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Tumour Targeting of the Anti-Ovarian Carcinoma X Anti-CD3/TCR Bispecific Monoclonal Antibody OC/TR and its Parental MOv18 Antibody in Experimental Ovarian Cancer

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Abstract. *The anti-tumour x anti-T-cell bispecific monoclonal antibody (biMAb) OC/TR is a biologically produced biMAb combining the anti-ovarian carcinoma activity of the MOv18 MAb with anti-CD3/T-cell receptor (TCR) complex activity. In this study, the in vitro binding characteristics of the OC/TR biMAb and its tumour targeting potential in nude mice with Hela tumours was studied. Scatchard analysis revealed that the affinity constant of the biMAb was 7 times lower than the affinity of the parental MOv18 antibody. Uptake of the OC/TR antibody in the Hela xenografts in nude mice was significantly higher than the tumour uptake of an irrelevant control antibody, indicating that the radioiodinated OC/TR biMAb specifically localized in the tumour xenografts. However, tumour uptake was significantly lower than the tumour uptake of the parental MOv18 antibody. This reduced tumour uptake most likely is a result of its reduced affinity. We conclude that, despite the loss of bivalent tumour cell binding, the biMAb OC/TR can still specifically localize in tumours. This indicates that the first prerequisite of an effective therapeutic approach using systemically applied biMAb can be met. Whether the interaction with human T-cells will affect the tumour targeting potential of the biMAb in patients remains to be investigated.*

Redirecting effector cells of the immune system towards tumour cells with bispecific monoclonal antibodies (biMAbs)

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has been proposed as a new approach of cancer treatment (1,2). In this concept a biMAb reactive with a tumour cell on one hand and an effector cell on the other is used to retarget the cytotoxic activity of the effector cell towards tumour cells. T-cell lysis of a target cell is generally restricted to major histocompatibility complex-associated (MHC) peptide recognition. When a biMAb provides the physical linkage between a CD3/T-cell receptor (TCR) complex and a tumour cell, specific tumour cell lysis can occur (3,4).

In most preclinical studies investigating the therapeutic potential of biMAbs, activated effector cells coated with biMAbs have been administered locoregionally (5-7). The first clinical studies have also focused on locoregional infusion of redirected effector cells. In a study in glioma patients biMAb-coated cells were administered intracranially (8), while Bolhuis and coworkers (9) administered *ex vivo* expanded and activated autologous peripheral blood lymphocytes (PBLs) intraperitoneally in ovarian cancer patients. However, only a minority of cancers can be treated locoregionally. In addition, *in vitro* studies have shown that CD3/TCR complexes on preactivated PBLs that had earlier been engaged in target cell lysis are no longer able to lyse another cell. Only biMAbs binding newly expressed CD3/TCR complexes were able to reengage the T-cell in another lytic cycle (10). This observation indicates that the administration of precoated effector cells could only have limited efficacy. Therefore, *in vivo* targeting of high doses of biMAbs to the tumour site in order to redirect the lytic activity of T-cells at the tumour site seems to be a more attractive approach. However, tumour targeting of anti-tumour x anti-T-cell biMAbs might be hampered by the loss of bivalent tumour cell binding, and/or the interaction of the biMAb with peripheral T-cells. The present study investigates the tumour targeting potential of an anti-tumour x anti-CD3

Table I. *In vitro* binding characteristics of the antibody preparations.

	Immunoreactive fraction		Affinity constant (M ⁻¹)	
	anti-gp38	anti-CD3/TCR	Displacement	Scatchard
MOv18 IgG	61 %	—	3.0 x 10 ⁸	20 x 10 ⁸
Anti-CD3 IgG	—	81 %	—	—
MOv18 x anti-CD3 biMAb	55 %	76 %	7.5 x 10 ⁷	2.9 x 10 ⁸
Anti-CD3/TCR x mismatch	n.d.*	69 %	—	—

*not detectable

biMAb OC/TR in a nude mouse model.

The biMAb OC/TR combines the anti-ovarian carcinoma activity of the MOv18 MAb with anti-CD3/TCR complex activity (11). In patients with epithelial ovarian cancer radioiodinated MOv18 has been shown to localize specifically in ovarian carcinoma lesions (12,13). The biMAb OC/TR was obtained after fusion of the MOv18-producing hybridoma cells with spleen cells from a BALB/c mouse immunized with a human T-cell clone (11,14). *In vitro* studies indicated that this biMAb is able to induce efficient tumour cell lysis (11,14). In nude mice with i.p. growing NIH:OVCAR-3 ovarian carcinoma cells, tumour growth inhibition was achieved by treating the mice with IL-2 and activated PBL coated with OC/TR F(ab')₂ (7). In addition, a phase I/II study in ovarian cancer patients treated with OC/TR coated autologous PBL and IL-2 has shown some remarkable responses (9). We plan to investigate the feasibility of a therapeutic approach based on the systemic administration of OC/TR biMAb in ovarian cancer patients. Therefore, we studied the *in vitro* binding characteristics and the *in vivo* tumour targeting potential of i.v. administered OC/TR biMAb in a nude mouse model.

Materials and Methods

Monoclonal antibodies. The MOv18 antibody (IgG₁) recognizes a 38 kD cell surface glycoprotein (gp38) antigen expressed on most ovarian carcinomas (15). The gp38 antigen was identified as a folate binding protein (16-18). MOv18 is also reactive with adenocarcinomas of the fallopian tube, endometrium and cervix (19).

The biMAb OC/TR (IgG₁ x IgG₁) is produced by a trioma cell line obtained after fusion of the MOv18 hybridoma with spleen cells from a BALB/c mouse immunized with a human T-cell clone (11,14). The biMAb was purified from trioma culture supernatant on a MONO-S cation exchange column (Pharmacia, Woerden, the Netherlands) as described previously (14). Inherent to the biological biMAb production process an IgG with an intact anti-CD3 binding site and a heavy-light chain pairing mismatch is produced. This 'anti-CD3 x mismatch biMAb' was also purified from the trioma supernatant and used as a control antibody in the biodistribution studies. Neither the OC/TR antibody nor the control antibody cross reacts with murine T-cells. The antibody preparations were prepared and supplied by Centocor Europe B.V. (Leiden, the Netherlands).

Cell lines. The HeLa human cervix carcinoma cell line (MOv18-positive) and the Jurkat human acute T-cell leukemia cell line (CD3-positive) were obtained from the American Type Culture Collection. Cells were

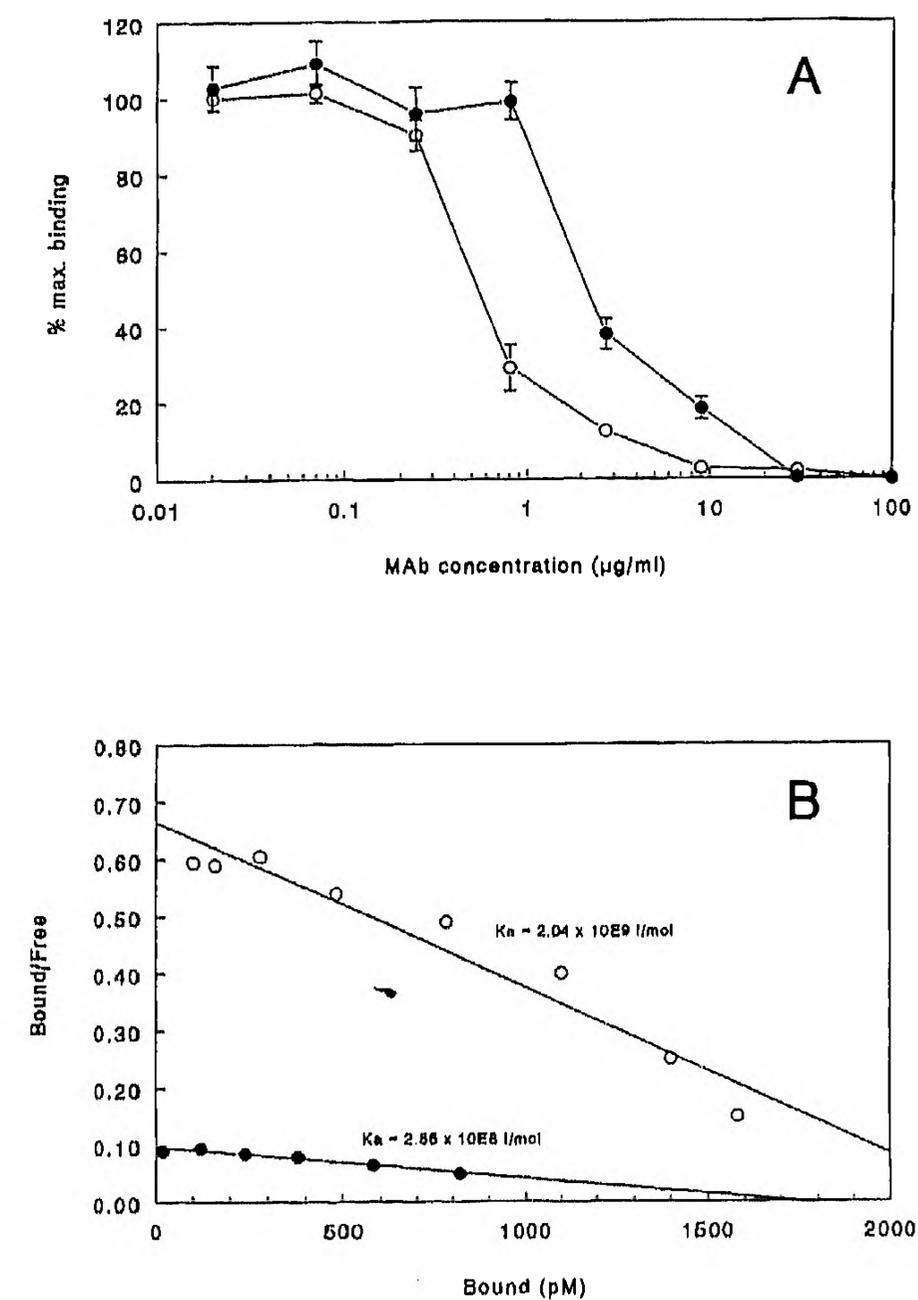


Figure 1. A. Homologous displacement assay for MOv18 (○) and OC/TR (●). B. Scatchard plot of radioiodinated MOv18 (○) and OC/TR (●) binding to HeLa cells.

cultured in RPMI-1640-based medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum.

Radiolabelling and quality control. The antibody preparations were labelled with ¹²⁵I or ¹³¹I using the enzymobead method (BioRad, Richmond, CA), according to the manufacturer's instructions. Briefly, 50 µg of IgG were mixed with 20 µl 0.5 M sodium phosphate (pH

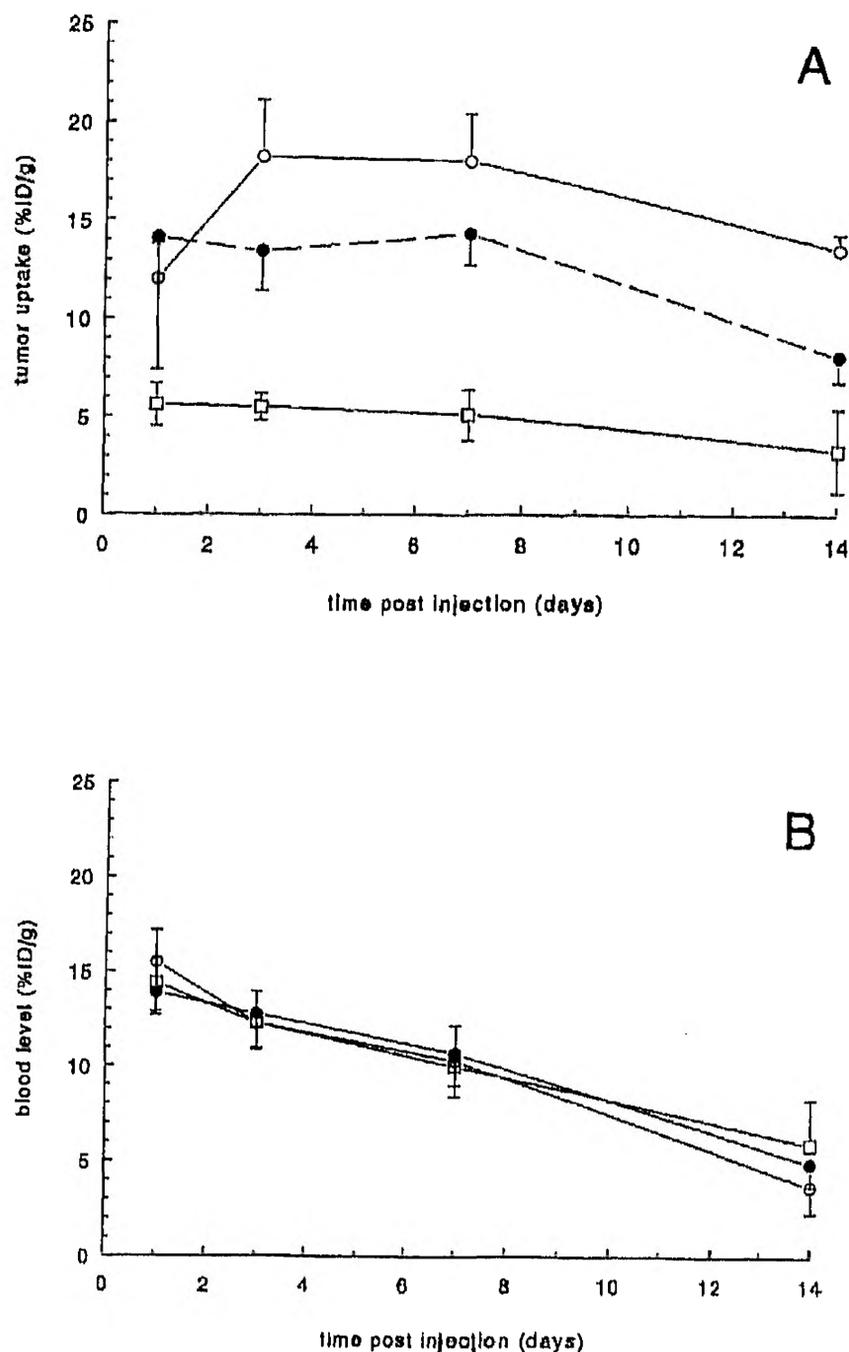


Figure 2. Tumour uptake (A) and blood levels (B) of radioiodinated MOv18 IgG (○), OC/TR IgG (●) and the control (anti-CD3 x mismatch) biMAb (□) after intravenous administration in mice subcutaneously bearing ovarian carcinoma.

7.2), 50 μ l enzymehead suspension and 500 μ Ci 125 I (Amersham International, UK) or 131 I (Medgenix, Fleurus, Belgium). The reaction was initiated by adding D(+) glucose to a final concentration of 0.3%. Following 15 minutes incubation, the reaction mixture was applied on a Sephadex G-25 column (Pharmacia, Sweden) and eluted with PBS, 0.5% BSA. The fractions containing the labelled antibody were pooled and used for *in vivo* studies within 16 hours. The amount of free iodine in the preparations was determined by instant thin layer chromatography (ITLC).

The immunoreactivity against the ovarian carcinoma-associated antigen gp38 (MOv18 reactivity) and/or against the CD3/TCR complex (anti-CD3/TCR reactivity) of the radioiodinated antibody preparations was determined on live Hela cells or Jurkat cells, respectively. A fixed amount of labelled antibody (10,000 cpm) was incubated with increasing numbers of cells (0.6×10^6 - 10×10^6) in 0.5 ml binding buffer (RPMI medium, 0.5% BSA, 50 mM HEPES, 0.05% NaN_3). After 4h of incubation at 4°C, the radioactivity in the pellet was counted in a well-type gamma counter. A duplicate of the lowest cell concentration was incubated in the presence of excess unlabelled antibody to correct for nonspecific binding. The data were graphically analysed in a modified Lineweaver-Burke plot: a double inverse plot derived from the

Table IIa. Tissue distribution of intravenously administered 125 I- MOv18 IgG in nude mice with s.c. Hela tumours (% ID/g, mean \pm SD, n=4).

Organ	Days post injection			
	1	3	7	14
Blood	15.5 \pm 2.6	12.3 \pm 1.4	10.3 \pm 1.3	3.7 \pm 1.4
Muscle	1.5 \pm 0.2	1.1 \pm 0.1	0.9 \pm 0.1	0.3 \pm 0.1
Liver	3.9 \pm 1.0	2.7 \pm 0.5	2.6 \pm 0.2	1.0 \pm 0.4
Intestine	1.9 \pm 0.2	1.3 \pm 0.2	1.1 \pm 0.2	0.4 \pm 0.1
Kidney	4.8 \pm 0.9	3.5 \pm 0.2	3.0 \pm 0.3	1.1 \pm 0.4
Tumour	12.0 \pm 1.8	18.2 \pm 2.9	18.0 \pm 2.4	13.5 \pm 0.8

Table IIb. Tissue distribution of intravenously administered 125 I- OC/TR IgG in nude mice with s.c. Hela tumours (% ID/g, mean \pm SD, n=4).

Organ	Days post injection			
	1	3	7	14
Blood	13.9 \pm 1.2	12.8 \pm 1.8	10.7 \pm 2.3	4.9 \pm 1.2
Muscle	2.2 \pm 0.9	1.4 \pm 0.2	1.0 \pm 0.2	0.5 \pm 0.1
Liver	4.7 \pm 1.3	3.6 \pm 0.7	2.6 \pm 0.7	1.1 \pm 0.3
Intestine	2.4 \pm 0.9	1.7 \pm 0.3	1.2 \pm 0.2	0.5 \pm 0.2
Kidney	5.1 \pm 1.9	3.7 \pm 0.7	3.1 \pm 0.8	1.4 \pm 0.3
Tumour	14.1 \pm 6.7	13.4 \pm 2.0	14.3 \pm 1.6	8.1 \pm 1.3

Table IIc. Tissue distribution of intravenously administered 131 I- anti-CD3 x mismatch IgG in nude mice with s.c. Hela tumours (% ID/g, mean \pm SD, n=8).

Organ	Days post injection			
	1	3	7	14
Blood	14.4 \pm 2.8	12.3 \pm 1.7	10.0 \pm 2.2	5.9 \pm 2.4
Muscle	1.6 \pm 0.2	1.2 \pm 0.1	0.9 \pm 0.2	10.6 \pm 0.2
Liver	4.2 \pm 1.0	3.2 \pm 0.6	2.8 \pm 0.6	1.7 \pm 0.9
Intestine	2.0 \pm 0.5	1.6 \pm 0.2	1.2 \pm 0.2	0.7 \pm 0.3
Kidney	4.3 \pm 0.8	3.8 \pm 0.7	3.1 \pm 0.6	1.8 \pm 0.8
Tumour	5.6 \pm 1.1	5.5 \pm 0.7	5.1 \pm 1.3	3.3 \pm 2.1

conventional binding plot (specifically bound activity versus cell concentration). The immunoreactive fraction was calculated by linear extrapolation to the Y-axis abscissa as described by Lindmo *et al* (20).

In vitro binding assays. The relative affinities of the unlabelled antibody preparations were compared in a homologous displacement assay. A serial dilution of cold antibody (0.01 - 100 μ g/ml) was incubated with

Hela cells (8×10^5 cells/vial) in the presence of a fixed amount of tracer antibody (15,000 cpm) in binding buffer. After overnight incubation at 4°C the cells were centrifuged (5 min, 2000 g), the supernatant was aspirated and the activity in the pellet was determined. The fraction of maximal bound tracer antibody (absence of cold competitor antibody) was plotted *versus* the concentration of cold antibody added. Relative affinity constants were calculated as the reciprocal concentration of unlabelled antibody required for 50% displacement of the radiolabelled antibody (21).

The affinities of the radioiodinated MOv18 and OC/TR preparations were compared in a Scatchard analysis (22). Briefly, live Hela cells were washed once with cold binding buffer and incubated with serially diluted ($10 - 10,000$ pM) ^{125}I -labelled antibody. Each vial was counted in the gamma counter to determine the total amount of activity added. For each antibody concentration the nonspecific binding was determined by incubating a duplicate sample in the presence of at least 500 fold excess of unlabelled antibody. After overnight incubation at 4°C cells were centrifuged (5 min, 2000 g), the supernatant was aspirated and the activity in the pellet was determined. The data were analysed by nonlinear weighted regression analysis.

Subcutaneous ovarian carcinoma model. The subcutaneous (s.c.) tumour model was established in nude mice by s.c. injection of 1×10^7 Hela cells. Tumours were resected aseptically, minced into small pieces of 2-3 mm diameter and serially transplanted subcutaneously at the left lumbar region of female BALB/c nude mice. Four to six weeks after transplantation, when tumours measured approximately 5 mm in diameter, mice were used for biodistribution studies.

Biodistribution studies. Female nude mice with s.c. Hela xenografts received 0.2 ml of a solution containing both ^{125}I - MOv18 IgG ($10 \text{ Ci}/10 \mu\text{g}$) and ^{131}I - control IgG (mismatch x anti-CD3) ($10 \mu\text{Ci}/10 \mu\text{g}$) via the tail vein. Another set of animals received a solution containing a mixture of both ^{125}I - OC/TR IgG ($10 \mu\text{Ci}/10 \mu\text{g}$) and ^{131}I - control IgG (anti-CD3 x mismatch) ($10 \mu\text{Ci}/10 \mu\text{g}$) via the tail vein. At least four mice were used per data point. The biodistribution of the radioiodinated antibodies was monitored during 14 days p.i., allowing not only the comparison of tumour accumulation (0 - 3 days), but also of tumour retention (3 - 14 days p.i.) of the biMAb and its parental antibody. The mice were bled under ether anaesthesia at 1, 3, 7 and 14 days post injection (p.i.). After cervical dislocation mice were dissected. Blood, liver, kidneys, intestine, muscle and tumour were removed. Tissues were weighed and radioactivity was measured in a well-type gammacounter (LKB-Wallac, Finland). A correction for scatter of ^{131}I in the ^{125}I channel was made. Tissue uptake was expressed as percentage of the injected dose per gram tissue (% ID/g).

Statistical analysis. Results are presented as mean \pm standard deviation. Differences in tissue uptake of the three antibody preparations (MOv18, OC/TR, control) were evaluated by Student's t-test.

Results

Radiolabelling and quality control. The labeling efficiency of the radioiodinations according to the enzymobead method was always between 60 and 80%. ITLC analysis of the radioiodinated preparations showed that more than 95% of the iodine-labels was protein-bound after Sephadex G-25 gel filtration. The immunoreactive fractions of the radiolabelled preparations used in the biodistribution studies are presented in Table I. The anti-tumour reactivity (55%) and the anti-CD3 reactivity (76%) of the bispecific MAb OC/TR were in the same range as the reactivity of the parental antibodies

(61% and 81%, respectively). In addition, the control antibody (anti-CD3/TCR x mismatch) revealed an immunoreactive fraction of 69% for the anti-CD3/TCR reactivity, combined with nondetectable anti-tumour reactivity, confirming its monospecific reactivity.

In vitro binding assays. The displacement of ^{125}I -labelled MOv18 binding to Hela cells with cold MOv18 is shown in Figure 1A. In this assay the unlabelled MOv18 antibody displays an apparent affinity of $3.0 \times 10^8 \text{ M}^{-1}$. The apparent affinity of the biMAb OC/TR in the same experiment is 4 times lower ($7.5 \times 10^7 \text{ M}^{-1}$).

The Scatchard analysis of the tumour cell binding of both antibody preparations is depicted in Figure 1B. The affinity constant of the radioiodinated MOv18 antibody was $2.04 \times 10^9 \text{ M}^{-1}$, while the affinity of the OC/TR biMAb was 7 times lower: $2.86 \times 10^8 \text{ M}^{-1}$. This would indicate that the loss of bivalent antigen binding of the bispecific OC/TR antibody causes a reduction of the affinity by a factor 7. The x-axis intercepts indicate that MOv18 could bind a maximum of 5.3×10^6 antigenic determinants on each Hela cell, while OC/TR could bind 4.5×10^6 determinants, confirming that both antibodies recognize the same epitope on the Hela cells.

Biodistribution studies. The blood clearance data are presented in Figure 2B. The blood clearance of the OC/TR biMAb was similar to the clearance of the parental MOv18 antibody and the control antibody. The elimination half-life ($T_{1/2\beta}$) of the three antibody preparations was 9 days. The label uptake of the normal tissues examined was lower than the blood level, but clearance of the radiolabel from these tissues followed the same pattern (Table II). Both for MOv18 and OC/TR the tumour uptake was higher than the blood level from three days p.i. onwards. In contrast, for the control antibody (anti-CD3/TCR x mismatch biMAb) tumour uptake was lower than the blood level at all time points. Tumour uptake of MOv18 and OC/TR plateaued between 3 and 7 days p.i. at 18 ± 3 and 14 ± 2 % ID/g, respectively (Figure 2A). At all time points, tumour uptake of both MOv18 as well as OC/TR was significantly higher ($p < 0.05$) than the tumour uptake of the control antibody, indicating that both antibody preparations showed specific, i.e. antigen-binding dependent, tumour localization. In addition, MOv18 tumour uptake was significantly higher than OC/TR tumour uptake from 3 days p.i. onwards ($p < 0.05$).

Discussion

The present study shows that the bispecific MOv18 x anti-CD3/TCR MAb OC/TR specifically localizes in human ovarian carcinoma xenografts in nude mice. However, the tumour uptake of the OC/TR biMAb was significantly lower than the tumour uptake of the parental MOv18 antibody. This reduction in tumour uptake can not be ascribed to a

more rapid blood clearance of the biMAb, since the blood clearance of both antibodies was identical. Most likely, the reduced tumour uptake of the biMAb is a result of its reduced affinity. Several studies in nude mouse models have demonstrated that antibody affinity is one of the important factors that determine tumour accumulation (23,24). This relationship is elegantly demonstrated in the comparative biodistribution experiments of a series of six second generation anti-TAG72 antibodies in nude mice with LS174T xenografts (25). It is known that the affinity of a monoclonal antibody as calculated from a Scatchard analysis is dependent not only on the antibody used, but also on other experimental conditions, *e.g.* antigen density on the target cell (26), assay volume (27) and ionic strength (28). Therefore, the absolute value of the affinity constant of an antibody has only limited value. This could also explain the fact that the K_a for MOv18 as derived from our Scatchard analysis is significantly different from the K_a reported by Miotti *et al* (15). Their determination on OvCa432 cells under different assay conditions revealed a 10 times lower affinity constant ($2.0 \times 10^8 \text{ M}^{-1}$).

Nevertheless, our results show that the OC/TR biMAb has a relatively lower affinity than the MOv18 antibody. Our observation that the loss of bivalency causes a reduction in affinity by a factor 4-7 is in line with other reports comparing the affinity of monovalent and bivalent antibody preparations (29,30). Van Dijk *et al* (29) studied the biodistribution of the anti-RCC x anti-CD3 biMAb in RCC xenografted nude mice. Tumour uptake of the biMAb was not significantly different from the tumour uptake of the parental anti-RCC antibody. From their data it appeared that the tumour uptake of an anti-tumour antibody is more dependent on antibody size (*i.e.* the blood clearance rate) than antibody affinity. However, in this study tumour uptake was monitored until 48 hr p.i., and possible differences in tumour retention between both antibody preparations could not be evaluated.

In patients the biodistribution of i.v. administered OC/TR biMAb might be significantly altered by the interaction of the biMAb with T-cells. However, it is difficult to study this phenomenon with this biMAb in an animal model, not only because nude mice lack mature T-cells, but also because the OC/TR biMAb does not cross react with murine T lymphocytes. It would be interesting to study the tumour uptake of an anti-T-cell x anti-tumour biMAb in a syngeneic model.

In conclusion, our data indicate that the biMAb OC/TR can preferentially localize in MOv18-positive tumours. Its reduced affinity as compared to the parental MOv18 antibody most likely caused decreased tumour uptake. As indicated in the introduction, systemic administration of biMAbs may have some important advantages in trying to redirect endogenous T-cells towards a tumour. A clinical study to investigate the tumour targeting potential and the therapeutic efficacy of OC/TR F(ab')₂ in ovarian cancer patients has been initiated (31).

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