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Criteria for selecting monoclonal antibodies with respect to accumulation in melanoma tissue

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Summary. Immunohistology provides a necessary but insufficient criterion for selecting monoclonal antibodies (MAbs) capable of tumour targeting in vivo. Additional selection procedures have been evaluated using a panel of anti-melanoma MAbs, including immunoreactivity of (labelled) MAbs, antibody affinity, kinetics of binding and release, apparent antigen density and accumulation in nude mouse transplants. According to these criteria, MAbs M.2.7.6 and M.2.9.4 showed the most favourable properties, i.e. high immunoreactivity and pronounced internalization into melanoma cells. With MAbs M.2.10.15 and KG 6–56, moderate immunoreactivity and a binding pattern characterized by temperature dependence in the absence of internalization was observed. According to the paired label assay, all four MAbs showed specific accumulation into solid melanoma tissue. However, application in the patient still requires evaluation of the side effects of antigen cross-expression on normal human tissues.

Introduction

Beyond testing hybridoma supernatants on cultured tumour cells, immunohistology is the most stringent criterion for evaluating the specificity of monoclonal anti-tumour antibodies. However, histological evidence together with immunochemical characteristics does not provide enough information to warrant application of individual monoclonal antibodies for tumour targeting in vivo. Immunohistology does not provide evidence as to the stability of antigen anchorage on the tumour cell surface, i.e. no visual evidence of antigen shedding or secretion can be obtained by this method. Furthermore, low density expression of the antigen on non-tumour tissues may not become apparent because of the inherent detection limit. Consequently, the suitability of individual MAbs for applicaton in vivo has to be tested explicitly, taking into account all features of the MAb molecule with potential influence on biodistribition and accumulation in the target tissue. [1] (1) A priori immunoreactivity, i.e. the proportion of immunoreactive molecules in a given preparation; (2) sensitivity of individual MAbs to "substitution damage" [12] including all negative influences of binding nuclides or other substituents onto the antibody molecule; (3) binding affinity [10]; (4) antigen density on cultured tumour cells of different morphotypes [5]; and (5) kinetics of antibody binding, internalization and release under physiological conditions [13]. This list does not include important parameters which can be evaluated exclusively in (tumour) patients, i.e. the antigen expression on normal (human) tissue, the actual antigen density in situ on the human tumour, the stability of antigen expression in heterogenous tumour cell populations, and the prevalence of high antigen density in the group of patients with a given tumour. In the present communication, the results of screening a series of anti-melanoma MAbs produced by one of us [J. B.; 3, 4, 15] along the above outlined criteria are reported.

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Materials and methods

Melanoma lines in vitro and in nude mice. Human melanoma cell lines were obtained through the courteousy of Drs. I. and K. E. Hellström (Seattle, USA; SK-MEL-25, SK-MEL-28), Dr. J. Fogh (New York, USA; MeWo), Dr. S. Ferrone (Valhalla, USA; Colo 38), and Dr. W. Tilgen (Heidelberg; MML-I). Cell lines were kept in RPMI 1640 media (GIBCO, Karlsruhe, FRG) supplemented with 10% fetal calf serum, antibiotics and 4 m*M* glutamine (GIBCO). Cells were detached from tissue culture flasks by short treatment with 0.25% trypsin (Sigma) containing 3 m*M* EDTA [7], or by EDTA alone. In some experiments, tumour cells were injected into nude mice obtained from either Bomholtgaard (Ry, Denmark; Balb background) or from Zentralinstitut für Versuchstiere (Hannover, FRG; NMRI background), which were kept under barrier conditions until the beginning of the experiments. Tumour cells (5×10^6 cells per animal) were inoculated s.c. into the flank and labelled MAbs were injected when tumour diameters exceeded 8–12 mm. Iodide uptake in the thyroid was blocked 1–3 days prior to injection of radio-iodinated MAbs by addition of Lugol's solution to the drinking water.

Monoclonal antibodies. Hybridomas secreting MAbs with operational anti-melanoma specificity were produced by immunizing mice with cultured melanoma line MeWo (MAbs M.2.2.4, M.2.7.6, M.2.9.4, and M.2.10.15), with biopsy material from primary melanomas (MAbs H.2.8.10, H.3.8.14, K.1.2.11), with the extract of a s.c. metastasis (MAb H.4·10·58) or with material from a brain metastasis

Table 1. List of monoclonal antibodies (MAbs) tested

Designation	Isotype	Antigen
Anti-melanoma MAbs[a]		
M.2.2.4	IgG1	Glycolipid[b]
M.2.7.6	IgG1	–
M.2.9.4	IgG2a	100 kilodaltons
M.2.10.15	IgG1	200 kilodaltons
H.2.8.10	IgG2b	–
H.3.8.14	IgG2a	–
H.4.10.58	IgG1	Glycolipid[b]
K.1.2.11	IgG1	–
KG 6–56	IgG1	Proteoglycan?[c]
Control MAbs[d]		
B40–7	IgG1	
HOPC–1	IgG2a	
142–23	IgG2b	

[a] Production and immunohistological characterization of MAbs in [3, 4]

[b] Glycolipid nature of the antigen detected by MAbs M.2.2.4 and H.4–10–58 suggested by heat stability and resistance to proteolytic treatment (J. Brüggen, unpublished evidence)

[c] Proteoglycan nature of the antigen suggested by concordance of antigen positivity on a panel of cell lines as compared e.g. to MAb 225.28S [17]

[d] B40–7 and 142–3 are anti-idiotype MAbs; HOPC-1 has no known specificity (G. Hämmerling, personal communication)

(MAb KG 6-56) as described previously [3, 4, 15]. Table 1 gives a list of the anti-melanoma MAbs tested, together with hitherto available biochemical data of the respective antigens. Hybridomas producing control antibodies of defined isotype but irrelevant specificity were kindly donated by Dr. G. Hämmerling, Heidelberg. MAbs were purified from ascitic fluid by Protein A chromatography (Pharmacia, Freiburg, FRG) according to Ey et al. [6] and ion exchange chromatography on a Mono Q column (Pharmacia). Purity of preparations was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Labelling of antibodies with either ^{125}I or ^{131}I was carried out according to the IODO-GEN method [8].

Immunoreactivity testing and Scatchard plot analysis. Immunoreactivity of products was tested by sequential absorption or by the Lineweaver Burk method [11] as described previously [12]. Binding affinity was determined from Scatchard plots performed exactly as described by Trucco and de Petris [16].

Determination of antigen density. Melanoma cells were detached by a 1 to 2-min treatment with a film of 0.25% trypsin and 3 m*M* EDTA in Ca^{2+} and Mg^{2+}-free Hanks medium [7]. Cells were washed, resuspended in complete medium, and subjected to incubation for 2 h at 37 °C, 5% CO_2, as a standardized preparatory treatment. This procedure was adopted in order to allow for a comparative evaluation of antigen density on various cell lines with different MAbs, yet being aware of the fact that maximal antigen expression could thereby be restored in most, but not all situations. After conditioning, cells were dispensed into u-shaped microtitre plates ($1.25–10 \times 10^4$ cells per well, 6–8 replicates), pelleted by centrifugation, and excess antibody (400000 cpm of ^{125}I-labelled MAb, sp. act. 0.7–3 μCi/μg) added. The reaction volume was 0.1 ml, except for one experiment wherein the effect of antibody dilution on binding was studied. After incubation at 0 °C for 2 h with continuous shaking, cells were washed 3 times with media containing 2% calf serum, quantitatively transferred into 0.5 ml tubes and counted in a gamma counter. From "counts bound" the number of bound MAb molecules per cell was calculated, and this was designated as "apparent" antigen density in the text, in order to account for the facts that under the given experimental conditions antibody binding might not have come to the highest possible value, and that one antibody molecule might have bound to one or two antigen molecules at a time.

Kinetics of binding, internalization and release. Methods have been described previously [13]. In short, uptake of ^{125}I-labelled MAbs by conditioned target cells was followed over a period of 150 min with repetitive sampling, cells being washed, counted and treated with iso-osmolar glycine/HCl buffer (pH 2.8) to dissociate externally exposed immune complexes. Release was followed by placing MAb-loaded cells into fresh medium. At predetermined time points, aliquots were withdrawn for determining residual radioactivity on tumour cells.

Biodistribution of labelled MAbs in vivo. This was evaluated by two methods: Paired label assay according to Pressman et al. [14]. Anti-melanoma MAbs and control MAbs labelled with different iodine nuclides were injected via the tail vein (2 μCi of each MAb per animal). At predetermined time points, animals were killed by cervical dislocation and various tissues including blood, liver, spleen, kindney, muscle, bone and tumour were removed. After weighing, tissue samples were counted in a gamma counter equipped with a dual-isotope program (LKB, München, FRG). MAb distribution was characterized by calculation of the Specificity Index (SI) according to:

$$SI = \frac{\text{cts/g(tumour) Mel.MAb : cts/g(organ) Mel.MAb}}{\text{cts/g(tumour) Contr.MAb : cts/g(organ) Contr.MAb}}$$

Immunoscintigraphy. Tumour-bearing mice were injected with 50–100 μCi of ^{131}I-labelled MAbs (50–125 μg per animal) via the tail vein. At various intervals thereafter, planar scintigrams were performed with a Pho/Gamma V camera (Siemens, Erlangen, FRG) fitted with a pinhole collimator. Data acquisition and handling was carried out with a Gamma 11 computer system (DEC, Offenbach, FRG). Scintigrams were photographed from the screen after subtracting 5%–10% of background.

Results

The experimental approach compared in vitro parameters with potential influence on MAb distribution to accumulation data obtained in vivo, i.e. in nude mice with melanoma transplants. Immunoreactivity, binding affinity, antigen density and stability of antigen expression on the cell surface were the parameters analyzed in vitro. Prior to studying any property of a given antibody, it was subject to a labelling experiment, wherein changes in immunoreactivity were analyzed over an iodination range of 0.5–20 μCi/μg [12]. By this procedure, the "safe" interval was found to be 2–5 μCi/μg, with the exception of M.2.9.4 showing maximal immunoreactivity at substituiton ratios of

Table 2. Immunoreactive fraction of MAbs after radio-iodination

MAb	Immunoreactive fraction[a]	Method[b]
M.2.2.4	7%	SA
M.2.7.6	51%	LB
M.2.9.4	89%	LB
M.2.10.15	95%	LB
M.2.8.10	6%	SA
H.3.8.14	6%	LB
H.4.10.58	70%	LB
K.1.2.11	17%	LB
KG 6–56	71%	LB

[a] MAbs labelled to an extent which had previously been shown to entail minimal damage

[b] SA, sequential absorption, cumulative binding in three runs; LB, Lineweaver Burk method, reciprocal of the intercept at the y-axis × 100

0.5–1 μCi/μg. In all subsequent experiments, MAbs were labelled according to this criterion.

Table 2 gives a list of immunoreactive fractions of individual MAbs, which were either determined by extrapolation to binding a infinite cell number (Lineweaver Burk plot), or by summation of relative binding in 3 consecutive cycles of absorbing labelled MAbs on cultured tumour cells. Both methods have been shown to yield concordant results [12]. As can be seen, MAbs M 2.7.6, M.2.9.4, M.2.10.15, H.4·10·58 and KG 6-56 had immunoreactive fractions above, 50%, while the other MAbs ranged between 6% and 17%.

In Lineweaver Burk analysis, the slope of the straight lines is determined by the reciprocal of the product of the immunoreactive fraction and the association constant [11]. However, we had modified the experimental conditions to achieve a rapid and simple determination of the immunoreactive fraction [12], so that they were no longer suitable for an exact evaluation of association constants. These were determined independently by Scatchard plots, provided the overall binding was high enough to meet the requirements of the assay. As can be seen in Table 3, the association constants ranged from 4.5×10^8 to 1.6×10^{10} M^{-1}. With the other MAbs, rough estimates of binding affinity were obtained by going back to the slopes of the Lineweaver Burk curves, as is demonstrated in Fig. 1, wherein M.2.9.4, H.4·10·58 and the weakly binding antibody H.2.8.10 are compared. H.2.8.10 is an example to show that binding levels close to the background of the method (note the scale at the right hand ordinate) imposed considerable uncertainty on the determination of both intercept

Table 3. Association constants as determined by Scatchard plots[a]

MAb	K_a[b] (M^{-1})
M.2.7.6	1.6×10^{10}
M.2.9.4	7.9×10^8
M.2.10.15	4.8×10^8
H.4–10–58	4.5×10^8
KG 6–56	1.6×10^9

[a] Binding assay at 0°C for 1 h according to Trucco and de Petris [16]

[b] Association constant

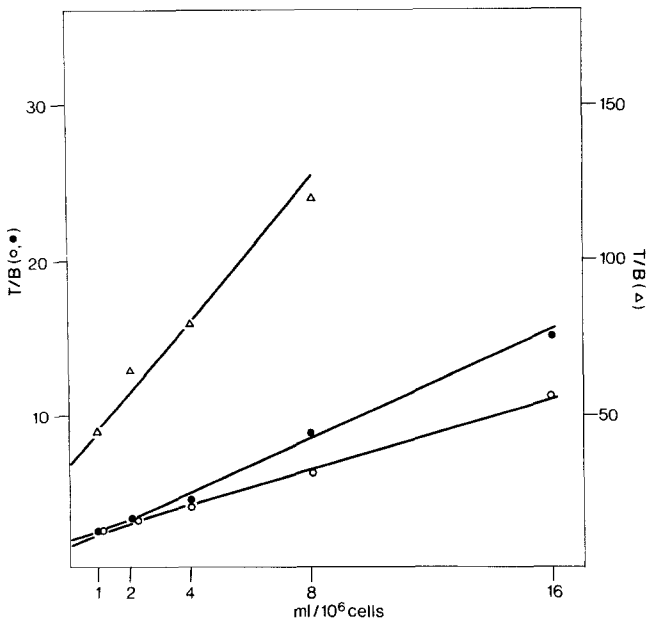

Fig. 1. Immunoreactivity of ^{125}I-labelled MAbs M.2.9.4, H.4·10·58, and H.2.8.10. Lineweaver Burk analysis of binding to SK-MEL-28 cells (2 h, 37 °C, 5% CO_2). ○, M.2.9.4; ●, H.4·10·58; △, H.2.8.10

at the ordinate and slope, and that no definite association constant could be calculated from data of this kind.

Apparent antigen density was measured using a panel of melanoma cell lines comprising lines with spindle-shaped (MeWo), epitheloid (SK-MEL-25, MML-I) and polydendritic morphology (SK-MEL-28), thus covering three major morphotypes [9]. The assay was run at 0 °C in order to minimize post-binding phenomena. M.2.9.4 bound to an antigen being highly represented on all cell lines tested, while MAbs M.2.7.6 and M.2.10.15 indicated high antigen density on some, but not all melanoma lines. This is reminiscent of immunohistology data showing that only a minority of patients with melanoma were positive for both MAbs [15]. MAbs M.2.2.4 and H.4·10·58 identifying the same antigen (putatively glycolipid; J. Brüggen, unpublished evidence), showed low antigen density despite strong immunohistological staining [2]. The latter finding, together with the impression that MAbs exhibiting low immunoreactive fractions and/or low binding affinity seemingly identified weakly expressed antigens (compare Tables 2, 3 and Fig. 1 to Table 4) did shed some doubt on the validity of the experimental set-up chosen for testing antigen density: antibody excess and sufficiently high binding rates at 0 °C may not have been warranted with those MAbs. In order to trace the putative influence of binding affinity on these data, MAbs with different association constants (see Fig. 1) were tested on SK-MEL-28 cells in parallel assays differing only in the total reaction volume (20 μl vs 200 μl; Fig. 2). In fact, the amount of bound antibody and, hence, apparent antigen density calculated from this parameter, was independent from the assay volume with the high affinity antibody M.2.9.4 ($K_a = 7.9 \times 10^8$ M^{-1}), while an increase in volume resulted in decreased binding of H.4·10·58 ($K_a = 4.5 \times 10^8$ M^{-1}) and even more so of H.2.8.10 (K_a markedly lower according to Fig. 1). Hence, antigen density was severely underestimated when using low affinity MAbs under the conditions of the standard procedure. This may explain why dif-

Table 4. Determination of antibody binding sites per cell

MAb	Antibody binding sites ($\times 10^4$ per cell)[a]			
	MML-I	SK-MEL-25	SK-MEL-28	MeWo
M.2.2.4[b]	0.1	0.2	0.6	0.3
M.2.7.6	0.5	3.5	3.8	7.0
M.2.9.4	16.0	10.0	21.0	12.0
M.2.10.15	0.6	9.0	6.0	13.0
H.2.8.10	0.1	0.3	0.5	0.5
H.3.8.14	0.05	n.t.	0.1	0.1
H.4.10.58	0.1	0.1	2.3	0.3
K.1.2.11	0.1	0.1	0.1	0.1
KG 6–56	1.3	0.2	4.3	0.8

[a] Calculation based on the assumption that every MAb molecule binds only to one antigen
[b] MAbs M.2.2.4 and H.4.10.58 bind to the same antigen

ferences in apparent antigen density were recorded with M.2.2.4 and H.4·10·58 on SK-MEL-28 cells (standard assay conditions), although both MAbs most likely identify the same antigen.

However, affinity considerations gave no full explanation for the discrepancy between apparently low antigen density and strong immunostaining as observed with M.2.2.4 [2] and H.4-10-58. A possible clue to this problem came from the kinetic analysis of binding and release, which was applied to living and fixed cells kept at 0 °C or 37 °C. In addition, internalization was characterized, taking advantage of the fact that externally exposed antibody will be desorbed by pH 2.8 buffer, while internalized antibody will be resistant to this treatment. Three distinct types of binding and internalization could be discriminated with the given panel of MAbs, which are described in Table 5. The assignment of individual MAbs to these binding types follows immediately from data published recently [13], which did not include M.2.10.15 and H.4-10-58 nor antibodies with very low cell binding not amenable to kinetic analysis. Binding features obtained with M.2.10.15 proved to be essentially similar to KG 6-56 (Fig. 3; Type II). Release of bound M.2.10.15 was found to be rapid at 37 °C, as was observed with other MAbs [13]. At 0 °C, bound radioactivity is usually retained over a long period of time. With M.2.10.15 an additional component of rapid release was observed, which may indicate a second compartment of antibody fixation being accessible for this MAb. Binding and release of H.4·10·58 was indistinguishable (data not shown) from that recorded with M.2.2.4 or MAb R24 [13; Type III]. Since Type III binding kinetics were associated with labile association of MAb-antigen complexes with the living cell (sehdding), low antigen densities might appear in the binding assay (including the antigen density experiment), but not in immunohistology using fixed or cryo-preserved sections.

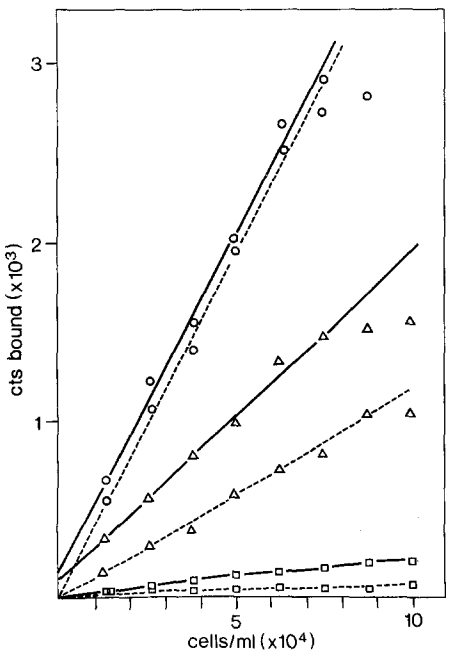

Fig. 2. Determination of apparent antigen density from antibody binding data: influence of the reaction volume. SK-MEL-28 cells were incubated with 4×10^5 cpm of labelled MAbs for 90 min at 0 °C in a total volume of 20 µl **(solid lines)** or 200 µl **(broken lines)**. ○, M.2.9.4; △, H.4·10·58; □, H.2.8.10. Number of binding sites as calculated from this experiment: M.2.9.4, 2.1×10^5 (20 µl) and 2.0×10^5 (200 µl); H.4·10·58, 4.0×10^4 (20 µl) and 2.9×10^4 (200 µl); H.2.8.10, 2.0×10^4 (20 µl) and 2.6×10^3 (200 µl). Note that the slopes obtained with different MAbs cannot be compared one with the other because of differences in the specific activity

MAb uptake in vivo was followed by paired label experiments, wherein anti-melanoma MAb concentrations per unit weight were compared to concentrations of irrelevant MAbs of matching isotype (Table 6). Non-specific distribution being reflected by a SI of 1, a contribution of binding specificity to accumulation in vivo was assumed to take place with SIs >2, which are *printed* in the Table. Specific accumulation in vivo was obtained with MAbs M.2.7.6, M.2.9.4, M.2.10.15 and KG 6-56, while all other MAbs showed accumulation data similar to those obtained with irrelevant MAbs. In absolute concordance with results in Table 5, clearly positive scintigrams of nude mice bearing melanoma transplants were produced with these MAbs only. This is illustrated in Fig. 4, showing scintigrams of the SK-MEL-28 tumour obtained with ^{131}I-labelled M.2.7.6 antibody.

Beside corroborating paired label assay data, Fig. 4 illustrates the point that native MAb, iodinated to a level at which high immunoreactivity is still conserved, will persist for a considerable period of time in the circulation, so that tumour accumulation emerges only slowly from a background of blood-borne radioactivity. Nevertheless, accumulation relative to irrelevant IgG of matching isotype in general had its maximum after 2 days, and, hence, these values have been included in Table 5.

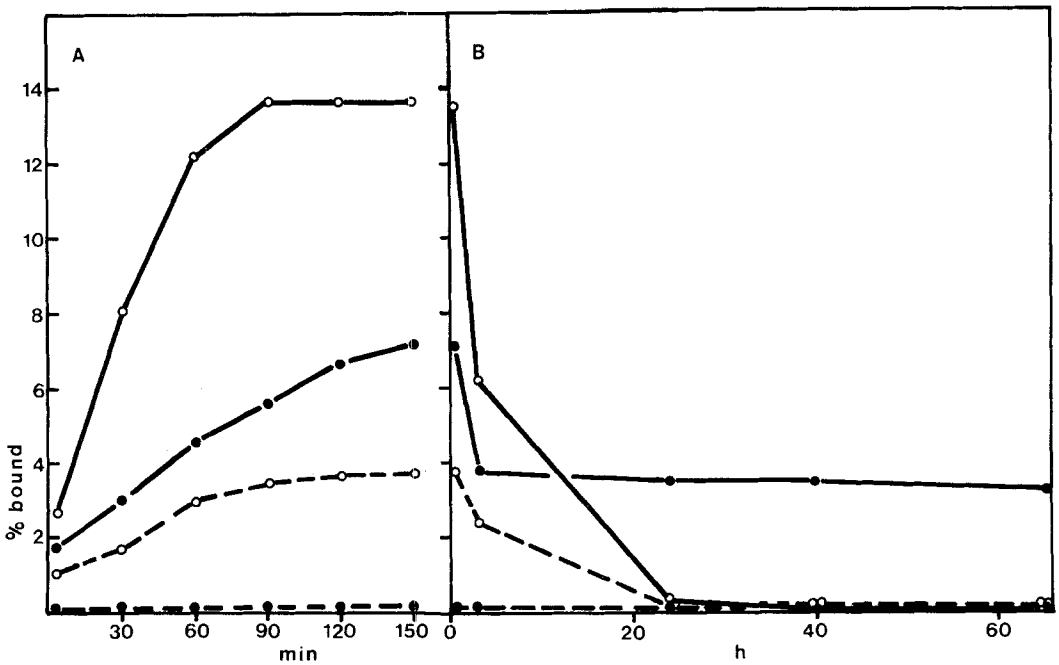

Fig. 3. Kinetics of uptake and release of M.2.10.15. Incubation conditions: O———O, 37 °C, 5% CO_2 ●———●, 0 °C. --, cells after pH 2.8 treatment. **A** MeWo cells were incubated with tracer amounts of M.2.10.15 at 0 °C or at 37 °C, 5% CO_2. Uptake was followed by withdrawing cells from the stock suspension, washing them and desorbing exposed labelled MAb by a 30-min treatment with an iso-osmolar buffer, pH 2.8. **B** Release. After the binding phase, cells were washed and transferred into fresh media containing no labelled antibody. Release was followed over a period of 64 h (incubation conditions as above) by determining the radioactivity remaining with pelleted cells

Table 5. Kinetics of MAb binding and internalization. Characterization of binding types and assignment of MAbs tested[a]

Type	Characteristic features	MAbs[b]
I	Temperature-dependent surplus in binding, high level of internalization. Binding to live cells > fixed cells	M.2.7.6 M.2.9.4
II	Temperature-dependent surplus in binding, low level of internalization. Binding to live cells = fixed cells	M.2.10.15 KG 6–56
III	Higher binding at 0°C than at 37°C, low level of internalization. Binding to fixed cells > live cells	M.2.2.4 H.4.10.58

[a] Methodology as described previously [13]
[b] MAbs showing low binding rates did not allow for an adequate analysis of kinetics

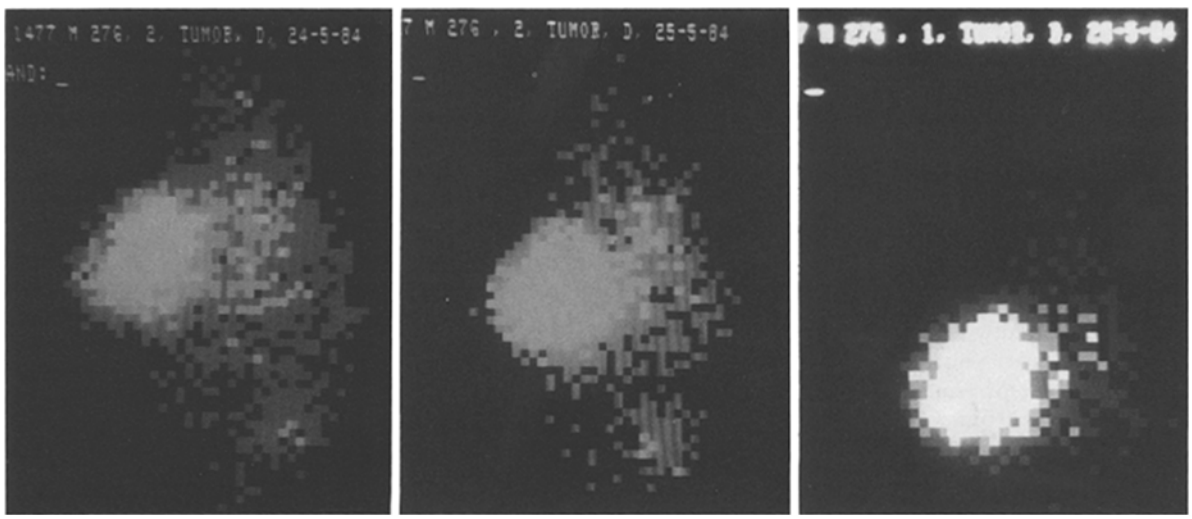

Fig. 4. Immunoscintigraphy of human malignant melanoma line SK-MEL-28 transplanted into a nude mouse. M.2.7.6 labelled with ^{131}I (0.8 μCi/μg) was injected i.v. (100 μCi per animal) and radioactivity distribution was recorded 2, 3 and 6 days after injection (from left to right). After 6 days, 25% of the initial radioactivity (not corrected for decay of ^{131}I) persisted in the animal, and this was concentrated in the tumour. Head of the animal on top of the scintigrams

Table 6. Accumulation of labelled MAbs in vivo. Paired label assay using nude mice with various melanoma line transplants

MAb	Tumour line	Number of animals	Specificity indices (SI)[a] after 48 h			
			T/Blood[b]	T/Liver	T/Bone	T/Muscle
M.2.2.4[c]	MML-I	3	1.1	1.0	1.0	1.1
	MeWo	3	1.7	1.4	1.4	1.6
M.2.7.6	MML-I	3	*5.5*	*5.7*	*6.3*	*5.1*
	MeWo	4	*6.1*	*5.8*	*5.0*	*5.8*
	SK-MEL-25	2	*9.8*	*9.0*	*6.3*	*6.8*
M.2.9.4	MeWo	6	*9.4*	*10.4*	*6.3*	*9.4*
M.2.10.15	MML-I	3	*3.4*	*4.0*	*3.9*	*2.6*
	MeWo	3	*6.5*	*7.9*	*8.7*	*7.4*
	SK-MEL-28	3	2.0	*3.3*	*3.5*	*2.6*
M.2.8.10	MeWo	3	1.3	*2.3*	1.5	1.2
	MML-I	4	1.2	1.8	1.6	1.1
H.3.8.14	MML-I	3	0.8	1.0	0.8	0.6
	MeWo	3	1.6	*2.6*	1.9	1.3
H.4.10.58[c]	MeWo	3	1.2	1.2	1.4	1.3
K.1.2.11	MeWo	3	1.2	1.6	1.3	1.0
	MML-I	4	0.9	1.0	0.7	0.9
	SK-MEL-28	2	1.5	1.7	1.6	1.3
KG 6–56	MML-I	2	*3.4*	*3.2*	*3.5*	*3.4*
	MeWo	2	*10.1*	*10.6*	*8.4*	*8.4*
	SK-MEL-28	5	*2.4*	*2.7*	*2.4*	2.0

[a] Calculation of SI see *Materials and methods*
[b] Normalized radioactivity ratios tumour:blood etc
[c] Both MAbs binding to the same antigen

Discussion

Our strategy consisted of comparing in vitro test procedures to in vivo distribution data (paired label assay), with the double goal of (1) elaborating the merit of individual test procedures, and (2) learning why a given MAb in an individual tumour transplant would accumulate either significantly or insignificantly. Hence, beyond evaluating the suitability of individual MAbs for application in vivo, we want to discuss the stringency of different selection procedures.

Immunoreactivity proved to be the most valuable parameter for prognosis of MAb accumulation in vivo. However, immunoreactivity of labelled antibody comprises at least two elements, which are the immunoreactive fraction [11], i.e. the relative number of ultimately reactive molecules in a given preparation, and the binding affinity of reactive molecules [12]. These elements may be influenced by the labelling process in a coordinate or an independent manner, what has to be analyzed beforehand in order to define labelling conditions allowing for maximal retention of immunoreactivity. On the basis of these precautions it was found that a high immunoreactive fraction and a high association constant correlated with high SIs of accumulation in tumour tissue, but it was not possible to elaborate the contribution of both factors individually. A positive correlation between binding affinity and accumulation in tumour tissue has also been observed in other systems [e.g. 10].

In addition, a methodical link between binding affinity and (apparent) antigen density was identified, in the sense that low affinity led to incomplete binding under the given experimental conditions and, hence, to a considerable underestimation of antigen density. Nevertheless, actual antigen density as an indirect parameter of MAb performance was found to be clearly independent from binding affinity. This can be realized in Table 4, showing that differences in antigen density on different melanoma lines could be visualized with one single antibody.

The extent of accumulation in tumour tissue (in vivo) as measured by the SIs was related to antigen density (in vitro), in that no significant assimilation was obtained when apparent antigen densities were below 10^4 sites per cell. This is in line with evidence obtained by Capone et al. [5], although no explicit evaluation of the number of antigen molecules per cell was given by the authors. In the present experimental series, the only exception was melanoma line MML-I, which showed considerable uptake in vivo despite low apparent antigen densities in culture. Taking this evidence together, it may be argued that antigen density is an ambiguous selection criterion, since on the one hand high density on cultured cells is essential for establishing meaningful test procedures, while on the other hand direct evidence for high density in vivo, especially on vital human tumour tissue, is hardly available. It may even be the case that some antigens will be weakly expressed on cell lines while being quite prominent in the patient. In such a case, selection procedures based on experimental systems will be erroneously discouraging. MAbs M.2.2.4 and H.4·10·58 might be representative of this constellation, since marked staining of histological sections [2] was contrasted by low apparent antigen densities in culture, which – at least in the case of H.4·10·58 – could not be explained by sub-threshold binding affinities.

This leads to the additional criterion of MAb fixation to the tumour cell, being reflected by the kinetics of binding, internalization and release. With MAbs M.2.2.4 and

H.4·10·58, Type III binding behaviour and rapid release even at low temperature indicated antigen (or immune complex) shedding in vitro, which did not go together with significant accumulation rates in vivo. Contrary to this, MAbs identifying an antigen which stays outside the cell (e.g. M.2.10.15, KG 6-56, ref. [13]), or an antigen subject to internalization (e.g. M.2.7.6, M.2.9.4, ref. [13]) showed SIs well above 2. Discrimination between the two latter options may gain far-reaching importance, because many MAbs' vehicle functions depend on whether the transported entity, be it a radio-nuclide or a toxin molecule, finally enters the cell or not. Thus, a comprehensive analysis of the binding behaviour may help in choosing the appropriate MAb for a given purpose.

When comparing in vivo distribution as assessed by the paired label assay and by immunoscintigraphy, concordance was recorded in all instances. An important aspect was found to be associated with immunoscintigraphic contrast, i.e. the amount of MAb retained in the tumour relative to the amount of non-bound MAb remaining in the circulation. In cases where sufficient levels of radioactivity were achieved only by using high amounts af antibody protein, small tumours "extracted" only a low portion of labelled MAb from the blood pool, and, hence, the tumour emerged only slowly from the background (e.g. M.2.9.4; data not shown). With MAbs tolerating higher levels of labelling, accumulation in the tumour and clearance from circulation were quicker, so that high contrast was achieved in a shorter period of time without intervention into the system (e.g. M.2.7.6, Fig. 4).

In the present study, benefits and limitations of preclinical testing of individual MAbs have been illustrated. According to out expectation, some of the MAbs showing excellent performance in immunohistology were found to localize only poorly in vivo, and this was either due to inadequate anchorage of the antigen (e.g. M.2.2.4, H.4·10·58), or to insufficient immunoreactivity (e.g. H.2.8.10, H.3.8.14, K.1.2.11), or both. But we also observed the opposite situation: some MAbs being "handicapped" by broad or restricted cross-expression of the antigen on normal human tissue (e.g. M.2.9.4 and M.2.7.6, respectively; ref [15]) impressed by adequate or even excellent accumulation in vivo. Yet, this result possibly pertains only to the nude mouse system, since no cross-expression of the respective antigens is to be excepted on normal tissues in this species.

Finally, mention should be made of some critical properties of individual MAbs, which became apparent only during the course of the experiments: (1) "shelf life" (at 4 °C) as well as integrity after freezing and thawing seem to differ from MAb to MAb. Yet, impairment of MAb function was mostly gradual and could not be reversed by e.g. removal of aggregates or by re-chromatography on Protein A Sepharose; (2) in vivo half-life showed differences which could not be explained by the isotype; and (3) enzymatic fragmentation was found to impair immunoreactivity of individual MAbs in a non-predictable way. These factors may be of great practical importance with respect to MAb performance and should be included into future selection protocols.

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