of chA7Fab in a competitive radioimmunoassay. Biotinylated chA7Fab retained a binding activity nearly identical to that of chA7Fab. ○, biotinylated chA7Fab-NCS; ●, chA7Fab-NCS; point, mean; bar, SD.

HPC-YS cells was compared to that of chA7Fab-NCS using a competitive radioimmunoassay. The results indicate that biotinylated chA7Fab-NCS retained a binding activity nearly identical to that of chA7Fab-NCS (Fig. 1). **Kinetics of ^{125}I-labeled chA7Fab-NCS accumulation after administration of avidin** The blood accumulation of ^{125}I-labeled biotinylated chA7Fab-NCS with and without the administration of avidin decreased linearly with time. ^{125}I-Labeled biotinylated chA7Fab-NCS was cleared more rapidly from the blood with the administration of avidin than without it, and significant differences were observed at 0.5, 6, and 12 h after the injection of avidin. In contrast, the tumor clearance pattern of ^{125}I-labeled biotinylated chA7Fab-NCS with the subsequent administration of avidin was similar to that when avidin was not administered. As for normal tissues, ^{125}I-labeled biotinylated chA7Fab-NCS was cleared earlier from the kidney with the subsequent administration of avidin than without avidin. Significant differences were observed between the two groups at 0.5, 6, and 12 h after injection ($P <$ 0.05). A larger amount of ^{125}I-labeled biotinylated chA7Fab-NCS was retained in the liver and spleen with the subsequent administration of avidin compared to that without avidin. Significant differences were observed between the two groups at 0.5 and 6 h after injection ($P <$ 0.05) (Fig. 2). The tumor tissue/blood ratio of radioac-
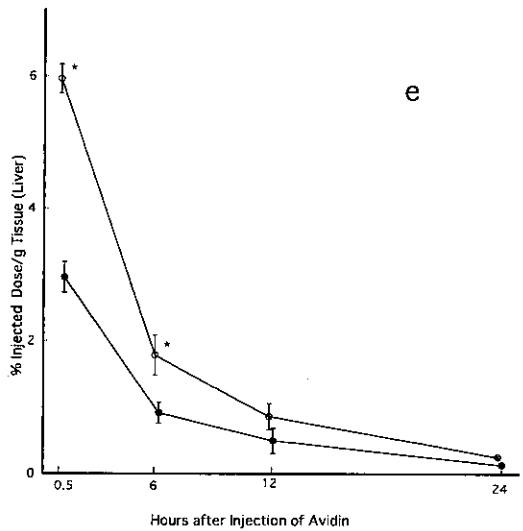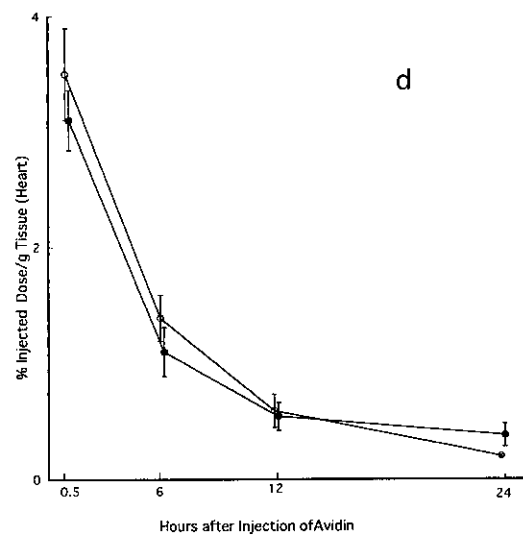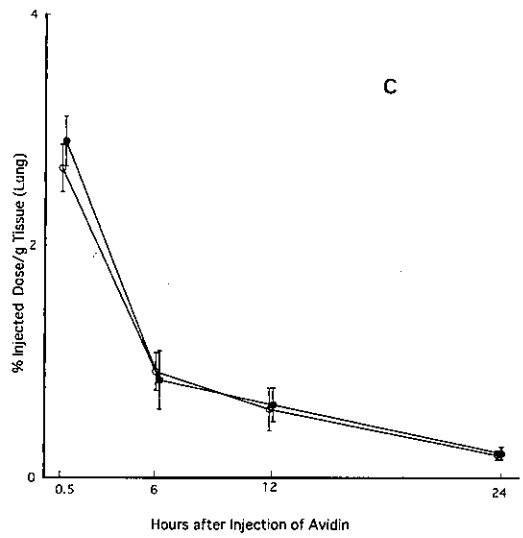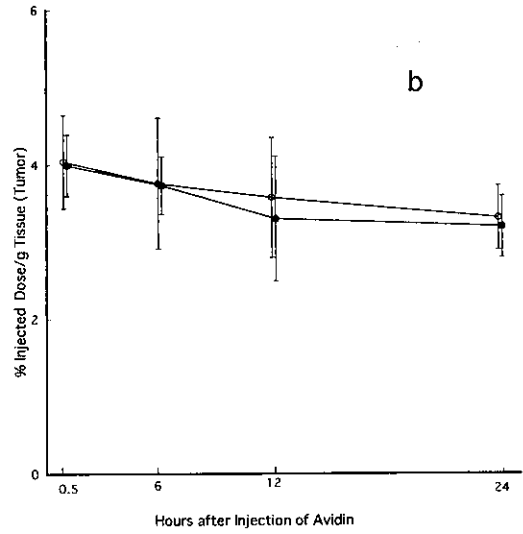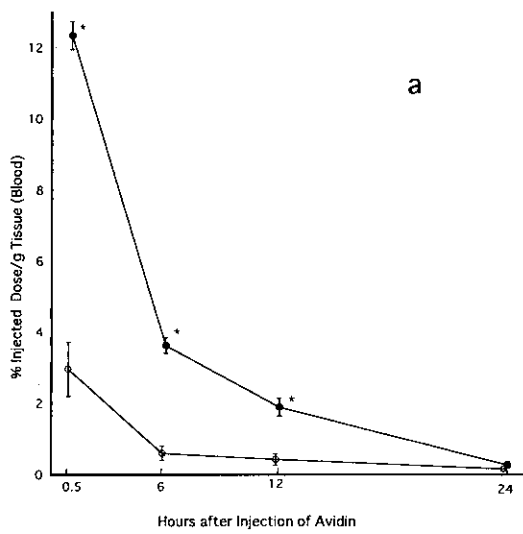
tivity of ^{125}I-labeled biotinylated chA7Fab-NCS was significant higher with a subsequent administration of avidin than that without avidin (Fig. 3). In the separate experiment, the concentration of ^{125}I-labeled biotinylated chA7Fab-NCS in the blood and the kidney decreased rapidly in the first 5 min after avidin administration and that in the spleen and the liver increased rapidly in the first 5 min after avidin administration (Fig. 4).

DISCUSSION

HAMA production should decrease when NCS conjugated to a human/mouse chimeric mAb is administered to humans because the origin of the Fc portion of the chimeric mAb, which is the most immunopotent region of intact mAbs, is human.[18] In this study we used chA7Fab-NCS in order to target NCS to human pancreatic carcinoma.

In general, the variable fragment of mAbs has the ability to leave the vascular space rapidly and to penetrate target tumor tissue.[19] Thus, the Fab fragment of mAb A7 may be able to deliver a large amount of a short-acting anticancer drug such as NCS[20] directly into the tumor. In a previous study, the antitumor effect of chA7Fab-NCS on the growth of the human pancreatic carcinoma grafted into nude mice was compared with that of A7-NCS, and the results suggested that only chA7Fab-NCS can completely suppress the tumor growth.[21] However, more ^{125}I-labeled chA7Fab-NCS than ^{125}I-labeled A7-NCS accumulated in the kidney[9] because most of the intravenously injected chA7Fab-NCS was cleared via the kidney. Because renal failure is a serious side effect of NCS therapy, renal accumulation of chA7Fab-NCS is a complication of chemotherapy with this conjugate.

The association constant of the avidin-biotin bond is 10^{15} M^{-1},[12] and as such, is 10^6-fold greater than most antigen-antibody interactions. The bond formation is completed within 15 min, and once formed, is extremely stable. The *in vitro* biotinylation of proteins has been well-described,[12] and any type of immunoglobulin can be easily biotinylated. ChA7Fab-NCS was biotinylated without loss of antibody-antigen binding activity and was injected into nude mice. Biotinylated chA7Fab-NCS bound to avidin forms a large aggregate, because avidin has four binding sites for biotin. As shown in Figs. 2 and 3, the accumulation of ^{125}I-labeled biotinylated chA7Fab-NCS in the spleen and the liver rapidly increased with the subsequent administration of avidin and a significantly greater amount of biotinylated chA7Fab-NCS was retained in the spleen and the liver in the group of mice injected with avidin compared to those which were not injected with avidin. The avidin-biotinylated mAb-NCS aggregate which formed in the serum were
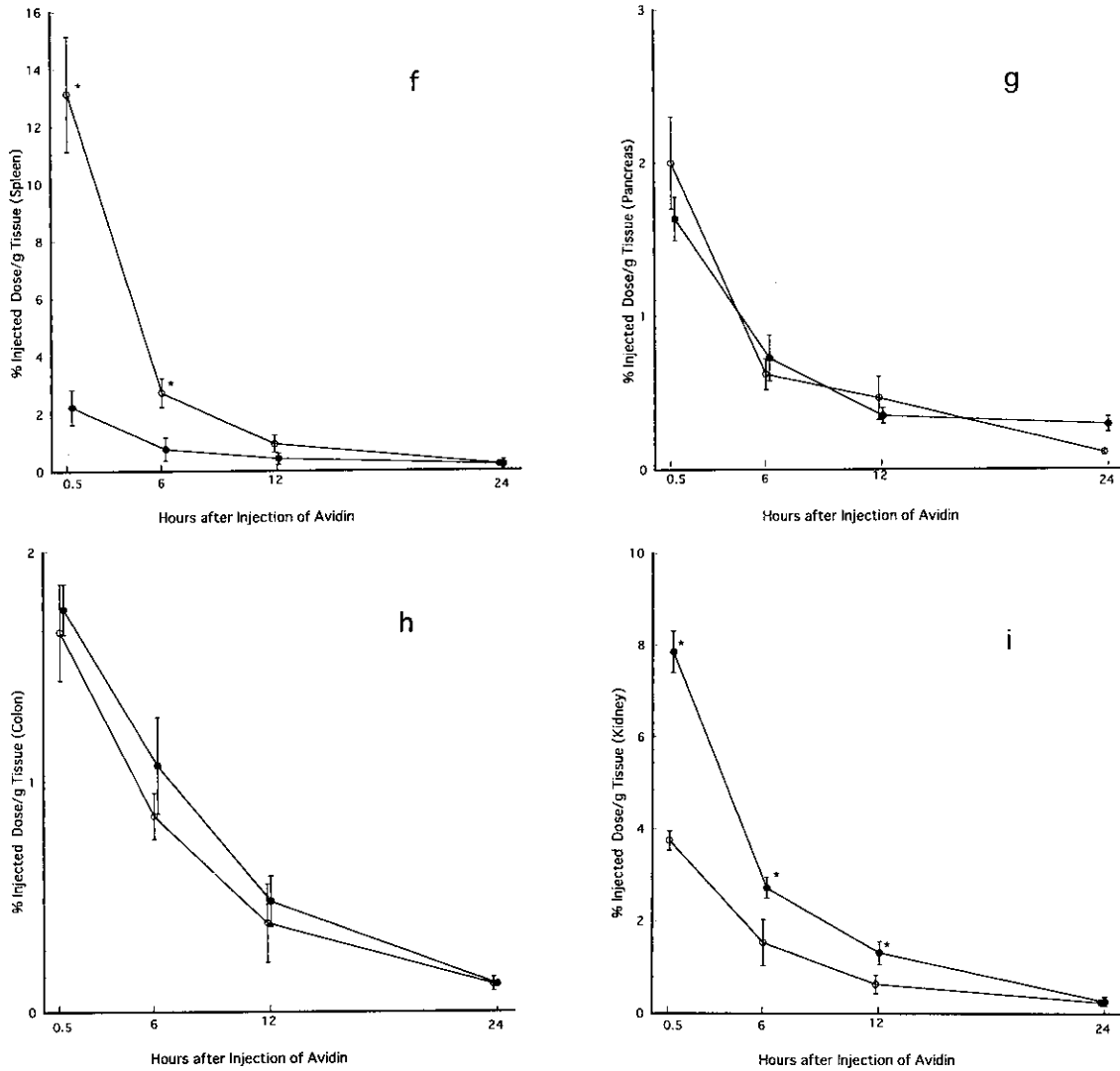
Fig. 2. The accumulation of ^{125}I-labeled biotinylated chA7Fab-NCS in the blood (a), tumor (b), and normal tissues (c, lung; d, heart; e, liver; f, spleen; g, pancreas; h, colon; i, kidney) of mice with or without the subsequent administration of avidin. The mice received an injection of avidin in 100 μl of 50 mM HEPES in 5% mannitol buffer or the same solution without avidin 30 min after the injection of ^{125}I-labeled biotinylated chA7Fab-NCS. The pattern of tumor concentration of ^{125}I-labeled biotinylated chA7Fab-NCS with the subsequent administration of avidin was similar to that without administration of avidin. The concentrations of ^{125}I-labeled biotinylated chA7Fab-NCS in the blood and kidney were significantly lower following the administration of avidin than without its administration at 0.5, 6, and 12 h after the injection of avidin. The accumulation of ^{125}I-labeled biotinylated chA7Fab-NCS in the spleen and the liver was higher following the administration of avidin than it was without the administration of avidin 1 and 6 h after the avidin injection. ○, with the subsequent administration of avidin; ●, no avidin administration; point, mean; bar, SD; $*P < 0.05$.
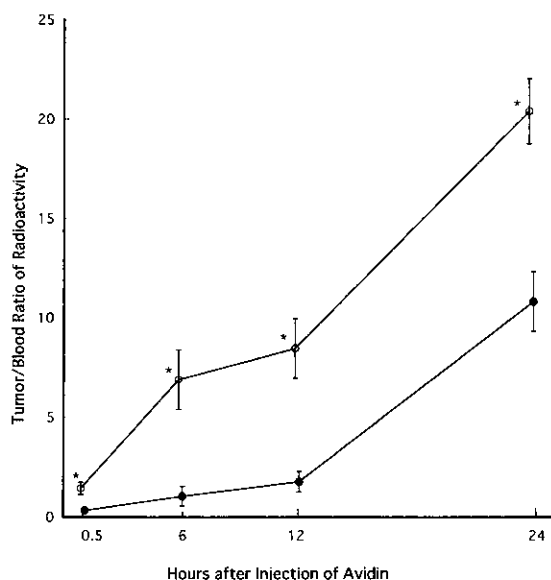
Fig. 3. The tumor tissue/blood ratio of radioactivity of ^{125}I-labeled biotinylated chA7Fab-NCS with subsequent injection of avidin was compared to that without avidin injection. The tumor tissue/blood ratio of radioactivity of ^{125}I-labeled biotinylated chA7Fab-NCS was higher with the subsequent administration of avidin than that without avidin ($P<0.05$). ○, with the subsequent administration of avidin; ●, no avidin administration; point, mean; bar, SD; $*P<0.05$.

Fig. 4. The kinetics of ^{125}I-labeled biotinylated chA7Fab-NCS accumulation in the blood, kidney, liver, and spleen were examined at 5 min intervals for 30 min after avidin administration. The concentration of ^{125}I-labeled biotinylated chA7Fab-NCS in the blood and the kidney decreased rapidly in the first 5 min after avidin administration and that in the spleen and the liver increased rapidly in the first 5 min after avidin administration. ○, blood; △, kidney; ×, liver; □, spleen; point, mean; bar, SD.

thought to be trapped by the reticuloendothelial system. In contrast, the concentration of ^{125}I-labeled biotinylated chA7Fab-NCS in the blood and the kidney decreased rapidly following the administration of avidin, and a significantly smaller amount of biotinylated chA7Fab-NCS was retained in the blood and kidney in the group of mice injected with avidin compared with those not injected with avidin. These results suggest that avidin administration may potentially decrease renal damage associated with biotinylated chA7Fab-NCS therapy.

Marked accumulation of biotinylated chA7Fab-NCS was seen in the spleen. Marshall *et al.* have reported the decreased splenic accumulation of ^{125}I-labeled antibody using a galactosylated form of streptavidin in tumor imaging.[22] However, the liver accumulation of ^{125}I-labeled antibody increased because of the binding of galactose and asialoglycoprotein receptor of hepatocytes. ChA7Fab-NCS was not galactosylated in the present study to avoid further accumulation of chA7Fab-NCS in the liver. The splenic accumulation of biotinylated chA7Fab conjugated to NCS is unlikely to be toxic to patients, because there have been no reports of adverse effects of NCS on splenic function. Although a relatively
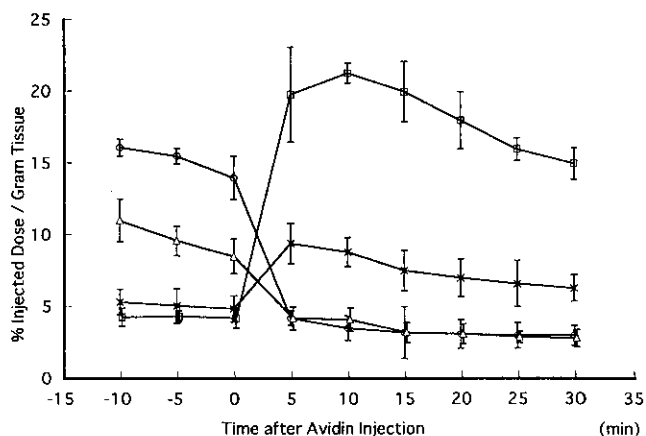
large amount of biotinylated chA7Fab-NCS also accumulated in the liver, NCS has been reported to be inactivated rapidly in the liver,[20] which may minimize its toxicity. Moreover, because biotinylated chA7Fab-NCS is rapidly cleared from the liver and the spleen, the adverse effects may be minimized.

Sung *et al.*[23] have reported that avidin-biotin complex was formed in the tumor as well as the normal tissues. Saga *et al.*[24] have also investigated the increased tumor localization of radiolabeled avidin after the injection of biotinylated antibody. Their results suggested that tumor accumulation and the tumor/blood radioactivity ratio of radiolabeled avidin in the pretargeted group were higher than those in the nontargeted group. In the present study, though the tumor/blood ratio of radioactivity was higher with than without avidin administration because the blood ^{125}I-labeled chA7Fab-NCS concentration decreased, tumor accumulation of ^{125}I-labeled chA7Fab-NCS was unchanged by administration of avidin. This discrepancy may be explained by the internalization of A7 antigen-antibody complex into the tumor cells and the decrease in the expression of cell-surface antigen due to antigenic modulation.[25]

Because avidin-biotinylated mAb aggregates were formed in the serum, the immunogenicity of this complex may be enhanced. However, we did not observe any side effects in the animals at the avidin doses used in this

study. Other researchers also have reported that such doses of avidin are not toxic.[13, 15, 26]

Based on these results, we conclude that biotinylated chA7Fab may be a suitable carrier of NCS and may show decreased renal toxicity when avidin is subsequently administered.

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