Acta Oncologica 29 (1990) Fasc. 8

FROM THE DEPARTMENTS OF SURGERY, UNIVERSITY HOSPITAL, LUND AND HELSINGBORG HOSPITAL, HELSINGBORG, AND THE DEPARTMENT OF RADIATION PHYSICS AND THE WALLENBERG LABORATORY, UNIVERSITY OF LUND, LUND, SWEDEN.

SUBCUTANEOUS INJECTION OF MONOCLONAL ANTIBODY 96.5

Biokinetics in the nude rat heterotransplanted with malignant melanoma

C. INGVAR, K. NORRGREN, S.-E. STRAND, T. BRODIN, P.-E. JÖNSSON and H.-O. SJÖGREN

Abstract

Nude rats heterotransplanted with human melanoma metastasis were injected subcutaneously on the hind paw with ^{125}I-labelled monoclonal antibody 96.5 and with control antibody ^{131}I-OKT3. The elimination from the injection site followed a biexponential function. The uptake in the inguinal lymph nodes on the side of the injection was initially high, but after 90 h it equalled the control side. The uptake in the tumour was slower than after i.v. injection but higher than in other tissues except blood. More than 80% of the activity in the dissected liver represented circulating blood. The uptake ratio of 96.5/OKT3 was c.3 in the tumours but c. 1 in all other tissues including blood. The capillary filtration coefficient was proportional to the uptake in organs like liver, lungs and muscle. It is concluded that subcutaneously injected radiolabelled monoclonal antibodies are initially transported via the lymph but then mainly distributed via the blood reaching the different tissues including tumours.

Key words: Monoclonal antibodies, subcutaneous injection, biokinetics, capillary filtration coefficient, melanoma, nude rats.

Scintigraphic imaging of lymph node metastases after administration of radiolabelled monoclonal antibodies might be a possible method to improve the diagnostics of such metastases. Most in vivo studies using monoclonal antibodies have used the intravenous route to administer antibodies with the assumption that access to the target sites could best be reached by the systemic circulation (1–3). Several authors, however, have suggested that subcutaneously injected antibodies concentrate in metastatic deposits in regional lymph nodes (4–6). Such an approach would maximize the tumour antibody uptake, and at the same time minimize the non-specific backgroud activity.

The debate on prophylactic lymph node resection in patients with malignant melanoma might be solved if antibodies can be localized specifically to metastatic deposits in lymph nodes. Pilot studies in man with radiolabelled antibodies have yielded conflicting results (2, 7). Nelp et al. (7) showed that 3 out of 6 patients showed positive images of lymph node metastases. However, among 5 patients without metastases one had a false positive image. In tissues from the metastatic lymph nodes, however, the concentration of specific and control antibody was similar. Lotze et al. (2) found an uptake ratio higher than unity in lymph nodes with metastatic deposits, but frequently also in lymph nodes without tumour. The explanation was cross reactivity with lymph node tissue.

A better knowledge of the pharmacokinetics of the monoclonal antibody (MAb) distribution after subcutaneous injection is needed to assess if this mode of administration might be advantageous for imaging of human tumours.

The nude rat model (8) offers favourable possibilities for in vivo studies of uptake parameters, including the corrected specific tissue uptake (STU_{corr}). We here report a study of the biokinetics of the subcutaneously injected radiolabelled monoclonal antibody 96.5 in nude rats heterotransplanted with human melanoma.

Material and Methods

The previously described model using nude rats (Rowett RNu/RNu strain) was used (8). The rats were bred and kept in cages with filter tops and provided with autoclaved food pellets, sterilized drinking water ad libitum, and sterile wood granulate bedding in a temperature-controlled unit, receiving particle filtered air. Four to five times

Submitted 29 December 1989.
Accepted for publication 1 May 1990.

during the experiment the animals were anesthetized with ether and 0.5-1 ml of blood was withdrawn. A human melanoma tumour was established by serial passages in the rats. The tumour transplants used were all from the same passage and all animals were inoculated with one tumour on each thigh, intramuscularly (right) and subcutaneosly (left).

The monoclonal antibody used in the present series was the 96.5 (IgG 2a) specific for p97, a cell surface glycoprotein with a molecular weight of 97 000. It is present in 60–80% of human melanomas but only in trace amounts in normal tissues (9). Antibodies were purified from ascites fluid of mice, bearing hybridoma ascites tumours, by affinity chromatography on a Protein A Sepharose CL-4B column (Pharmacia, Uppsala, Sweden) with a stepwise pH gradient elution (9, 10). The purity of the monoclonal antibodies was estimated by agarose electrophoresis. The antibodies were stored at −70°C.

Monoclonal antibody OKT3 (IgG1, specific for T-cell antigen, not present in these nude rats lacking thymus) was used as a non-specific control (11).

The monoclonal antibody 96.5 (350 μg) was labelled with 37 MBq ^{125}I according to the Chloramine-T method (12) (the iodine solution contained 3×10^{16} iodine ions per ml). Labelling efficiency was 65% with less than one iodine atom per molecule, which precluded interference with the immunoreactivity (13). Antibody OKT3 (300 μg) was labelled with ^{131}I (25 MBq) in the same way (labelling efficiency 62%).

The labelled antibodies were tested for binding to a suspension of cultured melanoma cells H-1477 (Mel-28) in the direct binding assay described by Brown et al. (9). The antibody activity of the labelled protein was expressed as the ratio of bound and total added activity (here 65% indicating retained binding capacity to melanoma antigen p97). In preceding titrations it was shown that the number of cells used represented a large antigen excess. The blood pool was estimated by labelling red blood cells (RBC) with ^{99}Tc^{m} in vivo 4 h before the rat was sacrificed (8). This procedure enables imaging of the blood pool and calculation of the corrected specific tissue uptake (STU_{corr}) in different tissues post mortem (for details, see ref. 8). Labelling efficiency was determined by measuring the ^{99}Tc^{m}-activity in the red blood cells and in the plasma, 15–30 min after injection.

Experimental design. A total number of 12 nude rats were used. With the rat under ether anesthesia on the scintillation camera a subcutaneous injection of labelled specific antibody was made on the dorsal side of one hind paw and the labelled control antibody on the other paw. The mean amount of antibody injected was 34.9 ± 3.8 μg (MAb 96.5) and 16.5 ± 2.1 μg (MAb OKT3). This corresponded to 7.8 ± 0.7 MBq and 0.8 ± 0.1 MBq respectively. Blood samples were taken from the periorbital venous plexa at regular intervals. Plasma and blood cells were separated by centrifugation.

The rats were sacrificed with an overdose of ether at different times (6, 24, 48, 72, 150 and 175 h) following the injections of antibodies. Tumours, liver, spleen, lungs, kidney, muscles and bone marrow were then removed. In each rat ten different lymph nodes were dissected: bilaterally one popliteal, one inguinal and two axillary nodes; one lymph node from the true pelvis and one node from the hilus of the liver. The rats were not bled and the different organs and tissues were not washed. Each tissue sample was weighed, m_j (g) and measured in an automatic NaI(Tl) well counter for radioactivity content, A_j(MBq).

The corrected specific tissue uptake (STU_{corr}) was calculated by the formula (8).

$$STU_{corr} = A_{m,t}(^{125}I) = \frac{A_j(^{125}I) - A_{blood}(^{125}I)}{m_j - m_{blood}} \quad (1)$$

This calculation takes two important factors into account: 1) the activity in the blood due to labelled antibodies, and 2) the mass of blood in every sample measured.

A standard scintillation camera (General Electric Maxi Camera I) was used, connected to a computer for storage of images (Gamma 11, Digital Equipment Corp., USA). For the ^{125}I-96.5 MAb biokinetics, static images were registered during 15 min at regular intervals with the rat under ether anesthesia in supine position on the collimator face. A 25% energy window was centered over the 28 keV photo peak for ^{125}I. The count rate in the 28 keV (^{125}I) window from ^{131}I was 6% of the count rate in the 365 keV (^{131}I) window for the parallel hole collimator. In the digital image, regions of interest (ROI) could be selected in which the count rate was estimated separately.

Results

All tumours used in the present experiments derived from the same passage. The intramuscularly implanted tumours gained a median weight of 0.79 ± 0.60 g ($n = 17$) and for those applied subcutaneously 0.49 ± 0.30 g ($n = 7$). Fast growing tumours showed a tendency for necrosis in central parts. Some subcutaneously inoculated tumours grew invasively into the muscles. This explains the uneven numbers of subcutaneous and intramuscular tumours.

In vivo biokinetics. The outflow of the ^{125}I-96.5 from the injection site (Fig. 1) can be described by a biexponential function:

$$A_{inj}(t) = 27.15\, e^{-0.69t} + 71.1\, e^{-0.06t} + 1.75 \quad (2)$$

t is expressed in hours.

After 50 h there was only 5% of the labelled antibody left at the injection site. At 150 h about 50% of the ^{125}I-activity still remained in the whole body.

The uptake (in percentages of injected activity) in subcutaneous and intramuscular tumours measured in vivo by scintillation camera as a function of time showed a high

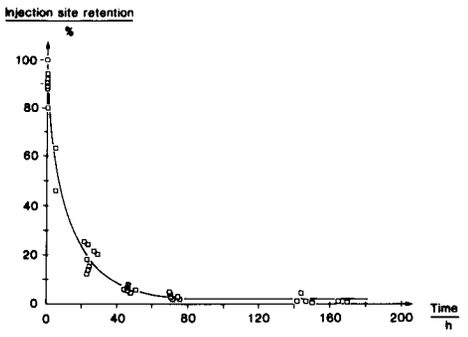

Fig. 1. The elimination of ^{125}I-96.5 (% of injected activity) from the injection site on the dorsal side of the hind paw in the nude rat, measured by the scintillation camera.

variability (data not shown) which was of the same magnitude as following intravenous injection of the same antibody (8). The blood plasma concentrations of the ^{125}I-96.5 and the ^{131}I-OKT3 were very similar during the whole study. The peak concentration in the plasma was 3%/g after 20 h (Fig. 2).

Specific tissue uptake (STU) in vitro. The specific tissue uptake of antibody 96.5 in the different dissected tissues and the tumours is shown in Fig. 3a and b. In the tumours, the uptake of antibody 96.5 reached a maximum after approximately 40 h, with peak values of 1.2%/g (i.m. tumours) and 0.9%/g (s.c. tumours) of injected activity. In the lungs high values (0.9%/g) were recorded about 20 h earlier than in the tumours. Muscle tissue showed a low and constant activity (0.1%/g). Other tissues (liver, kidney, bone marrow, spleen) showed an intermediate accumulation. The uptake in the lymph nodes (those from the

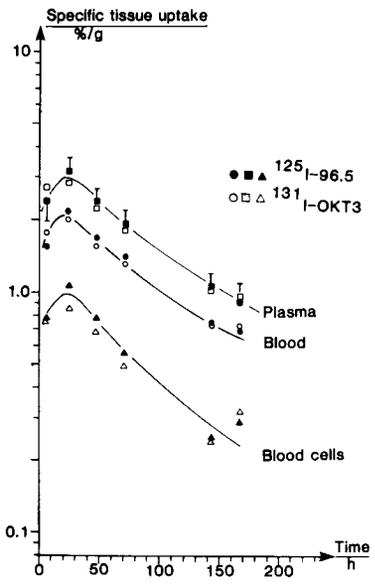

Fig. 2. Specific tissue uptake (% of injected dose per g tissue) of ^{125}I-96.5 (filled symbols) and control antibody ^{131}I-OKT3 (unfilled symbols) in plasma, whole blood and blood cells during 200 h following subcutaneous injection.

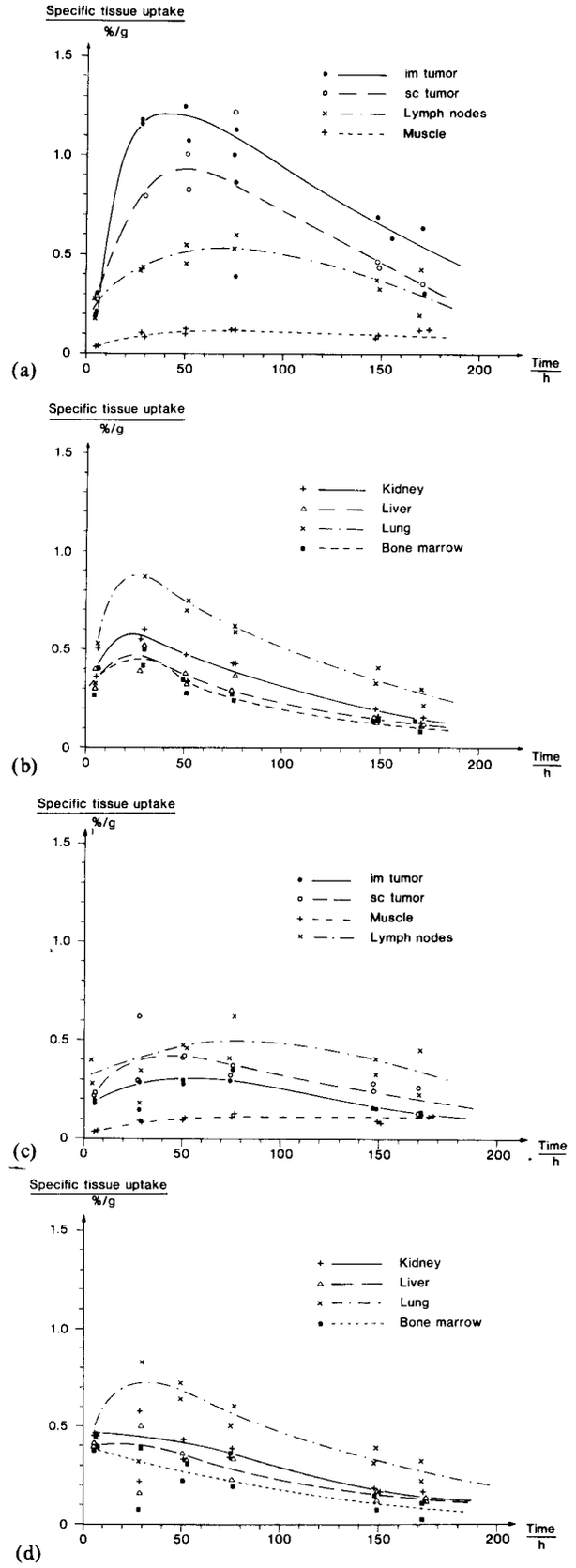

Fig. 3. Specific tissue uptake (STU) of ^{125}I-96.5 (a and b) and ^{131}I-OKT3 (c and d) in the different dissected organs and tumours.

injected extremity excluded) was much slower than in other tissues with the highest uptake at the end of the study.

Corresponding measurements of the control monoclonal antibody OKT3 did not show any tumour selectivity. The uptake curves were almost parallel for all organs including the tumours (Fig. 3c and d). The ratio between uptake values of MAb 96.5 and OKT3 clearly illustrated the tumour specificity of antibody 96.5 and, for all tissues except the tumour, the ratio equalled unity. The ratio for the tumours was, however, approximately three.

Lymph node biokinetics. A high uptake was initially registered in the poplietal lymph node on the injection side, but it later levelled out. After about 80 h the concentration was the same as on the contralateral side (Fig. 4). Other lymph nodes not draining the injection site had a peak activity after about 90 h. This uptake reached a peak later than the uptake in other organs. The control antibody OKT3 showed a similar uptake as the specific 96.5 antibody.

Corrected specific tissue uptake (STU_{corr}). In Fig. 5 the corrected values giving the STU_{corr} for the different tissues are shown. The tumour specific tissue uptake (STU) was only slightly reduced by this correction, i.e. the activity in the tumours registered in Fig. 3a is mostly due to true bound activity and not to blood activity. However, in the liver and lungs the specific tissue uptake was reduced to approximately 30% of the values in the initial registration in Fig. 3a and b.

The corresponding specificity of the labelled antibody uptake was calculated by the ratio of STU_{corr} values of the specific and control antibody 96.5/OKT3. This ratio equalled unity for all organs except the tumours which showed an even higher uptake than before the correction (mean 3.7).

In the bone marrow no initial high peak uptake was found in contrast to our previous findings after intravenous injection (8). The maximal specific tissue activity registered at 30 h in the marrow was 0.4% of the injected activity.

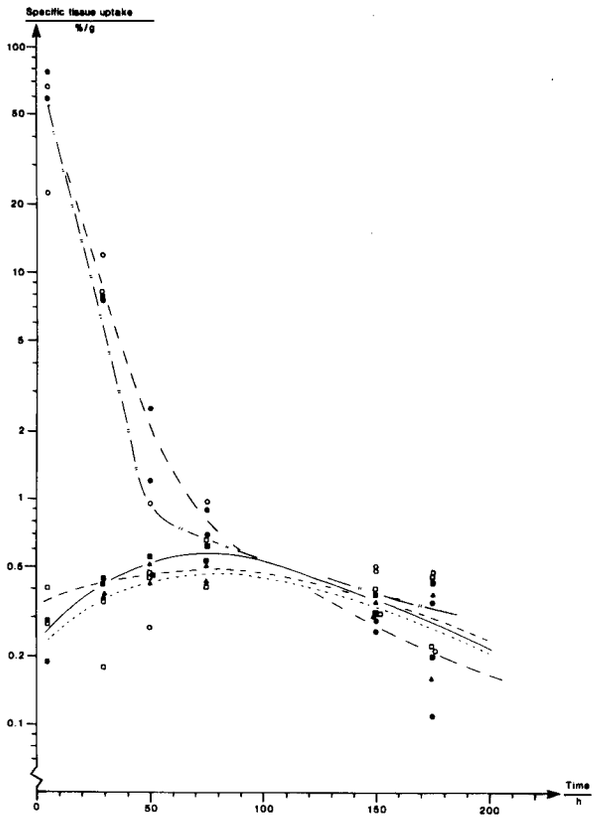

Fig. 4. Time-activity curves for lymph nodes on the injection side and at other locations (means of eight nodes). There was no sign of any higher accumulation in the lymph nodes of either 96.5 or OKT3 on the injection side as compared to the other lymph nodes after 80 h. Injection side:–·–● 96.5; –//–○ OKT3. Control side: ——■ 96.5; – – –□ OKT3 – – – –▲ 96.5 corrected.

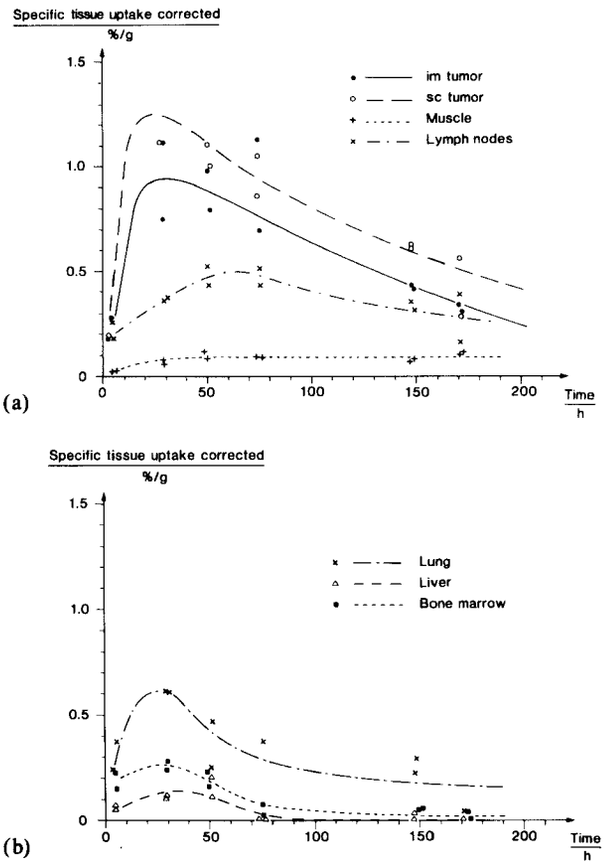

Fig. 5. Corrected specific tissue uptake (STU_{corr}) of ^{125}I-96.5 in the different dissected organs and tumours in the nude rat, calculated from data in Fig. 5a and b and corrected for blood content of labelled antibody 96.5. Still there is a high uptake in the tumours compared to Fig. 5a and b but the STU_{corr} in other organs are lower except for the lymph nodes where the uptake remains high.

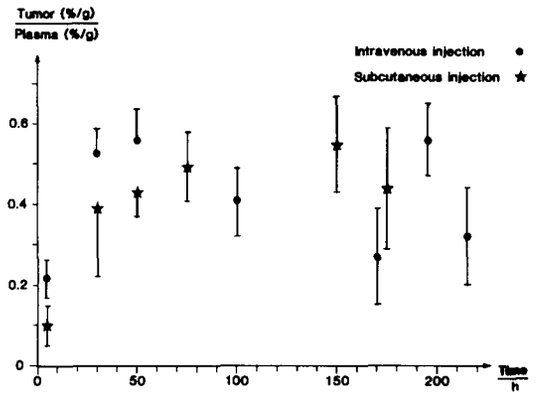

Fig. 6. Tissue to plasma activity ratios of ^{125}I-96.5 in tumours +/−SD after subcutaneous and intravenous injection during 200 h. Data concerning intravenous injection from ref. 10. Corrected activity ratio values are not shown since they did not differ significantly from the uncorrected ones.

Ratio between tissue uptake and plasma concentration. From our data we calculated two ratios.

$$TP(t) = \frac{A_{tissue}(t)}{A_{plasma}(t)} \quad (3)$$

and

$$TP_{corr}(t) = \frac{A_{(tissue)corr}(t)}{A_{(plasma)corr}(t)} \quad (4)$$

where A_{tissue} is measured in vitro (%/g) for different tissues at time t, and A_{plasma} is the corresponding plasma activity at time t. Both following intravenous (data from previous study, ref. 8) and subcutaneous injection of the same 96.5 labelled antibody, the tumour to plasma ratio was of the same order (Fig. 6). The lymph nodes and muscle tissue showed a slight increase over time while the lungs, liver and bone marrow had a constant ratio which remained so during 200 h (Table).

Discussion

When monoclonal antibodies were introduced, no definitive proof of selective uptake in metastatic deposits in lymph nodes was found following subcutaneous injection (4, 14). However, Weinstein et al. (5), showed that immunoscintigraphy could visualize small metastases (2–6 mg) in lymph nodes in guinea pigs. In nude rats, Strand et al. (15) showed that subcutaneous injection of monoclonal antibodies could image both tumours and single lymph nodes. A high uptake was found in an intramuscular tumour with a ratio to the normal muscle on the contralateral side of 40:1. In dogs an activity uptake in draining nodes a hundred-fold greater than in distal nodes has been demonstrated by Mulshine et al. (16).

In the present study the outflow of the whole antibody 96.5-^{125}I from the injection site could be described as a biexponential function. After 10 h only 50% of the activity remained at the site. This outflow rate is in accordance with what we and others have previously found (6, 17).

The total body retention of activity was the same when intravenous and subcutaneous injections were compared. This indicates that the degradation and excretion of the radiolabelled monoclonal antibody was similar after the two different modes of injection (8).

When patients with metastases of malignant melanoma were imaged, only 4 out of 14 patients with axillary or inguinal disease showed specific uptake in involved nodes. Only two of the four nodes were imaged (2). This finding and our previous work (8) indicate that the specific tissue uptake varied among tumours. The blood and plasma activity reached a peak between 20 and 30 h after injection, probably corresponding to the antibody transit time to the blood through the thoracic duct. Naturally, this transit time causes a slower activity uptake in the tumours as compared to the tumour uptake following intravenous injection (8).

Fig. 2 shows that the curves for the two antibodies in blood and plasma are completely overlapping, which

Table

Ratio between mean tissue uptake and mean plasma activity in the nude rat during 200 h after subcutaneous and intravenous injection of ^{125}I-96.5. Corrected values adjusted for activity due to blood still remaining in the measured tissue

Tissue	Subcutaneous injection		Intravenous injection*	
	TP (t)	TP_{corr}(t)	TP (t)	TP_{corr}(t)
Lungs	0.30	0.20	0.30	0.20
Bone marrow	0.14	0.05	0.15	0.10
Liver	0.13	0.0	0.20	0.0
Lymph nodes	0.002t + 0.10	0.001t + 0.11	0.001t + 0.19	0.001t + 0.08
	r = 0.87	r = 0.78	r = 0.83	r = 0.73
Muscle	0.001t + 0.01	0.001t + 0.01	0.001t + 0.02	0.001t + 0.03
	r = 0.97	r = 0.93	r = 0.99	r = 0.92

r = correlation coefficient
*/ data from Ingvar et al. (8)

means that concerning distribution, the OKT3 acts as a true control. After the peak values are reached, the slopes are identical to those obtained in our previous study in which the same antibody was injected intravenously (8).

In Fig. 3 the specific tissue uptake shows that there is a real specificity for the antibody 96.5 in comparison to OKT3. The ratios found here in tumours of specific and non-specific antibody activity uptake (between three and four), are of the same order of magnitude as those found in tumour biopsies from patients, subcutaneously injected with the same antibody (ratio 3.7 after 48 h and 3.4 after 72 h) (2). Compared to our intravenous studies we have in the present study found a tendency for the lymph nodes to have a different uptake kinetics of antibodies, both for 96.5 and OKT3. The peak uptake in the nodes came much later than in other organs like liver, lungs and kidneys (80 versus 30 h). A simple explanation for this delay is probably the fact that a substantial part of the activity reaches the lymph nodes via extravasation through openings in the capillary bed. It is then transported via the lymphatic vessel to lymph nodes and only to a smaller extent by the blood. The bone marrow showed no specific tissue uptake and the initial distribution followed all other organs and there was no high initial uptake as in our previous intravenous study in the nude rat (8).

When uptake values are corrected for activity in the blood still remaining in the measured tissue samples (STU_{corr}) (Fig. 5), it was found that the tumour activity was only slightly reduced. However, in the liver and bone marrow, most of the activity was due to activity in the blood. In lungs and lymph nodes the activity did not change significantly following correction. The reason for this is unclear, but it might be due to trapping of immune complexes or to unspecific binding of the Fc-part of the whole antibody. Such a mechanism has been demonstrated for another antimelanoma antibody 9.2.27 by immunohistology (2).

In melanoma patients, Bergqvist et al. (17) showed that there was a thousand-fold difference in the activity uptake of radiolabelled colloids in different draining lymph node glands measured in vitro following subcutaneous injection.

The capillary filtration coefficient, $K_{f,c}$, depends not only upon the hydrostatic and colloid osmotic pressure, but also on the porosity of the capillary wall (18). The $K_{f,c}$ varies for different organs, being smallest in the brain and muscle, and highest in the liver. The difference in capillary protein permeability parallels approximately the difference in $K_{f,c}$. The concentration of protein in the interstitial fluid of muscle is about 1.5 g/100 ml, 2 g/100 ml in subcutaneous tissue, and 6 g/100 ml in the liver. If the specific tissue uptake of the different tissue specimens is compared to the known values of the tissue concentration of protein, it is obvious that the so called 'non-specific binding' in organs like lungs and liver compared to muscles, to some extent is due to the capillary protein permeability and not to any active binding mechanism. The ratios between the tissues and the plasma confirm this statement (Table); the ratio being high for the lungs and lower for the lymph nodes and muscles. Both lymph nodes and muscles have a slow increase in the ratio, probably due to a lower $K_{f,c}$, giving a longer time period for saturation of the interstitial fluid, thereby causing a slow increase in the ratio even when the plasma activity is decreasing. This indicates that immunoglobulins behave as macromolecules and are transported passively through openings in the capillary wall and then distributed evenly according to the $K_{f,c}$ of the tissue. This first distributional step is probably the most important factor explaining why monoclonal antibodies do not reach such high ratios as was calculated from in vitro experiments in which the number of binding sites per tumour cell has been estimated to be more than 100 000 (9). The same mechanism could also explain why human monoclonal antibodies give even lower ratios. Such antibodies are namely often of the larger IgM type (MW 160 000).

It has been shown in vitro that the use of a 'cocktail' of monoclonal antibodies recognizing different antigenic epitopes on the tumour cell gives a better localization to the tumour cell. This may present a solution for the problem of low tumour uptake when there is variable expression of antigen related to the differentiation or to a heterogeneous tumour population (19). Still the use of a cocktail of antibodies does not solve the problem of penetration of the antibodies through the capillary walls to the tumour cells.

ACKNOWLEDGEMENTS

This study was supported by the Swedish Medical Research Council (project B86-16X-06573-04B), the Swedish Cancer Society (project 1625-B83-01X), the John and Augusta Persson Foundation for Medical Research, the Åke Wiberg Foundation, the Medical Faculty, Lund University, and the Royal Physiographic Society, Lund. The monoclonal antibody 96.5 was kindly supplied by Drs Ingegerd and Karl-Erik Hellström, Oncogen, Seattle, Wash., USA. We also thank Mrs Karin Wingårdh for technical assistance.

Corresponding author: Dr Christian Ingvar, Department of Surgery, University Hospital, S-221 85 Lund, Sweden.

REFERENCES

1. Larson SM, Brown JP, Wright PW, et al. Imaging of melanoma with I-131-labeled monoclonal antibodies. J Nucl Med 1983; 24: 123–9.
2. Lotze MT, Carrasquillio JA, Weinstein JN, et al. Monoclonal antibody imaging of human melanoma. Ann Surg 1986; 204: 223–5.
3. Rosenblum MG, Murray JL, Haynie TP, et al. Pharmacokinetics of 111In-labeled anti-p97 monoclonal antibody in patients with metastatic malignant melanoma. Cancer Res 1985; 45: 2382–6.

4. Deland FH, Kim EE, Goldenberg DM. Lymphoscintigraphy with radionuclide-labeled antibodies to carcinoembryonic antigen. Cancer 1980; 40: 2887–3000.
5. Weinstein JN, Steller MA, Kenan AM, et al. Monoclonal antibodies in the lymphatics: Selective delivery to lymph node metastases of a solid tumour. Science 1983; 222: 423–6.
6. Weinstein JN, Parker RJ, Holton OD, et al. Lymphatic delivery of monoclonal antibodies: Potential for detection and treatment of lymph node metastases. Cancer Invest 1985; 3: 85–95.
7. Nelp WB, Eary JF, Jones RF, et al. Preliminary studies of monoclonal antibody lymphoscintigraphy in malignant melanoma. J Nucl Med 1987; 28: 34–41.
8. Ingvar C, Norrgren K, Strand S-E, et al. Biokinetics of radiolabeled monoclonal antibodies in heterotransplanted nude rats. Evaluation of corrected specific tissue uptake. J Nucl Med 1989; 30: 1224–34.
9. Brown JP, Woodbury RG, Hart CE, et al. Quantitative analysis of melanoma associated antigen p97 in normal and neoplastic tissue. Proc Natl Acad Sci USA 1981; 78: 539–43.
10. Ey PL, Prawse SJ, Jenkins CR. Isolation of pure IgG, IgG2b immunoglobulins from mouse serum using protein A sepharose. Immunohistochemistry 1978; 15: 429–36.
11. Reinherz EL, Schlossman SF. The differentiation and function of human T-lymfocytes. Cell 1980; 19: 821–7.
12. Brown JP, Nishiyama K, Hellström I, et al. Structural characterization of human melanoma associated antigen p97 using monoclonal antibodies. J Immunol 1981; 127: 539–46.
13. Ferens JM, Krohn KA, Beaunier PL, et al. Highlevel iodination of monoclonal antibody fragments for radiotherapy. J Nucl Med 1984; 25: 367–70.
14. Engelstad BL, Spitler LE, Del Rio MJ, et al. Phase 1 immunolymphoscintigraphy with an In-111-labeled antimelanoma monoclonal antibody. Radiology 1986; 161: 419–22.
15. Strand SE, Bergqvist L, Ingvar C, et al. Lymphoscintigraphy with inert radiocolloids and monoclonal antibodies. In: Burcheil SW, Rhodes BA, eds. Radioimmunoimaging and radioimmunotherapy. New York: Elsevier, 1983: 307–22.
16. Mulshine JL, Keenan AM, Carrasquillo JA, et al. Immunolymphoscintigraphy of pulmonary and mediastinal lymph nodes in dogs: A new approach to the lung cancer imaging. Cancer Res 1987; 47: 3572–6.
17. Bergqvist L, Strand S-E, Persson B, et al. Dosimetry in lymphoscintigraphy of Tc-99m antimony sulfide colloid. J Nucl Med 1982; 23: 698–705.
18. Guyton AC. Textbook of medical physiology. Philadelphia: WB Saunders Co, 1981: 358–70.
19. Krizan Z, Murray JL, Hersh EM, et al. Increased labeling of human melanoma cells in vitro using combinations of monoclonal antibodies recognizing separate cell surface antigen determinants. Cancer Res 1985; 45: 4904–9.