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Immunology

Serum half-life and tumor localization of a chimeric antibody deleted of the C_H2 domain and directed against the disialoganglioside GD2

(mutant antibody/biodistribution/radioimmunodetection of human tumor)

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ABSTRACT **Recombinant techniques allow one to engineer an antibody molecule and, in this way, manipulate its properties and functions. We engineered a chimeric human/mouse antibody to the tumor-associated antigen ganglioside GD2, with the aim of decreasing its serum half-life, maintaining its full antigen-binding capacity, and deleting its effector functions, thus making it a potentially useful reagent for the radioimaging of tumors. To this end, the constant region of the human γ1 chain was mutated by deleting the second domain (C_H2). Here we show that the C_H2-deleted antibody (ch14.18-ΔCH2) was cleared from the blood of athymic (*nu/nu*) mice bearing human melanoma tumors with the same kinetics as human IgG F(ab')$_2$. At a β $t_{1/2}$ of 12 hr, 0.9% of the injected dose of ^{125}I-labeled ch14.18-ΔCH2 was found per milliliter of blood 24 hr after i.v. injection. In biodistribution experiments, ^{125}I-labeled ch14.18-ΔCH2 targeted specifically to melanoma xenografts, achieving optimal tumor-to-tissue ratios 12–16 hr after i.v. injection. ch14.18-ΔCH2 was localized to the melanoma tumors more rapidly and with better localization ratios than the intact chimeric antibody ch14.18. Sixteen hours after i.v. injection, the tumor-to-blood and tumor-to-liver ratios of ch14.18-ΔCH2 were 5 and 12, respectively, while optimal localization ratios obtained for ch14.18 were 1 and 5, respectively, but 96 hr after injection. A reagent such as ch14.18-ΔCH2 should be useful for radioimmunodetection of human tumors because of reduced immunogenicity, increased targeting specificity, and rapid clearance from circulation.**

Radiolabeled monoclonal antibodies (mAbs) against tumor-associated antigens have been shown to localize specifically to melanoma and other solid tumors and can be used in radioimmunoimaging for the identification of occult lesions (1–3). For tumor imaging, it is advantageous to use antibody fragments instead of intact antibodies, because fragments clear faster from circulation and have a better tumor accessibility due to their smaller size. This has been found in experimental animals (4–6) as well as in cancer patients (7).

Recombinant DNA techniques make it possible to engineer the antibody molecule and to obtain antibodies with novel properties and functions (8, 9). For use in human therapy, murine variable regions and human constant regions can be fused to construct chimeric antibodies that are considerably less immunogenic in man than murine mAbs (10). Also, site-directed mutagenesis in the variable region of a mAb can result in an antibody with higher affinity and specificity for its antigen (11), and exchanging constant regions of a mAb can improve its ability to mediate effector functions, such as complement-mediated cytotoxicity and antibody-dependent cytotoxicity by effector cells (12, 13). On the other hand, digestion with proteolytic enzymes is not applicable to all mAbs. If active fragments can be produced, this technique can be limited by problems such as low yield, contamination with enzyme, and others (14, 15). Therefore, our aim was to generate a mutant antibody with characteristics of a F(ab')$_2$ fragment that is useful for tumor imaging.

Earlier, we described the development of a chimeric human/mouse antibody, ch14.18, directed against the disialoganglioside GD2, an antigen expressed on human tumors of neuroectodermal origin (16). ch14.18 was used to study the effects of mutating and deleting the constant regions of the human γ1 chain. Although it was not possible to engineer a functional F(ab')$_2$ of ch14.18 [i.e., a mutant missing the second and third domains of the constant region of the human γ1 chain (C_H2 and C_H3) (17)], we were able to express an antibody that was deleted of C_H2. The mutant antibody deleted of C_H2 (ch14.18-ΔCH2) was characterized and compared with the intact chimeric antibody. By gel electrophoresis on SDS gels and by high-pressure exclusion chromatography under nondenaturing conditions, ch14.18-ΔCH2 appeared to have a molecular mass of 120 kDa. ch14.18-ΔCH2 bound to purified ganglioside GD2; however, unusual binding characteristics of ch14.18-ΔCH2 were observed in that it competes much more efficiently with ch14.18 for antigen than does ch14.18 with itself, especially at short incubation times. This is thought to reflect some conformational changes in the overall structure of the molecule rather than in binding affinity (17). In contrast to ch14.18, ch14.18-ΔCH2 did not mediate complement-dependent lysis of GD2$^+$ target cells and mediated very little cytotoxicity of human mononuclear effector cells. Here we describe the *in vivo* properties of ch14.18-ΔCH2 in athymic (*nu/nu*) mice bearing human melanoma tumors, comparing it with the intact chimeric antibody ch14.18 and with a nonrelevant F(ab')$_2$ fragment of human IgG.

MATERIALS AND METHODS

Cell Lines and Antibodies. The human melanoma cell line UCLA-SO-M21 was kindly provided by D. L. Morton, University of California, Los Angeles. A subclone of this cell line, derived in our laboratory (M21), expresses high levels of the disialoganglioside GD2. M21 cells were grown in RPMI 1640 tissue culture medium supplemented with 10% fetal calf serum.

Construction and expression of ch14.18 was reported previously (16). ch14.18 was purified from tissue culture supernatants by using protein A-Sepharose (Repligen, Cambridge, MA). ch14.18-ΔCH2 was generated as described (17). Briefly, an expression plasmid deleted of the C_H2 exon was

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Abbreviation: mAb, monoclonal antibody.

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transfected into the non-immunoglobulin-producing hybridoma cell Sp2/0 Ag14. ch14.18-ΔCH2 was purified from supernatants by using an anti-human κ light chain mAb coupled to Sepharose as affinity absorbent. The ch14.18-ΔCH2 that was used in this study consisted of at least 75% dimeric molecules when analyzed by SDS/PAGE under denaturing, nonreducing conditions and was resolved as a single component by high-pressure gel-permeation chromatography.

$F(ab')_2$ fragments of affinity-purified human IgG were purchased from Jackson Immunoresearch.

Indirect Immunofluorescence. M21 melanoma cells (10^6) were incubated for 1 hr on ice with 10 μg of ch14.18 or ch14.18-ΔCH2. The cells were washed three times with phosphate-buffered saline containing 1% fetal calf serum and 0.02% NaN_3 and stained with goat anti-human IgG conjugated to fluorescein isothiocyanate (Boehringer Mannheim). The cells were again washed three times, and 10^4 cells were analyzed in a FACS 440 flow cytometer (Becton Dickinson).

Radiolabeling. ch14.18, ch14.18-ΔCH2, and $F(ab')_2$ fragments of human IgG were labeled with ^{125}I as described (18). Briefly, 500 μg of antibody was incubated for 25 min on ice with 0.5 mCi of ^{125}I [100 mCi (3.75 GBq) per ml; Amersham] in polystyrene tubes coated with 100 μg of Iodo-Gen reagent (Pierce). Nonincorporated ^{125}I was removed by gel filtration on PD10 columns (Pharmacia). Specific activity was typically 0.5–1.5 nCi per ng of antibody.

Blood Clearance and Biodistribution. The 8- to 10-week-old female athymic (*nu/nu*) mice used in this study were purchased from the National Cancer Institute (Bethesda, MD). They were injected s.c. with 2×10^6 M21 tumor cells, resulting in tumors of 50–150 mg of weight within 10 days. At this time, the animals received i.v. injections into the lateral tail vein of 25 μg of antibody containing 3–4 μCi of ^{125}I-labeled antibody. At designated time points after injection, groups of three animals were anesthetized with halothane, and blood samples were obtained by retroorbital bleeding. For biodistribution of radiolabeled antibodies, groups of three animals were sacrificed at various time points after injection. Tumors and major organs (heart, skin, muscle, bone, lung, liver, spleen, thyroid, kidney, and intestine) were removed and weighed. All tissue samples were assayed in a γ counter for ^{125}I activity. The results were calculated as the percent of the injected dose recovered per gram of tissue and as localization ratios (cpm per gram of tumor/cpm per gram of tissue).

RESULTS

To verify the binding of ch14.18 and the mutant antibody (ch14.18-ΔCH2) to $GD2^+$ tumor cells, surface staining of viable M21 cells was compared by indirect immunofluorescence. It was shown previously that there are 15×10^6 binding sites for ch14.18 per M21 melanoma cell (13). After a 1-hr incubation, both antibodies stained all cells with the same intensity (Fig. 1), indicating no differences in the binding of the intact and mutant chimeric antibody to tumor cells.

In nude mice bearing M21 tumors, ^{125}I-labeled ch14.18-ΔCH2 (^{125}I-ch14.18-ΔCH2) was cleared very rapidly from the intravascular compartment (Fig. 2). The blood clearance curve for ^{125}I-ch14.18-ΔCH2 was biphasic, with an initial half-life ($\alpha\ t_{1/2}$) of 1.5 hr and a second half-life ($\beta t_{1/2}$) of about 12 hr. ch14.18-ΔCH2 was eliminated from the circulation with the same kinetics as a $F(ab')_2$ fragment of human IgG. At each time point measured, the concentration of tumor-specific ch14.18-ΔCH2 was lower than the concentration of the nonspecific $F(ab')_2$, most likely due to the rapid uptake of ch14.18-ΔCH2 into the tumor. We were not able to make a comparison with $F(ab')_2$ fragments of ch14.18 in the *in vivo*

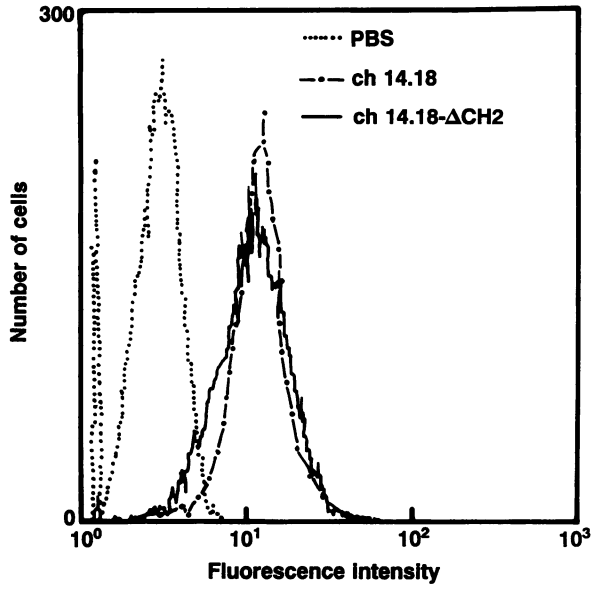

FIG. 1. Flow cytometric analysis of indirect immunofluorescence after staining of M21 melanoma cells with ch14.18 or ch14.18-ΔCH2 and goat anti-human IgG conjugated to fluorescein isothiocyanate. PBS, phosphate-buffered saline.

experiments described here, because the chimeric antibody was degraded by enzymatic digestion with pepsin (as well as bromelain), without resulting in a significant amount of $F(ab')_2$ fragment. In tumor-bearing animals that were injected with the same amount of ^{125}I-labeled ch14.18 (^{125}I-ch14.18), 28.5% of the injected dose was found per ml of blood 4 hr after i.v. injection, and 18% was found after 24 hr. Thus, ch14.18-ΔCH2 is cleared from the blood like a $F(ab')_2$ fragment and much more rapidly than the intact chimeric antibody.

The distribution of ^{125}I-ch14.18-ΔCH2 and nonspecific $F(ab')_2$ was compared 24 hr after i.v. injection into nude mice bearing s.c. human melanoma tumors (Fig. 3). ch14.18-ΔCH2 that binds to GD2 on the melanoma cells localizes specifically to the tumor. Nonspecific $F(ab')_2$ from human IgG was found at 0.5–1.5% of the injected dose per gram of the various tissues analyzed, with no preferential localization to the tumor. The tumor-to-blood ratio was found to be 3.25 for ch14.18-ΔCH2 but only 0.2 for the nonspecific $F(ab')_2$ fragment, demonstrating specific localization of ch14.18-ΔCH2 to $GD2^+$ tumors.

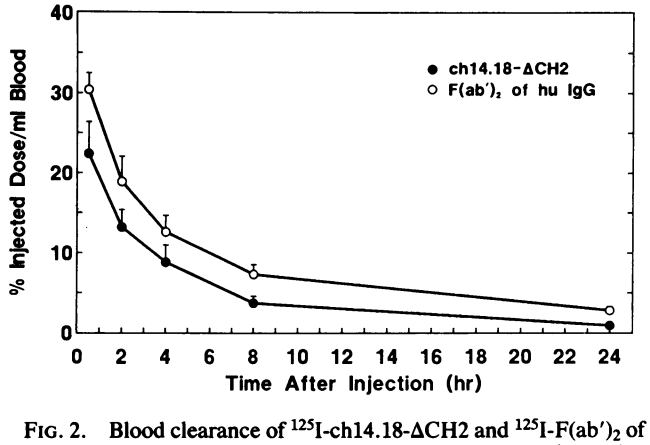

FIG. 2. Blood clearance of ^{125}I-ch14.18-ΔCH2 and ^{125}I-$F(ab')_2$ of human (hu) IgG following i.v. injection into athymic (*nu/nu*) mice bearing M21 tumors. Each animal was injected with 25 μg (3 μCi) of antibody. Data are given as the percent of the injected dose found per milliliter of blood, and each point and error bar represent the mean and standard deviation for three animals.

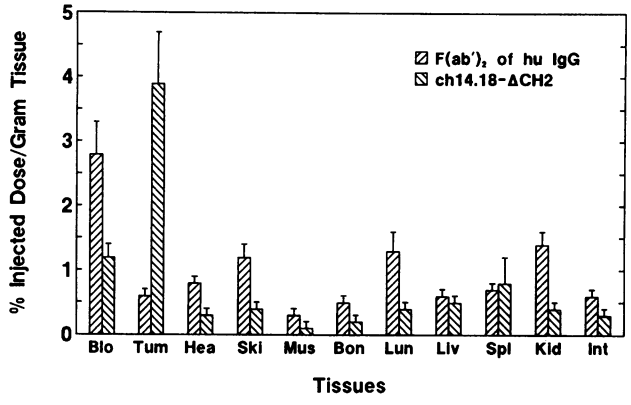

FIG. 3. Biodistribution 24 hr after i.v. injection of ^{125}I-ch14.18-ΔCH2 or ^{125}I-F(ab')$_2$ of human (hu) IgG [25 μg (3 μCi) per animal] into athymic (*nu/nu*) mice bearing M21 tumors. Data are given as the percent of the injected dose found per gram of tissue, and each value represents the mean and standard deviation for three animals. Blo, blood; Tum, tumor; Hea, heart; Ski, skin; Mus, muscle; Bon, bone; Lun, lung; Liv, liver; Spl, spleen; Kid, kidney; Int, intestine.

The biodistribution of ^{125}I-ch14.18-ΔCH2 in nude mice with human melanoma tumors is shown at different time points in Fig. 4. The amount of radioactivity in the s.c. tumors was the highest at the first time point after i.v. injection, reaching 14.8% of the injected dose per gram of tumor after 4 hr and dropping to 2.8% after 24 hr. At later time points, the selectivity of the localization increased as ^{125}I-ch14.18-ΔCH2 was eliminated more slowly from the tumor than from blood and the other organs. The biodistribution of ^{125}I-ch14.18-ΔCH2 was compared with that of the same amount of labeled intact antibody ch14.18 (Fig. 5). In addition to the 4- and 24-hr time points, the biodistribution after 4 days is also shown, because the intact antibody ch14.18 was cleared much more slowly from the blood than ch14.18-ΔCH2 and the tumor-to-tissue ratios improved at later time points. For ^{125}I-ch14.18, the highest uptake of radioactivity into the tumor was not found at the earlier time points but was found 24 hr after i.v. injection. Twenty-four hours after injection, the amount of ^{125}I-ch14.18 per gram of tumor was 3.5-fold higher than that of ^{125}I-ch14.18-ΔCH2. However, the ratio of cpm in the tumor to cpm either in the surrounding tissue (skin) or in major organs (liver) was clearly better for ch14.18-ΔCH2 than for ch14.18. As shown in Table 1, localization ratios of ch14.18-ΔCH2 to human tumors in nude mice were optimal

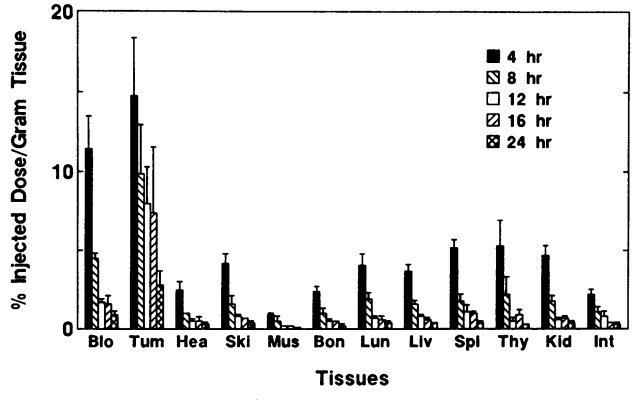

FIG. 4. Biodistribution of ^{125}I-ch14.18-ΔCH2 [25 μg (4 μCi) per animal] in athymic (*nu/nu*) mice bearing M21 tumors at 4, 8, 12, 16, and 24 hr after i.v. injection. Data are given as the percent of the injected dose found per gram of tissue, and each value represents the mean and standard deviation for three animals. Thy, thyroid. Other abbreviations are as in Fig. 3.

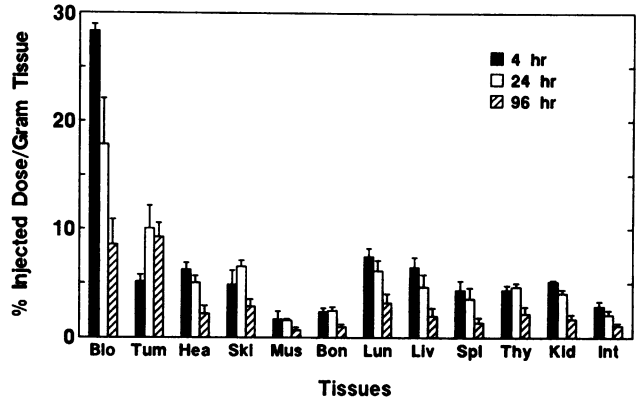

FIG. 5. Biodistribution of ^{125}I-ch14.18 [25 μg (4 μCi) per animal] in athymic (*nu/nu*) mice bearing M21 tumors at 4, 24, and 96 hr after i.v. injection. Data are given as the percent of the injected dose found per gram of tissue, and each value represents the mean and standard deviation for three animals. Abbreviations are as in Fig. 4.

between 12 and 16 hr after i.v. injection, whereas ch14.18 showed the best ratio after 96 hr. The C_H2-deleted mutant antibody, ch14.18-ΔCH2, localized to human tumor xenografts in athymic (*nu/nu*) mice more rapidly and with better ratios than the intact chimeric antibody.

DISCUSSION

In this study, we show that C_H2-deleted chimeric antibody was cleared from the blood of tumor-bearing athymic (*nu/nu*) mice with the same kinetics as human IgG F(ab')$_2$ and much faster than the corresponding intact chimeric antibody. The deletion mutant targets human melanoma xenografts in these mice specifically. It also localizes to the tumors more rapidly and with better localization ratios than the intact antibody.

The immunoglobulin molecule is divided into globular domains, and many of its functional properties can be localized to specific domains. The structural domains of the protein are encoded for by exons of the immunoglobulin genes. For the mutant antibody studied here, the C_H2 exon was deleted from the human Cγ1 gene and was used subsequently to produce a human/mouse chimeric antibody with specificity for the tumor-associated antigen ganglioside GD2. Blood clearance of the C_H2-deleted mutant antibody was studied in athymic (*nu/nu*) mice bearing human tumor xenografts, as it was shown previously that murine and chimeric human/mouse antibodies have identical pharmacokinetics in this model system (13, 19). The mutant antibody was cleared more rapidly from the blood of these mice. Clearance of F(ab')$_2$ fragments of murine mAbs has been reported to be similar to what we describe here for the C_H2-deleted mutant antibody and human F(ab')$_2$ fragments (20, 21). The C_H2-

Table 1. Localization ratios of ^{125}I-ch14.18-ΔCH2 and ^{125}I-ch14.18

Time after injection, hr	ch14.18-ΔCH2			ch14.18		
	T/B	T/L	T/S	T/B	T/L	T/S
4	1.29	3.99	3.51	0.18	0.80	1.05
8	2.23	6.25	6.04	ND	ND	ND
12	4.87	9.71	10.61	ND	ND	ND
16	4.64	12.19	10.18	ND	ND	ND
24	3.15	6.67	7.02	0.56	2.14	1.53
96	1.20	2.28	2.29	1.08	4.55	3.19

Athymic (*nu/nu*) mice with M21 xenografts were injected i.v. with ^{125}I-ch14.18 or ^{125}I-ch14.18-ΔCH2 [25 μg (3 μCi)]. Tumor-to-tissue ratios are given. T, tumor; B, blood; L, liver; S, skin. ND, not determined.

deleted mutant and the intact chimeric antibody were both expressed and produced in a murine hybridoma cell line. An obvious difference between the two mAbs is that the intact chimeric antibody is glycosylated, while the mutant lacking Asn-297, the sole site for N-linked glycosylation, is not. Therefore, it could be argued that the rapid elimination of the mutant antibody is due to the lack of carbohydrate side chains rather than to the deletion of the whole C_H2 domain. In this regard, plasma clearance of proteolytic fragments of human myeloma IgG has been studied in rabbits (22). A half-life of about 70 hr was found for whole IgG, Fc, and C_H2 fragments, while Fab and C_H3 fragments were eliminated with a half-life of 16 hr. Furthermore, carbohydrate-deficient antibodies have been generated by mutating the Asn-297 in the C_H2 region of another human/mouse chimeric antibody of the IgG1 isotype (23). No difference in blood clearance was found between the aglycosylated and the intact chimeric antibody in mice. Taken together, these findings and our data suggest that it is the C_H2 domain and the influence it has on the conformation of the whole molecule rather than the carbohydrate side chains that provides the dominant factor controlling the catabolic rate of IgG.

The C_H2-deleted antibody ch14.18-ΔCH2 was able to localize specifically to $GD2^+$ tumors. Twelve to 16 hr after injection, tumor-to-tissue ratios for the mutant antibody were optimal and higher at any time point than those observed with the intact chimeric antibody. Another advantage of the C_H2-deleted antibody as a potential imaging reagent is the fact that it is able to leave the vascular space and to bind to the tumor cells more rapidly than the intact chimeric antibody, suggesting that it is a less rigid molecule. The improvement of tumor uptake ratios achieved by the C_H2-deleted antibody is similar to that described by others, when comparing $F(ab')_2$ fragments with whole murine mAbs (5, 6, 20, 21). Rapid tumor localization and good localization ratios are properties that make C_H2-deleted antibodies good candidates for radioimaging of tumors, possibly allowing the use of radioisotopes with low energy and a short half-life, such as ^{123}I or ^{99m}Tc (24, 25). The use of C_H2-deleted antibodies to deliver cytotoxic agents such as toxins, drugs, or radionuclides for therapy may be limited by its fast elimination; however, a possible exception may be the targeting of α-particle-emitting radionuclides such as ^{212}Bi or ^{211}At, with half-lives of 61 min and 7.2 hr, respectively (26, 27).

In conclusion, by studying the pharmacokinetic properties of a C_H2-deleted chimeric antibody, we demonstrated that the catabolic rate of the antibody molecule is controlled by the C_H2 domain. At the same time, we engineered a molecule that because of its *in vivo* characteristics should prove useful for the radioimmunodetection of human tumors.

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