Radioimmunotherapy (RIT) is theoretically a useful way to selectively deliver therapeutic doses of radioisotopes to a tumor. Clinical applications of RIT in the treatment of malignant lymphoma patients showed encouraging results.\textsuperscript{1, 2)} In the therapy of solid tumors, however, RIT gave only limited success and was far from satisfactory.\textsuperscript{3)} For RIT to be effective, the uptake of radiolabeled antibody by the tumor should be high and the radioactivity should be diffusely distributed throughout the tumor. Therefore, small metastatic lesions, rather than large primary and recurrent lesions, may be the best target of RIT. We and others have recently shown that RIT is effective in controlling experimental liver micrometastases in nude mice.\textsuperscript{4, 5)} Some clinical success in the RIT of small lesions was also reported.\textsuperscript{6)} In the present study, in order to investigate how RIT functions in the therapy of small lesions, the pharmacokinetics of an intravenously injected therapeutic dose of radiolabeled antibody in nude mice bearing liver micrometastases were examined, and the dose absorbed by the micrometastases was evaluated, along with the long-term therapeutic effect of RIT.

**MATERIALS AND METHODS**

**Experimental liver micrometastases** Carcinoembryonic antigen (CEA)-expressing human colorectal carcinoma cells LS174T, obtained from the American Type Culture Collection (Rockville, MD), were grown in RPMI1640 medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY) and 0.03% L-glutamine, in a 5% CO\textsubscript{2} environment. Subconfluent cells were detached with calcium- and magnesium-free phosphate-buffered saline (PBS) containing 0.02% ethylenediaminetetraacetic acid and 0.05% trypsin. Female BALB/c\textsuperscript{nu/nu} mice were anesthetized with ether inhalation, and the spleen was exteriorized through a short left subcostal incision. A single-cell suspension of 3\times10^6 LS174T cells in 50 \mu l of serum-free RPMI1640 medium was slowly injected into the spleen through a 27-gauge needle, followed 2 min later by splenectomy. The left subcostal incision was

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closed with metal clips.7) With this procedure, all mice developed multiple liver metastases of several hundred microns in diameter within 1 week. 

**Monoclonal antibody and radiolabeling** The murine IgG 
monoclonal antibody designated F33-104 recognizes the CEA-specific proteinaceous part of the CEA molecule, and was purified from the ascitic fluid of the CEA-specific proteinaceous part of the CEA molecule and over normal liver by referring to the hematoxylin regions of interest were drawn over each metastatic nodules. 

Eight hundred micrograms of purified antibody, and 224.2 to 264.9 MBq of 131I (Du Pont, Wilmington, DE) were mixed with 8.0 μg of chloramine-T (Nacalai Tesque, Kyoto) dissolved in 0.3 M phosphate buffer. After 5 min, radiolabeled antibodies were separated from free iodine though PD-10 gel chromatography (Pharmacia LKB Biotechnology, Uppsala, Sweden). The specific activities of 131I-labeled antibodies ranged from 205.4 to 222.4 MBq/ mg, and the immunoreactive fractions, measured according to the method of Lindmo et al., were more than 70% for all preparations.11)

**Biodistribution study** One week after the intrasplenic injection of LS174T cells, mice received an intravenous injection of 131I-labeled F33-104 antibody (8.9 MBq/40 μg). The mice were killed 1, 2, 4, 6, and 10 days post-injection by ether inhalation. Livers with metastases were removed and quickly frozen in OCT compound (Tissue Tek; Miles Inc., Elkhart, IN) from which 15-μm thick sections were made and processed for a quantitative autoradiography (QAR) study. Blood and various organs were removed and weighed, and their radioactivity was counted. The percentages of injected dose per gram of tissue (%ID/g) were determined by QAR for the liver and liver metastases as described below, and by direct tissue counting for the other organs, and were normalized to a 20-g mouse. Metastases-to-normal tissue ratios of radioactivity were also calculated. All animal experiments were carried out in accordance with the Japanese government regulations regarding animal care and handling.

**Quantitative autoradiography** The uptake of radiolabeled antibody by the metastatic nodules and adjacent normal liver was evaluated by means of QAR. Dose estimation was done for tumors of three hypothetical sizes (1,000, 500, and 300 μm in diameter), using the conventional medical internal radiation dose (MIRD) schema. The mean absorbed dose in the target D (Gy) was calculated by use of the following formula; 

$$D = \frac{\Delta \beta \phi}{m},$$

where A is the cumulative radioactivity (Bq s), \(\Delta \beta\) the energy emitted by disintegration (J/Bq s), \(\phi\) the absorbed fraction and \(m\) the target mass. A was calculated as follows; uptake (Bq/g) at various time points determined by QAR was plotted against time, and the area under the curve (Bq s/g) was calculated. A was then obtained by multiplying by the hypothetical tumor weight. The ‘S’ factor (=\(\Delta \beta \phi/ m\)) was obtained from the table by Bardies and Chatel.13) From the results of autoradiography, the distribution of the radiolabel within the metastases was regarded as diffuse and the S factor of volume distribution was applied.

**RIT** Seven days after the intrasplenic grafting of LS174T cells, groups of mice received an intravenous injection of 1.85, 3.7, or 9.25 MBq of 131I-labeled F33-104. Protein dose was adjusted to 45 μg for each preparation by adding the unlabeled F33-104. The control group of mice received PBS instead of 131I-labeled antibody. All mice were monitored for survival up to 4 months after the injection of cancer cells. The survival was analyzed by the Kaplan-Meier method. Statistical analysis of the survival curve was done using the logrank test with Bonferroni correction.

**RESULTS**

**Pharmacokinetics of therapeutic dose of antibody and absorbed dose estimation** Fig. 1 shows typical autoradiograms of livers with micrometastases at various times after the injection of 131I-labeled F33-104. One week after the intrasplenic injection of cancer cells, multiple small metastases of less than 1 mm in diameter were formed in the liver. The radiolabeled antibody was localized diffusely at each metastatic nodule at each time point, whereas the radioactivity uptake by the normal liver was very low and decreased with time, resulting in high metastasis-to-normal liver uptake ratios (10.7 at day 1, 29.1 at day 10). The autoradiography also showed that the size and number of metastases decreased with time, confirming the short-term therapeutic effect reported previously.31) The area of intermediate grain density at the periphery of the liver was an area of necrosis; that this was an artifact caused by the injection of a large number
of cells was confirmed by H & E staining of the same section. In order to obtain a high metastasis yield, a larger number of cancer cells was injected as compared with that in previous studies, and the necrosis seems to be a consequence of peripheral circulatory disturbance by the cell aggregates.

The biodistribution data of the metastases, normal liver, and other normal tissues are summarized in Table I, and the clearance curve of radioactivity from the metastases, normal liver, and blood are illustrated in Fig. 2. The high uptake by the metastases (>20 %ID/g) was maintained until day 4, slightly decreased to 17.8 %ID/g at day 6,
and decreased further thereafter. The effective half-life of radioactivity in the metastases was calculated to be 3.6 days and that of the normal liver to be 2.3 days. The clearance of radioactivity from the blood was relatively slow, and the effective half-life was calculated to be 4.2 days, which was longer than that of the metastases.

The dose absorbed by the metastases was estimated from these data. From the distribution pattern visualized by autoradiography, dose estimation was done by hypothesizing that radioactivity was evenly distributed within 1,000-, 500-, and 300-µm metastatic nodules. The dose absorbed by metastases of 1,000 µm in diameter was estimated to be 19.10 Gy, that by metastases of 500 µm was 11.98 Gy, and that by metastases of 300 µm was 8.15 Gy.

**DISCUSSION**

The RIT of large solid tumors has been attempted with limited success, and recent efforts have focused on smaller sized-lesions such as metastases and minimal
residual disease after surgery. Small lesions are thought to possess several properties favorable for RIT; 1) the radioactivity uptake by smaller lesions is higher and more homogeneous than that by larger lesions,2,18 2) the radiation dose necessary to control smaller lesions is expected to be significantly lower than that needed for larger lesions.19

In the present investigation, the pharmacokinetics of a radiolabeled antibody in RIT were investigated to estimate the dose absorbed by the metastases. For absorbed dose estimation, data from a tracer study are usually used. In the case of small metastases, the growth of the metastases is rapid and the increase in tumor size during the experiment may significantly affect the pharmacokinetics of the radiolabeled antibody. In the present study, therefore, we injected a therapeutic dose, not a tracer dose, for the evaluation of the pharmacokinetics of radiolabeled antibody (under these circumstances, metastases are expected to decrease in size with time), and the dose absorbed by the metastases was estimated. The histological analysis of the liver showed that the number and size of the metastases did indeed decrease with time after the injection of the therapeutic dose of 131I-labeled F33-104, which was not observed after a tracer dose. In accord with the tracer studies,4,18 the initial radioactivity uptake by the metastases was high and was maintained until day 6, but decreased thereafter. Despite the high radioactivity uptake by the metastases, the clearance of radioactivity from the blood was retarded and slower than that from the metastases. As reported previously, radioactivity in the blood is significantly affected by the size and volume of metastases,4 and the retarded blood clearance in the present study reflects the small initial total volume of the metastases, which was further decreased by the RIT. This prolonged retention of radioactivity in the blood is problematic from the viewpoint of possible side-effects, since the circulating radiolabeled antibody is the major source of bone marrow irradiation. A therapeutic trial using 11.1 MBq of 131I-labeled F33-104 met with severe bone marrow toxicity (unpublished data). The facilitation of blood clearance will be necessary for clinical application, e.g., by the use of clearing agents. For example, after the injection of radioiodinated antibody conjugated with biotin, administration of avidin can significantly reduce the blood and normal organ radioactivity.20

In the micrometastases of submillimeter size in the present study, the radioactivity was distributed homogeneously within the metastatic nodules and the estimated absorbed dose was less than 20 Gy for the injected dose of 8.9 MBq, which is much lower than that necessary to control large solid tumors. However, the RIT experiment using 1.85 to 9.25 MBq of 131I-labeled F33-104 showed a significant dose-dependent therapeutic effect, and the intravenous injection of 9.25 MBq of 131I-labeled F33-104 gave apparently disease-free survival up to 4 months in 3 of the 8 mice, confirming that an absorbed dose of less than several thousand cGy can effectively control small-sized lesions. However, the injected dose of 9.25 MBq in a 20-g mouse corresponds, on a weight basis, to 27.75 GBq in a 60-kg human, which is unreasonably high. To reduce the necessary dose to a reasonable level, further potentiation of the therapeutic effect is needed, which can be achieved by combination therapy with various cytokines, radiosensitizers, vasomodulators, etc.21 As noted below, selection of a suitable radionuclide for the size of the target tumor and establishment of a more stable radiolabeling method to prolong the half-life in the tumor are also important for the effective irradiation of the tumor.

In RIT using 131I as a radiolabel, metabolism/dehalogenation at the tumor is a problem. In the present study, a high initial uptake was maintained until day 6, but the level then decreased significantly. If the initial high uptake could be maintained for a longer time period, the tumor absorbed dose could be increased without increasing the injection dose, or the injection dose could be reduced to obtain the same absorbed dose. Tumor uptake is the net result of the degradation of the antibody with release of radioiodine from the tumor and the supply of radioiodinated antibody from the blood. In the case of a radioiodinated antibody prepared by the chloramine-T method, which is sensitive to dehalogenation, prolonged tumor retention of the radioiodine is difficult to achieve. In the present study, the effective half-life of radioactivity in the metastases was shorter than that in the blood. If the radiolabeled antibody can be made more resistant to dehalogenation or can be retained at the tumor even after metabolism, the initial uptake can be maintained longer or the uptake can even be increased.22,23 Our previous study with 111In-labeled antibody with a stable chelate showed a much higher initial uptake, which increased with time (51.8 %ID/g on day 1 to 92.1 %ID/g on day 4).4

Another point to consider is that the effective range of the radiation should be matched to the size of the targeted tumor.24 The range of β-particles from 131I is long for the effective internal irradiation of submillimeter nodules, and only a small fraction of β-radiation of 131I is absorbed by the tumor. The present dosimetry results confirm this point. The dose absorbed by the 300-µm nodules was less than half of that by the 1,000-µm nodules, even though the radioactivity concentrations in these metastatic nodules were the same. Radioisotopes emitting β-particles with a shorter path length than that of 131I would be more suitable for the RIT of nodules of less than 1 mm.25

In conclusion, we have obtained further evidence that micrometastatic disease in the liver is a good target of RIT, and we have provided experimental evidence that an absorbed dose of less than 20 Gy can control small metastatic lesions (submillimeter in diameter) with a dose-
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dependent therapeutic effect on the survival of the mice, although the necessary dose of radiolabeled antibody is quite high. With further optimization of the conditions to give greater and more prolonged tumor uptake with rapid blood clearance, and with the selection of an optimal radioisotope, it should be possible to apply RIT successfully to patients.

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