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Immunology

Monoclonal antibody to an intracellular antigen images human melanoma transplants in *nu/nu* mice

(pigmentation-associated antigen/cancer immunotherapy)

S. WELT, M. J. MATTES, R. GRANDO, T. M. THOMSON, R. W. LEONARD, P. B. ZANZONICO, R. E. BIGLER, S. YEH, H. F. OETTGEN, AND L. J. OLD

Memorial Sloan–Kettering Cancer Center, 1275 York Avenue, New York, NY 10021

Contributed by L. J. Old, December 30, 1986

ABSTRACT **Mouse monoclonal antibody TA99 detects a 70-kDa pigmentation-associated glycoprotein in human melanoma cell lines. The antigen cannot be detected on the cell surface by sensitive rosetting techniques or absorption studies, nor can it be detected as a secreted product in culture fluids. Contrary to expectation, ^{125}I-labeled TA99 specifically localized to pigmented human melanoma transplants in *nu/nu* mice; no localization to nonpigmented melanoma or control tumors was found. Tumor imaging was initially obscured by circulating ^{125}I-labeled TA99 during the first 6 days after antibody injection. With clearance of ^{125}I-labeled TA99 from the blood (half-life, 4–7 days), specific tumor images could be clearly defined by day 13. Due to the persistence of ^{125}I-labeled TA99 at the tumor site (8.9% of the injected dose at 1 week and 4.6% at 8–10 weeks), images were obtainable for up to 10 weeks. At 8–10 weeks, the tumor/blood ratio was 10^4–10^5, and the tumor/normal tissue ratio ranged from 10^2 to 10^5. In view of these findings, antibodies detecting intracellular antigens may have a role in tumor imaging and therapy.**

Although there is much interest in the use of monoclonal antibodies as tumor imaging agents, a number of technical and theoretical issues need to be resolved before the value of this approach can be assessed (1–3). The importance of antibody characteristics, such as class/subclass and affinity, the development of optimal antibody labeling techniques, and the definition of effective antigenic targets in tumors require further detailed analysis to establish principles involved in tumor imaging by antibody. With regard to target antigens, most attention has focused on cell-surface antigens, because of their presumed greater accessibility to antibodies. Secreted tumor products that are not constituents of the cell surface, such as α-fetoprotein and human gonadotropin hormone, have been used as tumor imaging targets (4–6), the rationale being that the concentration of these products in the extracellular space of the tumor is greater than in the blood. In the present study, we examine the imaging characteristics of an antibody (TA99) that detects an intracellular antigen that is neither secreted nor represented on the cell surface.

TA99 recognizes a component of pigmented melanoma cells and melanocytes designated pigmentation-associated antigen (PAA) (7, 13). PAA is a 70-kDa glycoprotein that is strictly associated with melanin production in cultured cells. In tissue sections, PAA is found exclusively in regions containing melanin, including the basal layer of the epidermis and the pigmented cells of the eye. Immunofluorescence tests show that the antigen is detected in areas of the cytoplasm containing a high density of melanosomes (7), and PAA has now been localized to the interior of mature melanosomes by immunoelectron microscopy (unpublished data). As indicated by sensitive rosetting tests and absorption tests with cultured melanoma cells, no PAA can be detected on the cell surface. The antigen is found in the membrane fraction after cell disruption and is solubilized by nonionic detergents. In cells cultured with [^{3}H]glucosamine, PAA is found as a major labeled constituent (14). However, no soluble PAA is found in the culture medium of [^{3}H]glucosamine-labeled cells, indicating that PAA is not a secreted product.

MATERIALS AND METHODS

Cell Lines. Human melanoma cell lines SK-MEL-30 (heavily pigmented), MeWo (moderately pigmented), and SK-MEL-31 (nonpigmented), and a control colon carcinoma cell line, HT-29, were cultured in Eagle's minimal essential medium (MEM), supplemented with 2 mM glutamine, 1% nonessential amino acids, penicillin (100 units/ml), streptomycin (100 μg/ml), and 7.5% fetal bovine serum. Cells were detached by treatment with 0.1% trypsin and 0.02% EDTA (GIBCO).

Human Tumor Xenografts and Antibody Administration. Swiss *nu/nu* female mice (6–8 weeks old) were injected with 5×10^6 tumor cells i.m. in the right leg (antigen-positive melanoma) or left leg (control antigen-negative colon tumor). Labeled antibody was injected i.v. into the retroorbital venous plexus 10–14 days after tumor inoculation, when tumors weighed 300–500 mg. Local extravasation did not occur, as determined by external scintigraphy at <1 hr.

Monoclonal Antibodies: Characteristics and Labeling Procedure. TA99 (IgG2a) was isolated from mice immunized with the pigmented melanoma cell line SK-MEL-23; it was selected for its ability to precipitate PAA (7). MF116 (IgG2a) was isolated from mice immunized with the ovarian cancer cell line 2778 (8), and S4 (IgG2a) was from mice immunized with SK-RC-7 renal cancer cell line (9). MF116 and S4 detect cell-surface antigens and were used as control antibodies in this study. Antibodies were purified on protein A-Sepharose (Pharmacia) using 1 ml of beads per ml of serum, 0.05 M Tris·HCl/0.1 M NaCl, pH 8.6, as starting buffer and 0.05 M sodium acetate/0.1 M NaCl, pH 4.0, as eluting buffer. The eluted fractions were pooled and dialyzed against phosphate-buffered saline. Iodination was performed by the chloramine-T method using 20 μg of IgG, 1.0 mCi of ^{125}I (1 Ci = 37 GBq; New England Nuclear), and 10 μl of chloramine-T at 1.0 mg/ml. The buffer used for Sephadex G-25 chromatography was Dulbecco's phosphate-buffered saline (PBS) (GIBCO), 0.05% bovine serum albumin, 10 mM NaI. After iodination, antibody volumes were adjusted so the same number of cpm was injected into each mouse. Samples of ^{125}I-labeled antibody were analyzed by NaDodSO$_4$/PAGE under reducing

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Abbreviations: PAA, pigmentation-associated antigen; ^{125}I-TA99, ^{125}I-labeled monoclonal antibody TA99.

and nonreducing conditions; >90% of the cpm migrated as IgG heavy and light chains.

Radioimmunolocalization. Mice were divided into groups matched for tumor size and anesthetized with avertin. The thyroid was not blocked with free iodine. Mice were injected with equal volumes of labeled antibody containing equal amounts of radioactivity (10^8 cpm per mouse). After injection, scans were obtained immediately to obtain time 0 (<1 hr) whole body counts. Five percent of the injected cpm of each labeled antibody was used as a standard for scanning. Serial blood samples were obtained from the retroorbital plexus on the side opposite the injection site. A Technicare 410S LFOV (large field of view) Gamma Camera with an x-ray grid collimator was used for imaging. Mice were imaged for 5–15 min to collect a minimum of 600,000 counts per image. Quantitative image processing was performed on a Digital Equipment PDP 11/34 computer with Gamma-11 software. As the leg was used for the tumor site, an area of low background, quantitative external scintigraphy was possible comparing cpm localized in the tumor, whole body cpm, and total injected cpm (standard). Animals were sacrificed at various time intervals over the 80-day scan period (usually within 1–2 hr after scanning) to compare results of quantitative external scintigraphy with cpm in individual normal and tumor tissues. Prior to dissection, the mice were bled from the retroorbital plexus, collecting 0.5–1 ml of blood. After complete dissection of all organs in the thorax, abdomen, and pelvic cavity, the remaining carcass was homogenized and assayed separately. The liver was divided into four pieces, and each was counted separately to reduce tissue absorption of ^{125}I γ-ray emission.

Metabolically Labeled Antibody. TA99 and S4 hybridoma cells (10^7) were incubated for 1 week in MEM (GIBCO) containing 7.5% fetal bovine serum and 2 μCi of [^{3}H]leucine/[^{3}H]lysine per ml (New England Nuclear). Fetal bovine serum used to supplement the medium was passed through a protein A-Sepharose column (Pharmacia) to remove protein A-binding bovine globulins and was then resterilized by passage through a 0.22-μm micropore filter (Nalgene). Labeled antibody was purified from the supernatant by passage over a protein A-Sepharose column at pH 7 at 4°C and elution with acetate buffer at pH 4. The purity of the immunoglobulin fraction was confirmed by PAGE. ^{3}H-labeled antibody counts in tissues were determined after complete combustion in a Sample Analyzer (Packard Instruments, Downers Grove, IL).

RESULTS

Imaging SK-MEL-30 Melanoma with TA99. Serial imaging studies are shown in Figs. 1 and 2. Early after injection, more ^{125}I-labeled TA99 (^{125}I-TA99) localized to the control HT-29 tumor (left leg) than to SK-MEL-30 melanoma (right leg). MF116 control antibody (not reactive with either tumor) gave the same result. Initial accumulation of labeled TA99 and MF116 in the control HT-29 tumor appears to reflect circulating antibody in this highly vascularized tumor, an interpretation supported by two lines of evidence. First, the tumor/blood ratio (0.3) was equal for TA99 and for MF116. Second, quantitive external imaging indicated that the rate of radioisotope clearance from the control HT-29 tumor and from highly vascularized normal organs (heart, lung, and liver) was identical. Specific imaging of SK-MEL-30 melanoma at early time intervals is therefore obscured by large amounts of circulating ^{125}I-TA99. Clearance of labeled antibody from blood coupled with slow clearance from tumor permits distinct specific melanoma imaging by day 13 (Figs. 1 and 2), and clear images were obtained as late as 10 weeks after ^{125}I-TA99 administration. The proportion of retained radioactivity in the melanoma graft decreased slowly from 13.5% of the injected dose on days 6–9 to 4.6% on day 62 (Tables 1 and 2). In contrast, radioactivity in the blood decreased by a factor of 10^4–10^5 over the same time period (Table 2). After 60–80 days, >99% of the radioactivity remaining in the animals was concentrated in the melanoma graft, resulting in a tumor/blood ratio of >10^4–10^5.

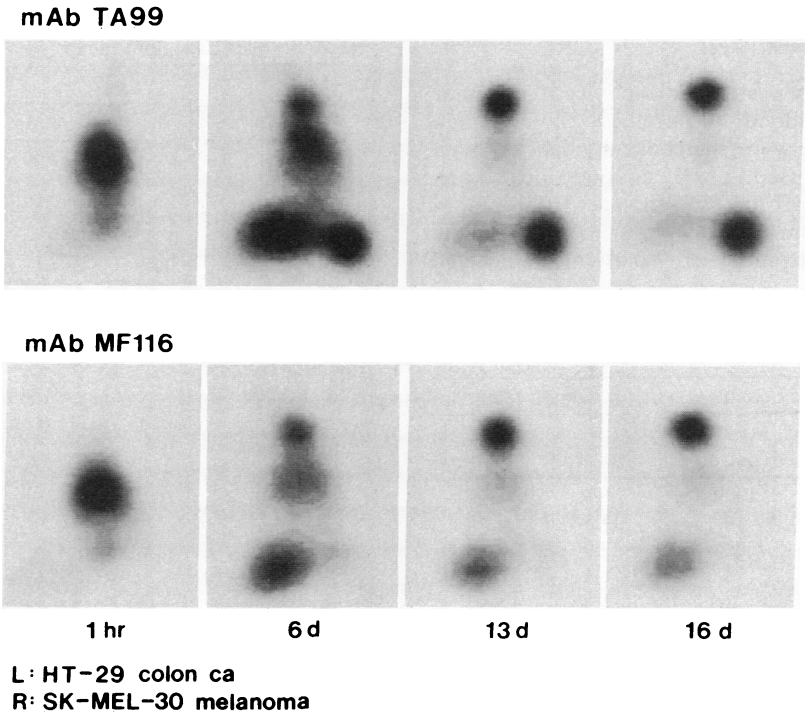

FIG. 1. Serial images of SK-MEL-30 melanoma (right leg) and HT-29 colon cancer (left leg) (control) after i.v. injection of ^{125}I-TA99 or MF116 (control) mouse monoclonal antibodies. ^{125}I uptake by thyroid was not blocked. Relative enhancement is approximately the same for each time period.

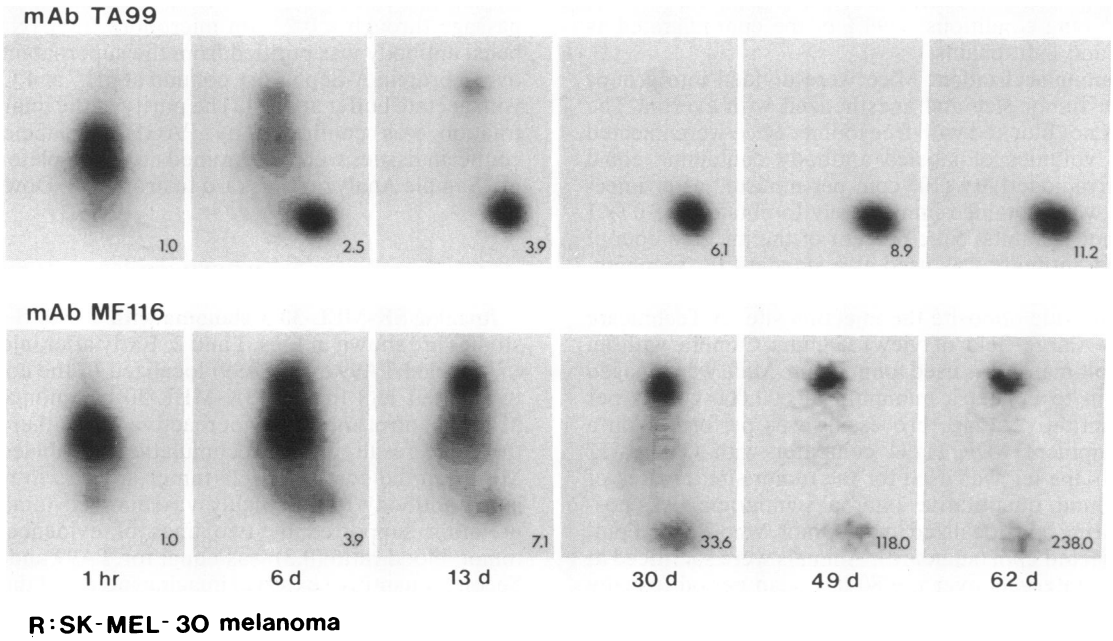

FIG. 2. Serial images of SK-MEL-30 melanoma (right leg) after i.v. injection of ^{125}I-TA99 or MF116 (control) mouse monoclonal antibodies. ^{125}I uptake by thyroid was not blocked. Figures in lower right corner of each image represent relative image enhancement compared to 1 hr scans.

Similar results were obtained with another pigmented melanoma (MeWo), although the more heavily pigmented SK-MEL-30 melanoma retained more than twice as many counts as the moderately pigmented MeWo tumor. A nonpigmented melanoma (SK-MEL-31), which did not express the TA99 antigen, showed no specific localization of ^{125}I-TA99.

With regard to the control MF116 antibody, some degree of retention by SK-MEL-30 melanoma (as compared with blood and normal tissues) was seen (Table 2). However, only 0.1–0.2% of the injected dose was retained, 1/50th of the amount observed with TA99. Most likely, this retention is nonspecific and reflects trapping of antibody in necrotic areas of the tumor.

To determine whether persistent radioactivity at the tumor site represents labeled antibody or detached ^{125}I, TA99 and a control antibody (S4) were metabolically labeled with tritiated amino acids and used to study antibody localization in *nu/nu* mice with SK-MEL-30 melanoma. ^{3}H-labeled S4 was rapidly cleared from the tumor, with only minute amounts of ^{3}H detectable on day 21 (<0.4% of the injected dose). In contrast, 4.6–6.1% of the injected ^{3}H-labeled TA99 was recovered from the melanoma graft at this time. From these data, we calculate that at least 460–610 ng of the 10 μg of TA99 injected was specifically concentrated in the melanoma graft at day 21.

Table 1. Localization of ^{125}I-TA99 in SK-MEL-30 melanoma transplants in *nu/nu* mice: Results of quantitative external scintigraphy

Time after injection	Whole body count*		Tumor count*	
	TA99	MF116 (control)	TA99	MF116 (control)
1 hr	100.0†	100.0	5.5	5.3
6 days	41.0	54.1	13.5	4.6
13 days	17.4	21.0	7.9	1.2
16 days	15.3	13.6	8.1	<1
23 days	9.8	2.1	6.5	<1
30 days	8.7	<1	6.4	<1
49 days	5.8	<1	4.9	<1
62 days	4.7	<1	4.6	<1

Data are from images shown in Fig. 2.
*Total injected dose (cpm) corrected for ^{125}I decay.
†Percent total injected dose (cpm) retained. Whole body count at 1 hr was defined as 100%. Uptake of ^{125}I by thyroid was not blocked.

Clearance of ^{125}I-TA99 and MF116. The distribution volume of intravenously injected ^{125}I-TA99 and ^{125}I-MF116 (control) antibodies in tumor-free mice was calculated (by radionuclide dilution) to be 1.6–2.1 ml; this is consistent with accepted standards for the intravascular blood volume of

Table 2. Localization of ^{125}I-TA99 and ^{125}I-MF116 in SK-MEL-30 melanoma transplants in *nu/nu* mice: Levels of radioactivity in blood, normal tissue, and tumor

	Day					
	3	9	18	22–28	38–50	60–80
	TA99					
Tumor/blood	0.5	2.3	6.7	29.7	889	1×10^4
Tumor/liver	1.9	3.1	11.8	82	830	1×10^5
Tumor/spleen	1.2	1.0	4.7	46	128	152
Tumor/kidney	2.0	2.2	8.8	52	573	666
Tumor/lung	1.4	3.5	12.9	59	371	423
% tumor bound/ total injected*	3.8	6.8	6.1	5.4	5.2	4.8
% tumor bound/ total retained†	9	33	58	78	97	>99
	MF116 (control)					
Tumor/blood	0.3	0.5	0.5	1.1	18	43
Tumor/liver	0.8	1.1	1.1	2.1	12	40
Tumor/spleen	0.7	0.8	0.7	3.4	7.7	6
Tumor/kidney	0.9	1.0	1.0	3.6	7.4	16
Tumor/lung	0.6	0.6	1.2	3.0	8.7	18
% tumor bound/ total injected*	2.0	1.8	0.4	0.3	0.2	<0.1
% tumor bound/ total retained†	5	3	4	2	7	8

Data calculated from four mice per time point. Ratio of (cpm per g of tumor)/(cpm per g of tissue).
*Corrected for radioisotope decay.
†Total retained cpm determined by total body countings, including blood, major organs, and carcass.

mice weighing 22–31 g (10). Tumor-bearing mice tended to have a larger volume of distribution (1.92 ml ± 0.22) than non-tumor-bearing mice (1.7 ml ± 0.16). Fig. 3 shows whole body ^{125}I counts (determined by external scintigraphy) and blood ^{125}I counts measured serially over a 4-day period in tumor-free mice. ^{125}I in the blood was shown to be >90% protein bound (by trichloroacetic acid precipitation) and associated with IgG light and heavy chains (by $NaDodSO_4$/PAGE analysis) for up to 43 days after injection. ^{125}I blood counts, but not whole body counts, decreased rapidly within the first 24 hr, indicating accumulation of radioisotope in a compartment outside the vascular space. In several experiments involving tumor-free mice and IgG2a mouse monoclonal antibodies (including TA99), the $t_{1/2}\alpha$ ranged from 14 to 22 hr, and the $t_{1/2}\beta$ ranged from 4.6 to 7.7 days. In the case of TA99, blood and whole body counts were cleared more rapidly from mice bearing pigmented antigen-positive tumors than from mice bearing nonreactive control tumors or from tumor-free mice (Fig. 4). This effect of TA99-positive tumors was clearly demonstrable as a shortening of $t_{1/2}\alpha$ (10–18 hr); $t_{1/2}\beta$ remained unaffected (Fig. 4). We considered the possibility that the more rapid disappearance of blood and whole body counts from TA99-positive tumor-bearing mice might be a consequence of antigen–antibody complex formation with clearance and breakdown by reticuloendothelial (RES) cells. However, tissue counting and quantitative external scintigraphy did not reveal significant accumulation of radioactivity in RES organs such as liver or spleen at any of the early time points studied.

DISCUSSION

The major conclusion from this study is that a mouse monoclonal antibody detecting an intracellular and nonsecreted antigen localizes effectively in tumors after intravenous injection. The site of antigen recognized by TA99 *in vivo* is not known, but we assume that it is necrotic areas of the tumor in which intracellular antigens are exposed. The precedent for this would be the imaging of myocardial infarcts with antibodies to myosin (11). Necrotic areas are common in human tumors and in human tumors growing in *nu/nu* mice. For example, some types of epithelial tumors grow characteristically as a sheath of viable cells, no more than 0.2 mm thick, surrounding a necrotic core (12). Antibodies to intracellular antigens present in areas of tumor necrosis may therefore be useful for antibody localization and therapy of a considerable range of tumors. Such antigens would not be exposed in normal tissues, and their concentration in tumor may be quite high. Moreover, intracellular antigens may be less affected by processes such as shedding, metabolic turnover, and degradation that might interfere with stable localization of antibodies reacting with surface antigens of viable cells. Antibodies reactive with other intracellular antigens need study to determine how general the finding of imaging with intracellular targets will be.

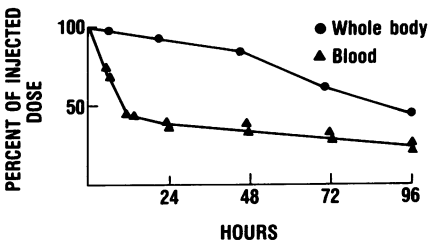

FIG. 3. Serial whole body counts and blood counts after i.v. injection of ^{125}I-TA99 in tumor-free CD1 Swiss mice. Similar results were obtained in three separate experiments; data shown here are from one experiment.

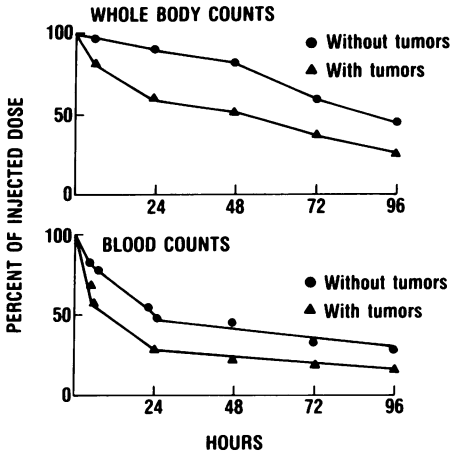

FIG. 4. Serial whole body counts and blood counts after i.v. injection of ^{125}I-TA99 in tumor-free *nu/nu* mice or in *nu/nu* mice with SK-MEL-30 melanoma. Similar results were obtained in three separate experiments; data shown here are from one experiment.

Two main parameters determine the ability of monoclonal antibodies to image tumors. First, a significant fraction of the administered radiolabeled antibody must localize to the tumor and, second, the circulating and extravascular pools of antibody must be cleared sufficiently to visualize the localized antibody. In the present study, early images of mice bearing melanoma and control tumors indicated that blood flow patterns dominate the picture during the first 6 days after antibody injection, obscuring any specific localization. A vascular control tumor was selected to illustrate this point. Ironically, certain antigenic systems, such as those associated with secreted antigens, previously thought to be more difficult to image, may actually show better images due to rapid clearance of antigen–antibody complexes. In the case of TA99, $t_{1/2}\alpha$ was clearly more rapid in tumor-bearing animals than in controls. Whole body counts also decreased more rapidly in tumor-bearing mice than controls, indicating that counts cleared from blood are cleared from the mice. The mechanism involved in the more rapid clearance of ^{125}I-TA99 from tumor-bearing mice is unclear; antigen in free form or bound to antibody may be released from the tumor site and complexed antibody may be degraded more rapidly.

In addition to excellent tumor imaging by TA99, the long persistence of TA99 in the tumor was another surprising finding of this study. Although the long retention of ^{125}I at the tumor site could be due to reincorporation of label released from the antibody, this seems unlikely because metabolically labeled antibody was also similarly retained. Conclusive proof that intact antibody rather than released and reincorporated label persists at the tumor site is difficult to obtain because of the low numbers of counts present at late time intervals. If, in fact, antibody retention is involved, there are several possible explanations for the long retention of TA99, including inefficient removal of antigen–antibody complexes from areas of tumor necrosis or unusually high affinity of TA99. In studies of other antibodies to cell surface and intracellular antigen, we find that each antibody has different retention characteristics, and no correlation with isotype of site of antigen has as yet emerged. In general, most labeled antibodies are retained for only a brief period. A second melanoma-reactive antibody showing prolonged retention by tumors (>2 mo) has been identified. In contrast to TA99, this antibody reacts with a cell-surface antigen and belongs to the IgG1 subclass. Clearly, much further information is required before the imaging and therapeutic potential of monoclonal antibodies can be assessed.

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