Regulation of Antibody-Dependent Cellular Cytotoxicity by IgG Intrinsic and Apparent Affinity for Target Antigen

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Unconjugated mAbs have emerged as useful cancer therapeutics. Ab-dependent cellular cytotoxicity (ADCC) is believed to be a major antitumor mechanism of some anticancer Abs. However, the factors that regulate the magnitude of ADCC are incompletely understood. In this study, we described the relationship between Ab affinity and ADCC. A series of human IgG1 isotype Abs was created from the anti-HER2/neu (also named c-erbB2) C6.5 single-chain Fv (scFv) and its affinity mutants. The scFv affinities range from \(10^{-7}\) to \(10^{-11}\) M, and the IgG Abs retain the affinities of the scFv from which they were derived. The apparent affinity of the Abs ranged from nearly \(10^{-10}\) M (the lowest affinity variant) to almost \(10^{-11}\) M (the other variants). The IgG molecules were tested for their ability to elicit ADCC in vitro against three tumor cell lines with differing levels of HER2/neu expression using unactivated human PBMC from healthy donors as the effector cells. The results demonstrated that both the apparent affinity and intrinsic affinity of the Abs studied regulate ADCC. High-affinity tumor Ag binding by the IgGs led to the most efficient and powerful ADCC. Tumor cells expressing high levels of HER2/neu are more susceptible to the ADCC triggered by Abs than the cells expressing lower amounts of HER2/neu. These findings justify the examination of high affinity Abs for ADCC promotion. Because high affinity may impair in vivo tumor targeting, a careful examination of Ab structure to function relationships is required to develop optimized therapeutic unconjugated Abs.

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Unconjugated mAbs have emerged as useful agents to treat different human cancer (1–11). Several clinically relevant mechanisms of the antitumor action of those unconjugated mAbs have been identified. These include complement fixation (6, 12), interference with ligand-receptor interactions (9, 13), modulation of cell signaling (10, 14, 15), and Ab-dependent cellular cytotoxicity (ADCC), which can occur when Abs simultaneously engage Ags on target cells through their Fab domains and Fc receptors on effector cells’ through Fc domains (16, 17). Although many clinically used unconjugated mAbs possess the human IgG1 isotype that promotes ADCC, the clinical relevance of this mechanism has been best demonstrated for rituximab, a CD20 specific murine-human chimeric mAb that is useful in the treatment of lymphoma. Patients with “high-responder” Fc receptor polymorphisms exhibited improved response rates in two separate studies (18, 19), suggesting the importance of ADCC for the clinical effectiveness of this Ab. Accordingly, many investigators have surmised that it is desirable to construct Abs that can efficiently mediate ADCC to maximize their clinical properties.

Much has been learned about the cellular mechanisms that regulate ADCC, including the relevance of distinct Fc receptors (20–22) and different effector cell populations (22–25). Cytokine modulation of ADCC has been demonstrated as well (26). Manipulation of the Fc domain structure by introducing mutations (27) or modifying fucosylation (28) has been shown to enhance ADCC. However, surprisingly little is known about how Ab affinity for target Ag affects ADCC, primarily because most of the compared low- and high-affinity Abs reported do not recognize the same epitope, as usually they are not derived from a single ancestor Ab. Hence, the improved ADCC seen with higher affinity Abs cannot be explained solely by the contribution of affinity (29).

We have previously designed and characterized the binding properties of a series of single-chain Fv (scFv) molecules that target an identical epitope on the extracellular domain (ECD) of HER2/neu. The original C6.5 scFv was isolated from a naïve human phage display library and bound to the ECD of HER2/neu with a \(K_D\) of 16 nM (30). This scFv was then subjected to chain-shuffling and site-directed mutagenesis to yield a series of mutants with affinities ranging from \(10^{-7}\) to \(10^{-11}\) M (31, 32). We have previously shown that higher affinity scFv do not target human tumor xenografts growing in immunodeficient mice better than their lower affinity variants (33), and in fact exhibit restricted trafficking through the tumors. In contrast, higher affinity does improve the ability of bispecific scFv heterodimers, prepared by fusing each of these scFvs to an anti-CD16 scFv (34), to promote ADCC against HER2/neu-expressing target cells (35). More recently we have shown that multivalent binding to the tumor Ag promotes more ADCC than does monovalent binding (36). However, the regulation of ADCC promotion by the affinity of IgG mAbs has not been examined previously using a rigorously derived panel of Abs that bind to an identical epitope of the targeted
Ag. To gain a balanced understanding of this issue, the scFv affinity mutants described were converted into human IgG1 Abs. We show in this study that IgG Abs with higher apparent affinities promote more ADCC by human NK cells than does a lower affinity variant. The Ag density on tumor cells must exceed a threshold in order for even high-affinity IgG Abs to mediate ADCC. We also demonstrate that high intrinsic binding affinity variants promote more ADCC, even when the affinity mutants possess essentially identical apparent affinities. These findings have important implications for the development of clinically relevant unconjugated Abs that mediate ADCC.

Materials and Methods

Commercial Abs and cells

Abs to FcγRI (197, IgG2a), FcγRII (IV.3, IgG2b), and FcγRIII (3G8, IgG1) were obtained from Medarex. Trastuzumab was obtained from the pharmacy of the University of California (San Francisco, CA) or Fox Chase Cancer Center (Philadelphia, PA). Monoclonal Ab 520C9 (37), a murine anti-HER2/neu IgG1, was purified from hybridoma supernatants by standard protein G affinity chromatography (GE Healthcare).

Three tumor cell lines with different expression levels of HER2/neu receptors were used in this study: MDA-MB-231, MDA-MB-361, and SK-OV-3 cells. All cells were cultured in proper medium based on American Type Culture Collection recommendations. Human PBMCs were isolated by density gradient centrifugation. Human NK cells were enriched by negative selection using the RosetteSep NK Cell Enrichment Cocktail (StemCell Technologies), consistently yielding >80% CD56+ CD16+ NK cells. Murine NK cells were isolated from CB-17 scid mouse splenocytes using lympholyte-M (Cedarlane Laboratories), and cultured in RPMI 1640 medium supplemented with 10% FBS, 1000 U/ml recombinant human IL-2 (Teceleukin, obtained from National Cancer Institute, Rockville, MD).

Conversion of C6.5 variants phagemic scFv to full-length human IgG1

The C6.5 IgG variants, derived from the G98A, C6.5, ML3-9, H3B1, and B1D2 scFv, were produced by stably transfected Chinese hamster ovary (CHO) DG44 cell lines. The adherent wild-type CHO DG44 cell line was a gift from Dr. D. Powers (PDL Biopharma, Freemont, CA). It was later adapted to serum-free medium and maintained in suspension culture using CHO-SFM-II medium (Invitrogen Life Technologies). All C6.5 variant IgG1 expression plasmids used for cell transfection were constructed on the basis of a full-length human IgG1 mammalian expression vector NSLG1Val-Lark, a gift from Dr. M. Reff (Biogen Idec, San Diego, CA). The plasmid construction process was similar to the published protocols (38, 39). Stable IgG1-producing cell lines for each C6.5 variants were established by selecting transfected cells in G418 first, then expanding selected cells step-wise into 1-L spinner flasks. The IgG1 production level of each C6.5 variants was increased by MTX amplification as reported (40). Cell culture supernatants containing each C6.5 IgG1 variant were collected, concentrated by ultrafiltration, loaded, and purified on protein G affinity columns. The purity and concentration of each C6.5 IgG variant was then assessed by native and denaturing SDS-PAGE, and the final concentration of each buffer-exchanged IgG was determined by DC protein assay (Bio-Rad).

C6.5 IgG variant affinity characterization by BIACore and FACS

The intrinsic binding affinity of each C6.5 IgG variant to HER2/neu was calculated using BIACore analysis. Briefly, binding kinetics with soluble HER2/neu ECD of each IgG were measured using surface plasmon resonance on a BIACore 1000 (GE Healthcare), and the association rate constant (ka) and dissociation rate constant (kd) data were used to calculate the Kd for each IgG. Approximately 2000–3000 response units of soluble HER2/neu ECD were coupled to a CMS sensor chip according to the manufacturer’s instructions. IgG samples in concentrations between 10 pM and 800 nM in HEPES-buffered saline (pH 7.4) were injected over the ECD surfaces of HER2/neu with a 20-min association phase followed by a 4-h dissociation phase. All experiments were conducted at 24°C. The Kd for each C6.5 IgG variant was measured under continuous flow of 15 ml/min, with the approximate captured IgG amount in the range from 600 to 1000 response units. The HER2/neu surface was regenerated after each cycle using 10 mM glycine (pH 1.7). The Kd value was determined from a plot of (ln(Rd/dt))/t vs Ab concentration. The Kd was determined from the dissociation part of the sensorgram at the highest concentration of Ab used, with a flow rate of 15 ml/min to prevent rebinding. Kd was calculated as Koff/Kon.

The apparent affinity of each C6.5 IgG variant was measured by FACS using the SK-OV-3 cell line, as described (38). All the measurements were performed at least three times for each IgG at 4°C, 25°C, or 37°C. Briefly, 105 to 106 tumor cells were stained with each C6.5 IgG variant at concentrations ranging from 1 pM to 500 nM. The total reaction volume (from 100 ul to 50 ml) and incubation time (from 1 to 24 h) were adjusted according to the HER2/neu density on the cell surface and the concentration of Ab being tested, so that all HER2 receptors on each cell were saturated by the testing Ab if equilibrium was reached during the staining process. FITC- or PE-conjugated goat anti-human IgG was used to detect the cell surface captured C6.5 IgG variant. The mean fluorescence intensity (MFI) of each sample was recorded after FACS analysis of the stained cells. The apparent affinity Kd value was then calculated using the equation: 

\[ Y = (m_1 + m_2)n/P(m_3 + m_0) \]

where Y is the MFI of cell samples stained with each concentration of C6.5 IgG variant, m0 is IgG concentration used, m1 is MFI of negative control cell samples without C6.5 variants Ab added, and m2 is MFI of positive control cell samples at saturation concentration of IgG, and m3 is Kd, with the unit reciprocal of IgG concentration used.

Characterization of HER2/neu expression level

Quantitative flow cytometry, using the QuantiBRITE PE kit (BD Immunocytometry Systems), was performed on each of the cell lines to characterize the level of HER2/neu expression. Briefly, cell lines were harvested by mild trypsin treatment, incubated with PE-conjugated anti-HER2/neu Ab, and subjected to flow cytometry using the manufacturer's recommended condition. Signal intensity was converted to molecules of PE based on direct comparison to a series of standards included in the kit and then converted to molecules of Ab bound to the cell surface based on the specific activity of the PE-conjugated Ab (i.e., the number of PE molecules coupled per molecule of Ab).

Ab labeling

The H3B1 IgG was labeled with FITC using the EZ-Label FITC protein labeling kit as instructed (Pierce). Briefly, 1 mg of H3B1 IgG was buffer changed to BupH Borate buffer, then mixed with 24-fold molar excess of FITC. The reaction mixtures were protected from light and incubated at room temperature for 1 h. Unbound FITC was removed from protein-bound FITC by dialysis against PBS. The binding of labeled H3B1 IgG to HER2/neu was shown by FACS analysis.

ADCC assay

This assay was performed as described previously (36). Briefly, human PBMC, NK cells, or murine NK cells served as the effector cells for this assay. The target cells were labeled with Na51CrO4 (100 μCi/106 targets; PerkinElmer) for 1 h at 37°C in RPMI 1640 medium followed by washed twice and resuspended at 2 × 105 cells/ml. A total of 50 μl of labeled target cells were added to individual wells of 96-well plates containing effector cells and/or Abs. Effector cells were added to yield a different E:T ratio in the presence or absence of various concentrations of Abs. Each well contained a total volume of 200 μl, and all assays were performed in triplicate. The plates were centrifuged at 300 × g for 3 min, incubated for 4 h in a 5% CO2 incubator at 37°C, then centrifuged again at 300 × g for 3 min. A total of 100 μl of supernatant were removed from each well for counting on a gamma-counter (PerkinElmer). Cytotoxicity was estimated by measuring the quantity of label released into culture supernatants using the formula: percentage of lysis = ((experimental release cpm − spontaneous release cpm)/(total added counts cpm/2 − spontaneous release cpm)) × 100, where the experimental release was defined as cpm released by target cells in wells in the presence of effector cells and/or Ab and the spontaneous release was defined as cpm released by target cells alone.

Complement-dependent cytotoxicity (CDC) assay

The SK-OV-3 target cells were labeled with 51Cr in the same way as described for the ADCC assay. Target cells (105) were mixed in varying concentrations of C6.5 IgG variants and 10% of cold human serum or 5% FCS as the m1 medium. After 2 h at 37°C, cells were sedimented, and 100 μl of the supernatant was transferred to counting tubes to measure radioactivity release using a gamma-counter. Cytotoxicity was calculated using the same formula as the ADCC assay. All experiments were performed in triplicate.
Table I. Binding properties of anti-HER2/neu scFv and IgG variants

| C6.5 Variants | ScFv $K_D$ Biacore/SC-OV-3 | IgG Intrinsic $K_D$ Biacore | IgG Functional $K_D$ | SK-OV-3 |
|---------------|-----------------------------|-----------------------------|---------------------|---------|
| G98A          | $3.2 \times 10^{-7}$ M      | $2.7 \times 10^{-7}$ M      | $5.0 \times 10^{-10}$ M |
| C6.5          | $1.6 \times 10^{-8}$ M      | $2.3 \times 10^{-8}$ M      | $5.4 \times 10^{-11}$ M |
| ML3-9         | $1.0 \times 10^{-7}$ M      | $7.3 \times 10^{-8}$ M      | $3.7 \times 10^{-11}$ M |
| H3B1          | $3.2 \times 10^{-10}$ M     | $5.6 \times 10^{-10}$ M     | $4.7 \times 10^{-10}$ M |
| BI1D2         | $1.6 \times 10^{-11}$ M     | $2.8 \times 10^{-11}$ M     | $2.5 \times 10^{-11}$ M |

Cell proliferation and apoptosis assay

Cell proliferation assays were performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit according to the manufacturer’s instructions (Promega). Briefly, cells were seeded into 96-well plates at 3000–6000 cells/well in varying Ab concentrations. The total volume for each well was 100 μL, and all assays were performed in triplicate. The cultures were incubated in a CO2 incubator for 2–4 h, and then 20 μL of CellTiter 96 AQueous One Solution Reagent was added to the culture medium. After incubating the plate 2–4 h in a CO2 incubator, the absorbance at 492 nm was measured with a 96-well plate reader. Each experiment was repeated at least twice.

Caspase-3/7 activities were measured using the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega). Cells were cultured in 96-well plates (ViewPlate-96, black; Packard) in varying Ab concentrations for 48 h at 37°C in a humidified, 5% CO2 atmosphere. Caspase-3/7 was released by lysing the cells with an equal volume of lysis buffer containing caspase substrate and incubating at room temperature for 1–2 h. Fluorescence was measured using Labsystems Fluoroskan Ascent FL (Thermo Scientific) at 485 nm excitation and 538 nm emission.

For flow cytometry analysis of apoptosis, cells were stained by FITC-conjugated annexin V and propidium iodide. Annexin V-positive and propidium iodide-negative cells are early apoptotic cells. Cells were cultured in 6-well plates for 24 h with or without 25 μg/ml Abs. Immediately after harvest, cells were washed twice with PBS and stained with Annexin V-FLUOS staining kit (Roche) according to the manufacturer’s protocol. Analysis was conducted using FACScan flow cytometer, and 10,000 events were analyzed using CellQuest software (BD Biosciences).

Cell surface retention assay

SK-OV-3 cells were incubated with varying concentrations of the C6.5 IgG variants in a 37°C, 5% CO2 incubator for 4 h, followed by washing three times with PBS supplemented with 1% BSA and 0.1% NaN3. The Abs retained on cell surface were detected by saturating amounts of FITC-conjugated anti-human IgG Ab, and measured on a FACScan flow cytometer. Analysis was conducted with FlowJo software, and the MFI was used to represent the amount of IgGs on the cell surface. Analysis was conducted with FlowJo software, and the MFI was used to represent the amount of IgGs on the cell surface.

For Ab blocking studies, SK-OV-3 cells were incubated with C6.5 IgG and H3B1 IgG, and then incubated with varying concentrations of soluble HER2/neu ECD at 37°C for 2 h. The retention of Abs on the cell surface was analyzed similarly after washing.

Results

C6.5 IgG variant production and characterization

Each of the IgG molecules used in the current study was obtained by converting the corresponding scFv into a full human IgG1. The C6.5 variant plasmid stably transfected CHO DG44 cell lines produced pure IgG at the level of 4–40 μg/cell/day (i.e., 10–100 mg/L) in 1-L spinner flasks. After protein G affinity column purification, all the C6.5 IgG variants were over 95% pure as judged by reduced and nonreduced SDS-PAGE (data not shown). All C6.5 IgG variants retained their corresponding scFv specificities toward HER2/neu as confirmed by BIAcore and FACS analysis (31, 32) (Table I and Fig. 1). The C6.5 IgG variants retained the relative intrinsic affinities of the scFv from which they were derived by BIAcore analysis. However, excepting the BI1D2, the apparent affinities of the corresponding IgG molecules were higher than the scFv when checked on higher receptor density cell lines such as SK-OV-3. Even the lowest intrinsic affinity variant G98A IgG has a functional $K_D$ value of $10^{-10}$ M, while the functional affinity was $10^{-11}$ M for the other IgGs that were constructed (Table I).

All the C6.5 IgG variants bound to cell surface-associated HER2/neu. Binding was dependent on the affinity of the tested Ab and HER2/neu copy numbers on the tumor cells. Based on quantitative flow cytometry, MDA-MB-231, MDA-MB-361, and SK-OV-3 cells possess 2.8 $\times$ 10⁴, 3.8 $\times$ 10⁵, and 1.3 $\times$ 10⁶ HER2/neu receptors per cell, respectively (Table II). As expected, each Ab bound better (i.e., yielded a higher MFI) to cells with higher levels of HER2/neu expression (Fig. 1) according to flow cytometry results.

Apparent affinity is an important determinant of binding of these IgG molecules to HER2/neu expressing tumor cells. For each tumor cell line tested, the two Abs with higher apparent affinity (e.g., C6.5 and H3B1) exhibited roughly equivalent tumor cell retentions that were greater than that achieved using G98A IgG (Fig. 1). G98A, which has 10-fold lower intrinsic affinity than C6.5 and 1000-fold lower intrinsic affinity than H3B1, also has a 10-fold lower apparent affinity than these two Abs, which have $10^{-11}$ M apparent affinity. These experiments were performed using relatively high concentrations of the Abs, and C6.5 IgG binding to MDA-MB-361 and SK-OV-3 cells was equivalent to its higher affinity counterparts. However, G98A IgG showed no specific binding to MDA-MB-231 cells, which express lower levels of HER2/neu, and C6.5 IgG bound less well than the higher-affinity
variants to this cell line, even though C6.5 shares the same apparent affinity for HER2/neu as H3B1. At lower concentrations of Ab, this relationship of affinity to cell retention was generally maintained in all three tested cell lines, which exhibit a 100-fold range of HER2/neu expression. When C6.5 IgG and H3B1 IgG were tested for binding to SK-OV-3 cells at a variety of concentrations, C6.5 IgG was bound as well as its higher-affinity counterpart (Fig. 2).

To determine whether the C6.5 IgG variants bind to a similar epitope on HER2/neu, a competition assay was performed. A total of 10⁶ SK-OV-3 cells were preincubated with 1 μg of either C6.5 IgG variants or trastuzumab, which binds to a distinct epitope on the ECD of HER2/neu. After washing the cells, 1 μg of the FITC-labeled 10⁻¹⁰ M affinity variant, H3B1 IgG, was added, and the binding of this Ab was assessed by flow cytometry. As shown in Fig. 3, preincubation with trastuzumab did not influence the ability of labeled H3B1 IgG to bind to the cells. Similar results were obtained using G98A IgG, which has an affinity of 10⁻⁷ M. Presumably, this low-affinity molecule cannot effectively compete with H3B1 IgG, which has 1000-fold higher intrinsic affinity and 10-fold higher apparent affinity for the ECD of HER2/neu. In other experiments (data not shown), we have found that unlabeled H3B1 IgG effectively competes with binding of FITC-labeled G98A IgG to SK-OV-3 cells. However, all of the other, higher-affinity variants blocked binding of labeled H3B1 IgG to the cells, in a roughly affinity-dependent manner. These results are consistent with interpretation that the tested affinity mutants bind to very similar, if not identical epitopes on the ECD of HER2/neu.

**AB affinity regulates the capacity to promote ADCC**

To determine whether Ab affinity affects the capacity of PBMC and NK cells to mediate ADCC, a standard ⁵¹Cr release assay was performed. Purified human PBMC from healthy donors were used as effector cells and MDA-MB-231, MDA-MB-361, and SK-OV-3 cells were used as targets. The results obtained with G98A, C6.5 and H3B1 IgG, which represent low, medium, and high intrinsic affinity IgG molecules, are representative of the relationships among affinity, binding and cytotoxicity promotion of the five examined affinity variants. Assays were conducted at E:T ratios of 1:1, 5:1, 25:1, and 50:1 using Ab concentrations ranging from 0.001 to 10 μg/ml. Monoclonal Ab 520C9 was used as an interassay control.

As might be expected from the binding studies (Fig. 1), ADCC increased 1) as a function of HER2/neu expression, 2) with increasing binding to HER2/neu, and 3) with higher affinity of the individual binding sites (Fig. 4A). Tumor cells with higher levels of HER2/neu expression were more susceptible to ADCC mediated by each tested Ab. MDA-MB-231 cell expresses low levels of HER2/neu, and is bound poorly by all tested C6.5 IgG variants (Fig. 1, upper row). Unsurprisingly, none of the affinity variants mediated significant cytotoxicity, even at a high E:T ratio and high Ab concentration (Fig. 4A, upper panel). MDA-MB-361 and SK-OV-3 cells were progressively more susceptible to ADCC mediated by all tested Abs (Fig. 4A, middle and lower panels).

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**Table II. Influence of tumor Ag density and affinity on ADCC promotion**

| Cell Line     | Receptor No./Cella | Conc. (μg/ml) Needed for 20% Cytotoxicity at 25:1 E:T Ratiob | Conc. (μg/ml) Needed for 20% Cytotoxicity at 50:1 E:T Ratiob |
|---------------|--------------------|------------------------------------------------------------|------------------------------------------------------------|
|               |                    | G98A IgG (10⁻⁷ M) | C6.5 IgG (10⁻⁸ M) | H3B1 IgG (10⁻¹⁰ M) | G98A IgG (10⁻⁷ M) | C6.5 IgG (10⁻⁸ M) | H3B1 IgG (10⁻¹⁰ M) |
| MDA-MB-231    | 2.8 × 10⁴          | >10              | >10              | >10              | >10              | >10              | >10              |
| MDA-MB-361    | 3.8 × 10⁵          | >10              | 1.1502           | 0.1018           | 0.0077           | 1.5082           | 0.1961           | 0.0394           |
| SK-OV-3       | 1.3 × 10⁶          | 0.2894           | 0.1018           | 0.0077           | 0.0568           | 0.0184           | 0.0028           |

a Statistical analysis was based on the experimental ADCC assay data, conducted as described in Fig. 4.
b The Ab concentrations required to achieve 20% cytotoxicity at 25:1 and 50:1 E:T cell ratio were calculated. All of the comparisons are significant at the 5% level. All the p values are <0.001. Two data sets were averaged for all comparisons.

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**FIGURE 2.** Ab retention on SK-OV-3 HER2/neu-expressing tumor cells. Cells were incubated with varying concentrations of C6.5 IgG variants at 37°C, 5% CO₂ for 4 h. The Abs retained on cell surface after washing were detected by FITC-conjugated anti-human IgG Ab. The MFI was used to represent the amount of IgGs on cell surface. Data represent mean ± SE of triplicate determinations.

**FIGURE 3.** The C6.5 IgG variants bind to an identical or similar epitope on the ECD of HER2/neu. SK-OV-3 cells were preincubated with the indicated unlabeled Ab for 30 min on ice, washed, and then incubated in 1 μg of FITC-labeled H3B1 IgG, as described in the Results. Data shown are the mean ± SE of triplicate determinations of the MFI.
In these assays, which used unactivated human PBMC, a higher E:T ratio increased the amount of cytotoxicity, but similar affinity-dependent patterns were observed at all E:T ratios that induced at least some tumor killing. The influence of affinity on tumor targeting was evident with both the MDA-MB-361 and SK-OV-3 cell lines, but the dynamic range was more restricted (e.g., G98A IgG induced more ADCC) in the setting of very high levels of HER2/neu expression. Interestingly, C6.5 IgG was more efficient than its lower-affinity variant, G98A IgG, but promoted less cytotoxicity than H3B1 IgG against both MDA-MB-361 and SK-OV-3 cells, even though the latter two Abs possess identical apparent affinity. To facilitate comparative analysis of the data, we calculated the concentrations of Ab needed for 20% cytotoxicity at E:T ratios of 25:1 (Table IIA) and 50:1 (Table IIB). As can be seen, at the 25:1 E:T ratio, none of the tested Abs promoted significant ADCC against MDA-MB-231 cells. However, the MDA-MB-361 cell line showed a clear affinity-dependent susceptibility to ADCC, as >10 μg/ml G98A IgG was required to achieve the targeted lysis level, as compared with 1.15 μg/ml C6.5 IgG and only 0.11 μg/ml H3B1 IgG. Similar patterns were observed using SK-OV-3 targets, which are more sensitive to ADCC due to the higher level of HER2/neu expression. In this experiment, 0.3 μg/ml G98A IgG was required to achieve the target level of lysis, as opposed to only 0.1 μg/ml C6.5 IgG and 0.01 μg/ml H3B1 IgG. Analysis of ADCC at a 50:1 E:T ratio yielded similar patterns (Table IIB). Identical studies performed using the ML3-9 IgG and B1D2 IgG affinity variants were consistent with the above findings and conclusions that intrinsic binding site affinity regulates the extent of ADCC (data not shown).

To determine whether NK cells are the predominant population in the effector cells that mediate ADCC promoted by these Abs, human NK cells were enriched from blood obtained from normal
donors using the RosetteSep NK Cell Enrichment Cocktail and used as effector cells in the ADCC assay. The NK cells were triggered to kill target cells by the C6.5 IgG variants, with a similar affinity-related pattern (Fig. 4B). This indicates that human FcyRIII is a key receptor in the ADCC initiated by the C6.5 IgG variants. Because all C6.5 IgG variants share the same human IgG1 constant region structure, we wished to determine whether the Abs can promote ADCC by murine NK cells because this property would be useful to test the antitumor properties of these Abs in mouse model. As shown in Fig. 4C, unactivated murine NK cells did not promote ADCC mediated by C6.5 IgG. However, prior activation of the NK cells by IL-2 led to the induction of significant tumor cell lysis.

FcyRIII is the primary Fc receptor responsible for ADCC promoted by the C6.5 series of human IgG

ADCC occurs when target and effector cells are bridged by Ab through Fab-Ag (target) interactions and Fc-Fc receptor (effector) interactions, respectively. Three human Fc receptors FcyRI, FcyRII, and FcyRIII interact with IgG (41). As shown in Fig. 5, blocking Abs to FcyRI (197) and FcyRII (IV.3) did not inhibit the binding of FITC-labeled H3B1 IgG Fc to human PBMC, in contrast to the anti-FcyRIII Ab 3G8, which inhibited the binding of H3B1 IgG Fc to all the NK cells and a small percentage of monocytes (Fig. 5). These findings were confirmed by cytotoxicity assays showing that human PBMC-mediated ADCC promoted by H3B1 IgG was inhibited by the anti-FcyRIII Ab 3G8, but not by the anti-FcyRI Ab 197 and anti-FcyRII Ab IV.3 (data not shown).

C6.5 IgG variants do not induce CDC, affect tumor cell proliferation, or promote apoptosis

Besides ADCC, CDC, signal transduction perturbation, apoptosis induction, and immunomodulation are also potential mechanisms of antitumor Ab action (17, 42). To determine whether the C6.5 series of IgG molecules uses other mechanisms of action, the ability of these Abs to induce CDC, inhibit proliferation, and induce apoptosis was tested. None of the tested C6.5 IgG variants initiated CDC to SK-OV-3 cells in the presence of human serum. None of the C6.5 IgG variants inhibited the proliferation of the HER2/neu-expressing tumor cell lines, MDA-MB-361, SK-OV-3, and BT-474 cells (data not shown). It has been reported that dimerized IgGs have significant antitumor activity against their target cells, whereas the monomers showed no effect (43). However, none of the C6.5 IgG variants showed antiproliferative activity when cross-linked by secondary Ab (data not shown). Two methods were used to detect tumor cell apoptosis. Caspase-3/7 activity in SK-OV-3 cells and MDA-MB-361 cells could not be detected after exposure to varying concentrations of C6.5 IgG variants for 48 h, and annexin V staining did not reveal a significant early apoptotic cell population (data not shown). These results indicate that the C6.5 IgG variants do not induce CDC, do not promote tumor cell apoptosis, and do not mediate direct antiproliferative effects.

The binding properties of C6.5 IgG and H3B1 IgG are different

Though C6.5 IgG and H3B1 IgG possess the same apparent affinity, and therefore similar cell surface retention properties (Fig. 2), they have differing tumor cell binding properties. When the IgGs were occupied (or blocked) by soluble HER2/neu ECD, they showed disparate binding patterns to SK-OV-3 (Fig. 6A). Low concentrations of soluble HER2/neu ECD did not block binding of either Ab to the tumor cells. As the soluble HER2/neu ECD concentration increased, the binding of H3B1 IgG to SK-OV-3 dropped more quickly than that of C6.5 IgG. Thus, the high affinity of H3B1 IgG for the soluble competitor saturates its binding sites and reduces binding to the cell surface-associated target. However, after the respective IgGs were bound to the cell surface, they showed different retention properties when soluble HER2/neu ECD was subsequently added (Fig. 6B). H3B1 IgG showed more stable binding to the cell surface in the presence of high concentrations of soluble HER2/neu ECD. In this setting the high affinity of H3B1 IgG promoted better tumor cell retention during the time course of the experiment.
Discussion

This is the first rigorous examination of the effects of human IgG affinity on the ability of Abs to mediate ADCC. The availability of a series of affinity mutants derived from a single human scFv makes this a valuable resource because analysis is not confounded by the uncertainties associated with Abs that bind to distinct epitopes on a targeted Ag, or possess differing isotypes that may influence their interactions with Fc receptors or complement. We show in this study that affinity for the target Ag clearly influences the extent and efficiency of ADCC, and that the relationships hold true in tumor cell lines with widely disparate levels of target Ag expression. Far lower concentrations of the H3B1 IgG (high-affinity Ab) are required to mediate ADCC than G98A IgG (the lowest affinity counterpart tested), and unlike G98A IgG, the high-affinity Ab is able to mediate efficient ADCC even when the target Ag is expressed in relatively low copy numbers. This set of results could be interpreted as evidence that the density of Ab on the tumor cell surface is the critical determinant of affinity-dependent effects on ADCC. However, this straightforward interpretation is confounded by the surprising observation that H3B1 IgG, with a higher intrinsic binding site affinity, increases cytotoxicity even when compared with a lower affinity Ab (C6.5 IgG) with a similar magnitude of apparent affinity.

Because these comparable functional affinities result in similar levels of Ab retention on targeted tumor cell surfaces over a broad range of Ab concentrations (Figs. 1 and 2), factors other than the actual density of Ab on the tumor cell surface must be another critical determinant of ADCC promotion. This possibility may be less true at lower Ab concentration ranges, or when there are low densities of target Ag on the cell surface. We found that C6.5 IgG and H3B1 IgG exhibited different cell surface retention when soluble HER2/neu ECD is added to the system (Fig. 6), though they possess similar apparent affinities (Fig. 2). These data suggest that when C6.5 IgG binds to the SK-OV-3 cell surface, it initially binds monovalently. Because C6.5 has a higher dissociation constant than H3B1 IgG, the binding arms of C6.5 IgG then dissociate from the cell surface more rapidly. Accordingly, the retention of C6.5 IgG to the cell surface is reduced in the presence of excess HER2/neu ECD (Fig. 6). We speculate that C6.5 IgG swaps its two binding domains on the cell surface if there is no competition from soluble Ag, resulting in a prolonged cell surface retention and high apparent affinity, based in part on sustained, but alternating monovalent binding. In contrast, HER2/neu ECD does not as effectively compete H3B1 off the cell surface, due to this Ab’s slower off-rate. We interpret these results to indicate that C6.5 IgG binds to the cell both monovalently and divaletly, whereas H3B1 IgG more likely binds to the SK-OV-3 cell surface divaletly (Fig. 7), and that binding valency is another important determinant of ADCC promoted by IgG molecules.

Besides Ab affinity, Ag expression level on the target cell surface is another factor that affects ADCC. As more IgGs are coated on the target cells, the potency of cytotoxicity increases accordingly. Trastuzumab-mediated ADCC against esophageal squamous cell carcinoma is correlated with the degree of HER2/neu expression (44), and cetuximab induces more ADCC against epidermal growth factor receptor-expressing esophageal squamous cell carcinoma (45). Similarly, our data indicate that target cells with higher Ag expression are more susceptible to ADCC. Irrespective of the tested IgG, more Ab molecules bound to SK-OV-3 cells than to MDA-MB-361 or to MDA-MB-231 cells (Fig. 1), and ADCC was most robust using SK-OV-3 cells as target cells. G98A IgG bound less well to MDA-MB-361 and SK-OV-3 cells, which express moderate and high levels of HER2/neu, respectively, than the other Abs, due to its lower apparent affinity. G98A IgG did not show any detectable binding to MDA-MB-231 cell, which expresses a low level of HER2/neu. C6.5 IgG bound less well to MDA-MB-231 cells than H3B1 IgG. Because the density of HER2/neu on MDA-MB-231 cell surface is so low, we speculate that the distance between two HER2/neu molecules extends beyond the span of the two Ab-binding arms of the Abs, precluding divalent binding. Though C6.5 IgG has the same apparent affinity as H3B1 IgG, it still showed less monovalent binding, presumably because its more rapid off-rate exerts a disproportional effect when low Ag density forces primarily monovalent binding.

Because ADCC is believed to be an important mechanism of Ab therapy of cancer, it is logical to design Abs that mediate improved ADCC against cancer cells. We show in this study that higher affinity Ab elicits higher ADCC. Other groups have demonstrated that increasing the binding of mAb to FcγR enhances ADCC (46). Shields et al. (27) reported a complete mapping of human IgG1 amino acids affecting binding to human FcγR. A number of IgG1 variants either improved binding only to specific receptors or simultaneously improved binding to one type of receptor and reduced binding to another type. The variants with improved binding to FcγRIIIA also exhibited enhanced in vitro ADCC. Carbohydrate optimization of mAb might also improve binding of the mAb to FcγR, consequently enhancing its ADCC (47–49). IgGs, either from serum or produced in hybridomas or mammalian cells, are heterogeneous with respect to the carbohydrate attached to the conserved Asn297 glycosylation site. Nonfucosylated mAbs exhibit stronger and more saturable in vitro and ex vivo ADCC with improved FcγRIIIA binding (50). The endogenous α-1,6-fucosyltransferase (FUT8) knockout CHO cell line makes it possible to obtain robust, stable production of completely nonfucosylated mAb. Apart from fucosylation, the terminal galactose residues have been reported to affect function, though the effect may be subtle and subclass-dependent. A recent study reported that higher levels of sialic acid can decrease mAb binding to both FcγRIIIA...
and cell surface Ag, thus adversely impacting its functionality of ADCC (51).

Unlike trastuzumab, the C6.5-based Abs have no antiproliferative or proapoptotic properties, and do not fix complement. However, these Abs undergo slow internalization and degradation in an affinity-dependent manner (manuscript in preparation). Thus, even though high affinity causes Ab degradation, high affinity still promotes efficient ADCC. Because degradation is a slow process, relatively little Ab is catabolized in the 4 h of incubation during an ADCC assay. However, if similar catabolism occurs in vivo, then the ultimate ability of high-affinity Abs to promote ADCC will be opposed by vigorous and prolonged catabolism of Abs at tumor sites. Further studies are needed to examine this possibility in more detail. It should be noted that internalization and Ab catabolism might also affect tumor cell viability by affecting the availability of cell surface-associated target Ags, and so might have additional benefits beyond the induction of highly efficient ADCC (52, 53).

Dissection of the relative contributions of these distinct affinity-related antitumor mechanisms will require in vivo studies that are ongoing.

In conclusion, mAbs with higher affinity for their target Ags more potently mediate ADCC, and target tumor cells with higher Ag expression are more susceptible to Ab therapy. These factors are more important than internalization into target cells. Thus, antibodies with improved ADCC efficacy will be required for the treatment of a variety of tumor types, especially those with high Ag expression. In vivo studies will be needed to determine the relative contributions of these mechanisms.

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