Engineered Humanized Dimeric Forms of IgG Are More Effective Antibodies

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Summary

Humanized IgG1 M195 (HuG1-M195), a complementarity determining region-grafted recombinant monoclonal antibody, is reactive with CD33, an antigen expressed on myelogenous leukemia cells. M195 is in use in trials for the therapy of acute myelogenous leukemia. Since biological activity of IgG may depend, in part, on multimeric Fab and Fc clustering, homodimeric forms of HuG1-M195 were constructed by introducing a mutation in the γ1 chain CH3 region gene to change a serine to a cysteine, allowing interchain disulfide bond formation at the COOH terminal of the IgG. Despite similar avidity, the homodimeric IgG showed a dramatic improvement in the ability to internalize and retain radioisotope in target leukemia cells. Moreover, homodimers were 100-fold more potent at complement-mediated leukemia cell killing and antibody-dependent cellular cytotoxicity using human effectors. Therefore, genetically engineered multimeric constructs of IgG may have advantages relative to those forms that are found naturally.

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CDR-grafted humanized mAbs have been constructed to improve immunological effector functions and reduce immunogenicity (1–5). The production of a CDR-grafted, humanized IgG1 construct (HuG1-M195) of the mouse M195 antibody, an anti-CD33 mAb that is specifically reactive with acute myelogenous leukemia (AML) cells and early myeloid progenitors, but not hematopoietic stem cells, has been described (6–9). M195 is currently being evaluated in the therapy of AML (10, 11). HuG1-M195 retains specificity of binding, the capability of internalization into HL60 leukemia cells, and the ability to fix human complement (7). In addition, HuG1-M195 shows superiority over its murine counterpart in its higher avidity and new ability to perform antibody-dependent cellular cytotoxicity (ADCC) with human effector cells against acute myelogenous leukemia cells (7).

The biological activities of IgG depend, in part, on the ability to crosslink antigen on the cell surface and to bind complement or Fc receptors on effector cells via multivalent interactions. Recently, the genetic engineering of a chimeric IgG reactive with a hapten to form a mutant Ig linked together as a covalent dimer, resulted in enhanced complement mediated cytotoxicity (CMC) (12). In an attempt to improve the CMC as well as other biological and immunological properties of the humanized M195 mAb, similar homodimers (Hd-IgG) were genetically designed. A mutation at the COOH end of the CH3 domain of the γ1 H chain was introduced in HuG1-M195 that results in enhanced CMC, and also a dramatic improvement in the ability to perform ADCC against leukemic target cells. In addition, the Hd-IgG shows more rapid modulation with markedly improved retention of targeted radioisotope within the target cells. These Hd-IgG may have therapeutic applications.

Materials and Methods

Construction of Homodimeric IgG (Hd-IgG). The construction of vectors to express humanized M195 L and H chains has been described (6). For expression of Hd-IgG, the H chain expression vector was mutagenized by changing the codon TCT to TGT which resulted in converting amino acid at position 444 of H chain from Ser to Cys (Fig. 1 *A*). The cys allows interchain disulfide bond formation which allows expression of Hd-IgG (Fig. 1 *B*). To allow formation of Hd-IgG, 87 mg of mAb was purified from culture supernatant and concentrated to 10 mg/ml in 0.1 M Tris, pH 8.6. 4 mg of Ellman's reagent (Pierce Chemical Co., Rockford, IL) was added and incubated at room temperature for 1 h to crosslink and then block the excess sulfhydryl sites. The sample was adjusted to 2.5 M NaCl and loaded onto a 50-ml phenyl-Sepharose column equilibrated with 2.5 M NaCl. Monomer antibody was eluted off the column in PBS. Crosslinked material (Hd-IgG) were eluted in 50% propylene glycol in water. SDS-PAGE analysis showed the dimers to be 90% pure.

Cell Surface Modulation. 5 × 10^6 HL60 cells were incubated at 37°C with 2 μg/ml of HuG1-M195 or Hd-IgG, and aliquots were taken at 0, 60, 150, and 300 min. The cells were washed twice in RPMI and pelleted at 500 *g*, and 50 μl of goat anti–human fluorescein conjugate was added for 30 min, followed by washing twice,

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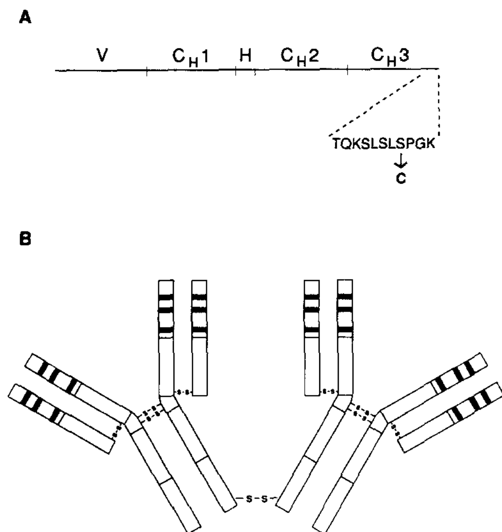

Figure 1. (*A*) Sequence at the site of the mutation of the constant region of the plasmid allowing interchain disulfide bond formation. (*B*) Diagram of homodimeric-HuG1-M195 (Hd-IgG).

and fixing in 0.5% paraformaldehyde before analysis. Ten thousand cells were analyzed on an Epics profile flow cytometer (Coulter Electronics Inc., Hialeah, FL), and the mean fluorescent intensity was measured. Hematopoietic cell lines were grown in RPMI 1640/5% newborn calf serum/10% serum-plus (Hazleton Biologics, Inc., Lenexa, KS).

Internalization Studies. HuG1-M195 or Hd-IgG were labeled with Na-^{125}I (New England Nuclear, Boston, MA) as described previously (8). Internalization of the HuG1-M195 or Hd-IgG was measured from 0 to 48 h by incubating 0.01–2 μg/ml of radiolabeled mAb with 1–2 × 10^6 HL60 cells/ml in RPMI 1640/2% human Ab serum. Cells were then washed twice in RPMI, and the surface-bound M195 was stripped with 1 ml of 50 mM glycine/150 mM NaCl, pH 2.8 at 24°C for 10 min. The amount (ng) of mAb per million cells remaining after the acid wash (i.e., internalized), or in the supernatant (i.e., cell surface) is shown.

CMC. For CMC assays, 25 μl of 5 × 10^6 HL60 cells/ml were incubated with 25 μl of diluted rabbit complement and 25 μl of serial dilutions of HuG1-M195 or Hd-IgG at 37°C for 1 h. mAb M31 (IgM anti-CD15) was used as a positive control. Live and dead cells were enumerated using trypan blue. Low toxicity rabbit serum was purchased from Pel-Freeze (Rogers, AR). Complement was used at the maximum concentrations not showing nonspecific lysis of the target cells, usually from 1:6 to 1:9 final dilution.

ADCC. Chromium release assays were conducted using PBMC from human volunteers as effector cells and HL60 cells as positive targets. Target cells were incubated in ^{51}Cr for 90 min and then washed of free ^{51}Cr. HuG1-M195 or Hd-IgG was added at E/T ratios of 10:1, 25:1, and 100:1. Cells were incubated at 37°C for 5 h and harvested using a cell harvester (Skatron, Inc., Sterling, VA), and released ^{51}Cr was counted in a gamma counter (Packard Instrument Co., Downers Grove, IL). Detergent-lysed cells were used as a 100% control. Effector cell only and mAb only treated target cells were used as negative controls. Samples were done in quadruplicate, and SD were always <10% of the mean value. Specific lysis = A − C/B − C; where: A = cpm release in the presence of mAb; B = total cpm released by detergent lysed cells; and C = cpm released in the presence of medium alone.

Results and Discussion

The ability of Hd-IgG to bind to CD33 expressing HL60 cells was determined by radiobinding assays in the presence of excess HL60 cells, as described previously (8). Total immunoreactivity (the fraction of the total radiolabeled Ig capable of binding to antigen) of Hd-IgG nearly doubled to 85% as compared with 50% for the HuG1 in these assays. The increased immunoreactivity may relate to the presence of multiple binding sites that were less likely to be inactivated by radiolabeling. Direct radioimmunoassay showed that binding of both constructs to HL60 cells was saturable and specific. Scatchard analysis of HuG1-M195 showed a slightly lower avidity of binding (K_a = 4.4 × 10^9 M^{-1}) than that of Hd-IgG (K_a = 6.1 × 10^9 M^{-1}).

Since direct radiobinding can be affected by damage to the mAb generated during the radioiodination of the Ig, the relative avidities of the HuG1-M195 and Hd-IgG were also compared by competition assays on HL60 cells in the presence of human serum to prevent nonspecific and human Fc receptor binding, as described previously (7). These experiments confirmed the Scatchard analysis that the binding avidities of Hd-IgG and HuG1-M195 were similar.

Cell surface modulation with subsequent internalization of M195 antibody and conjugated isotope has been seen in vitro and in vivo in patients, and is an important aspect of its therapeutic effect in humans for the treatment of AML (10, 11). Although Hd-IgG bound to the cell surface with

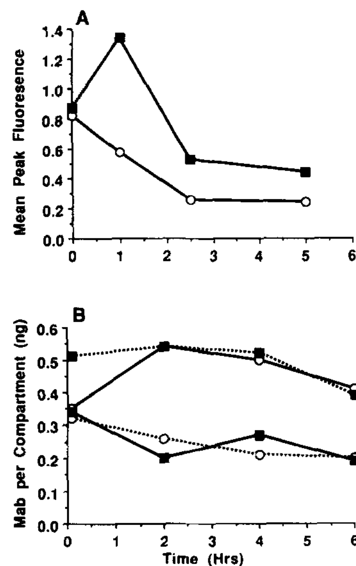

Figure 2. (*A*) Indirect flow cytometric analysis comparing cell surface modulation of HuG1-M195 (■—■) vs Hd-IgG (○—○) over 5 h. (*B*) Internalization and retention of HuG1-M195 in radiobinding experiments on HL60 cells at 37°C over 6 h. Cell surface HuG1 (■---■); cell surface Hd-IgG (○---○); internalized HuG1 (■—■); internalized Hd-IgG (○—○). Radiolabeled mAb at a final concentration of 2 μg/ml was incubated at 37°C with 10^6 HL60 cells/ml in a total volume of 200 μl. Internalization was measured as described in the text. Each time point was done in duplicate, and these results are representative of three independent experiments.

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similar avidity as HuG1-M195, we speculated that the oligomerization of the mAb might alter its ability to modulate. The kinetics of modulation were examined by indirect fluorescence flow cytometry on HL60 cells. HuG1-M195 accumulated on the surface of HL60 cells to reach a peak at 1 h, and then slowly modulated (Fig. 2 *A*). By 5 h, 40% of HuG1 remained on the cell surface. In contrast, Hd-IgG was immediately modulated from the cell surface without accumulation first, with 12% of the mAb remaining on the cell surface at 5 h. Thus, Hd-IgG modulated much faster and to a greater degree than HuG1-M195, apparently without requiring the threshold of binding shown by the HuG1-M195.

The fates of the rapidly modulated Hd-IgG and HuG1-M195 were evaluated in the same time period, and internalization was measured by acid washing the cells at various times after ^{125}I-mAb binding to remove residual cell surface mAb (Fig. 2 *B*). Studies done at 0°C showed that neither HuG1-M195 nor Hd-IgG entered the cell. At 37°C, the Hd-IgG at 2 μg/ml rapidly entered the intracellular compartment, with 70% of the radiolabeled mAb being retained inside the cell, and 30% staying on the surface. In contrast, only 30–40% of the HuG1-M195 was retained inside the cell over the same time period with the remainder on the surface.

An extended analysis of internalization out to 48 h showed persistence of the Hd-IgG within cells (Fig. 3). In contrast, HuG1 demonstrated the same pattern of limited internalization as seen in Fig. 2 (not shown). Thus, the rapid modulation resulted in efficient internalization of the radiolabeled Hd-IgG into the cell, and, most importantly, the Hd-IgG was not lost from the cell over long time periods.

Based on integration (areas under the curves) of these internalization data, radiation doses delivered by Hd-IgG to the inside of the cells was more than double that of HuG1 by 48 h. Therefore, presumably in vivo, total radiation doses to target cells would be twice as high for the same added dose and same toxicity. The differences in internalization kinetics may be attributed to the immediate clustering of CD33 achieved by the multivalent Hd-IgG. Such clustering may require time to accumulate with Hug1-M195, thus accounting for the threshold effect, the delay in internalization, and the poor efficiency.

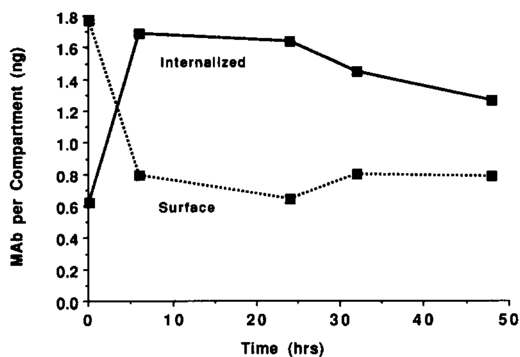

Figure 3. The amounts of radiolabeled Hd-IgG remaining on the surface (■---■) and internalized (■—■) over 48 h. Radiolabeled Hd-IgG at 1 μg/ml was incubated at 37°C with 2 × 10^6 HL60 cells/ml in a final volume of 200 μl, and internalization was measured as described in the text. Each time point was done in triplicate and SD was <10%.

Table 1. *Percent Internalization of HuG1 and Hd-IgG*

mAb Concentration	Percent internalization*	
	HuG1	Hd-IgG
nM		
6.70	43	67
2.23	40	70
0.74	39	64
0.25	41	58
0.08	38	45

* Radiolabeled mAb at concentrations shown were incubated with 2 × 10^6 HL60 cells at 37°C for 4 h in a total volume of 200 μl. Internalization was measured as described in the text. Each time point was done in triplicate and SD was <10% of the mean.

To determine whether mAb concentration affected internalization, HuG1 and Hd-IgG at similar molar concentrations were incubated with HL60 cells for 4 h at 37°C. Hd-IgG showed greater internalization than HuG1 at every concentration (Table 1). Whereas the total amount of bound radioactivity varied with the concentration of mAb added (not shown), the percentage of internalization of mAb did not change over the 2-log range of mAb concentrations. Thus, except possibly at very low concentrations, the percentage of either HuG1 or Hd-IgG internalized into HL60 cells was not dependent on mAb dose.

Although others (13–15) have found that crosslinked IgG bound to Fc receptors more avidly than monomeric Ig, our

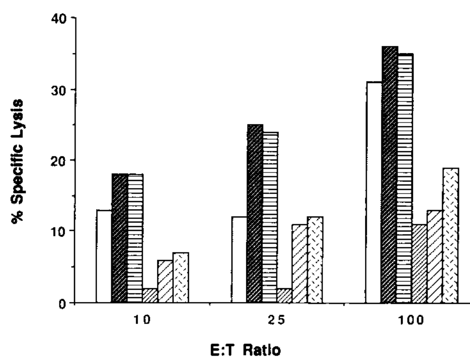

Figure 4. ADCC of HuG1-M195 and Hd-IgG is shown as a function of concentration and E/T ratio. Target HL60 cells were incubated at 37°C with mAb and PBMC as effectors, and ^{51}Cr-release was measured 5-h later as described in the text. Hd-IgG was 100-fold more potent (50 times on a weight basis) and 2–6 times more effective than the HuG1-M195 at ADCC. Hd-IgG at 0.2 μg/ml (▨); Hd-IgG at 1 μg/ml (▩); Hd-IgG at 10 μg/ml (▤); HuG1-M195 at 0.2 μg/ml (▨); HuG1-M195 at 1 μg/ml (▧); HuG1 at 10 μg/ml (▨). Results shown are from a representative experiment that was repeated on three separate days.

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system is unique in that binding and internalization occurred via specific Fab binding to antigen and not to Fc receptors. The metabolism of Ig after Fc receptor-mediated internalization has been reported to be both increased and decreased by oligomerization (13, 16, 17).

Hd-IgG was at least 100-fold more potent on a molar basis at cell killing with rabbit complement than the HuG1-M195 (not shown). In this and other experiments, no cytotoxicity of HuG1-M195 was seen below a concentration of .01 μg/ml, whereas Hd-IgG demonstrated a linear increase in cell lysis starting at .001 μg/ml. Since both HuG1-M195 and Hd-IgG have similar avidities of binding, this would not explain the enhanced ability of Hd-IgG to perform CMC. Moreover, the more rapid and efficient internalization of Hd-IgG would argue against its improved ability to perform CMC, since less mAb would be available on the cell surface to fix complement. Although HuG1-M195 (7) and Hd-IgG were capable of fixing human complement and killing AL67 fibroblasts that overexpress CD33, neither HuG1-M195 nor Hd-IgG lysed HL60 cells in the presence of human serum.

Several lines of evidence suggest that the proximity of Fc regions of multimeric IgG may explain its enhanced effectiveness in CMC as compared with monomeric IgG. Binding and activation of C1q requires the formation of doublet IgG on the cell surface (18). Since Hd-IgG intrinsically contains a doublet of Fc regions, it may allow more efficient binding of the polyvalent C1q. The concept that a cluster of IgGs allows for multiple points of C1 attachment (19–21) is consistent with our observations of enhanced CMC here. It has been suggested that another site of attachment to the CH1 domain is needed (22). Hd-IgG may facilitate such spatial rearrangements. Bispecific antibodies capable of binding to two antigens (23) and a chimeric antibody with dual Fc regions (24) were superior to conventional IgG in CMC, presumably because of the increased number of Fc regions and their close arrangement in pairs. A critical number of antigen sites or critical spacing of epitopes on the cell surface has been shown to be necessary for CMC to occur with monovalent IgG (7, 25, 26). Naturally occurring IgG3 mAbs to carbohydrate antigens, which exhibit intermolecular cooperativity through Fc region interactions, show potent complement-mediated effector functions (27). The Hd-IgG described here may achieve this via a genetic mechanism.

Hd-IgG was at least 100 times more potent on a molar basis than HuG1-M195 at ADCC (Fig. 4). Specific lysis with 0.2 μg/ml Hd-IgG was as much as twofold greater than 10 μg/ml HuG1-M195 at the same E/T ratios. Hd-IgG was two- to fivefold more effective over a 50-fold concentration range at each E/T ratio. Chimeric and humanized IgG have shown ADCC where the original murine mAb did not demonstrate that capability (28–30). It is not surprising that homodimeric humanized IgG would be even more effective than monomeric IgG because of the greater ability of dual Fc regions to bring together effectors and targets in close proximity to allow ADCC to occur. IL-2 has been shown to potentiate ADCC against human Tac-positive T cells with humanized anti-Tac mAb (28). We have evidence that IL-2 enhances both HuG1- and Hd-IgG–mediated ADCC against HL60 target cells (31). The combination of other cytokines and Hd-IgG to enhance effector cell function needs to be investigated.

Previously, a human polymeric IgG1 mAb against group B streptococcus, held together by noncovalent interactions, has been described which showed enhanced binding and opsonic activity compared with the original monomeric mAb (32). The demonstrated homophilic interactions with a mouse IgG3 mAb, R24, reactive with GD3 ganglioside, have also led to the suggestion that multimerization of Ig may be a mechanism to enhance antibody-mediated clearance of bacteria (33).

The improvement in internalization of homodimeric M195 IgG should be advantageous for its use as a carrier of radiolabels or toxins, and should allow the delivery of more cytotoxic agent to target cells for each injected dose. Although we are currently able to safely deliver therapeutic levels of radioisotope to leukemia cells in humans in vivo, this change in cellular kinetics should allow the same therapeutic effects with less injected dose. In addition, the markedly improved effector functions and potency of Hd-IgG alone seen in vitro may result in potentially effective cytotoxicity against AML in vivo without conjugated cytotoxins or isotopes.

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