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Rapid elimination of mouse/human chimeric monoclonal antibodies in nude mice

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Abstract At our laboratory we are currently evaluating the suitability of mouse/human chimeric monoclonal antibodies (cmAb) for use in radioimmunotherapy of patients with head and neck squamous cell carcinoma (HNSCC). We have developed cmAb containing the human constant IgG1 domain and the variable domains of murine mAb (mmAb) E48 and U36 respectively. We considered the tumour-bearing nude mouse to be a well-validated model for a first testing of the targeting capabilities of these cmAb in comparison with the mmAb. Therefore, 3 μg cmAb E48 (labelled with ^{125}I) and 3 μg mmAb E48 (labelled with ^{131}I) were simultaneously injected into HNSCC-bearing nude mice and, at various assay times, mAb uptake in blood and other tissues was assessed. Remarkably, while in roughly 50% of the animals the biodistribution of the conjugates was similar, in the other animals cmAb E48 showed a much higher blood clearance than mmAb E48. This resulted in a lower tumour uptake of cmAb E48 in comparison with mmAb E48. To determine whether this phenomenon was related to mAb E48 or to the animal model, other cmAb-mmAb combinations were evaluated in the same way: cmAbs SF-25, 17-1A and U36 (all IgG1) were tested and all showed a rapid elimination in about 50% of the animals. Besides a decrease in blood concentration, an increase of cmAb levels in liver and spleen was observed within 24 h after injection. Isotype-specific enzyme-linked immunosorbent assays showed that mice that demonstrated a rapid elimination of cmAb from the blood had much lower endogenous IgG1, IgG2b and IgG3 titres than mice showing normal clearance. IgG2a levels were low in all mice. Biodistribution experiments with 3 μg chimeric 17-1A isoforms showed high blood clearance in a proportion of the mice for IgG1, IgG3 and IgG4, but not for IgG2. Increase of the cmAb dose to 100 μg resulted in a similar cmAb and mmAb biodistribution in all mice. Moreover, the biodistribution of the $F(ab')_2$ fragment of an IgG1 cmAb was similar for all mice in contrast to that of coinjected whole IgG. On the basis of these results it can be hypothesized that, in mice with low endogenous IgG titres, cmAb with specific isotypes are rapidly removed from the blood (and ultimately from the body) by mediation of Fc-binding receptors. Apparently, in mice with high endogenous IgG titres or in mice receiving a high cmAb dose, these receptors are saturated. Furthermore, the rapid elimination of cmAb from nude mice, which may occur after injection at a low dose, is a phenomenon related to the nude mouse model.

Key words Chimeric antibodies · Tumour targeting · Tumour-bearing nude mice · Biodistribution · Squamous cell carcinoma

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Introduction

We previously described the development of a panel of mouse mAb (mmAb) directed against head and neck squamous cell carcinoma (HNSCC) [14, 15, 17]. Radioimmunoscintigraphy studies using mAb labelled with ^{99m}Tc demonstrated the capability of mmAb E48 and U36 to target HNSCC selectively in patients [3, 5]. Currently we are assessing the suitability of mouse/human chimeric monoclonal antibodies (cmAb) for use as targeting mAb in radioimmunotherapy (RIT). We have developed cmAb containing the human constant IgG1 domain and the murine variable domains of mmAb E48 or U36 [1]. These cmAb are expected to have the advantage of low immunogenicity in patients and of additional therapeutic effectiveness by mediating antibody-dependent cellular cytotoxicity [9, 20]. A prerequisite for use of the cmAb in antibody-targeted therapy is the preservation of the targeting properties and pharmacokinetics of the mmAb. To assess these characteristics, the biodistribution of cmAb E48, in comparison with

mmAb E48, was determined in nude mice bearing HNSCC tumour xenografts.

The tumour-bearing athymic nude mouse is a well-validated model for the preclinical in vivo evaluation of mAb and mAb conjugates. Although there has been substantial debate regarding the relevance of the model to predict clinical response, it is regarded as a suitable model for the testing of a number of radioimmunoconjugate characteristics of importance for clinical use. After analysis of several animal RIT studies, Wessels [22] pointed out that the applicability of the mouse model should be limited to: (a) initial radiolabelled antibody screening, (b) preliminary efficacy studies for target/non-target specificity, (c) evaluation of the suitability of a radiolabel, (d) toxicity studies, (e) comparison of the efficacy of various radiolabels in combination with different antibody types and fragments and tumour models and (f) basic dosimetry comparisons using animal target/non-target ratios extrapolated to humans.

With these limitations in mind, we considered the tumour-bearing nude mouse model suitable for the assessment of the targeting capabilities of cmAb E48 and U36. To compare both mAb E48 forms directly, the cmAb E48 (labelled with ^{125}I) and the mmAb E48 (labelled with ^{131}I) were injected simultaneously. For the initial comparison of the two mAb a tracer dose of both cmAb and mmAb was used. We report here an increased blood clearance of a low protein dose of cmAb in roughly 50% of the mice. We conclude that this effect is related to the nude mouse model. Factors that influence the blood clearance of cmAb like mAb isotype and mAb dose will be described, and measures to prevent this large variation in pharmacokinetics of cmAb in nude mice will be outlined. Moreover, the consequences of this phenomenon for the preclinical assessment of the targeting capabilities of cmAb will be discussed.

Material and methods

Monoclonal antibodies

All mAb used in these studies are considered candidates for tumour targeting in patients. The production and selection of mAb E48, mAb U36 and mAb K928 have been described previously [14, 15, 17].

mAb E48 recognizes a 16- to 22-kDa surface antigen, which is not shed from the membrane, and in normal tissues is present in stratified and transitional epithelium only. The antigen defined by mAb E48 was shown to be expressed by 94% of primary HNSCC (n = 196). In 70% of these tumours the antigen was expressed by the majority of cells. A comparable reactivity pattern was observed in 31 tumour-infiltrated lymph nodes from neck dissection specimens [4].

mAb U36 recognizes a 200-kDa surface antigen that in normal tissues is only present in stratified and transitional epithelium [17]. The antigen defined by mAb U36 was shown to be expressed by 99% of primary HNSCC (n = 196). In 96% of these tumours the antigen was expressed by the majority of cells. A comparable reactivity pattern was observed in 31 tumour-infiltrated lymph nodes from neck dissection specimens [4].

mAb K928 recognizes a 50- to 55-kDa protein expressed on the outer cell surface of head and neck as well as lung, breast and ovary carcinomas [15]. Among the normal tissues mAb K928 was found to be reactive with suprabasal cells of stratified squamous epithelium, pneumocytes, duct and acinar cells of salivary glands, bile ducts and canaliculi in liver, oviduct epithelium, all epithelial cells in mammae and urinary bladder and in cells of renal tubuli.

mAb SF-25 was developed and characterized by Takahashi et al. [21]. It recognizes a 125-kDa protein expressed by adenocarcinomas of the colon and among normal tissues in the distal tubule of the kidney.

mAb 17-1A was developed by Koprowski et al. [11] and recognizes an antigen expressed by endometrial, colon, thyroid, prostate and lung carcinoma. In normal tissues reactivity was observed with kidney, liver, thyroid and colon [4, 7].

mAb MOv18 was developed by Miotti et al. [13] and recognizes the human folate-binding protein that is preferentially expressed by gynaecological carcinomas. Some reactivity has been found with normal epithelium in the lung, ducts of the pancreas, epithelial cells of the salivary gland and epithelium of the fallopian tube [19]. cmAb 17-1A isotype variants IgG1, IgG2, IgG3 and IgG4 were developed by Steplewski et al. [20].

mmAb E48 and SF-25 are of the IgG1, mmAb K928 of the IgG2b and mmAb 17-1A of the IgG2a isotype. Chimeric mAb E48, U36, SF-25 and MOv18 are all of the human IgG1 isotype. The mmAb 17-1A, cmAb 17-1A (IgG1, IgG2, IgG3 and IgG4), cmAb SF-25 and cmAb MOv18 (IgG1 and the $F(ab')_2$ fragment of IgG1) were provided by Centocor Inc., Malvern, Pa., USA.

mmAb E48, K928, SF-25 and 17-1A and cmAb E48, U36 and SF-25 were used to evaluate the accelerated blood clearance of cmAb. Furthermore, cmAb 17-1A was used to assess the influence of the cmAb isotype on blood clearance. cmAb MOv18 IgG1 and $F(ab')_2$ were used to assess the influence of the Fc portion of the mAb on the blood clearance.

Radioiodination of antibodies

Iodination of IgG or $F(ab')_2$ was performed essentially as described by Haisma et al. [8]. Samples comprising 200–500 μg mAb IgG or $F(ab')_2$ in 500 μl phosphate-buffered saline, pH 7.4, and 2–5 μl ^{125}I or ^{131}I (100 mCi/ml; Amersham, Aylesbury, England) were mixed in a vial coated with 75 μg Iodogen (Pierce, Oud Beijerland, The Netherlands). After 10 min incubation at room temperature, a sample was taken to determine the amount of iodine incorporated by means of trichloroacetic acid precipitation. Free iodine was removed by gel filtration on a Sephadex-G25 column (Pharmacia-LKB, Woerden, The Netherlands). After removal of unbound $^{125}I/^{131}I$, the radiochemical purity always exceeded 96%. The specific activity of the various iodinated mAb ranged from 0.05 μCi/μg to 4.5 μCi/μg.

Immunoreactivity assay

In vitro binding characteristics of the various mAb labelled with ^{131}I or ^{125}I were determined in an immunoreactivity assay as described previously [3]. In short, cells expressing the appropriate antigen were fixed according to their specific protocols.

For binding assays of mAb E48, K928 and SF-25 UM-SCC-22A cells (kindly provided by Dr. T.E. Carey, Ann Arbor, Mich.) fixed in 2% paraformaldehyde were used; binding assays of cmAb MOv18 were performed using OVCAR3 cells fixed in 2% paraformaldehyde; for mAb U36 UM-SCC-11B cells (also provided by Dr. T.E. Carey) were fixed in 0.1% glutaraldehyde; for binding assays of mAb 17-1A unfixed cells of the colon carcinoma cell line WiDr-29 (kindly provided by Dr. H.H. Haisma, Free University Hospital, Amsterdam) were used. Six serial dilutions (ranging from 5 × 106 cells per tube to 3.1 × 105 cells per tube) were prepared with 1% bovine serum albumin (BSA) in PBS. mAb labelled with 10 000 cpm ^{125}I or ^{131}I was added to the tubes. The samples were incubated overnight at room temperature (mAb E48, U36, K928, MOv18 and SF-25) or for 2 h at 4 °C (mAb 17-1A). Excess unlabelled mAb IgG was added to the last sample to determine non-specific binding. Cells were spun down and radioactivity in the pellet and supernatant was determined in a gamma counter (LKB-Wallac 1282 CompuGamma, Kabi Pharmacia, Woerden, The Netherlands) and the percentage bound and free radioactivity was

calculated. The data were graphically analysed in a modified Lineweaver-Burk plot and the immunoreactive fraction was determined by linear extrapolation to conditions representing infinite antigen excess. The immunoreactive fraction of the various iodinated mAb preparations always exceeded 68%.

Biodistribution studies

The in vivo biodistribution characteristics of a mAb were determined in tumour-bearing or tumour-free female nude mice (Hsd, specified-pathogen-free athymic *nu/nu*, 25–32 g, Harlan/CPB, Zeist, The Netherlands). Tumour-bearing nude mice, as used for the experiments shown in Fig. 1 and Table 1, had two human HNSCC xenografts implanted subcutaneously. At the time of injection the tumour size was 226 ± 175 mm^3 (mean $\pm$ SD). In several experiments, tumour-free nude mice were used to avoid the influence of tumour uptake on the biodistribution characteristics of the mAb. Mice were 8–10 weeks old at the time of the experiments. ^{125}I- and ^{131}I-labelled mAb were intravenously injected in 0.9% NaCl. Injection volumes were 100 µl, while the total immunoglobulin dose ranged from 6 µg to 100 µg. At indicated assay times after injection, mice were anaesthetized, bled, killed and dissected. The urine was collected and the following organs were removed: liver, spleen, kidney, stomach, ileum, colon, sternum, muscle, lung. After weighing, radioactivity in organs, organ contents, blood and urine was counted in a dual-isotope gamma counter (Wallac CompuGamma 1282). Standards were included to correct for the contribution of both isotopes in the various window settings.

Radioactivity uptake in these tissues was calculated as the percentage of the injected dose per gram of tissue (%ID g^{-1}). Clearance of cmAb from the blood of mice coinjected with cmAb and the corresponding mmAb is arbitrarily called "high clearance" when the ratio of radioactivity level in the blood for cmAb to that for mmAb is lower than 0.7, irrespective of the time interval after coinjection of the cmAb and mmAb.

All animal experiments were performed according to the principles of laboratory animal care (NIH publication 85-23, revised 1985) and the Dutch national law *Wet op de Dierproeven* (Stb 1985, 336). Until the day of mAb administration mice were routinely housed under specified-pathogen-free conditions, in sterile cages standing in a humidity- and temperature-controlled clean room, classification 2000 according to the Federal Standard 209d. On the day of injection, mice were transported to the Radio Nuclide Center, Vrije Universiteit, and sterile radioimmunoconjugates were administered under aseptic conditions in a laminar-flow hood.

Isotype-specific enzyme-linked immunosorbent assay (ELISA)

To determine the endogenous mouse IgG levels, blood was heparinised, the cells removed by centrifugation and the plasma frozen until use in the ELISA. Ninety-six well U-bottomed plates were coated overnight at 4 °C with 100 µl goat heavy-chain-specific antisera against mouse IgG1, IgG2a, IgG2b and IgG3 (dialysed, fractionated serum; Sigma, St. Louis, Mo., USA) in carbonate buffer (0.01 M $NaHCO_3 \cdot H_2O$ adjusted to pH 9.6 with 0.025 M $Na_2CO_3 \cdot 10H_2O$). After three washes with phosphate-buffered saline (PBS)/0.05% Tween 20, plates were incubated for 60 min with 200 µl blocking solution (4% bovine serum albumin, BSA, in PBS) and subsequently 200 µl/well mouse plasma was added. Twelve serial dilutions were made of the mouse sera and added to the plates, starting with a 100-fold dilution; subsequent additions comprised 5-fold dilution steps. The assay buffer was used as a control. After incubation for 60 min at room temperature, three washes with PBS/0.05% Tween 20 and two with PBS, 150 µl peroxidase-labelled rabbit anti-(mouse immunoglobulin) (Dako, Lostrup, Denmark) diluted 1:400 in PBS/1% BSA was added. After incubation for 60 min at room temperature the plates were washed again three times with PBS/0.05% Tween 20 and twice with PBS, and 200 µl substrate solution containing 30 mg *o*-phenylenediamine·2HCl (2 tablets; Sigma, St. Louis, Mo., USA) in 20 ml citric acid/phosphate buffer (0.02 M $Na_2HPO_4 \cdot 2H_2O$ adjusted to pH 5.5 with 0.01 M citric acid) plus 45 µl 10% H_2O2 solution was added per well; the mixture was allowed to react at room temperature for 30 min. The reaction was stopped by adding 50 µl 2 M H_2SO_4, and the absorbance at 490 nm was measured in a plate reader. The reciprocal plasma dilution yielding an absorbance of 1.0 was used to quantify the endogenous IgG levels in the plasma. As standards, mouse IgG solutions of 1 mg/ml were used (IgG1: mAb E48, IgG2a: mAb αCD10, IgG2b: mAb K928, IgG3: mAb C153). Plasma concentrations are provided as means of triplicate analyses.

Results

Biodistribution of cmAb E48 and mmAb E48

The observation of a high blood clearance of chimeric mAb was made in an experiment in which the biodistribution of cmAb E48 and mmAb E48 was compared by coinjection of 3 µg of ^{131}I-labelled cmAb E48 (8.2 µCi) and 3 µg ^{125}I-labelled mmAb E48 (3.0 µCi) in HNSCC-bearing nude mice. The biodistribution was determined at 1, 2, 3, 5 and 7 days p.i. The calculated average radioactivity uptake (%ID g^{-1}) and standard deviations are given in Table 1. The mean level of cmAb E48 in blood was lower than the level of mmAb E48, with cmAb:mmAb ratios of 0.53, 0.67, 0.71, 0.71 and 0.27 at 1, 2, 3, 5 and 7 days p.i. respectively. Although the mean levels of the cmAb E48 in blood and all other organs were lower than the mean levels of mmAb E48, indicating an increased total body clearance of cmAb E48 in comparison with mmAb E48, the levels in the tumour were comparable. It is tempting to conclude that, on the basis of the biodistribution characteristics in mice, cmAb E48 is better suited for clinical application than mmAb E48. Owing to the large variation in blood levels of both mAb, no accurate estimates of the $t_{1/2}\alpha$ or $t_{1/2}\beta$ could be made.

A relatively large interanimal variation in the tissue levels of cmAb E48 was found in comparison with the levels of mmAb E48 (Table 1). Analysis of individual mice revealed that high clearance of cmAb E48 in comparison with mmAb E48 occurred in only a proportion of the animals. Because the elimination rates could not be determined accurately, the cmAb:mmAb ratio was calculated for each individual mouse and used to discriminate between mice with normal and high blood clearance of the cmAb. From Fig. 1, showing cmAb:mmAb levels in blood of individual mice 1–7 days after injection, it appears that, in 12 out of 23 mice, the cmAb was rapidly eliminated from the blood (ratio < 0.7) while in the other animals comparable levels were found for the cmAb and the mmAb (ratio > 0.7). At all assay times after injection a large variation in the blood levels was found.

In high-clearance mice the cmAb activity levels in the liver and spleen were relatively high 24 h after injection. For these mice liver:blood and spleen:blood ratios of respectively 0.83 and 1.01 were found for cmAb E48, while for normal-clearance mice these ratios were 0.19 and 0.20 respectively. At later assay times these ratios were similar for the high- and normal-clearance groups of mice. From this observation it can be concluded that a proportion of mice show a high blood clearance of cmAb E48, which

is accompanied by an increase in liver and spleen levels. As a result of this high blood clearance, tumour levels of cmAb were lower in high-clearance mice than in normal-clearance mice (8.0 and 29.3 %ID g^{-1} respectively 24 h after injection). The liver and spleen uptake takes place within 24 h after injection and is not observed for mmAb E48. For mmAb E48 the liver:blood and spleen:blood levels 24 h after injection were 0.20 and 0.18 respectively in normal-clearance mice, while in the mice demonstrating high clearance of cmAb these ratios were 0.23 and 0.18 respectively. The tumour levels of mmAb E48 were comparable in the two groups: 19.4 %ID g^{-1} in normal clearance mice and 18.8 %ID g^{-1} in high-clearance mice.

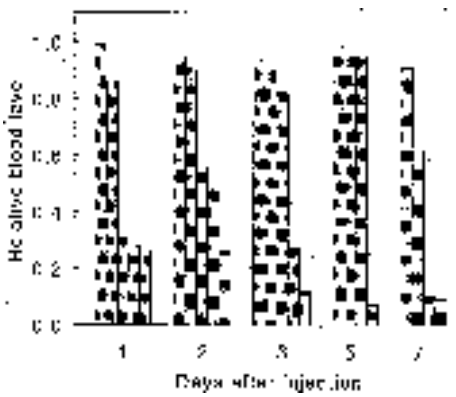

Fig. 1 Relative blood levels of mouse/human chimeric antibody (cmAb) E48, in nude mice bearing human head and neck squamous cell carcinoma xenographs, 1, 2, 3, 5 and 7 days after injection. The levels of all individual mice are given, expressed as the cmAb:murine mAb (mmAb) ratio of the radioactivity uptake (% injected dose ID g^{-1}) after coinjection of 3 μg of ^{131}I-labelled cmAb E48 (8.2 μCi) and 3 μg of ^{125}I-labelled mmAb E48 (3.0 μCi)

Blood clearance of various IgG1 cmAb

To test whether the variable elimination rate of cmAb in nude mice is a general phenomenon, four cmAb of the IgG1 isotype were evaluated for their biodistribution in tumour-free nude mice. When available, the murine IgG1 counterpart was injected simultaneously as a control. On the basis of the comparison of cmAb and mmAb E48 at various assay times, the biodistribution at one assay time 1–7 days after simultaneous injection of a cmAb and a mmAb was considered to be sufficient to discriminate between mice with normal and rapid clearance of cmAb. The following injections were performed: 3 μg (12.2 μCi ^{125}I) cmAb E48 with 3 μg (5.3 μCi ^{131}I) mmAb E48, 5 μg (5.6 μCi ^{125}I) cmAb SF-25 with 5 μg (3.1 μCi ^{131}I) mmAb SF-25, 3 μg (0.8 μCi ^{131}I) mmAb K928, 5 μg (5.8 μCi ^{125}I) cmAb U36, and 5 μg (7.6 μCi ^{131}I) cmAb 17-1A. For cmAb U36 and cmAb 17-1A the levels of activity in blood and various organs were measured 72 h after injection. For cmAb/mmAb couples of E48 and SF-25 this was at 96 h and 24 h respectively. For mmAb K928, the levels were determined 24 h after injection. The relative blood levels are given in Fig. 2. These data show that a similarly large variation in the elimination of cmAb E48 was seen in tumour-free nude mice and in HNSCC-bearing mice. Four out of five mice had cmAb E48 blood levels that were substantially lower than that of the other mouse, while the blood levels of the mmAb E48 were within a much narrower range. In one mouse the blood level of the cmAb was comparable to that of the mmAb E48. This variation was also seen for cmAb SF-25, U36 and 17-1A, but not for mmAb SF-25 and mmAb K928.

Table 1 Biodistribution of coinjected ^{131}I-labelled cmAb E48 (3 μg, 8.2 μCi) and ^{125}I-labelled mmAb E48 (3 μg, 3.0 μCi) in mice bearing human head and neck squamous cell carcinoma xenographs. *mmAb* murine mAb, *cmAb* mouse/human chimeric mAb

	Radioactivity uptake (% injected dose/g tissue)				
Tissue	1 day	2 days	3 days	5 days	7 days
mmAb					
Blood	12.59 ± 3.68	7.37 ± 1.34	4.91 ± 2.55	2.62 ± 1.38	1.74 ± 1.27
Tumour	19.05 ± 5.59	24.88 ± 5.41	17.68 ± 5.39	18.34 ± 6.87	15.03 ± 5.34
Liver	2.78 ± 1.10	1.44 ± 0.25	1.14 ± 0.57	0.64 ± 0.27	0.37 ± 0.32
Spleen	2.33 ± 0.91	1.22 ± 0.19	0.91 ± 0.42	0.47 ± 0.20	0.30 ± 0.24
Kidney	3.19 ± 0.79	1.65 ± 0.17	1.18 ± 0.59	0.67 ± 0.30	0.36 ± 0.26
Lung	4.41 ± 1.74	2.48 ± 0.51	1.57 ± 0.76	0.86 ± 0.39	0.46 ± 0.34
Sternum	1.37 ± 0.31	0.67 ± 0.34	0.50 ± 0.20	0.28 ± 0.12	0.15 ± 0.09
Muscle	0.87 ± 0.19	0.57 ± 0.09	0.37 ± 0.13	0.17 ± 0.07	0.09 ± 0.05
cmAb					
Blood	6.64 ± 4.68	4.93 ± 2.98	3.50 ± 3.39	1.87 ± 1.69	0.47 ± 0.46
Tumour	16.51 ± 13.77	28.65 ± 16.27	22.55 ± 19.61	27.53 ± 18.90	11.37 ± 15.00
Liver	2.65 ± 1.05	1.25 ± 0.44	0.79 ± 0.65	0.47 ± 0.39	0.08 ± 0.12
Spleen	3.13 ± 1.68	1.56 ± 0.47	0.93 ± 0.57	0.44 ± 0.34	0.12 ± 0.09
Kidney	2.12 ± 0.74	1.19 ± 0.49	0.87 ± 0.77	0.54 ± 0.45	0.13 ± 0.15
Lung	2.82 ± 1.05	1.81 ± 0.81	1.12 ± 0.98	0.61 ± 0.49	0.14 ± 0.15
Sternum	1.20 ± 0.20	0.48 ± 0.30	0.37 ± 0.31	0.22 ± 0.18	0.06 ± 0.06
Muscle	0.54 ± 0.20	0.43 ± 0.19	0.27 ± 0.22	0.13 ± 0.10	0.04 ± 0.04

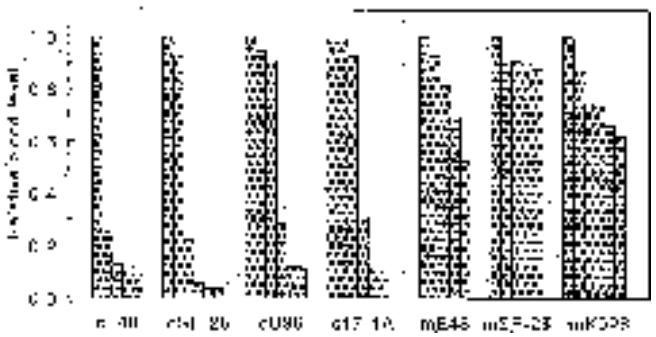

Fig. 2 Relative blood levels of four cmAb of the IgG1 isotype and three mmAb in tumour-free nude mice 1 day (cmAb SF-25, mmAb SF-25 and mmAb K928), 3 days (cmAb U36 and cmAb 17-1A) or 4 days (cmAb and mmAb E48) after injection. The following labelled mAb were injected: 3 μg ^{125}I-labelled cmAb E48 (12.2 μCi), 5 μg ^{125}I-labelled cmAb SF-25 (5.6 μCi), 5 μg ^{125}I-labelled cmAb U36 (5.8 μCi), 5 μg ^{131}I-labelled cmAb 17-1A (7.6 μCi), 3 μg ^{131}I-labelled mmAb E48 (5.3 μCi), 5 μg ^{131}I-labelled mmAb SF-25 (3.1 μCi); 3 μg ^{131}I-labelled mmAb K928 (0.8 μCi). The mmAb E48 and cmAb E48 were injected simultaneously as well as the mmAb and cmAb SF-25. The levels in all individual mice are given as the radioactivity uptake (%ID g^{-1}) divided by the uptake (%ID g^{-1}) of the mouse with the highest blood level in the group

Biodistribution of various isotypes of cmAb 17-1A

To determine if the increased blood clearance of cmAb is related to the isotype, four isotypes of chimeric 17-1A were tested for their biodistribution in nude mice. In three groups of six tumour-free nude mice 5 μg ^{131}I-labelled cmAb 17-1A of the human IgG1 isotype (7.6 μCi) was coinjected with 5 μg ^{125}I-labelled cmAb 17-1A of the human isotypes IgG2, IgG3 or IgG4 (6.7 μCi, 2.2 μCi and 5.7 μCi respectively). Three days after injection the levels of activity in blood and various organs were measured. The highest blood levels of cIgG1, cIgG2, cIgG3 and cIgG4 in the particular groups were 6.84, 14.98, 1.89, 9.21 %ID g^{-1}. No abnormal uptake was seen in any organ. In Fig. 3A–C the relative blood levels of both coinjected iodine labels are given for the individual mice. The levels are given as percentages of the highest blood level for each mAb isotype in the particular group to facilitate the comparison. A variation in blood levels similar to that of cIgG1 was found for the cIgG3 and cIgG4 isotypes (Fig. 3B, C). Furthermore, mice that demonstrated a fast elimination of cIgG1 also showed a fast elimination of these two isotypes. In contrast, the cIgG2 levels were similar for all mice tested although the blood levels of the coinjected cIgG1 did show a large variation in these mice (Fig. 3A).

Biodistribution of cmAb IgG1 and mmAb IgG2a at a low dose

A similar large variation in the blood clearance of mAb has been reported by Sharkey et al. for murine mAbs [18]. They found an extremely high blood clearance of mmAb of the IgG2a and IgG2b isotype in Swiss *nu*/*nu* mice after injection of a low mAb dose (less than 30 μg). Similar to the phenomenon described here, they found an increased elimination from the blood in only a proportion of the animals while in other mice of this strain the clearance was comparable to the clearance in other mice strains. They could not give an explanation for this phenomenon.

To test whether the effect described here is related to the phenomenon described by Sharkey et al., we compared the biodistribution of cmAb 17-1A IgG1 and mmAb 17-1A IgG2a by means of coinjection of 3 μg (6.9 μCi ^{125}I) cmAb 17-1A with 3 μg (9.9 μCi ^{131}I) mmAb 17-1A in tumour-free nude mice. The blood levels of the individual mice 24 h after injection are given in Fig. 4A. On the basis of the blood levels of mIgG2a, we divided the mice into two groups: a normal-clearance group consisting of mice 1, 2 and 3 and a high-clearance group consisting of mice 4, 5 and 6. The mean levels of activity in blood and other tissue are given for both groups in Fig. 4B for cmAb 17-1A IgG1 and in Fig. 4C for mmAb 17-1A IgG2a. It appears that high blood clearance of cmAb 17-1A IgG1 and mmAb 17-1A IgG2a occurred in the same mice and, in both cases, was accompanied by an increase of the activity levels in liver, spleen and ileum.

Influence of mAb dosage on the elimination of cmAb

To determine the influence of mAb dosage on the elimination of cmAb, 100 μg ^{125}I-labelled cmAb SF-25 (11.4 μCi) was injected together with 2 μg of ^{131}I-labelled mmAb E48 (8.7 μCi) as a reference in six nude mice, and the biodistribution was determined after 3 days. Levels of activity in blood and various organs are given in Fig. 5. The blood levels of the chimeric and murine mAb were similar and, for the whole group, only a small variation was found between animals. This demonstrates that a mAb dose of 100 μg is sufficient for complete saturation of the uptake of cmAb by the liver and spleen and thereby the fast elimination from the blood. This experiment was repeated with various cmAb/mmAb combinations with similar results.

Analysis of IgG titres in mouse plasma

The blood clearance of chimeric IgG1 was determined by coinjection of 5 μg ^{125}I-labelled cmAb SF-25 (5.6 μCi) and 5 μg ^{131}I-labelled mmAb SF-25 (3.1 μCi) and analysis of the distribution of the activity 24 h after injection in tumour-free nude mice. Plasma of both high-clearance (n = 3) and normal-clearance (n = 3) mice was analysed by means of HPLC on a size-exclusion column. For both groups of mice, the plasma did not contain high-molecular mass complexes containing iodine label. Comparison of endogenous IgG levels, assessed by means of an isotype-specific ELISA, in plasma of normal- and high-clearance mice, revealed much lower IgG levels in high-clearance mice (Fig. 6.). A 30-fold difference was seen for IgG1 while for IgG2b and IgG3 levels this difference was a factor

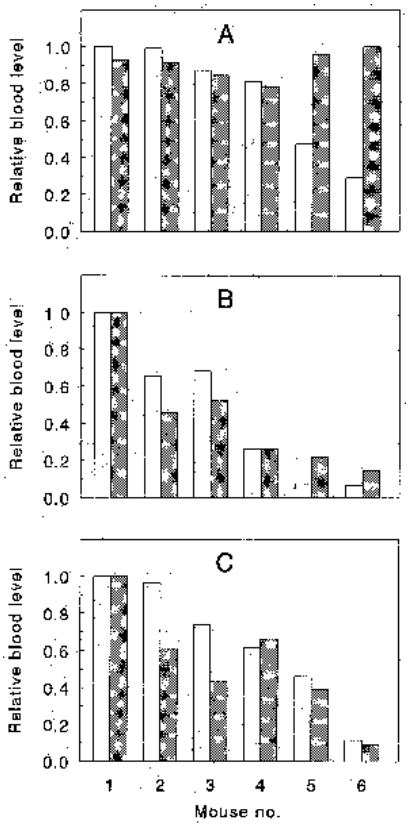

Fig. 3A–C Relative blood levels of various isotypes of cmAb 17-1A in tumour-free nude mice 3 days after injection. (**A**) 5 µg of IgG2 labelled with 6.7 µCi ^{125}I, (**B**) IgG3 labelled with 2.2 µCi ^{125}I or (**C**) IgG4 labelled with 5.7 µCi ^{125}I (*grey bars*) was injected simultaneously with 5 µg IgG1 labelled with 7.6 µCi ^{131}I (*open bars*). The blood levels of both isotypes in all individual mice are given, expressed as the radioactivity uptake (%ID g^{-1}) divided by the uptake (%ID g^{-1}) of the mouse with the highest blood level in the group

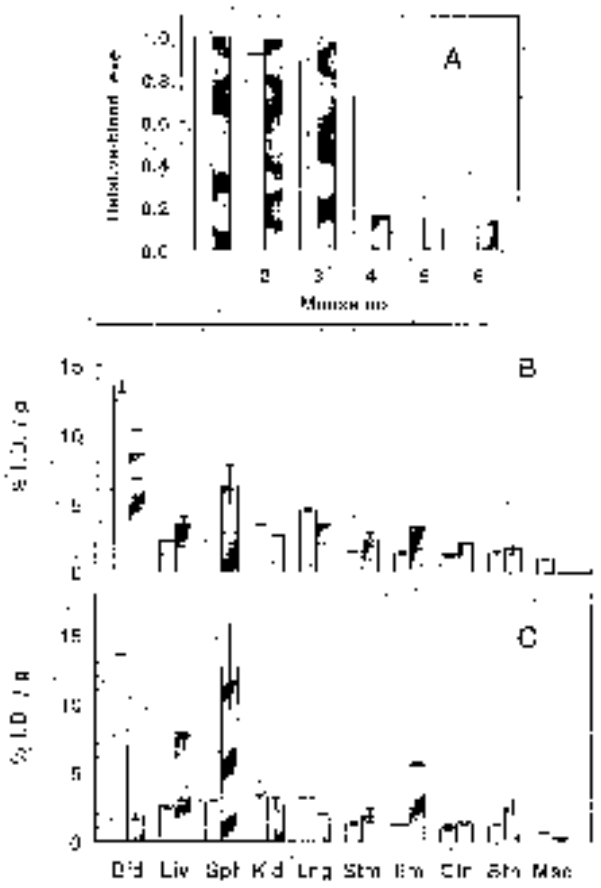

Fig. 4A–C Relative blood levels (**A**) of 3 µg ^{125}I-labelled cmAb 17-1A IgG1 (6.9 µCi; *open bars*) and 3 µg ^{131}I-labelled mmAb 17-1A IgG2a (9.9 µCi; *grey bars*) in tumour-free nude mice at 24 h after injection. The levels of both mAb in individual mice are given as the radioactivity uptake %ID·g^{-1} relative to the uptkae for the mouse with the highest blood level in the group (%ID g^{-1}: %ID g^{-1}). Biodistribution of (**B**) cmAb 17-1A IgG1 and (**C**) mmAb 17-1A IgG2a after simultaneous injection into tumour-free nude mice. On the basis of the blood levels given in **A**, the mice were divided into a high-clearance group (n = 3; *hatched bars*) and a normal-clearance group (n = 3; *open bars*). The mean blood and tissue levels are given for each group as radioactivity uptake (%ID g^{-1}). *Bld* blood, *Liv* liver, *Spl* spleen, *Kid* kidney, *Lng* lung, *Stm* stomach, *Ilm* ileum, *Cln* colon, *Stn* sternum, *Msc* muscle

14 and 6, respectively. No significant difference in IgG2a plasma levels between normal- and high-clearance mice was found.

Biodistribution of whole IgG1 and F(ab′)$_2$ fragment of cmAb MOv18 at a low dose

Eccles et al. described “non-specific” isotype-related accumulation of rat immunoglobulins in tumour, liver and spleen of immunocompetent and athymic syngeneic rats, which appeared mediated by Fc binding [6]. To test whether the Fc portion of the cmAb is important for the effect described herein, we compared the biodistribution of whole IgG1 and the F(ab′)$_2$ fragment of cmAb MOv18. A 3- µg sample of MOv18 IgG labelled with 3.4 µCi ^{125}I and 3 µg of MOv18 F(ab′)$_2$ labelled with 5.2 µCi ^{131}I were injected simultaneously in seven tumour-free nude mice. The relative blood levels 20 h after injection of all individual mice are given as percentages of the highest blood level in the group for the whole IgG and the F(ab′)$_2$ fragment in Fig. 7. While cmAb MOv18 IgG showed a large variation in elimination similar to that found for other IgG1 cmAb (Fig. 2), the cmAb MOv18 F(ab′)$_2$ blood levels were similar for all mice tested. These data indicate the involvement of the Fc portion in the rapid elimination of cmAb from the blood.

Discussion

In this study we describe the rapid elimination of mouse/human chimeric antibodies in nude mice in comparison with the mouse analogues of these cmAb. This phenomenon is observed in roughly half of the mice, while in the other half the blood levels of the cmAb and mmAb tested

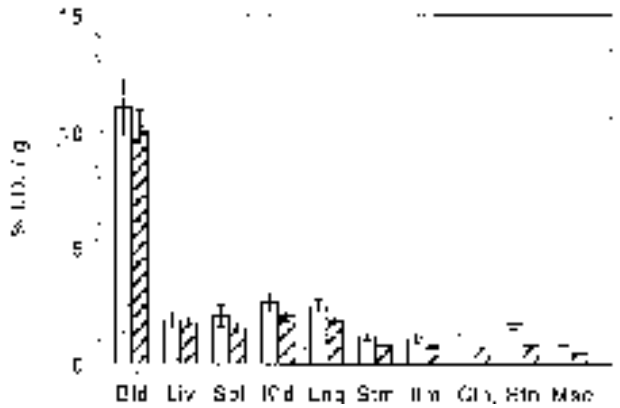

Fig. 5 Biodistribution of 100 μg ^{125}I-labelled cmAb SF-25 (11.4 μCi; *open bars*) and 2 μg ^{131}I-labelled mmAb E48 (8.7 μCi; *hatched bars*) after simultaneous injection into six nude mice. The mean blood and tissue levels are given for each group as radioactivity uptake (%ID g^{-1}). *Bld* blood, *Liv* liver, *Spl* spleen, *Kid* kidney, *Lng* lung, *Stm* stomach, *Ilm* ileum, *Cln* colon, *Stn* sternum, *Msc* muscle

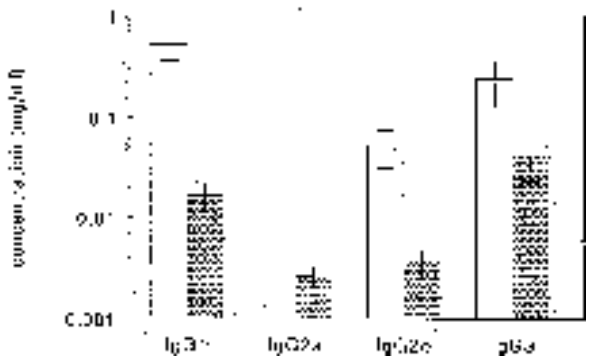

Fig. 6 Endogenous IgG levels of normal- and high-clearance mice. On the basis of the blood levels of cmAb SF-25 24 h after coinjection of ^{125}I-labelled cmAb SF-25 (5 μg; 5.6 μCi) and ^{131}I-labelled mmAb SF-25 (5 μg; 3.1 μCi), the mice were divided in a normal-clearance group (*open bars*; relative level >0.7; $n = 3$) and a high-clearance group (*grey bars*; relative level <0.7; $n = 3$). The mean blood levels of the various endogenous mouse IgG titres in plasma are given for each group as measured by means of an isotype-specific enzyme-linked immunosorbent assay

did not show a significant difference. The lower blood levels of cmAb probably resulted in the low tumour uptake that was found for mice with high blood clearance of cmAb E48. Owing to the large variation in the blood and tissue levels of cmAb E48 in the individual mice, no conclusions could be drawn from this experiment concerning the targeting properties of cmAb E48. This phenomenon has, to our knowledge, not been reported before for cmAb, although many studies have already been done in the nude mouse model, using tracer doses to determine the pharmacokinetics and targeting capabilities of cmAb. It could be that in a number of these studies the phenomenon was not noted as such, but attributed to in vivo variation. Another possibility is that a deviation in the biodistribution has been explained to be the result of a misinjection. In this study we determined whether this large variation in the biodistribution could have consequences for the suitability of the cmAb for clinical use. Most experiments were done in tumour-free nude mice because the variation in the uptake of mAb by the tumour increases the variation in the blood levels.

The increased clearance of cmAb was found to be independent of the variable domain but dependent on the isotype of the constant domain and of the mAb dose. Several other cmAb of the same IgG1 isotype were tested and, for all mAb, the phenomenon was seen. Determination of the influence of the isotype of the cmAb on the clearance of a tracer dose showed that cIgG1, cIgG3 and cIgG4 behaved in a similar fashion while cIgG2 did not demonstrate increased blood clearance in any of six mice. A strong correlation was found between the blood clearance of cmAb and the endogenous IgG titres of the mice, demonstrating that the variation in the elimination of cmAb is an experimental artifact related to the nude mouse.

Several studies have been published describing the influence of the mAb dose on the pharmacokinetics and liver uptake of various mAb [10, 16]. Most mAb used in these studies, however, recognized antigens that are shed by the xenografts in the bloodstream of the mice. The cause of liver uptake in the cases of antigen shedding was explained by the formation of immune complexes and subsequent uptake and catabolism by the liver. However, the effect we describe here must have a different cause because (a) HPLC analysis of plasma of high-clearance mice did not reveal any iodine-labelled immune complexes (data not shown), (b) the phenomenon was not observed with mmAb and (c) the high clearance was also seen in tumour-free nude mice. It is, therefore, safe to assume that the whole and uncomplexed mAb is taken up from the blood by the liver and spleen and subsequently degraded. In this respect the phenomenon seems to be similar to that described by Sharkey et al. for mIgG2a and mIgG2b [18]. They described the high blood clearance of mIgG2a and mIgG2b in various strains of outbred Swiss *nu*/*nu* mice. They found this high clearance, which was dependent on the age of the mice and on the mAb dose, in three-quarters of the mice tested while the other mice demonstrated a “normal” clearance rate. To test whether this phenomenon is similar to the one we found for cmAb, we injected ^{125}I-labelled cmAb 17-1A IgG1 and ^{131}I-labelled mmAb 17-1A IgG2a simultaneously into nude mice. The fact that the pattern of the biodistribution of both mAb was similar irrespective of the blood clearance indicates that the high clearance of cmAb we describe here is caused by the same mechanism.

The mechanism behind the increased clearance of cmAb described here remains unclear and hypothetical. The fact that it is independent of the variable domain but dependent on the isotype of the human IgG (hIgG) suggests involvement of Fc-binding receptors. These receptors should have a high affinity for both monomeric mIgG and monomeric hIgG. The observation that, for mIgG2a, there was a similar variation in clearance to that found as we found for cIgG1, strengthens the hypothesis that Fc-binding receptors are involved. The structural resemblance of hIgG1, hIgG3 and

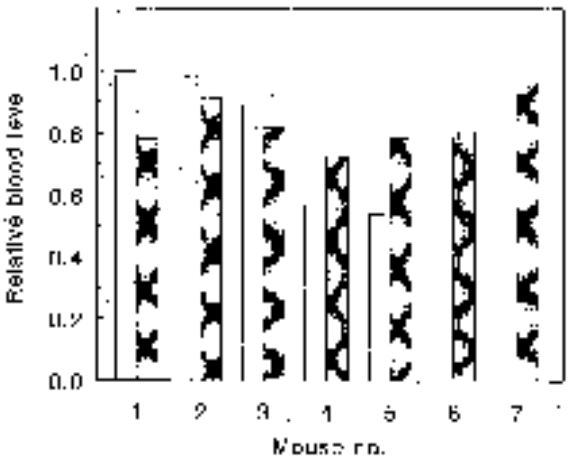

Fig. 7 Relative blood levels of 3 μg ^{125}I-labelled cmAb MOv18 (3.4 μCi; *open bars*) and 3 μg ^{131}I-labelled cmAb MOv18 F(ab′)$_2$ (5.2 μCi; *grey bars*) 20 h after simultaneous injection into seven nude mice. The levels of the individual mice are given as the radioactivity uptake relative to the uptake of the mouse with the highest blood level in the group (%ID g^{-1}: %ID g^{-1})

mIgG2a that leads to the comparable affinity of hIgG1 and mIgG2a for human Fc receptors might also cause cross-reactivity of mouse and human IgG isotypes with mouse Fc receptors. In fact, the pattern seen in the affinity of, for example, the human Fc γ I receptor to the various monomeric hIgG isotypes (IgG1 > IgG3 > IgG4 > > IgG2) corresponds roughly to the pattern of increased clearance seen in this study. Furthermore, the organs of uptake of the cmAb in the higher-clearance mice are the liver and spleen, organs with high numbers of Fc γ receptor-bearing cells like macrophages.

To test the role of Fc-binding receptors in this phenomenon, the whole IgG and the F(ab′)$_2$ fragment of cmAb MOv18 were injected simultaneously into nude mice and the biodistribution was determined. The F(ab′)$_2$ fragment demonstrated comparable blood levels for all mice 20 h after injection while, for the whole IgG, four out of seven mice showed a fast elimination. Some variation was found in the case of the F(ab′)$_2$ fragment but this was not correlated with the variation in the blood levels of the IgG. This is in agreement with the findings of Sharkey et al., who found no increase in the blood clearance rate of the F(ab′)$_2$ of the mmAb EPB-2 (mIgG2a) in mice that did have a rapid elimination of the whole IgG. Similar results for another mIgG2a and its F(ab′)$_2$ fragment have been described by others [2]. The results on the pharmacokinetic behaviour of mmAb and cmAb in nude mice appear to be comparable with the results described by Eccles et al. on the biodistribution of rat mAb in normal and athymic syngeneic rats. They also found an influence of the immunoglobulin subclass on the blood clearance (IgG2b > IgG2a > IgG1). Faster elimination from the blood was found to be related to higher uptake levels in the liver and spleen. This phenomenon was abolished by the use of F(ab′)$_2$ fragments, a reason for the authors to conclude that Fc receptors are involved [6].

The question remains why the increased elimination is only seen in a proportion of mice. Measurement of the IgG levels showed lower endogenous antibody titres in the plasma of mice with increased clearance. Significantly lower levels of IgG1, IgG2b and IgG3 were found in these mice. IgG2a levels were low in all mice. For *nu*/*nu* mice it has been shown that the endogenous IgG levels are low in young mice, but increase to levels comparable to those of their heterozygous littermates thereafter [12].

We hypothesize that, in mice with low endogenous levels, there could be an excess of free Fc-binding receptors, capable of binding monomeric cIgG1, cIgG3, cIgG4 or mIgG2a. During the increase in endogenous IgG levels, these free receptors become saturated with mIgG, thereby preventing binding of cIgG. Indeed, when mice of 14 weeks of age were given low doses of cmAb IgG1 or mmAb IgG2a, no excessive variation in the blood clearance or uptake by spleen or liver was seen (data not shown). This observation corresponded with similar endogenous mIgG titres in all mice. This hypothesis explains the fast removal of the injected cIgG from the blood, but it does not explain the fast degradation of the cIgG after binding to these receptors.

The phenomenon described here appears to be related to the nude mouse model and there is no evidence that increased uptake by liver and spleen of these cmAb will occur in the human situation. For a correct preclinical evaluation of the targeting capabilities of a chimeric, humanized or human IgG1, IgG3 or IgG4, one out of three measures, as described by Sharkey et al. for the evaluation of mIgG2a, can be taken: (a) an mAb dose can be given to nude mice that is sufficient for complete saturation of the uptake by the liver and spleen (preferably 100 μg), (b) only older mice (at least 4 months of age) can be used or (c) BALB/c nude mice or outbred nude mice, which do not show this phenomenon, can be used.

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