Stability and Immunoreactivity of the Monoclonal Anticytokeratin Antibody TS1 after Different Degrees of Iodination

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The immunoreactivity, stability and in vivo kinetics of an anticytokeratin 8 monoclonal antibody, TS1, were investigated following different degrees of labeling with ^{125}I (0.2, 1 and 2–3 ^{125}I/TS1 MAb). By testing with ELISA, it was demonstrated that a high degree of iodination, i.e. >2 ^{125}I/TS1, caused a rapid decrease in immunoreactivity to almost zero within 10 days. Furthermore, a complete degradation to low molecular weight fragments and free iodine was seen, as shown by SDS PAGE and autoradiography. The differently labeled radionuclide conjugates were injected into nude mice inoculated with HeLa Hep2 cells and tumor doses (estimated by MIRD formalism), tumor:non-tumor dose ratios, % I.D./gram tissue, Gy/MBq and in vivo kinetics of the differently labeled MAbs were determined. Despite the in vitro instability of the highest iodinated radionuclide conjugate, it was possible to deliver high doses to the tumors if the conjugate was injected into the animal immediately after completion of the iodination procedure. Increases from 1.4 Gy to 15.2 Gy delivered tumor dose were obtained with a tenfold increase in the specific activity, without alterations in the tumor:non-tumor tissue dose ratios. There is room for significant improvements in efficacy at radioimmunotherapy, which can be gained by optimizing the degree of iodination. For therapeutical applications a high degree of iodination may be an advantage.

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Since the introduction of monoclonal antibody (MAb) technology in 1975, antibody-mediated targeting of tumors has been a putative cancer treatment modality that has undergone significant improvements both diagnostically and therapeutically. In order to improve the radioimmunotargeting (RIT) of solid tumors, the relatively low level of accumulated radioactivity in the tumor has hampered further developments. The proportion of accumulating radiolabeled antibodies in the tumor versus normal tissues is generally not high enough to sterilize tumors without risking lethal bone-marrow toxicity, despite efforts to improve the tumor-targeting efficacy (1–8). Tumor doses of 70 Gy have been postulated to eradicate experimental tumors completely, but this level has been difficult to reach (9).

One way to increase the accumulation of radionuclide in the tumor might be to increase the specific activity of the conjugate, i.e. the number of iodine atoms per MAb, if saturation of the tumor occurs. However, too high a specific activity may exert a deleterious effect on the MAb as a result of radiolysis causing decreased immunoreactivity and deiodination during the actual labeling procedure, over time because of the storage of labeled antibody under disadvantageous conditions, or in the in vivo situation. The degree of iodination is also of importance, as the probability of attaching iodine to the antigen-binding site of the MAb increases with an increased total number of iodine atoms per MAb, which may cause a decreased immunoreactivity. By increasing the average degree of iodination from 0.5 to 2 iodine atoms per MAb, a fourfold increase in delivered dose to the tumor can be achieved given that the immunoreactivity is unaffected. Hence, it is of interest to investigate the putative improvement that can be made by optimizing the properties of the radionuclide conjugate.

The aims of this study were to investigate the effects of different degrees of iodination of the anticytokeratin MAb TS1 using ^{125}I, both in vitro and in vivo.

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MATERIAL AND METHODS

The monoclonal antibody

The monoclonal anticytokeratin antibody TS1, of the $IgG_{1\kappa}$ subclass, was obtained from InRo Biomedtek (Umeå, Sweden) (10).

Radiolabeling and storage

The MAbs were radiolabeled with ^{125}I (IMS 30, Amersham) using the chloramine-T method (11). Free iodine was removed on a Sephadex G 50 medium column (Pharmacia). For the in vivo study, the specific activity of the lowest iodinated TS1 was 82 MBq/mg TS1, which corresponds to 0.2 ^{125}I/TS1, for the moderately iodinated TS1 545 MBq/mg TS1, which corresponds to 1.1 ^{125}I/TS1 and, finally, for the highest iodinated TS1 1 273 MBq/mg TS1, which corresponds to 2.3 ^{125}I/TS1. Furthermore, the moderately and lowest iodinated MAbs were also stored in 4°C for one week prior to usage. For the in vitro study, the lowest iodinated MAb had 0.3 ^{125}I/TS1, the moderately iodinated MAb had 1 ^{125}I/TS1 and, finally, the highest iodinated MAb had 3 ^{125}I/TS1.

Enzyme-linked immunosorbent assays

ELISAs were performed on labeled TS1 (0.3, 1 and 3 ^{125}I per TS1) using unlabeled TS1 as control in order to follow the changes of immunoreactivity over time (2 months). 96-well microtiter plates were coated overnight with tissue polypeptide antigen (TPA) at a concentration of 5 μg/ml in 0.1 M $NaHCO_3$ at 4°C. After washing, TS1 and ^{125}I-TS1 was diluted to 1 μg/ml in PBS-Tween and incubated for 3 h on the microtiter plates at room temperature. Rabbit anti-mouse antibody at a ratio of 1:1000 in PBS-Tween (150 mM NaCl, 10 mM Na_2HPO_4, pH 7.4, Tween 20 0.05%) coupled to horseradish-peroxidase (HRP) was used for detection. Absorbance was measured at 450 nm.

Gel electrophoresis and autoradiography

SDS PAGE was carried out according to Laemmli (12) in mini-Protean 2 electrophoresis cells from Bio-Rad. The lowest (0.3 ^{125}I/TS1), moderately (1 ^{125}I/TS1) and highest (3 ^{125}I/TS1) iodinated MAbs were run on an SDS PAGE (6% gel) which was performed at room temperature in 25 mM Tris/190 mM glycine, pH 8.6. The moderately iodinated MAb was run on days 7, 16 and 65 after iodination. The lowest and highest iodinated MAbs were run on day 100 after iodination only. Autoradiographies, using Hyperfilm™ MP (Amersham), were performed on all gels.

Animal model

Twenty-three female nude mice (BALB/c nu/nu, Bomholtgaard, Denmark), aged 8–10 weeks, were inoculated with approximately 5×10^6 HeLa Hep2 cells, subcutaneously, near the right hind leg. The cells had been cultured in RPMI 1640 medium (Gibco, Scotland), containing 5% (v/v) fetal calf serum and 1% (v/v) penicillin-streptomycin, prior to inoculation. HeLa Hep2 cells originate from a human cervix adenocarcinoma line which expresses the cytokeratins 8, 18 and 19.

Radioimmunolocalization

The mice were divided into groups A1, A2, B1, B2 and C. Group B1 received 50 μg ^{125}I-TS1 with a specific activity of 545 MBq/mg while group A1 received 50 μg ^{125}I-TS1 with a specific activity of 82 MBq/mg. Group A2 received 50 μg of the ^{125}I-TS1 from the same batch as group A1, with the exception that it had been stored at 4°C for one week, and group B2 received 50 μg from the same batch as group B1, which also had been stored at 4°C for one week. Group C received 50 μg ^{125}I-TS1 with a specific activity of 1 273 MBq/mg. The injected dose per mouse in groups B1 and B2 was on average 18 MBq, in groups A1 and A2 2.4 MBq and finally, in group C, 48 MBq. All mice were injected with the MAb intraperitoneally and the MAb had been diluted in 0.25 M phosphate buffered saline, pH 7.5. The animals were fed ad libitum with pellets and water. The water was supplemented with potassium iodide (Lugol's solution; 10 mM $NaHCO_3$ supplemented with 1 mg KI/ml) in order to block thyroid uptake of free iodine.

A scintillation camera (Porta camera, General Electric) connected to a Hermes (NUD, Stockholm, Sweden) evaluation system was used to determine the distribution of radioactivity in the mice. A holder was used to situate the anesthetized animal at a distance of 9 cm from the pinhole collimator, in order to maintain constant geometric conditions. The mice were anesthetized intraperitoneally prior to scintigraphy using 0.1–0.2 ml of a 1:1:2 cocktail of Dormicum (Roche, Midazolam 5 mg/ml), Hypnorm (Janssen Pharmaceutica, Fluanisonum 10 mg/ml and Fentanylum 0.2 mg/ml) and sterile water.

The study period was 47 days in total and each animal was subjected to 22 consecutive radioimmunoscintigraphic measurements.

RESULTS

In vitro stability of TS1

Enzyme-linked immunosorbent assay. The ELISA measurements are presented in Fig. 1 and demonstrate that the unlabeled TS1 completely maintained its immunoreactivity over the two-month study period. The highly iodinated TS1 demonstrated a reduced immunoreactivity (70%) compared to the unlabeled MAb (100%) immediately after termination of the iodination procedure. After approximately 10 days the immunoreactivity had diminished to less than 2% of the unlabeled TS1, as determined from the ELISA optical density (O.D.) values. The moderately iodinated TS1 showed no initial decrease in immunoreactivity. However, 10 days after iodination the immunoreactivity began to decrease. After two months the immunoreactivity

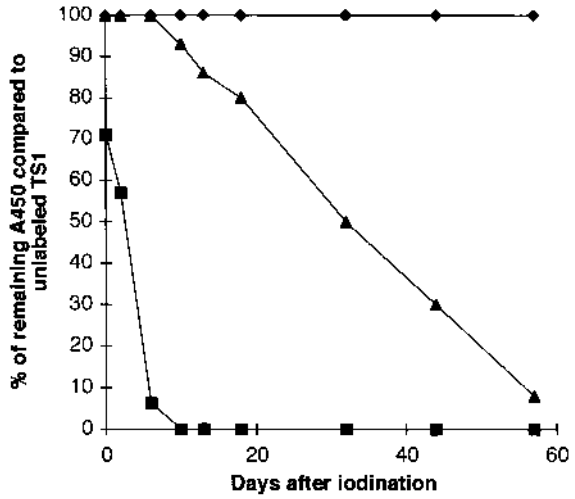

Fig. 1. Differences in immunoreactivity determined by ELISA technology. Non-labeled TS1 (◆) was set to 100% while TS1 iodinated to a high (■) and low degree (▲) were calculated as percentage of remaining absorbance measured at 450 nm as compared to non-labeled TS1. ELISAs were run at certain intervals for a total of 57 days, after performance of the labeling procedure.

was severely compromised, with less than 10% preserved as compared with the unlabeled TS1.

Gel electrophoresis and autoradiographies. When SDS PAGE was used to evaluate the properties of TS1, the purified unlabeled MAb displayed one single band following Coomassie staining for protein, as seen in Fig. 2 (control). When the same MAb with approximately 1 ^{125}I/TS1 was investigated 7, 16 and 65 days after iodination, degradation of the protein was seen and low molecular weight bands appeared in increasing numbers (Fig. 2). These gels were also subjected to autoradiography, which confirmed the appearance of radiolabeled low molecular weight fragments of the antibody after 7 days (data not shown). In order to illustrate the degradation differences between one low (0.3 ^{125}I/TS1) and one high (3 ^{125}I/TS1) labeled TS1 over time, Fig. 3 demonstrates the complete degradation of the highly iodinated antibody to low molecular weight fragments and free iodine, while the low-labeled MAb shows less severe degradation, 100 days after labeling.

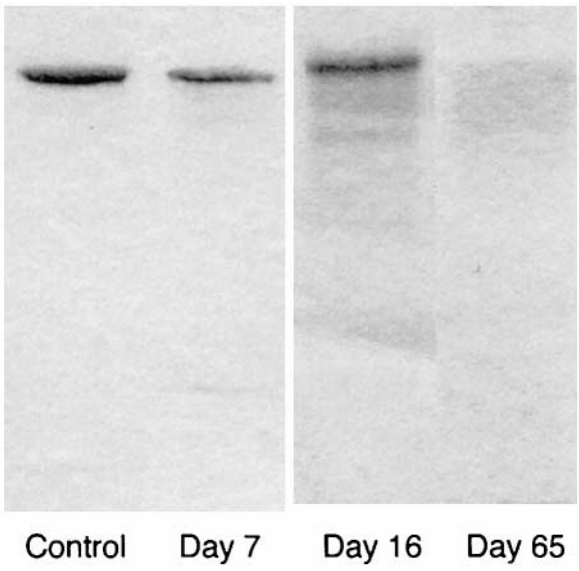

Fig. 2. SDS PAGE (6%) stained for protein, performed on ^{125}I-labeled TS1 MAb (1 ^{125}I/TS1) on days 7, 16 and 65 after the labeling procedure. Appearance of low molecular weight degradation products can be seen over time.

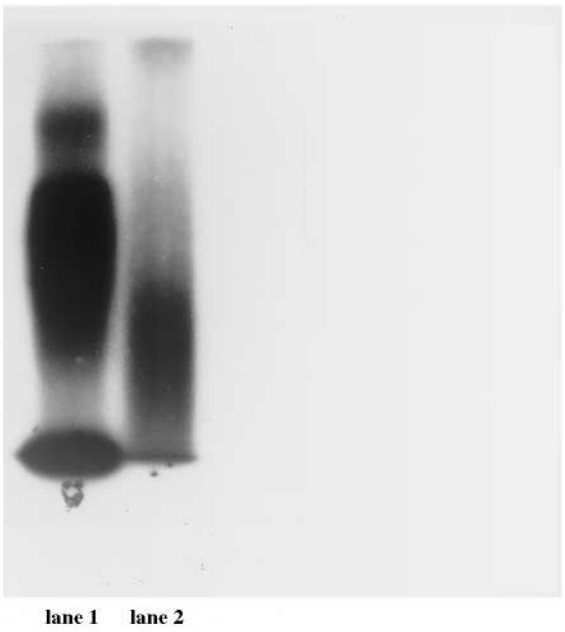

Fig. 3. Autoradiography 100 days after performance of the labeling procedure, demonstrating the complete degradation of a highly iodinated MAb to low molecular weight fragments and free iodine (lane 2), while the low-labeled MAb shows less severe degradation (lane 1).

In vivo properties of the conjugates

Dose calculations. As illustrated in Fig. 4a the median tumor doses (Gy), estimated using MIRD-formalism (13), in the different groups varied widely throughout the study. The group A1 mice reached a maximum value of 1.4 Gy, and the corresponding value for group A2 was 0.5 Gy on day 47. Group B1 mice had reached a median dose in the tumor of approximately 7 Gy on day 47, while the corresponding value for group B2 was 0.06 Gy. Group C accumulated the highest median tumor dose at 15.2 Gy on day 47.

The tumor:non-tumor dose ratios (Gy/Gy) of the different groups can be seen in Fig. 4b. The results indicated no major differences between groups A1, B1 and C during the course of the study. On day 47 the dose ratio varied between 4.5 and 5.5 for these groups. The ratios for the

groups which received 1-week-old MAbs demonstrated a lower dose ratio compared with the freshly iodinated MAbs. Group A2 had a dose ratio of 3.3, which should be compared to 5.5 for group A1. Group B2 had a low ratio, only barely climbing above 1, compared with approximately 4.5 for group B1.

The tumor antibody uptake (% Injected Dose (I.D.)/gram tissue) is presented in Fig. 5. There were only slight differences between the freshly iodinated groups (groups A1, B1 and C), with peak values varying between 6.5 and 8% I.D./gram tumor tissue, reached between days 9 and 16 after injection of the radionuclide conjugate, respectively. Group A2 had a peak value of 4.6, which was reached on day 8 after injection, while group B2 peaked at 0.4 on day 2.

The decrease in relative radioactivity in groups A–C was estimated by the ratio counts per second (c.p.s.) of non-tumor tissue/maximum c.p.s. value (day 1). The results presented in Fig. 6 indicate that there were no major differences between groups A1, A2, B1 and C, although group A1 demonstrated the slowest decrease in non-tumor tissue radioactivity and group A2 the fastest. Group B2 showed a pronounced, rapid decrease in relative radioactivity, clearly distinguishable from the other groups.

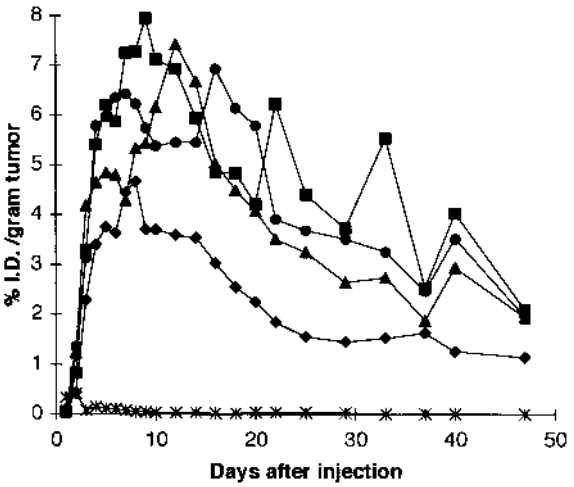

Fig. 5. The tumor antibody uptake (% I.D./g) shown as the mean value for groups A1 (■), A2 (◆), B1 (▲), B2 (×) and C (●), from days 1–47 after injection of radionuclide conjugate.

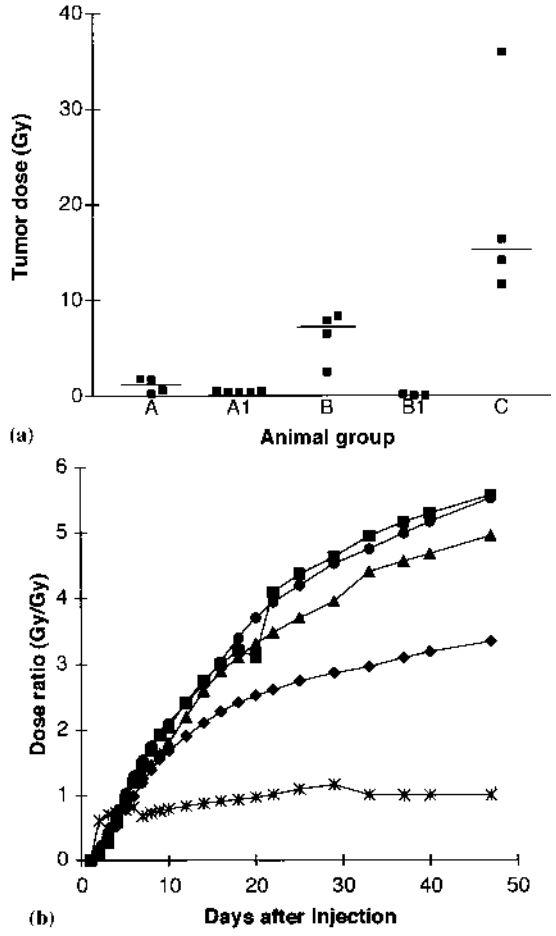

Fig. 4. (a) The tumor doses of each individual animal in groups A1 (■), A2 (◆), B1 (▲), B2 (×) and C (●). The median tumor dose values for each group are indicated by a horizontal line. (b) The mean dose ratios of groups A1 (■), A2 (◆), B1 (▲), B2 (×) and C (●) estimated as mean tumor dose in tumor tissue (Gy) divided by mean tumor dose in non-tumor tissue (Gy), on days 1–47.

The highest efficacy in tumor dose per injected MBq (Gy/MBq) was seen in the freshly iodinated groups (A1, B1 and C), and did not display significant variability (Fig. 7a). The stored moderately iodinated MAb (B2) displayed almost no efficacy, while the stored lowest iodinated MAb presented intermediate efficacy. The plot of the ratio between Gy/MBq in tumor:non-tumor tissues is presented in Fig. 7b, and reached values between 5 and 5.5 for the freshly iodinated groups, while again, group B2 had values

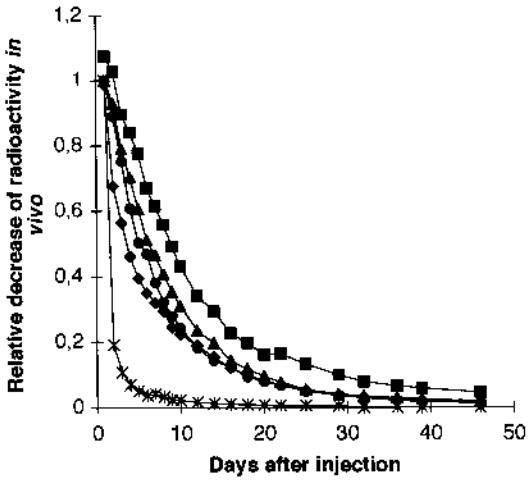

Fig. 6. The relative mean decrease in radioactivity in vivo for groups A1 (■), A2 (◆), B1 (▲), B2 (×) and C (●) The values were estimated as counted c.p.s. in non-tumor tissue on days 1–47 divided by the maximum c.p.s. count, i.e. day 1.

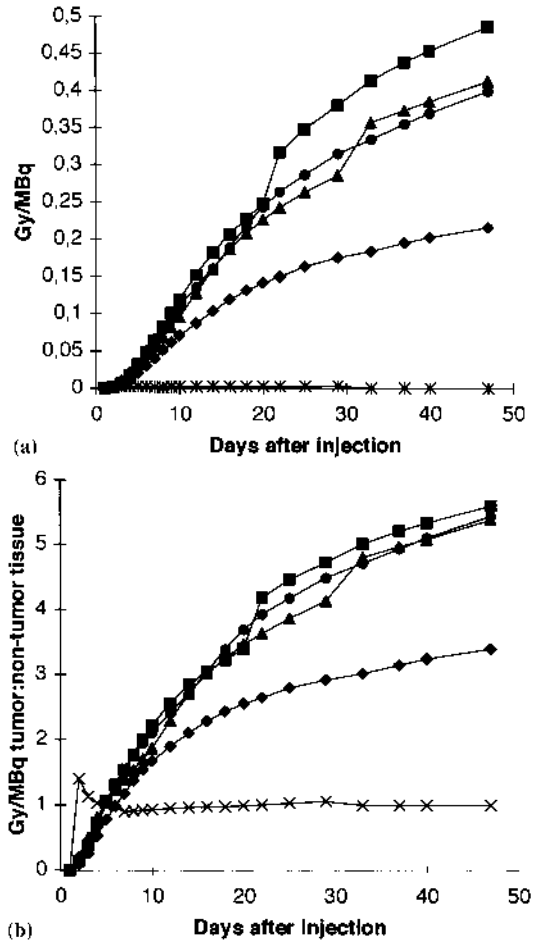

Fig. 7. (a) The delivered dose to the tumors for groups A1 (■), A2 (◆), B1 (▲), B2 (×) and C (●) in relation to degree of iodination (Gy/MBq), from days 1–47 after injection of radionuclide conjugate. (b) The ratio between the delivered dose to tumor and delivered dose to non-tumor tissues for groups A1 (■), A2 (◆), B1 (▲), B2 (×) and C (●) in relation to degree of iodination (ratio Gy/MBq), from days 1–47 after injection of radionuclide conjugate.

close to 1 indicating no specific targeting. Group A2 presented intermediate results.

DISCUSSION

Several major conclusions can be drawn from the results obtained in this study: First, heavily iodinated antibodies (3 ^{125}I/TS1) lose immunoreactivity rapidly after the labeling procedure. ELISA studies showed that the reactivity had decreased to undetectable levels after 10 days (Fig. 1). The moderately iodinated MAb (1 ^{125}I/TS1) demonstrated more stability compared to the highest iodinated MAb, and was operational 10 days after iodination with partially preserved reactivity, as shown in ELISA. Long-term storage of iodinated MAbs caused a degradation of the protein into small molecular weight fragments and free iodine in all groups irrespective of labeling degree, which was shown by SDS-PAGE and autoradiographies (Figs. 2 and 3). Concerning the number of iodine atoms that can be attached to a MAb without affecting the immunoreactivity, it seems reasonable that the fewer the tyrosine residues situated in the complementarity-determining regions (CDR) of the MAb, the smaller the probability of attaching an iodine atom to this sensitive location. Erlandsson et al. (unpublished data) have shown that there are 16 tyrosine residues in the CDRs of TS1, out of an estimated total number of 120 in the entire molecule. This is a considerable number, and there may be a risk of affecting the antigen-binding site during heavy labeling, thus rendering the MAb less efficient.

The in vivo study demonstrated that the tumor:non-tumor dose ratio (Fig. 4b) was relatively unaffected by the different degrees of iodination, demonstrating unaffected immunoreactivities. The dose ratio reached values between 4.5 and 5.5 for all three groups which were injected with freshly iodinated MAb. From these results it could be argued that the degree of iodination within these frames is of lesser importance concerning the efficacy of the RIL procedure. However, both groups injected with stored MAb (1 week old) illustrated lower dose ratios in vivo, and decreased reactivity in vitro, emphasizing the point that radionuclide conjugates should not be stored under such conditions. It has been shown in several reports that the damaging effect is caused by decay-generated free radicals (14). Furthermore, it has been established that free radical scavengers such as human serum albumin (HSA), cysteinamine and ascorbic acid can reduce these deleterious effects, but also strategies like cryopreservation can improve the shelf life of radiolabeled antibodies (14–16). An indication of how rapidly the injected radioactivity is cleared from the animals is given in Fig. 6 and that the differences between the freshly iodinated groups (A1, B1 and C) and group A2 were small, i.e. no major kinetic differences between the differently iodinated MAbs were identified in vivo. Group B2, however, showed a very rapid decrease, the major reason probably being in vitro radiolysis prior to injection, resulting in free iodine which was excreted in the urine.

From an RIT perspective it could be valuable to increase the specific activity of the utilized MAb, from, for example, 1 to 2 ^{125}I/TS1. In this study the results indicated a doubled tumor dose in the highest iodinated group compared to the moderately iodinated group, i.e. it is possible to increase the delivered dose by modifying the degree of iodination. Furthermore, the total amount of cytokeratins in these tumors has been determined to 1–3 μg/g tumor tissue, indicating that it might be possible to saturate the target sites (Stigbrand, pers. comm.). In order to avoid a comparatively low accumulation of radionuclide in the tumor, saturation of a large proportion of the target sites with unlabeled MAb should be avoided. This could be achieved by using radionuclide conjugates with

high specific activity. The increase in tumor dose from 7 Gy to 15 Gy, entirely delivered by modifications in the degree of iodination, is of utmost importance as it significantly affects the degree of tumoricidal capacity. A tumor dose of 70 Gy has been postulated to eradicate tumors and optimization of degree of iodination can influence the result. This improvement, however, does not change the dose ratio between tumor and non-tumor tissues, and further investigations concerning removal of non-localizing MAb by using, for example, anti-idiotypic antibodies or extracorporeal immunoadsorption (17–19) in order to spare the non-tumor tissues from radiation damage are of interest in order to optimize this technique further.

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