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# RADIO-IODINATED AND INTERNALLY LABELLED ($^{35}$S) IgM MONOCLONAL ANTIBODIES IN A SYNGENIC RAT MODEL

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**Abstract**

To simulate the human situation concerning human monoclonal antibodies (MAbs), we have introduced a new syngenic rat model with an implanted rat colon carcinoma. Rat IgM MAbs (10B12), labelled by the chloramine-T method with $^{125}$I or internally with $^{35}$S, were injected intravenously into the rats and the biodistribution was studied for 8 days. The radioactivity uptake in the tumours of the $^{35}$S label was higher than that of the $^{125}$I label and the retention of $^{35}$S in the tumours gave tumour/blood ratios 8 times higher than those of $^{125}$I at 48 and 96 h after injection. In this model we have shown that dehalogenation of iodinated IgM MAbs is a serious problem. We therefore suggest that internally labelled MAbs should be used and that further investigations should be carried out in a syngenic rat model, since this probably reflects the clinical situation better than the nude mouse model.

*Key words:* Monoclonal antibodies, colon carcinoma, rat.

Radiolabelled monoclonal antibodies (MAbs) offer a powerful tool in cancer diagnosis and therapy. By studies of animal models the clinical hypothesis of antibodies and their conjugates are tested. Better models for biokinetic studies of MAbs are needed before such antibodies can be considered the optimal agents for radio-immunoimaging and radio-immunotherapy. The heterotransplanted nude mouse as an in vivo animal model has been extensively used for testing tumour antibody specificity. Due to limitations in the nude mouse model, we previously introduced the nude rat model for investigating the biokinetics of radiolabelled mouse MAbs and for the calculation of specific tissue uptake and corrected specific tissue uptake (1). In the present study we have eliminated the species differences and used a new syngenic rat tumour model, which theoretically reflects the clinical situation better.

The majority of MAbs so far studied in vivo are murine IgG MAbs. Much less frequently IgM MAbs labelled with different radionuclides have been studied (2–6). The labelling of MAbs has mostly been done with radionuclides of iodine, but also $^{111}$In and $^{99}$Tc$^{m}$ have been used. Concern has been voiced about these labelling techniques, and the question is to what extent the in vivo activity measurements reflect the antibody distribution. In order to enhance this correlation, biosynthetically incorporated radionuclides, $^{75}$Se and $^{35}$S, have been suggested and tested. Halpern et al. (2) reported on the distribution of human and mouse monoclonal IgM antibodies radiolabelled with $^{75}$Se, $^{111}$In, and $^{125}$I in murine models and found that the $^{75}$Se and $^{111}$In MAbs had similar distribution and kinetic patterns while the $^{125}$I-labelled MAbs were dehalogenated after 4 h. In a methodology review, Sands & Jones (6) reported the work of Pollack et al., who used internally $^{35}$S-labelled MAbs to establish that the blood clearance of internally labelled IgM MABs was much more rapid than the clearance of IgG MABs. Sands & Jones suggest that the internally labelled antibody ($^{35}$S, $^{75}$Se) represents a 'gold standard' to which other labelling techniques can be compared.

In this study, we report the use of a new, completely syngenic model to compare the biodistribution of the radioactivity after intravenous injection of monoclonal IgM antibodies, radiolabelled by the chloramine-T method with $^{125}$I or internally with $^{35}$S in rats with syngenic colon carcinomas.

## Material and Methods

*Cells and antibodies.* The 10B12 hybridoma cell line was prepared as described elsewhere (7) and was cultured in

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RPMI 1640 supplemented with 10% fetal calf serum (FCS) and antibiotics. The 10B12 rat pentameric IgM was produced in ascitic fluid in syngenic rats. It was precipitated from the ascitic fluid by dialysis against distilled water (euglobulin precipitation) and further purified by hydroxyl-apatite chromatography. The antibody recognizes a high-molecular-weight glycoprotein in rat colorectal carcinomas and, by immunohistochemistry, it also stains the lower colonic crypts, some acinar cells in the pancreas and the mucus in the antrum (Brodin et al.: Unpublished data).

*Radioiodine labelling.* The 10B12 antibody was labelled with $^{125}I$ by the chloramine-T method (7). The specific activity following iodination was in the range of 24 MBq/mg. After labelling, the antibody fraction was eluted from a Sephadex G-25 column. About 25–50% of the MAb retained its ability to bind the antigen after the iodination process, as determined by binding to cultured tumour cells (see below).

*Internally labelling with $^{35}S$.* The hybridoma cells were washed twice by centrifugation-resuspension in trypsin buffer and preincubated for 20 min at $6 \times 10^6$ cells/6 ml in methionine-free RPMI 1640 medium containing 10% dialyzed fetal calf serum. The cells were then resuspended at $6 \times 10^6$ cells/1.4 ml in the same medium and incubated with 37 MBq $^{35}S$-methionine (Amersham Ltd) for 24 h in a 6% $CO_2$ incubator. After 24 and 48 h the cells were replaced with fresh cells treated as above. After three days the internally labelled proteins in the supernatant were separated from low-molecular-weight material on a Sephadex G-25 column. The purity of the antibody was confirmed by SDS-polyacrylamide gel electrophoresis and autoradiography. The specific activity was in the range of 50 MBq/mg. About 30% of the labelled proteins demonstrated specific binding to the tumour cells, as determined in the assay described below. The $^{35}S$ activity was assayed in a Beckman 1801 liquid scintillator.

*Cell-binding assay.* The immunoreactivity was determined by a cell-binding assay. The labelled antibody was tested for binding to a suspension of cultured colon carcinoma cells BN-7005. An excess of colon carcinoma (determined by previous titration) or the same number of antigen negative control cells were incubated with undiluted or diluted, labelled protein in 1% BSA (bovine serum albumine) in PBS (phosphate buffered saline) for 1 h on ice. Cells were washed three times by low-temperature centrifugation-resuspension with 1% BSA 1 ml. The antibody activity of the labelled protein was expressed as the percentage of the added radioactivity bound to the cells.

*Solid-phase inhibition radio-immunoassay for the determination of immunoglobulin concentration.* The radioimmunoassay was performed as previously described (8). Briefly, a 96-wells' plate was coated with antigen (50 $\mu$l of 5 $\mu$g/ml in each well) by passive adsorption. In each well, 30 $\mu$l of purified 10B12 (100–640 $\mu$g/ml) or an unknown sample, mixed with 30 $\mu$l rabbit antimouse IgG antiserum (Cappel, Downington, PA) diluted to 1:1 200 in 10% FCS, were incubated for 30 min at room temperature. The supernatant was removed, and the plate was washed twice with 1% BSA in trypsin buffer. Then $^{125}I$-labelled staphylococcal protein A with an activity corresponding to a count rate of about $1.7 \times 10^3\ s^{-1}$ per well was added and the incubation for 30 min at room temperature was repeated. The supernatant was again removed, and the plate was washed twice, as before.

Finally, the plate was sealed using tape and cut. The individual wells were counted in the Packhard Autogamma 500C system. The concentration of 10B12 in the sample was calculated from the inhibition standard curve.

*Animal model.* In this study 24 female Wistar Furth (W/Fu) rats weighing 160–214 g were used. A chemically-induced, syngenic rat colon carcinoma BN 7005 was transplanted onto the thighs of these rats, intramuscularly (right) and subcutaneously (left). The rats were studied for 13 to 20 days after tumour inoculation. The i.m. tumours had gained a median weight of 2.0 g (range 1.0–5.0, n = 24) and the s.c. tumours 1.7 g (range 0.5–5.1, n = 24) at the time of dissection. The growth of the tumours was locally invasive without any distant metastases.

The rats were divided into two groups; twelve rats received $^{35}S$-10B12 and twelve $^{125}I$-10B12. In both cases 200 $\mu$l of labelled material, corresponding to 5 $\mu$g of the antibody, was injected into the femoral vein. After each of six intervals, at 6, 12, 24, 48, 96 and 192 h after injection, two rats from each group were sacrificed and dissected. Tissue samples were taken of blood, muscle, lymph nodes and bone marrow. Also, the liver, spleen, kidneys, heart, lungs, stomach, pancreas, thyroid, the s.c. tumour and the i.m. tumour were excised. All organs and samples were cleaned to remove blood, weighed and counted, together with standards prepared from the injected material.

The data are presented as specific tissue uptake, STU, as a percentage of the injected activity per gram tissue (% ID/g) and as tissue/blood ratios, which were calculated from the STU-values. In addition, biopsies of the tumours were frozen immediately in isopenthane (Aldrich, Belgium), cooled in liquid nitrogen and stored at −70°C for the preparation of frozen sections.

*Corrected specific tissue uptake, $STU_{corr}$.* By calculating the blood volume in the dissected tissues using $^{99}Tc^m$-labelled red blood cells (1), the activity in the tissue due to blood content can be estimated and corrected for, thereby giving the corrected specific tissue uptake, $STU_{corr}$.

*Immunohistochemical assay of rat MAb's binding to syngenic tissue.* The binding of rat monoclonal antibody to antigen, expressed in an isograft of rat colon carcinoma in the rat, was demonstrated in a modified two-step immunohistochemical assay (Unpublished data by Brodin et al.). Briefly, 4 $\mu$m frozen sections of the tumours were picked

up and thawed on multitest slides. The sections were dried for 1–14 days at +20°C, fixed in aceton for 10 min at −20°C and dried just before use. Twenty per cent FCS in 0.05 mol/l Tris-buffer (pH 7.6) was added to rehydrate the sections for 20–30 min. The biotinylated primary antibody was added and incubated on the tissue sections for 1 h. Finally avidin-biotinylated HRPO complexes were incubated for 30 min before the slides were immersed and incubated for 15 min in 0.5 mg/ml of the substrate solution (diaminobenzidine) in washing buffer with 0.01% $H_2O_2$. All steps were performed at room temperature and three washings followed each incubation.

## Results

Fig. 1 gives the STU time versus activity curves in different organs and tumours after injection of $^{35}$S-10B12 and $^{125}$I-10B12 respectively. The values are the means of the results of two rats. There is a difference in the in vivo activity uptake between $^{35}$S and $^{125}$I. The activity uptake in the i.m. and the s.c. tumours was higher when $^{35}$S was the label, and the amount of $^{35}$S remained relatively constant at 1%/g over the period from 6 to 48 h.

For most of the organs, the curves in Fig. 1 show the corrected $STU_{corr}$-values. Exceptions are the stomach, pancreas, thyroid and the lymph nodes, for all of which the STU-values are given uncorrected (marked unc in the figure). With the $^{35}$S label, the correction for the blood content hardly influenced the STU-value for the tumours, which implies that the activity in the tumours is due to true tumour uptake. STU-values for liver and bone marrow were higher than those for tumours and were only slightly influenced by the blood content correction.

For the thyroid, a much higher activity uptake occurred with the $^{125}$I label. No blocking of the thyroid was used.

Fig. 2 shows organ/blood ratios for the two radionuclides. The two labels exhibit rather different patterns with generally higher values for the $^{35}$S label (except for the stomach and thyroid, as can be understood from Fig. 1).

Fig. 3 shows tumour/blood ratios for the two radionuclides. The uptake of $^{35}$S in tumours is much higher than of $^{125}$I. Because of the higher uptake of $^{35}$S in tumours, the tumour/blood ratios increased with time and the ratios at 48 and 96 h after injection were 8 times higher than those of $^{125}$I.

## Discussion

For many years it has been known that antibody biodistribution and catabolism is dependent upon the

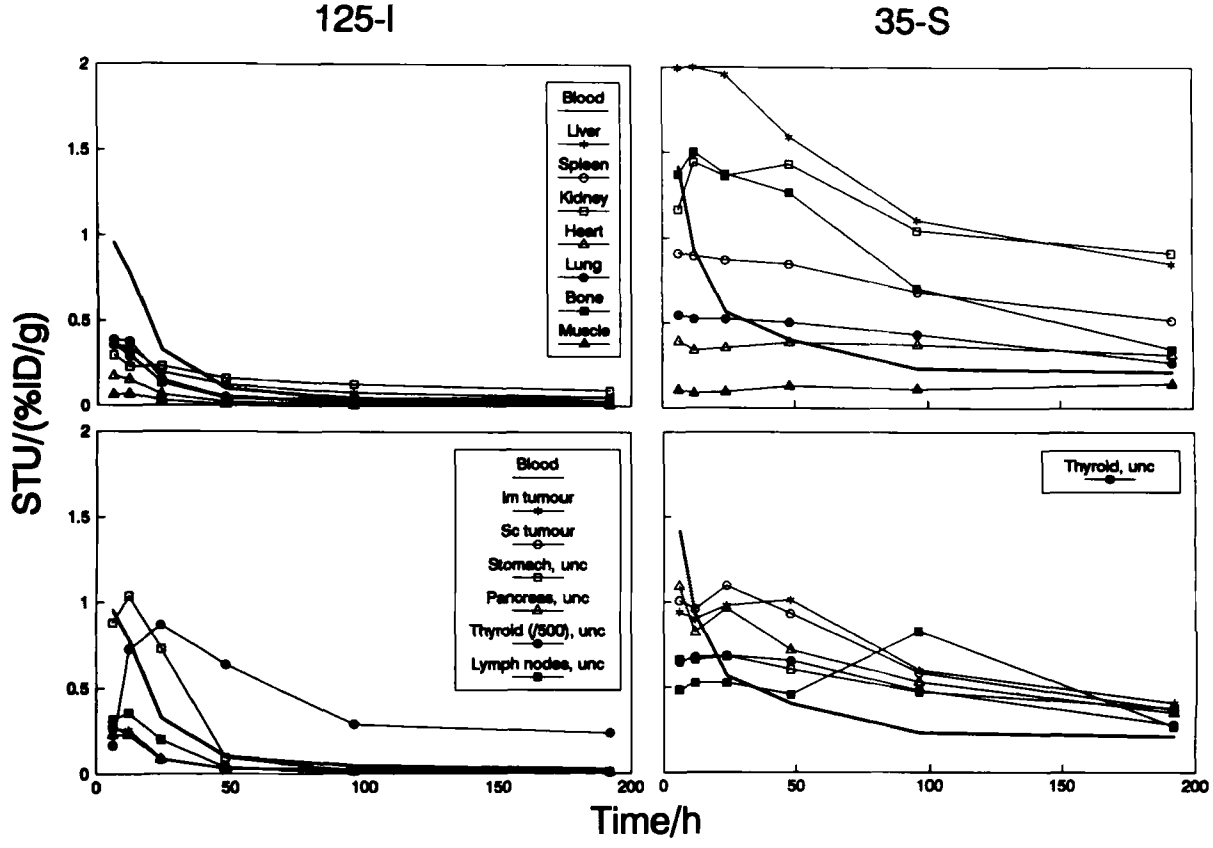


Fig. 1. Uncorrected (marked unc) and corrected STU time versus activity curves for different dissected organs and tumours. The curves illustrate the means of two rats per group. Please note the different scale for the $^{125}$I uptake in the thyroid.

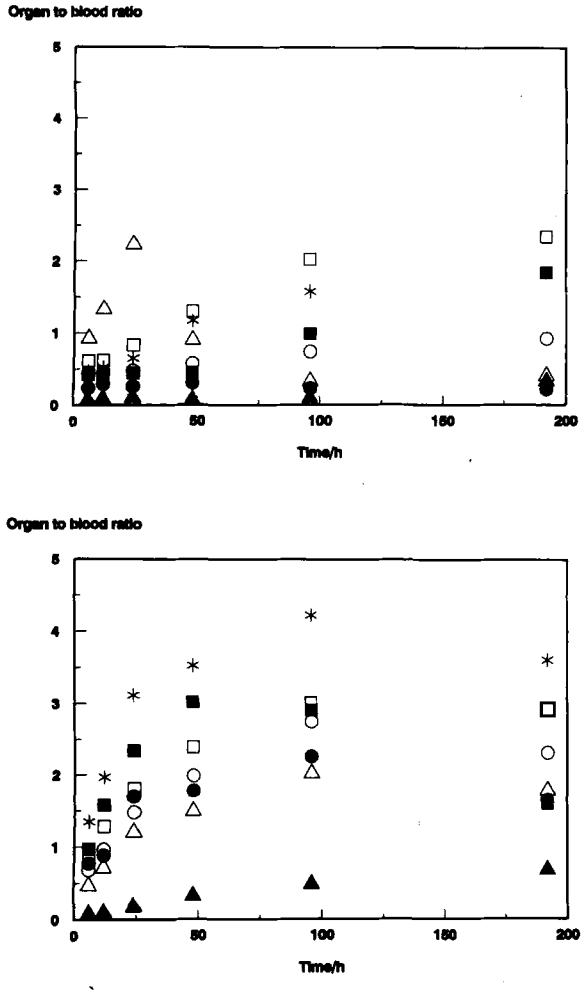


Fig. 2. Time versus activity curves for the organ/blood ratios of $^{125}I$ (a) and $^{35}S$ (b). *liver; ○ spleen; □ kidney; △ stomach; ● pancreas; ■ bone marrow; ▲ muscle.

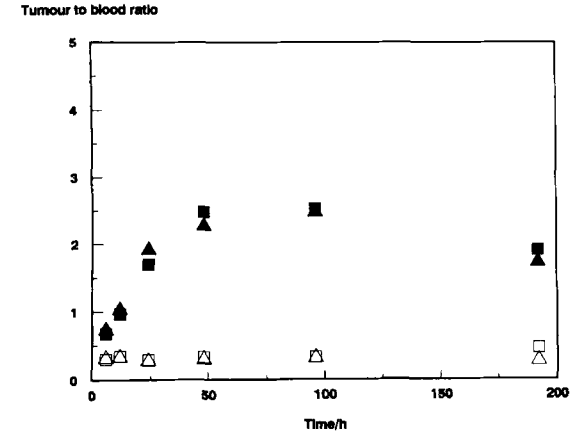


Fig. 3. Time versus activity curves for the tumour/blood ratios of $^{125}I$ and $^{35}S$. □ i.m. tumour, $^{125}I$; △ s.c. tumour, $^{125}I$; ■ i.m. tumour, $^{35}S$; ▲ s.c. tumour, $^{35}S$.

species from which the antibody is obtained and the species in which it is studied (9). Some data have been published concerning radiolabelled IgM MAbs in either mice or humans (2–6). Our previous work has shown that the nude rat model is superior to the nude mouse model for investigating the biokinetics of radiolabelled MAbs (1). In that model we used human xenografts in the nude rat injected with mouse MAb. In the present study we use a new syngenic rat model to compare the biokinetics of rat monoclonal IgM antibody 10B12 radiolabelled with $^{125}I$ and internally with $^{35}S$ in rats bearing rat colon carcinoma. As in our previous rat model, we studied the rats for 8 days, a period during which fewer than 21% of the tumours had reached an unrealistic tumour/host ratio ($>0.02$) (1).

In the present study, Fig. 1 shows the rapid clearance of $^{125}I$ from the blood such that low levels of activity were observed in all tissues and tumours, except in the thyroid and in the stomach (thyroids were not blocked with KI). Similar results were obtained in our laboratory when using radio-iodinated 4D7 IgM (data not shown). When the STU of tumours and other organs for the $^{125}I$ conjugates are compared with the $^{35}S$ conjugates (Fig. 1), the results suggest that the dehalogenation in vivo is indeed fast (less than 6 h). In contrast to the $^{125}I$ uptake, the uptake of $^{35}S$ in the tumours increased rapidly and the amount of $^{35}S$ remained relatively constant at 1%/g over the period from 6 to 48 h after injection. In the s.c. and the i.m. tumours, the peak values were 1.1%/g at 48 h and 1.0%/g at 24 h respectively. This cannot be accounted for by the immunoreactivity of 10B12 being stronger when labelled with $^{35}S$ than with $^{125}I$, since $^{35}S$-10B12 and $^{125}I$-10B12 exhibited similar binding to an excess of rat colon carcinoma cells BN-7005 in vitro nor can it be explained by the tumour size. The tumours used with the $^{35}S$ and $^{125}I$ labels weighed $2.2 \pm 1.2$ g ($n = 24$) and $2.1 \pm 1.0$ g ($n = 24$) respectively. In addition, it is unlikely that different amounts of antibody could account for the difference, since the animals received the same dose, 5 μg/rat, both of the $^{35}S$- than $^{125}I$ labelled antibody. The explanation of the higher levels of $^{35}S$ than $^{125}I$ in the tumours must thus be a consequence of dehalogenation and/or $^{35}S$ incorporation in the tumour cells.

Investigators have reported $^{131}I$-MAb dehalogenation after labelling by the Bolton-Hunter, the lactoperoxidase and the chloramine-T methods (10). Markkanen et al. (11) found progressive change with time in the size of proteins iodinated by the chloramine-T technique, and suggested molecular alterations and denaturation secondary to the labelling process. However, denaturation caused by in particular the chloramine-T labelling process might lead to decreased half-lives and increased degradation in vivo thus mimicing effects of dehalogenation per se. Further, the degree of dehalogenation appears to vary with some factors, especially different classes of MAbs. Halpern et al. (2) compared the reports of several authors with

their own data and indicated that $^{125}$I-labelled IgM MAbs were dehalogenated in vivo after 4 h, the most rapidly dehalogenated MAb of all types studied. Our data from the comparison of the IgM radiolabelled with $^{125}$I and internally with $^{35}$S in a syngenic rat tumour model support the view of Halpern et al.

Buchsbaum et al. (5) have compared the distribution and binding of the IgM and the IgG1 subclass labelled with $^{131}$I and $^{111}$In in normal mice and noted that IgM was larger and catabolized more rapidly than IgG1. Pimm & Baldwin (3) failed to localize the tumour in a murine system using a radio-iodinated IgM, and indicated that it was more likely a reflection of the poor extravasation of IgM antibodies. This factor, together with the short whole-body half-life of IgM, could account for inefficient tumour localization. To all appearances, IgM MAbs labelled with radioiodine seem to be undesirable in future use of monoclonal antibodies.

The catabolism of antibody by the liver has received much attention. Sands & Jones (6) reviewed the mechanisms by which radiolabelled antibodies were taken up and accumulated in the liver and indicated that differences in the content of radioactivity in the liver are due to inherent differences in the manner by which the radioisotope is subsequently handled. They believe that the total accumulation of antibody in the liver may appear similar regardless of the cell type (Kupffer cells, hepatocytes, or both) which initiated the uptake of antibody-antigen complexes. In our study, results have shown that the liver uptake occurs with $^{35}$S-labelled material during the 6–48 h period, a fact not obvious when $^{125}$I is the label. This is probably due to the fact that iodine leaves the liver, whereas the $^{35}$S-methionine is accumulated in the liver after catabolism. As shown in Fig. 2, the pancreas rapidly acquired more $^{35}$S than $^{125}$I. This could be due to a specific antibody binding as supported by the finding by Brodin et al. (unpublished results) using immunohistochemistry, that 10B12 stains some acinar cells in pancreas. However, antibody catabolism in the liver could lead to free $^{35}$S-methionine in the circulation, a suspicion aroused by the general difference between the labels in Fig. 2. If so, according to the well-known behaviour of $^{35}$S-methionine, one would expect increased activity uptake in some normal tissues such as the pancreas and the stomach, and certainly also in the fast growing tumours. Naturally, such uptake of non-MAb activity could also be caused by a deficient purification technique. None of these courses is confirmed, but since it is of great importance for the correlation between the MAb distribution and the radioactivity measurements, it is desirable that further studies on these circumstances are carried out.

In summary, we use a new syngenic model to verify the existence of the problem with dehalogenation of iodinated MAbs, especially IgM MAbs. Internally radiolabelled $^{35}$S-10B12 results in activity uptake in tumour and some normal tissues. We suggest that internally labelled MAbs should be used for biokinetic studies in the future and that these studies should be carried out in a syngenic model. This may better reflect in vivo antibody biodistribution and metabolism.

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