Light Chain Variants of an IgG3 Anti-GD3 Monoclonal Antibody and the Relationship among Avidity, Effector Functions, Tumor Targeting, and Antitumor Activity

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ABSTRACT

R24 is an IgG3 mouse monoclonal antibody which recognizes the ganglioside GD3. Two variants of R24, in which one (V2-R24) or both (V1-R24) light chains were substituted by MOPC-21 light chains, were isolated and characterized. R24 had a 40-fold higher avidity for GD3 than either variant, suggesting that high avidity binding required the presence of two R24 light chains and, thus, divalency. R24 and both variants mediated antibody-dependent cellular cytotoxicity but antibody-dependent cellular cytotoxicity mediated by variants was weak compared to R24. The presence of at least one R24 light chain was required for complement-dependent cytotoxicity; complement-dependent cytotoxicity was mediated by R24 and weakly by V2-R24 but not by V1-R24. R24, but not V1-R24 or V2-R24, inhibited attachment of melanoma cells to plastic and activated T-lymphocytes, suggesting a threshold of avidity required for these biological effects. In a human melanoma xenograft model in nu/nu mice, radiolabeled R24, variants, and isotype-matched control monoclonal antibodies all appeared to localize in tumors (based on tumor:nontumor tissue ratios), but specific tumor targeting by R24 was generally 3- to 6-fold higher. R24 prevented melanoma outgrowth in nu/nu mice, while V2-R24 induced partial tumor protection. V1-R24 and the negative control monoclonal antibody did not inhibit tumor outgrowth. Antitumor activity of R24 corresponded to avidity and ability to mediate complement-dependent cytotoxicity in vitro.

INTRODUCTION

Antibody avidity is determined by the variable region of the immunoglobulin molecule, while effector functions such as complement fixation and ADCC are dependent on constant domains within the Fc region. These Fc-dependent functions require binding of the antibody to the target cell but the relationship between avidity and effector function has been difficult to define. In the past, mAbs have been compared to nonreactive, isotype-matched control mAbs. Such control mAbs can differ substantially from the test mAbs within the variable and constant regions. It is difficult to assess the relative role of avidity since isotype-matched control mAbs have no avidity for the antigen. The role of avidity in antitumor activity is further complicated since: (a) the correlation between effector functions and antitumor activity remains unclear, (b) even mAbs with no avidity can target tumors (1-4), and (c) the Fc region plays an important role in antibody biodistribution (5-7). To investigate the role of avidity would require the analysis of mAbs that are constructed with nearly identical constant and variable regions but with a range of avidities.

R24 is a mouse IgG3 mAb directed against the ganglioside GD3, a prominent glycolipid on human melanoma cells (8, 9). R24 mediates a variety of biological effects such as complement fixation, ADCC, prevention of attachment of melanoma cells to plastic, and activation of T-lymphocytes (10-12, 14). R24 is of interest for the therapy of cancer because in preliminary clinical trials it has induced tumor regressions in patients with metastatic melanoma (13). We report the identification and isolation of two variants of R24 with an approximately 40-fold decrease in avidity for GD3. Decreased avidity of the variant mAbs was due to the presence of MOPC-21 light chains derived from the NS-1 myeloma fusion partner. We were interested in studying the effect of these replaced light chains on avidity and on in vitro effector functions, in vivo biodistribution, tumor localization, and antitumor activity.

MATERIALS AND METHODS

Media. Cell cultures were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mm glutamine, 1% nonessential amino acids, penicillin (100 units/ml), and streptomycin (100 units/ml).

Monoclonal Antibodies and Cell Lines. R24, an IgG3 mouse mAb raised against human melanoma cell line SK-MEL-28, was the product of a fusion between the NS-1 mouse myeloma cell line and [BALB/c x C57BL/6] F1 splenocytes (8). mAb 34-417, an IgG3 mAb raised in New Zealand black mice against human colon carcinoma, recognizes sialylated Lewis blood group A (15) and does not bind to human melanoma cells. MOPC-21 (Sigma Chemical, St. Louis, MO) is an IgG1 (a) produced by the BALB/c P3X63Ag8 myeloma cell line. NS-1, the myeloma cell line used to produce R24, is a subclone of P3X63Ag8 which continues to produce the MOPC-21 light chain but not the heavy chain (16). FLOPO-21 (IgG3) was purchased from Sigma Chemical. SK-MEL-28 and SK-MEL-30 are GD3 human melanoma cell lines. SK-MEL-30, which was used as a target for ADCC assays, is resistant to natural killer cell lysis.

Purification of R24 and R24 Variant mAbs. Ascites of mice bearing hybridoma tumors was purified by Protein A affinity chromatography. Briefly, delipoproteinated ascites equilibrated with 0.05 m Tris/0.15 m NaCl, pH 8.6, was applied to a Protein A-Sepharose column. After extensive washing, antibody was eluted off the column in 0.05 m citrate/0.15 m NaCl, pH 4. The Protein A-purified preparation was applied to a Mono S cation-exchange fast protein liquid chromatography column (Pharmacia, Piscataway, NJ) in a buffer of 0.05 m 2-morpholino)ethanesulfonic acid/5 m NaCl, pH 5.6. R24 and the variants were eluted using an increasing NaCl gradient. Eluted protein was monitored by absorbance at a wavelength of 280 nm and fractions were collected and pooled.

IEF and Western Blotting. IEF was performed in tube gels as previously described (17). After soaking in transfer buffer, gels were arranged on a nitrocellulose filter (Schleicher & Schuell, Keene, NH) and electrophoresed at 50-70 V for 1-2 h. The filters were blocked using 5% nonfat milk (Carnation, Los Angeles, CA) and then incubated with 125I-labeled goat anti-mouse light chain (Fisher Scientific, Springfield, NJ) which had been radioiodinated using the chloramine-T method.

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(18). After extensive washing, the filter was exposed to Kodak X-Omat film.

N-Glycanase Digestion of mAbs. mAbs (10–20 μg) were digested overnight with N-glycanase (Genzyme, Boston, MA), at a final enzyme concentration of 60 units/ml, according to manufacturer's recommendation.

Measuring Binding to G03 by ELISA. G03 was purified from human melanoma tissue as previously described (14). For ELISA assays, either pure melanoma G03 or G03 mixed with other melanoma gangliosides was adsorbed to 96-well plates (Nunc, Roskilde, Denmark) to yield 100 ng G03/well. Since R24 does not bind to other gangliosides, results were similar whether we used pure G03 or G03 mixed with other gangliosides. Wells were blocked with 5% γ-globulin-free bovine serum (GIBCO, Grand Island, NY) in phosphate-buffered saline with 0.05% Tween 20 (γ-globulin-free buffer). mAbs were diluted in γ-globulin-free buffer and added to wells for 1 h at room temperature. Binding of mAbs was detected using either alkaline phosphatase-conjugated Protein A or biotinylated goat anti-mouse immunoglobulin followed by alkaline phosphatase-conjugated avidin. The reaction was visualized by adding p-nitrophenyl phosphate in a diethanolamine buffer. Absorbance at 410 nm was read on a plate reader (Dynamech).

CDC Assay. SK-MEL-28 cells were plated in Terasaki plates at 200–1000 cells/well and cultured for 48–72 h. mAb was diluted in Veronal buffer containing normal human serum, diluted 1:4, as a complement source. Various concentrations of mAb were added for 3–4 h and the plates were incubated at 37°C. After washing with Veronal buffer without serum, the wells were stained with trypan blue and scored for percentage of viable cells.

ADCC Assay. Normal PBMC from healthy donors were isolated by Ficoll-Hypaque gradient centrifugation. SK-MEL-30 cells were labeled with 250 μCi of 51Cr (New England Nuclear, Boston, MA) and plated (5000 cells/well), in at least triplicate, either in flat-bottomed 96-well plates (Falcon, Oxnard, CA) and allowed to adhere overnight or in U-bottomed 96-well plates (Costar, Cambridge, MA) and used immediately. mAb was added at various concentrations. PBMC effectors were added to achieve an effector to target ratio of 25:1. Plates were incubated for 4 h at 37°C. Supernatants were collected using a supernatant collection system (Skatron, Lier, Norway) and "Cr release was measured using a Beckman Model LS9000 liquid scintillation counter. Percentage of specific lysis was calculated as follows:

\[
\text{% lysis} = \frac{\text{cpm [supernatant]} - \text{cpm [spontaneous]}}{\text{cpm [total]} - \text{cpm [spontaneous]}} \times 100
\]

where total cpm were the counts released by incubation of labeled target cells in 0.5% Nonidet P-40 and spontaneous cpm were the counts released by incubation of labeled target cells in medium and added to wells of a 24-well plate (1.5 ml/well). Skatron Model LS9000 liquid scintillation counter. Total cpm were recorded. After 34 days, the experiment was terminated and any mouse free of tumor or with tumor less than 5 mm was subjected to necropsy to confirm tumor status.

RESULTS

Isolation and Purification of R24 Variants. Fig. 1 shows the purification of R24 and R24 variants by cation-exchange chromatography. Three protein peaks were collected (occasionally the second peak eluted as two distinct peaks) and each peak was analyzed by SDS-PAGE (Fig. 2). The first peak, designated V1-R24, contained a single Mr, 27,000 light chain species which co-migrated with the light chain of MOPC-21. The second peak, designated V2-R24, contained the MOPC-21 light chain and a second light chain of Mr, 26,000. On SDS-PAGE both light chain bands of V2-R24 stained with equal intensity (Fig. 2, lane C), showing they were present in approximately an equimolar ratio. Under nonreducing conditions, V2-R24 migrated as a single band on SDS-PAGE between V1-R24 and R24, confirming that each V2-R24 antibody molecule contained two distinct light chain species (data not shown).

To confirm that the M, 26,000 band represented the MOPC-21 light chain and the M, 26,000 band the native R24 light chain, we analyzed the κ-chains of MOPC-21, V1-R24, V2-

![Fig. 1. Elution profile of R24 and variants from Mono S cation-exchange chromatography. V1-R24 eluted as the first protein peak, V2-R24 as the second peak, and R24 as the third peak.](cancerres.aacrjournals.org)
light chain. The IEF patterns of the various light chains were not affected by treatment with N-glycanase (data not shown), demonstrating that differences in IEF patterns were not due to detectable differences in N-linked glycosylation. We conclude, therefore, that V1-R24 is composed of two R24 heavy chains and two MOPC-21 light chains. The V2-R24 molecule has two R24 heavy chains, one covalently linked to a MOPC-21 light chain and the other to a R24 light chain. R24, purified in the third peak from the cation-exchange column, contains two R24 heavy chains and two R24 light chains.

Avidity of R24 and R24 Variants for GD$_3$. We could not assay the true affinity of R24 or R24 variants for GD$_3$ in solution because purified GD$_3$ is not soluble in an aqueous environment. We therefore compared the avidities of R24 and variants for GD$_3$ adsorbed to plastic 96-well plates by ELISA. Saturation of mAb binding to GD$_3$ could be demonstrated, and the apparent dissociation constant, $K_D$, was estimated from the mAb concentration which gave half-maximum binding to GD$_3$.

Fig. 4 illustrates typical binding curves of R24 and the R24 variants. The estimated $K_D$ (mean of triplicate experiments) of R24 was $2.13 \times 10^{-9}$ M, while the estimated $K_D$ values of V1-R24 and V2-R24 were considerably lower and were approximately equal to one another, $8.7 \times 10^{-10}$ and $8.2 \times 10^{-8}$ M, respectively. Thus, R24 bound to GD$_3$ with an approximately 40-fold higher avidity than V1-R24 and V2-R24. Similar avidities were obtained if mAb concentrations giving a fixed absorbance value (i.e., at 0.1 or 0.2 absorbance units) were compared. These results showed that replacement of a single R24 light chain by MOPC-21 light chain (in V2-R24) resulted in a marked decrease in avidity for GD$_3$. Replacement of the second R24 light chain (V1-R24) resulted in little or no further decrease in avidity for GD$_3$. These results suggest that (a) R24 heavy chains determine avidity for GD$_3$ but R24 light chains further enhance avidity and (b) high avidity binding requires divalency.

CDC against Human Melanoma. R24 is known to activate complement from a variety of species, including humans (10, 12). We studied the ability of R24 and the two variants to mediate CDC using human complement (Fig. 5). V1-R24 did not mediate CDC of SK-MEL-28 melanoma cells. V2-R24 was able to mediate weak CDC, but even in the presence of 100 µg/ml V2-R24 only an average of 30% of target cells were killed (70% viability). In contrast, only 12 µg/ml R24 was required for 50% killing of target cells, and 100% killing could be achieved at 40–100 µg/ml. Since V1-R24 and V2-R24 have about the same avidity for GD$_3$ and identical Fc regions, it is unclear why V2-R24 was active in CDC. While ability to mediate CDC did not strictly correlate with avidity for GD$_3$, it appears that at least one R24 light chain is crucial for effective CDC.
LIGHT CHAIN VARIANTS OF AN ANTI-GD3 mAb

Induction of T-Lymphocyte Proliferation. We have previously shown that a subpopulation of peripheral blood T-lymphocytes expresses GD3 and that R24 stimulates proliferation of this GD3+ subpopulation (14). Proliferation peaks on days 4–5 and does not require addition of exogenous growth factors. We were interested to know whether either R24 variant was able to stimulate lymphocyte proliferation. The results of two experiments are shown in Table 1. In contrast to R24, neither V1-R24 nor V2-R24 stimulated PBMC proliferation. Thus, as in the prevention of attachment to plastic, there was a threshold of avidity for GD3 necessary for lymphocyte activation.

**In Vivo Pharmacokinetics of R24 and R24 Variants.** R24 and R24 variants were radiolabeled with 125I and injected by i.v. bolus into nu/nu mice bearing SK-MEL-30 human melanoma xenografts. Fig. 7 illustrates pharmacokinetics of radiiodinated V1-R24 and R24 in peripheral blood. At the first time point, 20 min, the blood level of radiolabeled R24 was already 50% below the level of V1-R24. Subsequently the two curves were parallel. Pharmacokinetics fit a two-compartment model, with a t1/2 of 2.7 h and a t1/2 of 50 h. Because equivalent amounts of labeled mAb were injected and there was no evidence of extravasation at injection sites (see “Material and Methods”), these data suggest that up to 50% of radiolabeled R24 left the blood pool within 20 min, presumably into the extravascular compartment. This also suggested that there was early targeting of radiolabeled R24 either to tumor or to GD3+ normal tissues, or both. Because we could find little information on the GD3 content of normal tissues in the nu/nu mouse, we examined the ganglioside content of brain, blood, liver, spleen, kidney, and muscle from non-tumor-bearing nu/nu mice by thin layer chromatography alone or thin layer chromatography with immunostaining by R24. Both brain and muscle contained significant amounts of GD3. Thus, it is possible that radiolabeled R24 rapidly left the intravascular space and bound to tumor and normal tissues in vivo.

**Table 1** Activation of PBMC by V1-R24, V2-R24, and R24

<table>
<thead>
<tr>
<th>Experiment</th>
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<th>V2-R24</th>
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<td><strong>cpm</strong></td>
<td><strong>Fold stimulation</strong></td>
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<tr>
<td>V1-R2</td>
<td>332 ± 27</td>
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<td>1,415 ± 343</td>
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<td>V2-R2</td>
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<tr>
<td>R24</td>
<td>4,339 ± 38</td>
<td>15.22</td>
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</tr>
<tr>
<td>No mAb</td>
<td>285 ± 86</td>
<td>1,333 ± 460</td>
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*cpm represents [3H]thymidine incorporation expressed as the mean ± SE of triplicate wells.

* Fold stimulation was calculated by dividing observed cpm by cpm of unsimulation PBMC.

ADCC against Human Melanoma. All three R24 antibody species were found to mediate ADCC. The result of a representative assay is presented in Fig. 6. In this experiment, 51Cr-labeled target cells were allowed to adhere to the bottom of wells overnight before mAb and effector cells were added, but similar results were obtained when labeled target cells were added to U-bottomed wells and used immediately. Significant ADCC was seen with 0.1 µg/ml R24 at an effector:target ratio of 25:1, while 100-fold more V1-R24 and V2-R24 were required for equivalent cytotoxicity. It is especially interesting to note that V1-R24 was able to mediate ADCC and was approximately equivalent to V2-R24 in activity. Thus, in contrast to CDC activity, ability to mediate ADCC correlated with the avidity for GD3, since the low avidity R24 variants mediated equivalent but low level ADCC.

Inhibition of Cell Attachment. GD3 is expressed within cell adhesion plaques and is thought to play a critical role in cell attachment to substrates, in part through regulation of receptors for GD3. GD3 is expressed on cell adhesion plaques and is thought to play a critical role in cell attachment to substrates, in part through regulation of receptors for GD3.

**Fig. 5.** CDC of melanoma by V1-R24 (O), V2-R24 (■), and R24 (△). Each point represents the mean ± SE of seven experiments performed in duplicate wells.

**Fig. 6.** ADCC mediated by V1-R24 (O), V2-R24 (■), and R24 (△). Target cells (SK-MEL-30) were labeled with 51Cr and allowed to adhere overnight to flat-bottomed 96-well plates. Effector to target ratio was 25:1 and spontaneous lysis of target cells was 17.5% of total lysis by Nonidet P-40. Specific lysis by effector cells in the absence of mAb was 3.5%. Each point represents the mean ± SE of five replicates.

**Fig. 7.** Blood pharmacokinetics of 125I-V1-R24 (O) and 125I-R24 (△). Each point represents the mean ± SE of five mice.

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**Table 2** Activation of PBMC by V1-R24, V2-R24, and R24

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**Table 3** Activation of PBMC by V1-R24, V2-R24, and R24

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**Table 4** Activation of PBMC by V1-R24, V2-R24, and R24

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**Table 5** Activation of PBMC by V1-R24, V2-R24, and R24

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muscle (brain would not be targeted due to the blood-brain barrier). This hypothesis is supported by biodistribution data at day 3, which shows specific localization of R24 to muscle and tumor (see below).

Biodistribution and Tumor Targeting. Biodistribution of radioiodinated R24, V1-R24, V2-R24, and an isotype control, 34–417, was analyzed on days 3, 8, and 15 after injection of mAb. On day 3 (Fig. 8, upper), R24, V2-R24, and mAb 34–417 had similar tumor:tissue ratios, except for slightly higher tumor:ki cney and tumor:lung ratios seen with R24. Tumor: muscle ratios were substantially lower for R24, consistent with specific targeting of R24 to GD3 on nu/nu mouse muscle, as discussed above.

At day 8 (Fig. 8, middle), tumor:tissue ratios for R24 generally were 3- to 6-fold higher than for V1-R24 or 34–417. Two exceptions were tumor: liver ratios, in which R24 targeted 21-fold better than 34–417 but only 2-fold better than V1-R24. The consistently low tumor:kidney and tumor: lung ratios seen with 34–417 raised the possibility that 34–417 reacts with mouse kidney and lung, although we have not tested this hypothesis directly. By day 8 there was no longer any evidence of specific localization of R24 to muscle, demonstrating a relatively short half-life of radiolabel in muscle, approximately 2.5 days. In comparison, radiolabel had a half-life of approximately 16.5 days at tumor sites. Thus, despite specific targeting of R24 to GD3 on both tumor and normal muscle, the relative half-life of radiolabel was >5 times longer at tumor sites compared to normal muscle.

By day 15 (Fig. 8, lower), the majority of tumor:tissue ratios for all mAbs, including control mAb 34–417, were greater than 3. The R24 tumor: blood ratio continued to increase as R24 cleared from the blood pool and was substantially higher (5- to 16-fold) than tumor: blood ratios of V1-R24, V2-R24, or 34–417. For tumor: tissue ratios other than tumor: blood, relative targeting of R24 was only modestly increased (approximately 2- to 3-fold) compared to either R24 variant. With the exception of tumor: kidney and tumor: lung ratios for 34–417 as noted above, the localizations of V1-R24, V2-R24, and 34–417 in nontumor tissues were generally equivalent, although by day 15, tumor: tissue ratios for the R24 variants were approximately 2-fold higher than for 34–417.

Values for maximum percentage of injected dose/g of tumor were similar for R24, V2-R24, and control 34–417 mAb and ranged from 0.34 to 1.2% of injected dose/g. The relatively low percentage of injected dose at the tumor may be explained, in part, by the fact that only a low dose of R24, approximately 3 μg was injected per mouse. We observed no correlation between the percentage of injected dose/g of tumor and tumor size (data not shown).

Prevention of Human Melanoma Xenograft Outgrowth in the nu/nu Mouse Model. Mice were inoculated s.c. with 5 × 10⁶ viable SK-MEL-30 human melanoma cells and immediately started on treatment with either R24, V1-R24, V2-R24, or 34–417 injected i.p. The time course of tumor outgrowth is presented in Fig. 9. Neither V1-R24 nor 34–417 prevented tumor outgrowth; 8 of 10 and 9 of 10 mice developed tumors, respectively. This incidence was the same as the incidence of tumor outgrowth in untreated mice (data not shown). Six of 10 mice treated with V2-R24 developed tumors by day 34, while only 3 of 10 mice treated with R24 developed tumors. Measurements of mean tumor size (determined as the product of greatest tumor diameter and its perpendicular, expressed in mm²) were smallest in R24-treated mice (mean diameter, 25 mm² at day 20), intermediate in size in V2-R24-treated mice (29 mm²), and largest in mice treated with V1-R24 (48 mm²) and 34–417 (34 mm²), but differences between groups were not statistically significant (data not shown). These results show that (a) neither the control IgG3 (34–417) nor V1-R24 prevented tumor outgrowth, (b) V2-R24 seemed to induce partial tumor protection, and (c) R24 abrogated the outgrowth of tumors in the majority of animals.
DISCUSSION

The antigen binding site of immunoglobulin molecules is created by three-dimensional interactions of variable regions of heavy and light chains. A crucial role for the light chain in affinity has been demonstrated in experiments where minor alterations of the light chain, including single amino acid substitutions, have been shown to result in complete loss of antigen binding. On the other hand, antibodies have been identified that retain specificity and similar affinity despite complete substitution of light chain (21). Therefore, while variable regions of light chains can play a critical role in determining affinity, the relative contribution of heavy and light chains in determining affinity varies from antibody to antibody. In our studies with R24, it appeared that the R24 heavy chain determined the specificity for Gd, since even V1-R24 binds to Gd3. We cannot yet rule out the possibility that the MOPC-21 light chain and the R24 light chain are related (e.g., come from the same family) and that MOPC-21 could contribute to avidity for Gd3 in the R24 variants. Surprisingly, the presence of two, but not one, R24 light chains enhanced avidity for Gd3 40-fold. This suggests that divalent binding enhances avidity for Gd3 and that valency is involved in the higher avidity of R24.

In most in vitro assay systems that we studied (ADCC, T-lymphocyte stimulation, prevention of melanoma attachment), activity correlated with relative avidity for Gd3. In general, results with V1-R24 and V2-R24 were equivalent and showed either no activity or activity which was markedly less than R24. A striking exception was the ability to mediate CDC, which did not strictly correlate with avidity for Gd3. V2-R24 could mediate CDC, although less than R24; V1-R24 showed no activity.

Both V1-R24 and R24 demonstrated similar pharmacokinetics, with a t1/2 of 50 h. It is interesting to note that, in patients with melanoma injected with 111In-labeled R24, we have observed a nearly identical terminal half-life (22). The fact that V1-R24 and R24 showed identical blood clearance rates suggests that the Fc region regulates R24 and V1-R24 clearance from the blood.

Tumor targeting is of special interest since it has been presumed that efficient targeting is a prerequisite for therapeutic efficacy. However, many factors other than avidity are known to influence the ability of mAb to target tumors (23–26). Studies in our model system have suggested that ADCC correlates well with antitumor effects (27, 28). In our model, the in vitro effector function which best correlated with antitumor effects in vivo was CDC. In support of this, previous animal studies have shown that depletion of serum complement by cobra venom factor abrogates the antitumor effects of R24 (12). The mechanism of antitumor activity in our nu/nu mouse model is unclear but could involve specific induction of inflammatory response at tumor sites.

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Light Chain Variants of an IgG3 Anti-GD3 Monoclonal Antibody and the Relationship among Avidity, Effector Functions, Tumor Targeting, and Antitumor Activity

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