[Methyl-Carbon-11]Thymidine for In Vivo Measurement of Cell Proliferation

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[Methyl-^{11}C]thymidine and PET provide an in vivo, noninvasive, quantitative approach for studying nucleoside uptake in cells on condition that the fraction of metabolites in the total accumulated activity is known. **Methods:** Using an animal model (Wistar rats), two independent approaches were followed. In the first approach, total accumulated activity in rapidly dividing tissue after intravenous injection of [methyl-^{11}C]thymidine, respectively, [methyl-^{11}C]thymine (first metabolite), was compared. In the second approach, the liver was surgically isolated to avoid thymidine catabolism. **Results:** After injection of [methyl-^{11}C]thymidine, tissue activity consists of both labeled thymidine and metabolites, while after injection of [methyl-^{11}C]thymine, it consists only of metabolites. The fraction of metabolites ranged from 9% to 44%. Comparing the specific activity with and without liver function yielded similar results. The calculated amount of metabolites was about 10%. **Conclusion:** In spite of the intense in vivo catabolism, major activity in rapidly dividing tissue consists of [methyl-^{11}C]thymidine.

Key Words: thymidine; positron emission tomography; cell proliferation

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PET offers the opportunity to perform dynamic measurements of uptake, distribution and clearance of radiolabeled molecules. It also allows the quantification of functional metabolism in normal and diseased tissues.

PET measures total activity retained in each tissue at any point of time, regardless of the chemical species with which the positron emitter is associated. To interpret the images and the quantitative data, a detailed knowledge of the metabolic fate of the studied compound is required (*1*).

Thymidine (^{3}H- or ^{14}C-labeled) is commonly used to evaluate DNA synthesis during cell proliferation (in vitro test). [Methyl-^{11}C]thymidine is presumed to be a suitable candidate for non invasive studies of cell proliferation using PET on condition that the nature of the accumulated activity is known. Besides incorporation in DNA, thymidine is also metabolized as depicted in Figure 1 (*2*). Assuming that the blood pool activity, which mainly consists of labeled metabolites, is also representative for the rest of the body, the accumulated activity in rapidly dividing tissue cannot be correlated with cell proliferation (DNA synthesis).

The present study has investigated the contribution of all the ^{11}C-labeled metabolites of [methyl-^{11}C]thymidine to the total activity of the intestine, a tissue with a high cell proliferation rate, acting as surrogate for tumor tissue.

A pilot study in normal Wistar rats indicated that the activity after a bolus injection of [methyl-^{11}C]thymidine accumulated mainly in liver and intestine. Two different approaches to quantify more accurately the fraction of the labeled metabolites to the overall accumulated ^{11}C activity in the intestine were followed:

1. First, the uptake of ^{11}C activity in the intestine of normal rats after intravenous injection of [methyl-^{11}C]thymidine was compared to that of [methyl-^{11}C]thymine (the first metabolite of thymidine).
2. Second, an attempt was made to block thymidine catabolism which occurs mainly in the liver. Comparing ^{11}C activity in the intestine without and with elimination of the hepatic catabolic function, the ^{11}C activity associated with the metabolites could be calculated.

MATERIALS AND METHODS

Radiopharmaceutical Production

[Methyl-^{11}C]thymidine was produced as described previously (*3*). About 9.3 GBq of pure [methyl-^{11}C]thymidine dissolved in 8 ml of an isotonic phosphate solution (pH 4.5; 3% ethanol) was routinely produced within 45 min.

For the production of [methyl-^{11}C]thymine, 7 ml of this isotonic phosphate solution containing about 8.1 GBq of [methyl-^{11}C]thymidine was transferred to a vial and evaporated to dryness. Hydrochloric acid (0.5 ml; 12 M) was added and heated to boiling point for 3 min to split off the deoxyribose group. Purification of the [methyl-^{11}C]thymine was performed with HPLC (reversed phase: C_{18}-column 25 * 1 cm; eluent: isotonic phosphate solution, flow: 4 ml min^{-1}) resulting in a solution ready for intravenous injection (activity: 3.0 GBq; specific activity: 3.4 GBq μmole^{-1}).

Biodistribution Studies in Wistar Rats

The animal experiments were undertaken according to the "Guiding Principals in Care and Use of Animals" (American Physiological Society), the Belgian Law for Animal Protection and Welfare and the guidelines of the European Community for the use of animals in laboratory investigations. All studies were performed in male Wistar rats, weighing between 275 and 425 g (mean: 340 g). The animals were anesthetized with sodium pentobarbital (Nembutal^{R}, 50 mg kg^{-1} intravenous). A tracheostomy was performed and the right jugular vein was catheterized for intravenous injection of the radiopharmaceutical (4 to 80 MBq in 0.7 ml of phosphate solution and 0.2 ml of 0.9% sodium chloride).

There were three experimental groups: the pilot study, the study for thymidine and thymine distribution and the hepatectomized rats.

The first group was the pilot study. Pilot experiments in normal rats allowed the study of the time-dependent uptake of total ^{11}C activity in liver, large intestine and other organs and tissues (kidneys, heart, lungs and gonads). These normal rats were sacrificed at 7, 15, 20 and 30 min postinjection by an overdose of Nembutal^{R}. All organs and tissues were removed, cleaned, blotted

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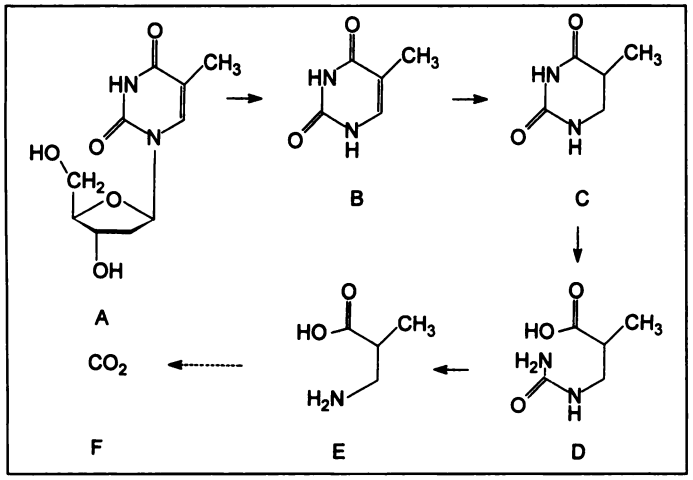

FIGURE 1. Metabolization scheme of thymidine: (A) thymidine, (B) thymine, (C) dihydrothymine, (D) ureidoisobutyric acid, (E) aminoisobutyric acid and (F) carbon dioxide.

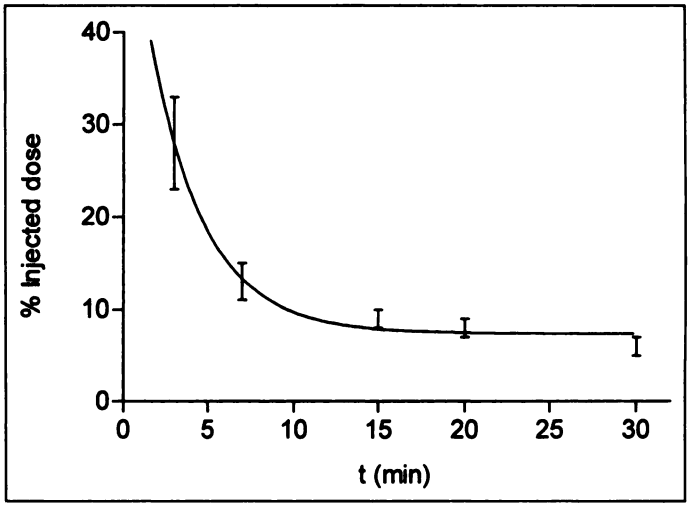

FIGURE 2. Total ^{11}C blood-pool activity versus time after intravenous injection of [methyl-^{11}C]thymidine (n = 3; range).

to minimize the adhering blood, and weighed. The decay corrected results were expressed as percentages of the total injected dose (%ID) and/or as differential absorption ratios (DAR) to correct for the differences in body weight and injected dose.

The second group displayed thymidine and thymine distribution in normal rats. In two groups of six normal rats, the ^{11}C activity distribution at 20 min postinjection of [methyl-^{11}C]thymidine resp. [methyl-^{11}C]thymine was studied. Emphasis was put on the uptake of total ^{11}C activity by the intestine and the liver at 20 min postinjection. Other organs and tissues like lungs, heart, kidneys were considered to be of minor interest for this study.

The third group was a study of hepatectomized rats. In seven normal Wistar rats, prior to the injection, the blood perfusion of the liver was reduced by clamping both the hepatic artery and the portal vein by surgical intervention to reduce the enzymatic formation of ^{11}C-labeled metabolites. The animals were sacrificed at 20 min postinjection. The intestinal activity was considered to be mainly due to uptake of [methyl-^{11}C]thymidine while the liver values yielded information on the efficiency of the decrease in blood flow.

Measurement of [Methyl-^{11}C]Thymidine and Radiolabeled Metabolites in Plasma

Blood samples (2–3 ml) were obtained by heart puncture, mixed with heparin (0.05 ml) and cooled. The total radioactivity of a fraction (0.3 ml blood) was measured with a calibrated well-type NaI-detector and expressed as percent of administered dose. The main fraction (1.7 to 2.7 ml) was immediately centrifuged for 5 min at 2000 rpm to separate the plasma. The plasma samples (1.0 ml) were purified to eliminate proteins and lipids and analyzed as described previously (*2*).

The blood clearance of the total activity and the time course of the [methyl-^{11}C]thymidine in the blood pool versus the total ^{11}C activity were studied in three rats of the pilot group. In the same number of animals of the other groups (thymidine, thymine and hepatic series), blood samples were taken at 20 min postinjection and analyzed for the different ^{11}C-labeled metabolites. In three normal rats, prior to the injection of [methyl-^{11}C] thymidine, blood samples of 1 ml were taken using the catheterized jugular vein. After adding 37 kBq of [methyl-^{11}C]thymidine, these samples were incubated at 37°C during 20 min to study the degradation of thymidine in blood (in vitro test).

RESULTS

Biodistribution Studies

In the pilot study (n = 3), the decay-corrected time-activity curve of total ^{11}C-blood activity from 3 to 30 min postinjection is depicted in Figure 2. Data (range; n = 3) are expressed as % injected dose (%ID) versus time after injection. In the blood pool, the total ^{11}C-radioactivity following the bolus injection of [methyl-^{11}C]thymidine rapidly decreased (initial distribution phase), thereafter the level of activity remained essentially constant from the 10th min postinjection on. The mean values at 15, 20 and 30 min were not significantly different. The blood pool activity at any point of time consisted of different labeled metabolites (catabolization scheme, Fig. 1). Quantification of the amounts of activity present as thymidine and as total metabolites using HPLC (with 8% of ethanol in the eluent) demonstrated that already after 3 min 60% of total plasma activity was due to metabolites; after 20 min this value increased to 90% (Fig. 3). For the blood samples taken after 30 min, the ^{11}C activity associated with thymidine was too low to obtain reliable and reproducible results (range 1 to 5%; n = 3).

The time-dependent uptake of activity in the liver and intestine, is depicted in Figure 4. For both tissues, the highest uptake of activity was observed between 15–20 min postinjection.

In the group of thymidine and thymine in normal rats, the results of two groups of six rats (%ID and DAR), injected with

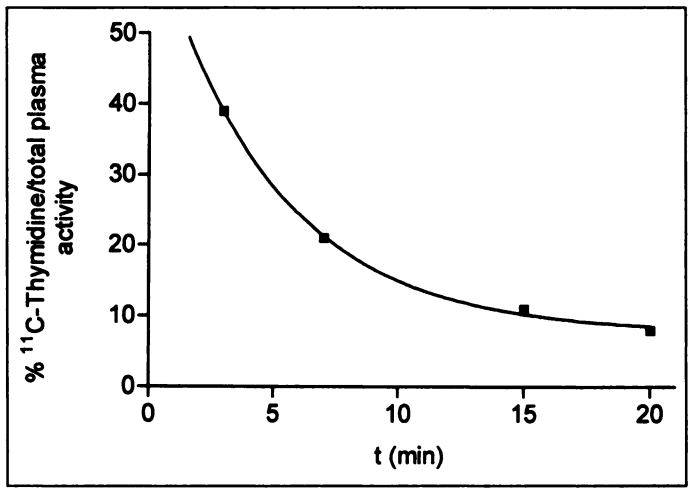

FIGURE 3. Fraction of [methyl-^{11}C]thymidine in the total plasma ^{11}C activity versus time.

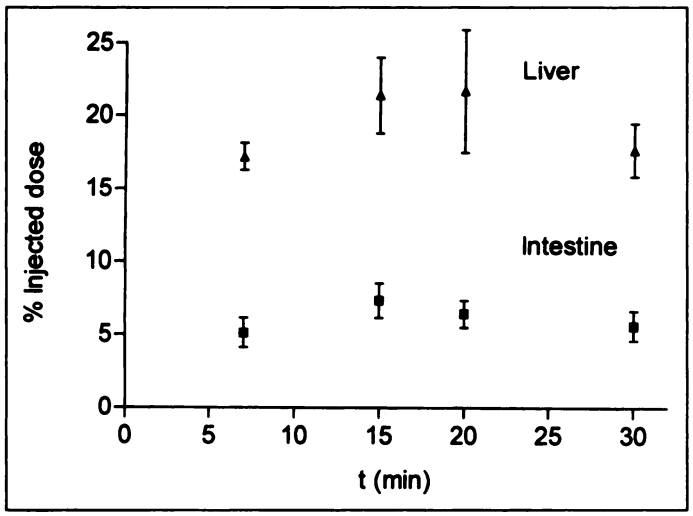

FIGURE 4. Uptake of total ^{11}C activity in the liver and in the intestine versus time (n = 3; mean ± s.d.).

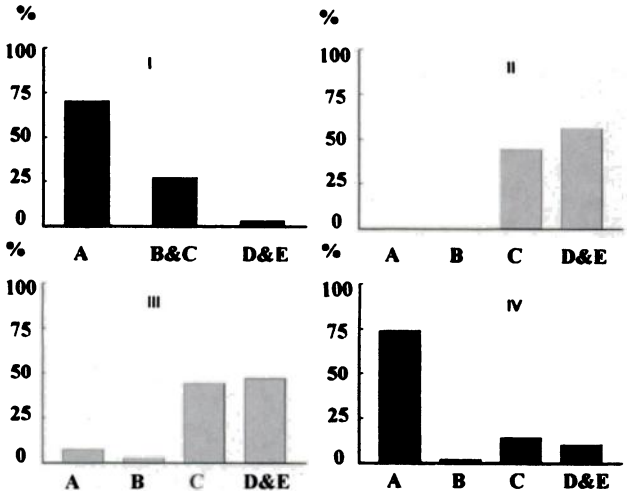

FIGURE 5. Percentages of the different labeled metabolites at 20 min postinjection in the blood pool of normal rats (III), normal rats with thymine (II), hepatic rats (IV) and the in vitro results (I). Samples were analyzed by HPLC. (a) thymidine, (b) thymine, (c) dihydrothymine and (d, e) open chain metabolites.

either [methyl-^{11}C]thymidine or [methyl-^{11}C]thymine killed 20 min after injection, are summarized in Table 1. For the liver uptake, there was no significant difference between the DAR values for both radiopharmaceuticals. On the other hand, the uptake of activity after injection of labeled thymidine in the large intestine was much higher. The blood values for the thymine rats were not significantly higher.

The results of the experiments on hepatectomized ("clamped" liver) rats (n = 7) are also summarized in Table 1. In six animals, the uptake in the liver was reduced to less than 10% of the normal level, illustrating that the hepatic blood flow was decreased by at least 90%. In one rat, the blood flow was apparently only partially interrupted to a level of 50% corresponding with a significant higher uptake of activity in the liver. In all animals (n = 7), the values obtained in the intestinal tissue (%ID and DAR) were not significantly different.

The total blood activity of the two groups of rats in the presence or absence of hepatic perfusion, 20 min after injection of [methyl-^{11}C]thymidine, was not significantly different (DAR values: 5.7 ± 1.0 versus 5.5 ± 1.1).

In three rats of each group (two groups of normal rats with thymidine and thymine and the hepatic group) and in an in vitro control group, all the different metabolites were determined quantitatively by HPLC at 20 min postinjection of the radiopharmaceutical. The results showed a totally different pattern (Fig. 5). In the blood pool of normal rats (Fig. 5, III), the main activity consisted of labeled metabolites (93%) while for those with reduced liver perfusion (Fig. 5, IV), the total fraction of the metabolites was only 26%. This low value for the metabolites corresponds with the data obtained in the in vitro (ex vivo) test (Fig. 5, I). In the group of rats injected with [methyl-^{11}C]thymine (Fig. 5, II), no activity associated with thymine could be detected in the blood pool. The main fraction consisted of dihydrothymine and the open chain structures (Fig. 1, D-E).

TABLE 1
Total Accumulated Carbon-11 Activity in the Liver and Intestine 20 Minutes after Intravenous Injection of Labeled Thymidine or Thymine

		Normal rats	
		[Methyl-^{11}C]thymidine	[Methyl-^{11}C]thymine
Intestine	DAR	1.63 ± 0.18	0.72 ± 0.06
	%ID	6.40 ± 0.90	3.04 ± 0.28
Liver	DAR	5.48 ± 0.78	5.83 ± 0.98
	%ID	21.67 ± 4.17	20.87 ± 3.10
Blood	%ID	5.72 ± 0.97	8.33 ± 0.94
		Liver-clamped rats	
		[Methyl-^{11}C]thymidine	(*)
Intestine	DAR	1.50 ± 0.24	1.49
	%ID	5.85 ± 0.74	4.87
Liver	DAR	0.45 ± 0.15	3.46
	%ID	1.62 ± 0.48	9.18
Blood	%	5.52 ± 1.09	—

* Data are expressed as DAR values and as % ID (mean ± s.d.; n = 6) partially clamped liver, n = 1.

DISCUSSION

Many radiopharmaceuticals were used to obtain biochemical information on tumor behavior (*4–6*). The most accurate information about cell proliferation can be obtained by studying the nucleoside incorporation during the S-phase of the cell cycle into DNA. This can be performed in vitro by thymidine labeling index (*7*) and flow cytometry (*8*) or in vivo using a labeled nucleoside. [Methyl-^{11}C]thymidine has already been successfully used for qualitative imaging of human tumors (*9,10*). Studying the metabolic fate of [methyl-^{11}C]thymidine in human plasma of a group of patients with head and neck tumors demonstrated that the ratio of [methyl-^{11}C]thymidine versus total ^{11}C activity in plasma decreases rapidly and that the labeled metabolites become dominant soon after the injection (*2*). Thus, to interpret the PET quantitation data accurately, a detailed knowledge of the in vivo behavior of all the metabolites is mandatory.

The short half-life of ^{11}C (20.4 min) does not permit a destructive analysis of the tissues of interest. Homogenizing tissue and isolation of the DNA takes at least 2.5 hr by which the ^{11}C activity is too low to obtain reproducible results within an acceptable standard deviation.

The data of the pilot study (control group) of normal rats confirmed the results obtained in dogs (*1*). Soon after intravenous injection of the rats with [methyl-^{11}C]thymidine, the ^{11}C

activity was rapidly cleared from the blood pool and stabilized at a level of about 6%–8% of the injected dose (Fig. 2). In the blood pool, the fraction of [methyl-^{11}C]thymidine was inferior to the total labeled metabolite fraction from 3 min postinjection on (Fig. 3). For the tissues and organs, the highest uptake of activity was observed in the liver, followed by a moderate uptake in intestine (Fig. 4). The lowest (around 1%) was in the other organs and tissues (e.g., kidney, heart, lungs, gonads). For the liver, the highest uptake reaching a value of about 25% was observed between 15 to 20 min postinjection. The liver curve in Figure 4 can be considered as the result of two processes: an uptake and a release of activity by the liver. On the curve, one notices that between 20 and 30 min postinjection, there is no significant decrease over a period of 10 min. One can suppose that in the period 0 to 20 min postinjection, the uptake was strongly superior to the release of activity. This idea is also confirmed by the blood-pool profile (strong decrease followed by constant level) visualized in Figure 2. Assuming that there was an important release of activity in the first minutes from the liver to the blood pool, the slope of the blood-pool activity curve would be significantly less-shallow.

A collaborative patient study of the Louvain-la-Neuve and the Gent group (*12*), comparing thymidine labeled in either the methyl or 2-C position, demonstrated that in the case of the methyl-labeled thymidine the initial blood activity clearance was faster and that at 60 min postinjection both blood activities were similar. The higher blood profile in the case of the 2-C labeled was due to liberation of labeled [^{11}C] CO_2 from the liver in the blood pool. In the same study a seven-fold higher uptake in the liver for the methyl labeled compared to the 2-C labeled was noticed, suggesting higher accumulation of methyl-labeled metabolites.

Studying the biokinetics and dosimetry for [methyl-^{11}C]thymidine by Thierens et al. (*13*), a similar activity profile for the liver in humans as found for the rats in the present study was obtained.

Using the blood analyses, it can be assumed that the liver is mainly responsible for this metabolism (Fig. 5). In the normal rats, the main fraction of the blood pool activity (Fig. 5, III) after intravenous injection of [methyl-^{11}C]thymidine or [methyl-^{11}C]thymine consists of metabolites. In contrast, in the animals without or at least markedly reduced liver perfusion (Fig. 5, IV) (less than 10% of the normal uptake), 74% of the blood activity at 20 min postinjection consisted of original [methyl-^{11}C]thymidine. This value corresponded with the results of the in vitro experiment (Fig. 5, I).

Previous experiments have shown that the degradation of thymidine could also be due to blood platelets (*11*). Our results confirm and clarify previous data obtained in human blood (*3*).

Since the maximum accumulated activity in the liver and in the intestine is reached in the 15–20-min interval, the latter one was taken as the point of time for further, more detailed experiments. Comparing the liver data (%ID and DAR) after intravenous injection of thymidine and thymine, respectively, we did not find significant differences as expected for an organ involved in metabolism. On the other hand, for the intestine, the ^{11}C activity accumulation after intravenous injection of thymine was 44% to 48% compared to thymidine. This may be explained by the fact that for the "thymidine" experiments (normal rats), the intestinal ^{11}C activity consisted of both thymidine and its metabolites. In contrast, for the thymine rats, the total ^{11}C activity was only due to metabolites. To estimate the amount of thymidine actually retained in the intestine one could simply subtract both DAR values. This resulted in an underestimation of the real thymidine fraction. Multiplication of the thymidine percentage and DAR values with 0.25 would therefore give a more realistic estimation (11%) of the metabolite fraction in the intestine. With and without taking into account the correction coefficient of 0.25, the estimation based on the thymine behavior led to a range between 11% and 44% of metabolites in the total tissue activity (between 89% and 56% for thymidine). In order to narrow these broad thymidine fraction, a second experiment was set up. To suppress the activity associated with metabolism (metabolites), the blood flow to the liver was interrupted. This did not result in significantly lower results for the intestine (Table 1). To explain these data correctly, two confounding parameters associated with the experimental protocol must be taken into account:

1. Interruption of the hepatic blood perfusion was produced by clamping the portal vein and the hepatic artery, resulting in reduced intestinal blood flow.
2. Since liver perfusion is absent and the accumulation of the activity is only 10% of the normal value, about 25% more of the activity is presented to the rest of the body.

While the blood flow reduction in the intestine is quite difficult to estimate, the contribution of the latter confounding parameter can more easily be corrected. Only normalization for the injected dose and not for reduction of the intestinal blood flow, the activity associated with [methyl-^{11}C]thymidine in the intestine was at least 69%. The real value is in the interval 91% to 69%.

In conclusion, two different experimental approaches were developed to evaluate the actual [methyl-^{11}C]thymidine activity retained in a tissue with a high cell proliferation rate. We are aware of the fact that the proposed procedures only permit to determine the range (upper and lower limits) in which the real value is situated. Both independent approaches reveal that, in spite of the rapid in vivo catabolization, the main ^{11}C activity retained in the intestine comes from originally accumulated [methyl-^{11}C]thymidine. The metabolites generated in the liver seemed to have a minor impact. Assuming that [methyl-^{11}C]thymidine was completely incorporated in the DNA of rapidly proliferating cells, this provides a basis for noninvasive quantification of cell proliferation in tissues using PET with [methyl-^{11}C]thymidine as radiotracer. Several issues still need to be investigated: repeating the same experiments in tumor-bearing rats and the correlation of the accumulated activity in tumor tissue with real cell proliferation rates.

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Enhancement of Radiation Dose to the Nucleus by Vesicular Internalization of Iodine-125-Labeled A33 Monoclonal Antibody

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In radioimmunotherapy, the emission characteristics of the radioisotope is critical in determining the radiation dose to the tumor compared to normal organs. If antibodies internalize and transport low-energy electron emitting isotopes close to the tumor cell nucleus, an improved therapeutic advantage is achieved. **Methods:** Using fluorescent microscopy, we studied the subcellular distribution of an internalizing antibody, A33, which detects a restricted determinant on colon cancer cells. We developed a physical model to assess the dose deposited in the nucleus by electrons emitted from radiolabeled A33 accumulated inside vesicles. This model is based on the energy-range relationship of electrons in water. Similarly, another model was developed to calculate the radiation dose to the nucleus from electrons emitted from extracellular space. The percentage of A33 bound to membrane and internalized was determined in vitro at various time points. Cytotoxicity experiments were performed with ^{125}I- and ^{131}I-labeled A33 at various concentrations and specific activities. **Results:** A33 accumulates in cytoplasmic vesicles (40% of total bound) which transport the activity close to the nucleus. This increases the radiation dose to the cancer cell nucleus by a factor of 3 compared to the average dose calculated based on the assumption of a uniform distribution on the cell membrane. The cytoplasm of antigen-negative normal cells shields the nucleus from the electrons emitted from extracellular ^{125}I. This shielding is 30 times less for ^{131}I. Cytotoxicity data show 10% cell survival with 10 μCi/ml of ^{125}I-A33, but 90% survival with up to 100 μCi/ml of ^{125}I-A33 in the presence of a blocking dose of 100-fold excess of cold A33. Similar experiments with ^{131}I showed cytotoxicity in both cases. **Conclusions:** The results of the cytotoxicity experiment are in agreement with the physical model and suggest a basis for improved tumor-to-marrow radiation dose by clinical use of ^{125}I-A33.

Key Words: Auger electrons, cell-level dosimetry; monoclonal antibody; vesicular internalization

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Isotope selection strategies for the radioimmunotherapy (RIT) of cancer have been greatly influenced by the general observation that antigen expression in tumors is heterogeneous. In addition, attention has been focused on the apparent poor penetrability of tumors by monoclonal antibodies (MAbs) (*1–4*). For these reasons, the majority of clinical studies have utilized long-range beta emitters (^{131}I, ^{90}Y), which irradiate cells up to 1 or 2 mm from the site of the radioisotope decay. As a consequence of the long-range electrons, the bone marrow receives a high radiation dose from radiolabeled MAb in the blood, limiting the amount of radioactivity which can be administered (*5–8*).

The A33 antigenic system was selected as a model for low-energy emitter RIT because it shows high uniform expression on colon cancer-tissue sections by immunohistochemical techniques. Binding studies on colon cancer cell lines demonstrate relatively high expression (800,000 MAb A33 molecules bound per cell), and radiolabeled MAb A33 is internalized into cells (*9*). In clinical studies, autoradiographs of biopsied colon cancer metastases show relatively uniform distribution of the intravenously administered radiolabeled antibody within these tumors with excellent tumor to normal tissue ratio (*10*).

The prevalent electron emissions from ^{125}I are Auger electrons ranging in energy from a few electron volts to 28 keV (*11*). In addition, there is a small yield (7%) of internal conversion electrons at 32 keV. Photon emissions do not locally deposit their energy and therefore can be neglected in dose calculations to individual cells. Their contribution, however, will not be negligible in patient studies. The radiotoxicity of ^{125}I, when directly incorporated into DNA, is quite high; as few as 100 decays will kill a cell (e.g., ^{125}I-labeled IUDR) (*12–14*). This radiotoxicity is attributed to the prolific emission of very short-range electrons (<20 nm) which acts as high LET radiation (*12*). The radiotherapeutic effects of Auger-emitting isotopes has been studied both experimentally and theoretically (*15,16*).

It is widely believed that the cell nucleus, and more specif-

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