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In Vivo Targeting of Human Neuroblastoma Xenograft by Anti-G_{D2}/Anti-FcγRI (CD64) Bispecific Antibody

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Antidisialoganglioside (G_{D2}) monoclonal antibodies can target *in vitro* and *in vivo* neuroblastoma cells. However, their *in vivo* use is limited by the presence of high levels of circulating IgG which hamper the recruitment of effector cells through the high affinity FcγRI (CD64). A bispecific Fab′ × Fab′ antiG_{D2}/antiFcγRI antibody (7A4 bis 22), which binds outside the IgG binding site of FcγRI, was therefore developed. This antibody binds both human G_{D2+} neuroblastoma and FcγRI$^+$ activated macrophages *in vitro*. It can localise a G_{D2} positive neuroblastoma xenografted on Nu/Nu mice. Scintigraphy tumour/muscle ratios showed that targeting with this antibody has an excellent selectivity for the tumour over normal tissues. Furthermore, although its whole body clearance is more rapid than that of the 7A4 parental antibody over the first 48 h, its selective tumour uptake is similar, as shown by immunoscintigraphy imaging. Thus, such a bispecific antibody may represent an efficient tool for *in vivo* therapy of neuroblastoma through its ability to recruit FcγRI$^+$ effector cells even in presence of circulating IgG and to bind concomitantly G_{D2+} tumour cells.

Key words: disialoganglioside, FcγRI, bispecific antibody, neuroblastoma, xenograft, scintigraphy
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INTRODUCTION

SEVERAL ANTIDISIALOGLANGLIOSIDE (G_{D2}) monoclonal antibodies (MAbs) have been successfully used for immunolocalisation in human neuroblastoma xenogeneic tumour models [1–3]. This is due, at least in part, to the fact that G_{D2} is expressed at high levels on neuroblastoma (10^7 molecules per cell) [4], is poorly expressed or absent from most normal tissues and is only very slowly down-modulated from tumour cell surface following its interaction with specific MAbs [1], if any [5]. Clinical trials have also demonstrated that partial and even complete remission could be achieved with antiG_{D2} MAbs [6, 7]. This has been correlated with their ability to trigger *in vitro* antibody-dependent cell cytotoxicity (ADCC) by activated mononuclear cells and neutrophils [5–9]. In the case of neutrophils and activated macrophages, ADCC is mediated, at least in part, through the engagement of the high affinity FcγRI (CD64) [10, 11]. However, *in vivo* binding of antitumour MAbs to FcγRI is likely to be hampered by the presence of high levels of circulating IgG, since this high-affinity FcγR binds monomeric IgG [12]. One alternative approach is to use bispecific antibodies (BsAb) binding to FcγRI outside its IgG binding site in addition to G_{D2}.

In the present study, a MAb, termed 22, directed against an epitope distinct from the IgG binding site of FcγRI and able to trigger cytotoxicity [11, 13], was used in combination with an antiG_{D2} MAb, 7A4 [14] to generate a BsAb (7A4 bis 22). *In vitro*, this BsAb binds efficiently to a human G_{D2}+ neuroblastoma cell line (LAN 1) and to FcγRI+ macrophages activated by recombinant human interferon gamma (rHuIFNγ). Studies on the *in vivo* targeting to LAN 1 tumour xenografted in Nu/Nu mice indicate that this engineered BsAb reacts with neuroblastoma tissue as efficiently as its parental counterpart (7A4). An irrelevant bispecific antibody, 520C9 bis 22, directed against the same epitope of FcγRI and the c-erbb2 product of HER-2/*neu* proto-oncogene, showed no tumour uptake by scintigraphy. Thus, this antiFcγRI/antiG_{D2} BsAb may provide a useful tool to develop a new specific immunotherapeutic approach based on the recruitment of FcγRI+ activated cytotoxic effector cells.

MATERIALS AND METHODS

Activated human macrophages

Peripheral blood mononuclear cells derived from a healthy donor were incubated for 5 days at 37°C in 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% heat-inactivated normal human AB serum. Macrophage activated killer (MAK) cells were obtained from this preparation by elutriation of macrophages, which were incubated (5×10^6/ml) overnight with 400 IU/ml of rHuIFNγ (Roussel-Uclaf, Romainville, France).

Neuroblastoma LAN 1 cell line

The G_{D2}+ LAN 1 neuroblastoma cell line was kindly provided by Dr D. Beck (CHUV, Lausanne, Switzerland). LAN 1 cells were cultured in RPMI 1640 medium (Seromed, Berlin, Germany) containing 10% heat-inactivated fetal calf serum (FCS) (Seromed), 1% L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin. Experiments were performed on trypsinised cells (Trypsin-EDTA, Gibco, Paisley, Scotland) subsequently washed in 10% FCS-containing culture media. These cells have been previously selected for their ability to form G_{D2}+ tumours *in vivo* on Nu/Nu mice by Dr N.K. Cheung (Memorial Sloan Kettering, New York, U.S.A.).

Antibodies

The hybridoma B cell lines 7A4 (mouse IgG3, κ, antiGD_2), and P51. 1 (IgG3, κ, antiphosphorylcholine) were kindly provided by Drs D. Beck and N. Gross (CHUV, Lausanne, Switzerland) and by Pr. M.D. Scharff (Albert Einstein College of Medicine, New York, U.S.A.), respectively. They were purified from cell culture supernatants by affinity chromatography on to Protein G-Sepharose (Pharmacia, Uppsala, Sweden), and their purity was checked by SDS-gel electrophoresis and ELISA.

Alkaline phosphatase-labelled antihuman IgM and antimouse IgG3 goat antibodies were purchased from Southern Biotechnology Associates (Birmingham, U.S.A.). Fluorescein isothiocyanate-labelled F(ab)'2 IgG goat antimouse immunoglobulin (GAM-FITC) (Southern Biotechnology) were used in indirect immunofluorescence assays. FITC-labelled mouse IgG1 was obtained from Immunotech (Marseille, France). FITC-coupled MAb 22 (IgG1, κ), directed against an epitope distinct from the IgG binding site of FcγRI was supplied by Medarex, Inc. (Annandale, U.S.A.).

Bispecific anti-G_{D2}/antiFcγRI (7A4 bis 22) and anti-c-erbb-2/antiFcγRI (520C9 bis 22) antibodies

The 7A4 bis 22 BsAb (Fab' × Fab') was prepared as previously described [15] at Immunotech using 7A4 and 22 MAb Fab' fragments and bis-maleimide as the cross-linking reagent. It was purified by preparative gel filtration on Superdex 200 (Pharmacia). The purity was over 90% after analytical Superdex 200 chromatography. The 520C9 bis 22 BsAb (Fab' × Fab') was prepared at Medarex. 520C9 is a mouse MAb directed against the c-erbb2 protein product of the HER-2/*neu* [11]. The BsAb stock solutions (1.0 mg/ml in PBS, pH 7.3) were stored at 4°C.

Biochemical characterisation of BsAbs

^{125}I-7A4 bis 22 and ^{125}I-520C9 bis 22 preparations were analysed by SDS-7.5% PAGE in non-reducing conditions using minigels (MiniProtean II, Biorad, Richmond, U.S.A.). Autoradiograms were obtained after exposure of the dried gels to X-Omat AR films (Kodak, Rochester, U.S.A.). Unlabelled BsAbs were also run on 7.5% polyacrylamide-SDS gels subsequently stained with Coomassie blue.

FcγRI-μ fusion protein

A cell culture supernatant of transfected COS 7 cells containing a fusion protein exhibiting the extracellular domains of FcγRI and the constant region of human IgM (FcγRI-μ fusion protein), supplied by Medarex, was used to detect the 7A4 bis 22 BsAb binding to both G_{D2} and FcγRI molecules by ELISA.

ELISA

LAN 1 cells were harvested from culture flasks after trypsinisation, washed and resuspended in phosphate buffered saline (PBS) at 10^7 cells/ml. Cells were then sonicated and the cell extract was stored at −20°C until further use. ELISA microplates (Maxisorp, Nunc, Rockslide, Denmark) were coated with 50 μl/well of cell extract (corresponding to a concentration of 5×10^4

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sonicated cells/well) for 2 h at room temperature. After washing, the plates were saturated with 1% low-fat milk-PBS overnight at 4°C. 50 μl/well of MAbs (7A4 bis 22, 7A4, or P51.1) were two-fold diluted in 50 μl of PBS containing 1% bovine serum albumin (BSA) (Fraction V, Sigma, St Louis, U.S.A.). After 2 h incubation at 20°C, plates were washed and 50 μl/well of FcγRI-μ COS 7 supernatant (1:4 diluted in PBS-1% BSA) were added. After 2 h incubation at 20°C, plates were washed and incubated for 90 min at 37°C with alkaline phosphatase-labelled antihuman IgM (1:250 in PBS-1% BSA) goat antibodies before the addition of substrate (*p*-nitro-phenyl phosphate disodium). Optical density was read at 405 nm using an ELISA microplate reader (Titertek Multiscan, Labsystems, Les Ulis, France).

Immunofluorescence assays

Direct immunofluorescence was performed by incubating on ice 10^6 cells for 30 min with FITC-labelled antibodies diluted in 50 μl of PBS-1% BSA. Indirect immunofluorescence was performed by incubating for 30 min on ice 10^6 cells with the first antibody (10 μg/ml). Cells were then washed twice in RPMI 1640 containing 5% FCS and incubated for another 30 min with the GAM-FITC diluted in PBS-1% BSA. After washing, cells were fixed with PBS-1% formaldehyde. 5000 cells per experiment were analysed with a FACScan cytometer (Becton-Dickinson, Pont de Claix, France), using the Lysis program.

Tumour imaging

LAN 1 human neuroblastoma tumour was grafted in female Nu/Nu mice. G_{D2} expression on tumour cells was assessed after 15 consecutive grafting by indirect immunofluorescence. Sixteen Nu/Nu mice bearing LAN 1 tumours were studied by scintigraphic imaging by *in vivo* binding of ^{125}I-7A4, ^{125}I-7A4 bis 22, and ^{125}I-520C9 bis 22 antibodies. Tumours were surgically implanted 3–4 weeks before imaging analysis into the left scapular area of 5–6 week old mice. A first experiment was performed with two groups of three mice each that were injected intraperitoneally with either ^{125}I-7A4 (991.6 ± 44.4 kBq or 26.8 ± 1.2 μCi/mouse, 1.7×10^6 cpm/μg) or ^{125}I-7A4 bis 22 (817.2 ± 25.9 kBq or 22.1 ± 0.7 μCi/mouse, 2.4×10^6 cpm/μg) on day 0. A second experiment was performed with two groups of 5 mice each that were injected intraperitoneally with either ^{125}I-7A4 bis 22 (540.2 ± 81.4 kBq or 14.6 ± 2.2 μCi/mouse, 8×10^6 cpm/μg) or ^{125}I-520C9 bis 22 (654.9 ± 66.6 kB or 17.7 ± 1.8 μCi/mouse, 9.5×10^6 cpm/μg) on day 0. The biodistribution pattern (whole body, tumour area, hindleg—used as muscle control—liver and thyroid gland) was analysed 24, 48 and 120 h after injection using a General Electric GE 400 gamma camera equipped with a pin hole collimator and a SOPHA Medical SIMIS III console. After the last imaging analysis, the animals were sacrificed. Hindlegs, livers and tumours were carefully removed, weighed and radioactivity was counted using a gamma counter (Cobra II, Packard, Meriden, U.S.A.).

RESULTS

Polyacrylamide gel electrophoresis analysis of the 7A4 bis 22 BsAb

The SDS-PAGE analysis (Figure 1) of the 7A4 bis 22 BsAb preparation showed a major component with an apparent molecular mass of 110 kDa, the expected molecular mass of a bispecific Fab' × Fab' antibody, as well as some additional molecules with apparent molecular masses of 85–100 kDa and of 50 kDa (Figure 1A, lane 3 and Figure 1B, lane 1). The 520C9 bis 22 BsAb preparation also showed a major 110 kDa

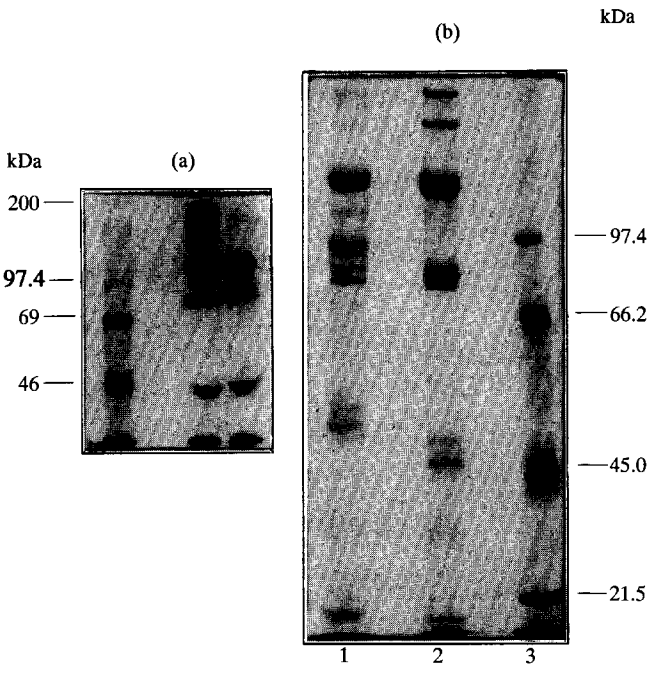

Figure 1. Analysis of the 7A4 bis 22 and 520C9 BsAb preparations by polyacrylamide gel electrophoresis. (a), ^{125}I-7A4 bis 22 (lane 3) and ^{125}I-520C9 bis 22 (lane 2) antibody preparations were analysed on a 7.5% polyacrylamide-SDS minigel in non-reducing conditions. Markers (lane 1) are Myosin (200 kDa), Phosphorylase b (97.4 kDa), bovine serum albumin (69 kDa), and ovalbumin (46 kDa). (b), 7A4 bis 22 (lane 1) and 520C9 bis 22 (lane 2) BsAbs were run on a 7.5% polyacrylamide-SDS 20 cm gel in non-reducing conditions which was subsequently stained with Coomassie blue. Markers (lane 3) are Phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and Soybean Trypsin Inhibitor (21.5 kDa).

component, as well as the presence of molecules with apparent molecular masses of 150 kDa, 140 kDa, 85–90 kDa, and 46–47 kDa (Figure 1A, lane 2 and Figure 1B, lane 2). The 85–100 kDa molecules probably correspond to intermediate bispecific forms lacking one of the light chains

Binding of 7A4 bis 22 to FcγRI$^+$ macrophages and $G_{D2}$$^+$ LAN 1 cells

Figure 2 indicates that the 7A4 bis 22 BsAb (10 μg/ml) binds to FcγRI+ MAK (Figure 2A) as the 22-FITC antibody (Figure 2B) and to $G_{D2}$$^+$ LAN 1 cells (Figure 2C) as the 7A4 antibody (Figure 2D), as detected by an indirect immunofluorescence assay using a F(ab)$_2$ GAM-FITC.

Bispecificity of the 7A4 bis 22 BsAb

The ability of 7A4 bis 22 BsAb to bind both to G_{D2} and to FcγRI was investigated by an indirect ELISA using the FcγRI-μ fusion protein. Microplates were coated with $G_{D2}$$^+$ LAN 1 cell extracts for capture, and the binding of the 7A4 bis 22 to G_{D2} was detected with the FcγRI-μ fusion protein. The binding of the latter was revealed using alkaline phosphatase-coupled goat antihuman IgM antibodies. Figure 3 shows that the 7A4 bis 22 preparation does contain molecules that exhibit a functional bispecificity, that is, a binding to both G_{D2} and FcγRI. The binding of the parental 7A4 antibody on LAN 1 cell extracts was also revealed using the FcγRI/μ fusion protein (Figure 3).

In vivo *tumour binding*

A comparison was made of the *in vivo* biodistribution of either ^{125}I-7A4 or ^{125}I-7A4 bis 22 antibodies to LAN 1 neuroblastoma

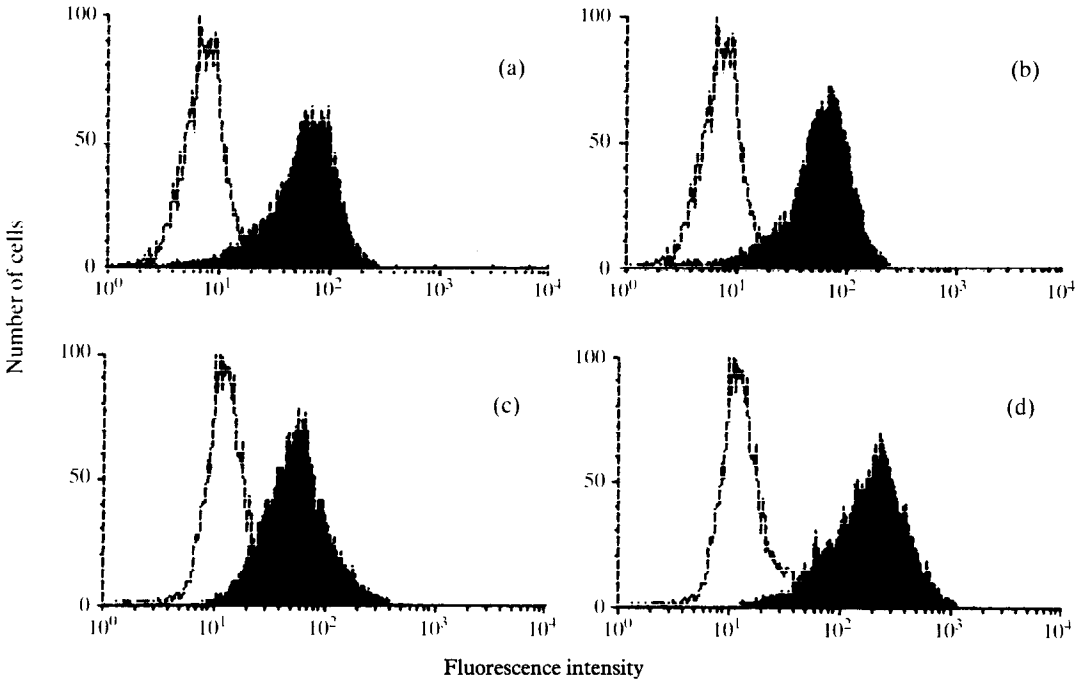

Figure 2. Immunofluorescence staining of FcγRI$^+$ MAK and G_{D2}+ LAN 1 neuroblastoma cells. MAK were stained with the 7A4 bis 22 BsAb revealed using a F(ab)$'_2$ GAM-FITC (a), or with the 22-FITC anti-FcγRI MAb (b). LAN 1 neuroblastoma cells were stained with the 7A4 bis 22 BsAb revealed using a F(ab)$'_2$ GAM-FITC (c) or with the 7A4 antiG_{D2} MAb (d). Control labelling (clear histogram) are those obtained with F(ab)$'_2$ GAM-FITC (a, c and d) or with mouse IgG1-FITC (b).

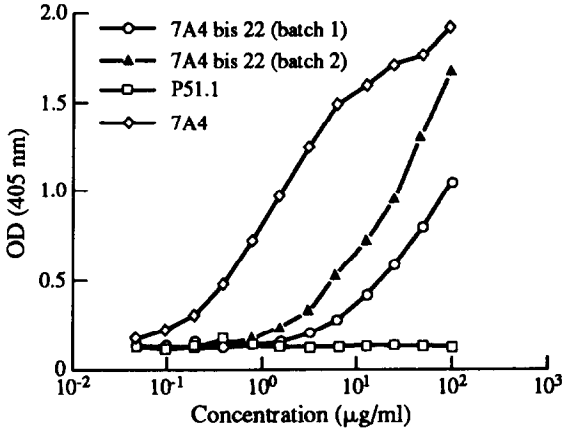

Figure 3. Bispecificity of 7A4 bis 22 BsAbs preparations: binding of 7A4 bis 22 BsAb to both G_{D2}+ LAN 1 neuroblastoma cell extracts and recombinant FcγRI. The G_{D2}+ LAN 1 cell extracts were coated on to ELISA microtitre plates and the binding of 7A4 bis 22 BsAb (two different batches), of the whole antiG_{D2} 7A4 MAb, and of an irrelevant IgG3 mouse MAb (P51.1) was revealed by adding the recombinant fusion human protein FcγRI-μ, followed by an incubation step with an alkaline phosphatase-coupled goat antihuman IgM.

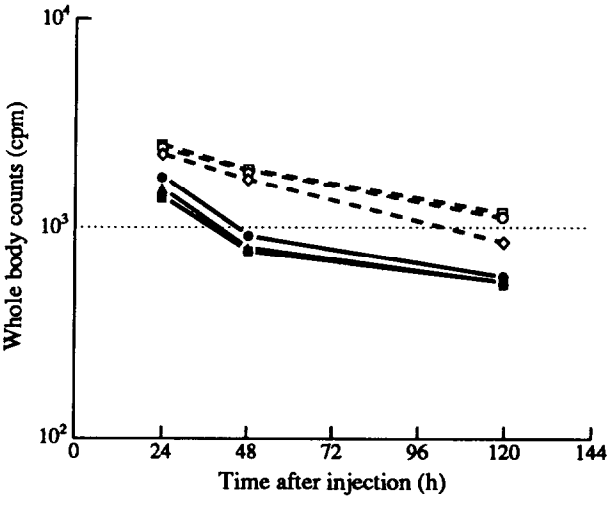

Figure 4. Comparison of the antibody whole body clearance after injection of the ^{125}I-7A4 MAb (open symbols) and of the ^{125}I-7A4 bis 22 BsAb (closed symbols) into LAN 1 tumour-bearing Nu/Nu mice. Groups of three mice were injected for each antibody.

tumours. As shown in Figure 4, whole body clearance of the ^{125}I-7A4 bis 22 BsAb was more rapid than that of the ^{125}I-7A4 antibody over the first 48 h, while these whole body clearances were similar between 48 and 120 h after injection. The selective tumour uptake of the two antibodies was very similar, as shown by the immunoscintigraphy imaging (Figure 5). ^{125}I-7A4 (Figure 5A) and ^{125}I-7A4 bis 22 (Figure 5B) binding to the tumour could be detected up to 120 h after the injection, with a maximum contrast at 48 h. A thyroid gland uptake of radioactivity corresponding to free ^{125}I was observed whichever antibodies were used.

The *in vivo* binding of the 7A4 bis 22 BsAb was then compared with that of an irrelevant BsAb, 520C9 bis 22. ^{125}I-7A4 bis 22 or ^{125}I-520C9 bis 22 were injected intraperitoneally in LAN 1 tumour-bearing nude mice (5 mice/Ab). As already observed in the first experiment with the 7A4 bis 22 BsAb, the whole body clearance of bispecific antibodies was very rapid over the first 48 h (with a half-life lower than 6 h) and slowed thereafter (data not shown). The selective tumour uptake of the two antibodies was very different. The ^{125}I-520C9 bis 22 BsAb tumour uptake

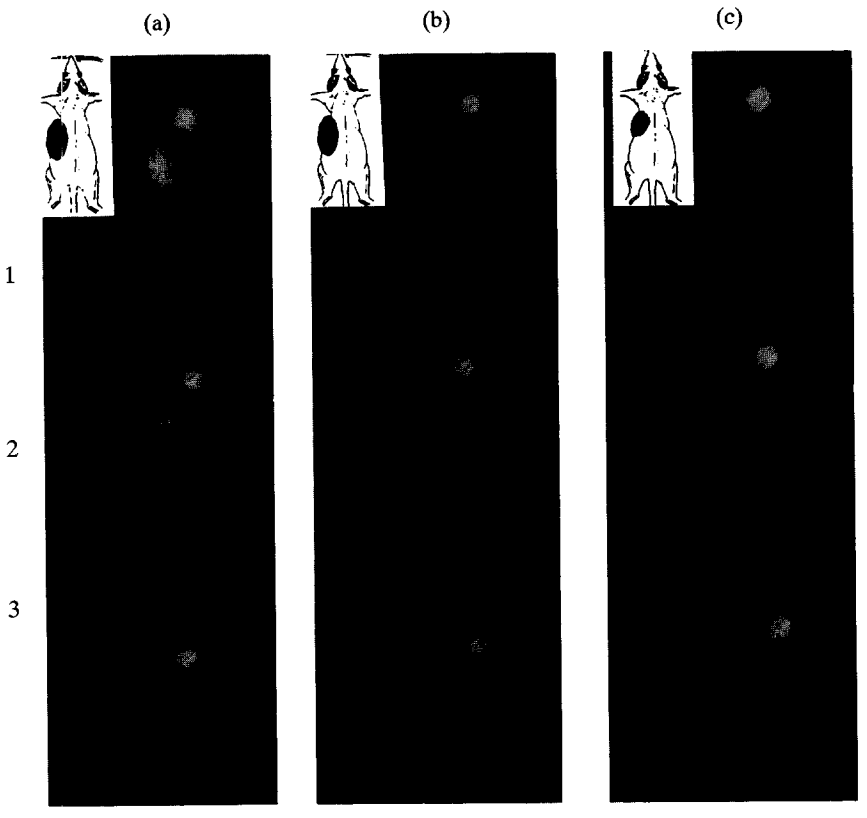

Figure 5. Immunoscintigraphy imaging of LAN 1 neuroblastoma tumours in Nu/Nu mice 24 h (1), 48 h (2), and 120 h (3) after injection of either (a) ^{125}I-7A4 MAb, (b) ^{125}I7A4 bis 22 or (c) ^{125}I-520C9 bis 22 BsAbs.

could not be detected even 24 h after the injection (Figure 5C), while the binding of ^{125}I-7A4 bis 22 was similar to that observed in the first experiment (not shown). The tumour uptake difference between the two bispecific antibodies could be evaluated by calculating the tumour/hindleg count (24, 48 and 120 h after injection) ratios (Figure 6). Notably, it was 2-fold higher with ^{125}I-7A4 bis 22 compared with ^{125}I-520C9 bis 22 BsAb 48 h after injection.

In these experiments, mice were sacrificed after the last tumour imaging, and tumours, livers, and hindleg muscles were excised, weighed and counted (Table 1). A strong binding of ^{125}I-7A4 or ^{125}I-7A4 bis 22 antibodies was demonstrated by the high tumour/hindleg muscle ratio. In addition, the binding of these two antibodies was found to be almost identical when the counts were normalised per gram of tissue. It should be pointed out that a significant radioactivity uptake by the liver was observed in both cases, which accounts for the lower tumour/liver ratios (Table 1). By contrast, a striking difference was observed between the two bispecific antibodies in the second experiment. A high ^{125}I-7A4 bis 22 tumour binding was again

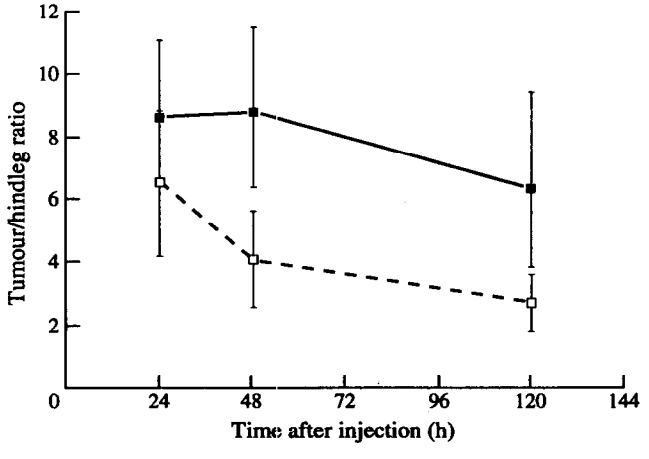

Figure 6. Kinetic of the tumour/hindleg radioactivity uptake ratio 24, 48 and 120 h after injection of either ^{125}I-7A4 bis 22 (closed squares) or ^{125}I-520C9 bis 22 (open squares) BsAbs. Each ratio value represents the mean value and S.E. derived from the counts of tumours and hindlegs of 5 LAN 1 tumour-bearing mice.

Table 1. Comparison of MAb and BsAbs binding to tumour, muscle and liver

	Tumour/muscle	Tumour/liver
Experiment 1		
^{125}I-7A4	8.7 ± 3.1*	2.1 ± 0.75
n = 3		
^{125}I-7A4 bis 22	8.7 ± 5.8	2 ± 2
n = 3		
Experiment 2		
^{125}I-7A4 bis 22	9.1 ± 1.7†	2.8 ± 1.2†
n = 5		
^{125}I-520C9 bis 22	3.4 ± 1.7	0.7 ± 0.2
n = 5		

* Results are expressed as the mean ratios (±S.D.) of tumour/hindleg muscles and tumour/liver cpm uptake per gram of tissue from nude mice sacrificed 120 h after injection.
† Significantly lower: $P \leq 0.01$ (Mann–Whitney U test).

observed, whereas ^{125}I-520C9 bis 22 antibody did not show any significant binding (Table 1, experiment 2). As already observed in the first experiment, a lower tumour/liver ratio was observed, due to a significant radioactivity uptake by the liver. However, a significantly lower tumour/liver ratio was found with the irrelevant bispecific antibody.

DISCUSSION

The 7A4 MAb directed against G_{D2} [14] has been previously used for *in vivo* imaging and biodistribution studies in Nu/Nu mice bearing a human xenografted neuroblastoma [3]. The analysis of the scintigraphy tumour/muscle ratio obtained showed that targeting with this antibody has an excellent selectivity for the tumour over normal tissues, is specific for G_{D2}^+ tumours and allows good imaging quality. The tumour uptake reached up to 13% of injected dose per gram of tumour. Thus, these results suggest that this antibody, which mediates efficient *in vitro* antibody-dependent cell cytotoxicity (ADCC) (Michon *et al.*, submitted), could also be used for *in vivo* therapy against neuroblastoma. It has been demonstrated that clinical responses could be achieved with anti-G_{D2} MAbs which are able to trigger *in vitro* ADCC through the engagement of FcγRI, II and III on various effector cells. However, the *in vivo* efficiency of MAbs is likely to be hampered, at least partially, by the presence of high levels of circulating monomeric IgG which saturate the high-affinity FcγRI [12]. To overcome this difficulty, the 7A4 bis 22 BsAb described here was developed which binds both to FcγRI outside its Fc binding site and to G_{D2}, as demonstrated by ELISA and immunofluorescence assays.

In vivo biodistribution studies of 7A4 bis 22 BsAb indicated that this antibody can target G_{D2}^+ neuroblastoma engrafted on Nu/Nu mice. Scintigraphy imaging showed a slightly better contrast with the 7A4 antibody compared with the 7A4 bis 22 BsAb (Figure 5). This could be due to the fact that 7A4 bis 22 exhibits a faster whole-body clearance over the first 48 h. Its smaller molecular mass and/or its lower *in vivo* stability could account for this observation. However, as shown by the comparison of the tumour/muscles and tumour/liver ratios obtained with the parental and the BsAb antibodies (Table 1), the 7A4 bis 22 BsAb binds as efficiently as the parental 7A4 antibody to neuroblastoma tumours. The failure of an irrelevant bispecific antibody (520C9 bis 22) to bind neuroblastoma indicate that the antiFcγRI arm of the 7A4 bis 22 antibody is not responsible for tumour targeting. Whether this bispecific antibody will be trapped *in vivo* in humans by circulating FcγRI$^+$ cells and will therefore allow an efficient targeting of tumour cells remains to be determined.

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