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Influence of Circulating Antigen on the Biodistribution and Tumour Localization of Radiolabelled Monoclonal Antibody in a Human Tumour:Nude Mouse Xenograft Model

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Abstract—*A monoclonal antibody, against a colorectal carcinoma tumour-associated antigen, was radioiodinated and its biodistribution studied in comparison with that of control immunoglobulin in nude mice with colon carcinoma xenografts. Tumour localization of the antibody in comparison with normal tissues was poor, and in absolute terms more control IgG than antibody was present per gram of tumour. This failure to achieve localization could not be ascribed to poor immunoreactivity of the antibody nor to the failure of the xenografts to express the appropriate antigen. Analysis of serum from mice with xenografts showed the presence of circulating tumour-derived antigen. This serum-borne antigen was found to form immune complexes both* in vitro *and* in vivo *with the monoclonal antibody, and this complex formation is probably the limiting factor in tumour localization of the antibody. This is one of only few examples where mice with human tumour xenografts have levels of circulating antigen sufficient to perturb biodistribution of antibody. These findings are relevant to the biodistribution of monoclonal antibodies in the clinical situation, since circulating antigen is often found in cancer patients.*

INTRODUCTION

RADIOLABELLED monoclonal antibodies directed against antigens associated with human tumours can localize *in vivo* in tumour deposits sufficiently for tumour detection by external imaging [1] and this is the basis for the proposed use of antibody–drug conjugates for tumour therapy [2]. Although some of these tumour-associated antigens are shed into the circulation [3–8], it is unclear whether this shed antigen can prevent accretion of antibody into tumours. Athymic nude mice with human tumour xenografts have been widely used for preclinical evaluation of monoclonal antibodies, but in these animals circulating tumour antigen is rarely detectable. In the studies to be reported here, a monoclonal antibody against a colorectal carcinoma antigen was found to show only poor localization into tumour xenografts. Investigation of this phenomenon indicated that this was due to the presence of tumour-derived antigen in the circulation which complexed with the monoclonal antibody.

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MATERIALS AND METHODS

Antibody and control immunoglobulin

The hybridoma producing the monoclonal antibody, designated 505/4/6, had been produced in this Laboratory by fusion of P3NSI myeloma cell line with spleen cells from a BALB/c mouse immunized with cultured colon carcinoma cell lines. Immunoperoxidase staining of cryopreserved colorectal tumour and normal tissue sections with 505/4/6 revealed that all tumours stained strongly. Gastrointestinal tract mucosa stained moderately and was exclusively associated with luminal contents whereas breast and sweat glands stained weakly. Liver, lung, kidney, heart, brain and blood tissues were all negative. Immunofluorescence staining of fresh primary tumour cell suspensions showed strong cell surface staining of 70% of colorectal tumours.

The antibody (IgG1 isotype) was purified from hybridoma culture supernatant by Protein A affinity chromatography. Control mouse IgG1 immunoglobulin was purified from normal BALB/c mouse serum by Protein A affinity chromatography by elution at pH 6.0 following adsorption of immunoglobulins from the serum [9].

Antigen

The 505-defined epitope is known to be expressed on the glycoprotein bearing the Lewis Y-hapten. This glycoprotein was isolated from human sputum by affinity chromatography as described by Price *et al.* [10].

Radiolabelling

Antibody and control IgG1 were radiolabelled with ^{125}I or ^{131}I to specific activities of about 30 MBq/mg, using $Na^{125}I$ and $Na^{131}I$ (Amersham International, Bucks, U.K.) using an Iodogen method [11] to specific activities of about 30 MBq/mg.

Determination of immunoreactivity

The immunoreactive fractions of two labelled antibody preparations was determined in a cell binding assay. A constant amount of antibody (100 ng in 1 ml of Eagle's medium containing 2% calf serum) was incubated in replicates of three with a range of concentrations of Colo-205 cells ($0.3–3 \times 10^6$/ml) for 4 h at 4°C with constant agitation. Cells were subsequently sedimented by centrifugation and the fraction of total radioactivity bound to the cell pellet was determined. The analytical technique of Lindmo *et al.* [12] was used for data extrapolation to assess the proportion of ^{125}I binding to cells at a theoretical infinite cell concentration. Antigen negative control cells (osteosarcoma 791T cell line) were tested in parallel.

Gel filtration chromatography

Sephacryl S300 (Pharmacia, Uppsala, Sweden) gel filtration chromatography of preparations was carried out on a 90 cm × 1.5 cm column, eluted in phosphate buffered saline pH 7.2. Gel filtration was carried out on the initial labelled preparation added to normal mouse serum, or to serum from mice with Colo-205 or 791T xenografts and on serum from mice previously injected with radiolabelled preparations. Eluate was monitored continuously for protein by measurement of absorption of u.v. light at 280 nm. Fractions of 2 ml were collected and analysed for radioactivity.

Tumour xenografts

Cells of the colon carcinoma line Colo-205 and osteosarcoma 791T were used to initiate a tumour xenograft in athymic nude mice (Harlan Olac, Oxon, U.K.). The xenografts were maintained by routine subcutaneous passage. Mice were housed in isolators (Vickers Pathoflex Isolator, Vickers, Hampshire, U.K.) with sterile bedding, food and water.

Serum from tumour-bearing mice

Serum from Colo-205 and 791T tumour-bearing mice was collected from animals with large (~ 1.5 cm diameter) tumours and stored at −20°C.

Biodistribution studies

Biodistribution studies were carried out by intraperitoneal injection of radiolabelled antibody or antibody/control IgG1 into mice with Colo-205 xenografts. Mice were killed 1–4 days later and radioiodines counted in weighed samples of blood, tumour, visceral organs and carcass.

The proportion of the injected dose of radioiodine per gram of tissue was calculated. Data were also expressed as a tissue to blood ratio =

$$\frac{\text{Count rate of radioiodine/g tissue}}{\text{Count rate of radioiodine/g blood}}$$

A localization index was calculated in some cases =

$$\frac{\text{Tumour to blood ratio of antibody}}{\text{Tumour to blood ratio of control IgG}}$$

Radioimmunoprecipitation assays

Antiserum precipitation assays on the radiolabelled preparations was carried out by adding a trace (5 kBq) to normal mouse serum (20 μl) followed by 200 μl of rabbit anti-mouse Ig antiserum. The immune precipitate formed after incubation at 4°C for 18 h was sedimented by centrifugation and radioiodine in the precipitate measured. Similar tests were carried out with the serum of mice injected with radiolabelled antibody but without the addition of further radiolabelled preparation.

Immunofluorescence assay of antigen expression

The expression of the 505-defined antigen on cells of Colo-205 xenografts was examined by an indirect immunofluorescence assay. Cell suspensions were prepared by digestion of tumour tissue either with trypsin (0.25% w/v) or with collagenase (0.1% w/v). Tumour cells (2×10^5) were incubated with 505 antibody or control IgG1 (5 μg in 0.5 ml of Eagles medium containing 2% calf serum) for 30 min in ice. Cells were then washed three times and incubated again with 0.1 ml of 1/40 dilution of fluorescein labelled rabbit anti-mouse IgG antisera (Dako, Bucks, U.K.).

After washing, cells were examined in a Becton Dickinson (California, U.S.A.) FACS IV flow cytometer. Fluorescence of cell populations was expressed as a mean linear fluorescence value (MLF) [13].

Serum antigen ELISA assay

Microtitre plates (Flow, Irving, U.K.) were coated overnight at 4°C with 505 monoclonal anti-

body (250 ng/well). The plates were blocked with 5% BSA for $2\frac{1}{2}$ h at 4°C prior to addition of 50 μl 10-fold dilutions of mouse sera or purified glycoprotein (40, 8, 1 μg/ml) as a positive control. After 1 h the plates were washed extensively and incubated for a further hour at 4°C with 50 μl of 10 μg/ml of 505 monoclonal antibody which had been chemically conjugated to biotin. Plates were washed and incubated for a further hour with strepavidin peroxidase (BRL, Gathersburg, MD, U.S.A.) and finally developed with ABTS (2,2'-azino-di-[3-ethylbenzthiasoline]6-sulphonic acid diammonium salt, Boehringer, Mannheim, F.R.G.). Optical densities were read at 405 nm (Titertek, Flow Labs, Irving, U.K.).

Determination of catabolic half times

Three control nude mice and three mice with Colo-205 xenografts were injected with 1 μg of each of ^{125}I-labelled antibody and ^{131}I-labelled IgG1. Radioactivity of both radiolabels was counted immediately and at daily intervals for 5 days in a 7.5 cm × 7.5 cm well crystal scintillation counter (John Caunt Scientific, Oxon, U.K.). Count rates of the two radioiodines, corrected for physical decay, were expressed as the log of the proportion of the immediately post-injection count rates. Whole body half-times were calculated by least squares regression analysis.

RESULTS

Biodistribution tests

In the first biodistribution test the immunoreactive fraction of the labelled antibody was 50% against antigenic Colo-205 cells (Fig. 1). There was less than 1% binding to antigen negative 791T cells. Five mice were injected with 1 μg (30 μg/kg) of the ^{125}I-labelled antibody together with 1 μg of ^{131}I-labelled control IgG1. Two mice were dissected after 24 h. Blood levels of antibody at 3.6% of the dose/g were about 2-fold lower than those with control IgG (7.7%/g). There was no localization of antibody in comparison with normal organs (Table 1). Thus tumour levels were a mean of 1% of the dose/g, but spleen and liver had 2.8% and 2.4%/g respectively. The disproportionate level of antibody in liver and spleen, compared with control IgG was even more apparent when the data was expressed as a tissue:blood (T:B) ratio. T:B ratios for antibody in spleen and liver were 0.81 and 0.68, compared with 0.26 and 0.32 for control IgG1. The T:B ratio for antibody in tumour was 0.33. The whole body retention of radiolabel was a mean of 42% for the ^{131}I-control IgG1 and 31% for the ^{131}I-antibody.

Sephacryl S.300 gel filtration of serum from these mice showed the antibody radiolabel (^{125}I) running

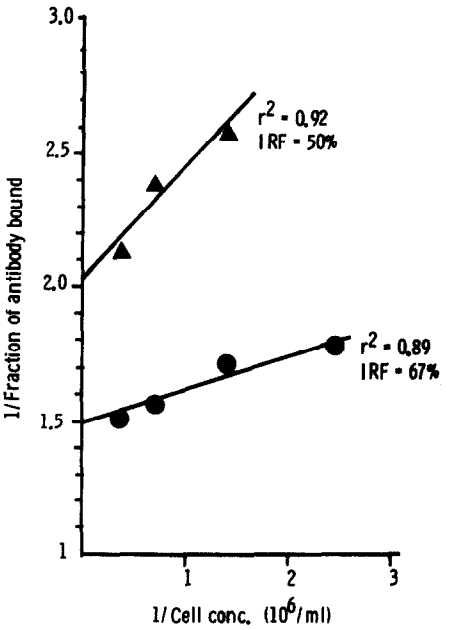

Fig. 1. Determination of the immunoreactive fractions of the two ^{125}I-labelled 505 antibody preparations against Colo-205 cells. The data are expressed as a double reciprocal plot [12]. Extrapolation to 1/cell conc. = 0 (i.e. infinite antigen excess), gave immunoreactive fractions for Prep. 1 (▲—▲) of 50% and Prep. 2 (●—●) of 67%. Both gave only about 1% binding to antigen negative cotrol cells.

in high molecular weight form in the excluded fractions (Fig. 2B), that of the IgG1 radiolabel (^{131}I) running co-incident with the second peak of the serum protein fractionation (Fig. 2B). A control run with ^{125}I-labelled 505 antibody added to normal mouse serum showed radiolabel eluting in the second serum protein peak (Fig. 2A). Antiserum precipitation tests demonstrated that the ^{125}I in the serum of these tumour bearing mice was attached to immunoglobulin. Thus virtually 100% of the ^{125}I (and that of the co-administered ^{131}I-labelled IgG1) was precipitated, as were the radiolabels in the injection material (Table 2).

The remaining three mice which had been injected with antibody and IgG1 were dissected after 5 days (Expt 2, Table 1). Tumour localization, in comparison with visceral organs, was now apparent (Table 1) with the exception of the spleen. However, the average tumour level of antibody radiolabel was 0.7% of the dose but that of control IgG1 was 1.75%. The average whole body retention of ^{125}I-labelled antibody was 6.0%, that of ^{131}I-labelled IgG1 was 26.2% and blood levels were 4.95% of the dose/g for control IgG1 but only 0.8%/g for the antibody.

In a second test with another labelled batch of antibody the immunoreactive fraction of ^{125}I-labelled 505 antibody was 67% against antigen positive Colo-205 cells (Fig. 1). Again there was less than 1% binding to antigen negtive 791T cells.

Table 1. *Biodistribution of* ^{125}I-*labelled* 505 *antibody and* ^{131}I-*labelled IgG*1 *in mice with Colo*205 *xenografts*

Expt No.	Tumour weight (g)	Radiolabel	Dose (μg)	Day killed	Whole body retention (%)	Percentage dose of radioiodine/g of								
						Blood	Tumour	Spleen	Intestine	Kidney	Liver	Heart	Lung	Carcass
1	0.74	^{125}I-505	1	1	36	4.60	1.10	3.30	0.81	1.23	2.45	1.36	1.97	1.00
		^{131}I-IgG1	1		44	8.50	1.48	1.75	0.71	1.90	2.39	2.42	3.39	1.19
	2.0	^{125}I-505	1	1	27	2.60	1.10	2.40	0.70	0.89	2.23	0.71	1.06	0.71
		^{131}I-IgG1	1		41	6.93	1.84	2.24	0.59	1.86	2.52	1.88	2.49	1.11
2	1.3	^{125}I-505	1	5	5.8	0.71	0.62	0.38	0.12	0.20	0.21	0.24	0.50	0.17
		^{131}I-IgG1	1		29	5.20	2.00	1.60	0.50	1.30	1.90	1.80	2.10	0.90
	2.0	^{125}I-505	1	5	4.8	0.52	0.68	0.94	0.05	0.12	0.13	0.18	0.23	0.13
		^{131}I-IgG1			16	2.90	1.00	0.70	0.20	0.60	0.67	0.91	1.20	0.50
	2.6	^{125}I-505	1	5	7.5	1.13	0.76	0.46	0.17	0.27	0.33	0.36	0.52	0.27
		^{131}I-IgG1	1		33	6.70	2.30	1.90	0.65	1.50	2.00	1.80	3.00	1.17
3	0.9	^{125}I-505	10	4	8.4	1.68	0.67	0.63	0.10	0.34	0.40	0.46	0.68	0.30
	1.6	^{125}I-505	10	4	4.8	0.86	0.42	0.55	0.10	0.24	0.21	0.24	0.39	0.17

Two mice with Colo-205 xenografts were injected intraperitoneally with 10 μg of labelled antibody (400 μg/kg) and killed for dissection after 4 days. There was no clear cut localization into tumour tissue (Expt 3, Table 1) and overall the retention of radiolabel by mice was low (mean 6.5% of dose).

Antigen expression in xenografts

To confirm that the xenograft line of Colo-205 was expressing the 505-defined antigen, cells were prepared from tumour tissue from other mice with Colo-205 xenografts implanted at the same time as those in mice receiving antibody. Indirect immunofluorescence reaction following by FACS analysis showed virtually identical reaction of 505 antibody with cells prepared by trypsin digestion (MLF 1839, normal IgG1 control 75) or with collagenase (MLF 2088, normal IgG1 control 94).

Detection of circulating antigen

To examine serum of mice with Colo-205 xenografts for tumour antigen capable of forming immune complexes, gel filtration chromatography was carried out with serum after addition of radiolabelled 505 antibody and IgG1. The antibody radiolabel was found predominantly in the excluded fractions (Fig. 2C). This was not seen with serum from mice with xenografts of the antigen negative 791T osteosarcoma (Fig. 2D). Control IgG1 elution profiles were undisturbed with serum from mice with Colo-205 and 791T xenografts (Fig. 2C, D). In the serum antigen assay, antigen was detected in serum from nude mice bearing Colo-205 xenografts even at dilutions of $1/10^3$ (Table 3). Antigen was also detected in the glycoprotein extract but not in normal nude mouse serum or serum from mice with 791T xenografts.

Rate of catabolism of antibody

The half-times of catabolism of control ^{131}I-labelled IgG1 in control and tumour-bearing nude mice were virtually identical at 5.7 days and 5.1 days respectively. The half-time of ^{125}I-labelled 505

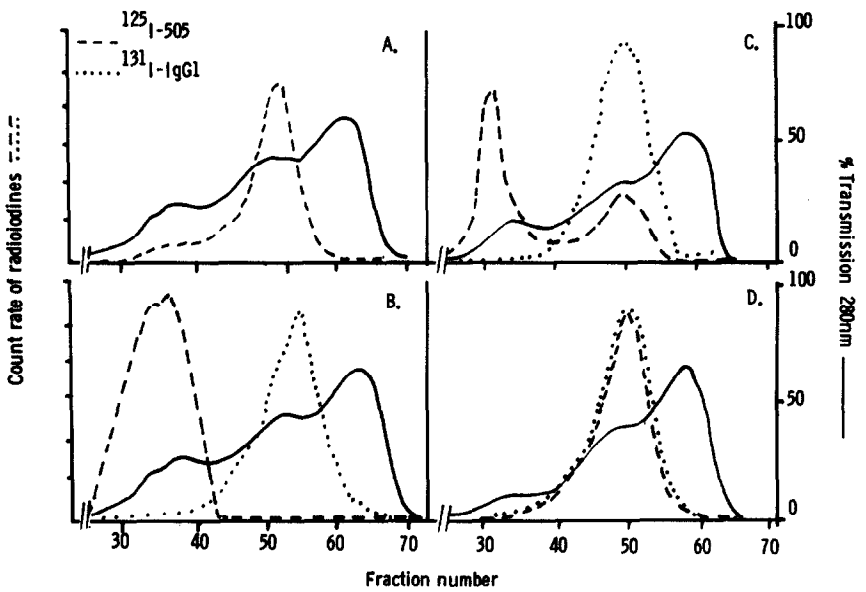

Fig. 2. Sephacryl S.300 gel filtration of radiolabelled antibody (Prep. 1) and control IgG. A. ^{125}I-labelled 505 antibody added to normal mouse serum. Radiolabel elutes in the second serum protein peak. B. ^{125}I-labelled antibody and ^{131}I-labelled IgG1 present in the pooled serum of two mice injected 24 h beforehand with the preparation. Antibody elutes in high molecular weight form in the excluded volume of the gel. Control IgG1 is not affected. C. Antibody and control IgG1 added to serum collected from mice with Colo-205 xenografts. Antibody radiolabel is moved predominantly to high molecular weight complex form. Control IgG1 is not. D. As C but serum from mice with 791T xenografts. The elution profiles of neither antibody nor control IgG1 is pertubed. Radioiodine count rates (left hand vertical axis) are shown on an arbitrary scale, since count rates of the two radioiodines in the four tests varied considerably. The right hand vertical axis shows change in transmission at 280 nm.

Table 2. Immune precipitation of radiolabelled 505 antibody and control IgG1

Radiolabels	Precipitation (%)* of ^{125}I-505	^{131}I-IgG1
Added to mouse serum	93	94
In serum from mice injected 1 day before	99	99
In serum from mice injected 5 days before	99	100

*200 μl of rabbit anti-mouse Ig antiserum added to 20 μl of test serum.

Table 3. ELISA assay for 505-defined antigen in serum of mice with colon carcinoma xenografts

Test preparation*	Dilution/conc.	Optical density (420 nm) ± S.E.†
Colo-205 serum pool	10^{-1}	1.62 ± 0.06
	10^{-2}	0.90 ± 0.05
	10^{-3}	0.25 ± 0.005
	10^{-4}	0.09 ± 0.01
Colo-205 serum pool	10^{-1}	1.58 ± 0.07
	10^{-2}	0.94 ± 0.12
	10^{-3}	0.32 ± 0.003
	10^{-4}	0.11 ± 0.01
Normal serum pool	10^{-1}	0.068 ± 0.006
	10^{-2}	0.087 ± 0.004
	10^{-3}	0.054 ± 0.009
	10^{-4}	0.052 ± 0.005
791T serum pool	10^{-1}	0.094 ± 0.012
	10^{-2}	0.072 ± 0.006
	10^{-3}	0.095 ± 0.012
	10^{-4}	0.079 ± 0.002
Glycoprotein‡	40 μg/ml	0.51 ± 0.02
	8	0.19 ± 0.05
	1.6	0.10 ± 0.003
	0.32	0.09 ± 0.001

*Pools of serum from mice with Colo-205 xenografts of 791T xenografts, or no tumour.
†Replicates of four.
‡Glycoprotein extract from human sputum.

antibody was 4.6 days in control mice, but only 0.9 days in mice with xenografts.

DISCUSSION

Following intravenous injection of anti-CEA antibodies several clinical studies have shown the formation of circulating immune complexes [3, 8]. Similar findings have been reported with monoclonal antibodies to a number of other tumour-associated antigens, for example, with alpha foetoprotein in patients with germ cell tumours [4], the B72-3-defined antigen in colo-rectal cancer [7] and the OC-125-defined antigen in ovarian cancer [6]. In none of these situations was immune complex formation thought to have any deleterious effect on tumour localization, at least as assessed by gamma camera imaging. However, the data obtained with the 505 antibody and Colo-205 xenografts are consistent with the interpretation that at least in this model system circulating antigen can perturb the biodistribution of radiolabelled antibody.

The 505 antibody retained immunoreactivity following radioiodination. Cells from tumour xenografts expressed the target antigen, and as this was seen with both collagenase and trypsin prepared cells it is unlikely that antigen had been exposed only following enzyme treatment. The half-time of the antibody in control nude mice was similar to that of control immunoglobulin and therefore failure to localize cannot be ascribed to intrinsically poor bioavailability. It is probable that in xenografted mice immune complexes were formed in the circulation, cleared from the circulation, probably to the liver or spleen, and the antibody catabolized and the radiolabel excreted with consequently little localization into tumour tissue. The presence of antigen in the circulation was confirmed in the ELISA assays with serum from tumour-bearing mice. Whether the antigen is actually secreted by the tumour or released into the circulation only after cell death is unclear. One problem with such xenografts is that by the time tumours are established they represent a high proportion of the animal's body weight and there may be necrosis resulting in release of tumour material into the circulation. However the effect was seen in animals whose tumours were of only about 0.7 g (i.e. about 3% of the animal's body weight).

There is little other data on the influence of circulating antigen on the biodistribution and tumour localization of radiolabelled antibodies in tumour xenograft systems. One exception is the

report by Hagan *et al.* [5] with CEA-producing colon carcinoma xenografts and anti-CEA antibody where it was shown that radiolabelled antibody, putatively as immune complexes, was cleared particularly to the liver and spleen. Tumours producing the highest level of circulating CEA showed the most pronounced clearance of radiolabelled antibody. In addition with the 19.9 anti-colorectal carcinoma monoclonal antibody, Douillard *et al.* [14] found that localization into HT29 xenografts was poor, with uptake of radiolabel into liver and spleen. The 19.9-defined antigen was shed into the circulation and it was assumed, but not formally demonstrated, that immune complexes were formed and cleared.

It is feasible that the apparent dichotomy between mouse xenograft systems and clinical findings is caused by different handling of immune complexes in these species. In particular it is possible that the complexes formed between mouse antibody and antigen are cleared more readily from the blood by the mouse's reticulendothelial system than they are in man. It is pertinent in the present context that immune complexes formed between mouse monoclonal antibodies and anti-mouse antibodies generated in patients *are* cleared from the circulation, particularly to liver and spleen [5, 15, 16]. This may be due to easier recognition in man of complexes containing human antibody. Alternatively it could be due to the formation of complexes of larger size than those with tumour-derived antigen, although an examination of the relative sizes and the composition of these two sorts of complexes has not been reported.

It is possible that clearance of antigen–antibody complexes could be avoided if antibody fragments (Fab or $F(ab)_2$) were used in place of intact antibody, since these would produce smaller complexes and be devoid of the Fc portion probably involved in complex clearance. Experimental systems such as those described here and by Hagan *et al.* [5] could be used to examine this and to determine whether such complexes if formed, but not cleared, could still localize in tumour tissue. With $F(ab')_2$ fragments at the 19.9 antibody, Douillard *et al.* [14] reported more effective localization than with intact antibody into colon carcinoma xenograft. They suggested that immune complexes formed with intact antibody were cleared to Fc-receptor bearing cells in liver and spleen, whereas if complexes had been formed at all with the $F(ab')_2$, they were not cleared due to the lack of the Fc receptor in these complexes.

In conclusion, the available animal data suggest that tumour localization of antibodies for radioimaging or therapy might be markedly perturbed in the presence of circulating antigen. Although complexes do not appear to be removed from the circulation in man as easily as in mouse, at least with the antibody–antigen systems reported, the tumour uptake of antibody might be being retarded with a decrease in the tumour uptake of antibody. Antigen–antibody systems in which the antigen does not circulate may be the most suitable to avoid the possibility of reduced tumour localization.

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