

Biological and Immunological Features of Humanized M195 (Anti-CD33) Monoclonal Antibodies

Philip C. Caron, Man Sung Co, Marcia K. Bull, et al.

Cancer Res 1992;52:6761-6767.

Updated version	Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/52/24/6761
------------------------	---

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org .

Biological and Immunological Features of Humanized M195 (Anti-CD33) Monoclonal Antibodies¹

Philip C. Caron, Man Sung Co, Marcia K. Bull, Nevenka M. Avdalovic, Cary Queen, and David A. Scheinberg²

Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [P. C. C., M. K. B., D. A. S.], and Protein Design Laboratories, Mountain View, California 94043 [M. S. C., N. M. A., C. Q.]

ABSTRACT

Human-mouse chimeric immunoglobulins G1 and G3 (IgG1 and IgG3) (ChG1, ChG3) and "complementarity-determining region"-grafted, humanized IgG1 and IgG3 (HuG1, HuG3) constructs of the mouse monoclonal antibody (mAb) M195 were characterized. M195 is a murine immunoglobulin G2a (IgG2a), anti-CD33 mAb, specifically reactive with acute myelogenous leukemia cells, that is active as an antileukemia agent in humans. The new mAb constructs maintained specificity and biological function, including rapid internalization after binding to the cell surface, which has been important for delivery of therapeutic isotopes in patients. Although previously reported complementarity-determining region-grafted mAbs had reduced avidities, the HuG1 and HuG3 M195 showed up to an 8.6- and 4-fold higher binding avidity, respectively, than the original murine mAb. All constructs were effective at mediating rabbit complement-mediated cytotoxicity against HL60 targets. Fibroblasts transfected with *CD33* genes and expressing high levels of CD33 antigen were also lysed in the presence of human complement, but HL60 cells or fibroblasts with lower CD33 levels were not killed. Thus, the inability of M195 and constructs to kill HL60 targets with human complement is due to the much lower antigen density on HL60 cells compared to CD33⁺ fibroblasts. Unlike the murine M195, the chimeric and humanized M195 demonstrated antibody-dependent cell-mediated cytotoxicity using human peripheral blood mononuclear cells as effectors. Because the chimeric and humanized M195 have improved avidities as compared to the original M195 and have, in addition, the potential to avoid human anti-mouse antibody responses and to recruit human effector functions, these new constructs may be useful therapeutically, either alone or conjugated to toxins or isotopes, in the treatment of acute myelogenous leukemia.

INTRODUCTION

Mouse monoclonal IgG2a³ antibody M195 (mAb M195) is specifically reactive with the CD33 antigen on AML cells and early myeloid progenitor cells, but not with normal tissues or hematopoietic stem cells (1-5). This sparing of normal stem cells makes it an ideal candidate for leukemia therapy and for *ex vivo* bone marrow purging of AML cells (6-8). M195 reacts with the CD33 antigen, a *M_r* 67,000 glycoprotein, and is rapidly internalized into target cells upon binding. M195 is not intrinsically cytotoxic *in vitro*. Although M195 kills target cells with guinea pig and rabbit complement, it is not effective with human complement or effector cells. However, the efficient binding and internalization of M195 have allowed the use of radiolabeled mAb in trials for AML therapy (9, 10). These trials

have demonstrated that specific targeting of ¹³¹I-M195 can result in marked leukemia cytoreduction, therapeutic responses, or marrow ablation with no toxicity.

Genetic engineering techniques are now available to produce new human-mouse chimeric mAbs that combine mouse variable regions with human constant regions (Fig. 1) or more fully humanized mAbs that have human framework and constant regions. These humanized mAbs have been "CDR-grafted" so that only the original mouse antigen binding sites are retained. The new constructs offer advantages over murine mAbs in being more effective in recruiting human effector functions and in avoiding neutralizing HAMA responses. Avoidance of HAMA may allow for repeated treatments without loss of effectiveness and a longer circulating time for the mAb. The added features may allow us to avoid the use of radiolabels and toxins in clinical therapeutic trials (11-16).

One humanized IgG1 mAb, CAMPATH-1H, derived from CDRs from a rat mAb together with human framework regions, has been used in a clinical situation (15). CAMPATH-1H produced remissions in two patients with refractory non-Hodgkin's lymphoma. Mathieson *et al.* (16) have also used CAMPATH-1H in one patient with systemic vasculitis.

A second humanized mAb, anti-Tac-H, reactive with the IL-2 receptor, has recently been constructed using computer-aided three-dimensional structural design to maintain avidity at its binding site (17). Humanized anti-Tac, as well as the chimeric version, blocks T-cell activation and has the added advantage over the original murine mAb of performing ADCC against human tumor targets (18). More recently, humanized versions of two murine mAbs against herpes simplex virus gB and gD glycoproteins have resulted in similar binding, virus neutralization, and cell protection as seen with the original murine mAb (19).

This paper describes four new reconstructed M195 mAbs in comparison to the original mouse M195. The four new constructs compare favorably to the parental M195 with respect to their specificity, immunoreactivity, and internalization, and they showed increased avidity of binding and improved immunological functions, such as ADCC. Therefore, these constructs hold potential promise for a new approach to the treatment of AML.

MATERIALS AND METHODS

MAB. Mab M195 (anti-CD33) originated in BALB/c mice immunized with leukemia cells from a patient with AML and was produced from hybridomas and purified as described previously (4, 5). Human-mouse chimeric IgG, with human-derived constant regions for IgG1 and IgG3 (ChG1, ChG3), and CDR-grafted humanized M195, retaining only the CDRs and other sterically important amino acids from the mouse IgG (HuG1, HuG3), were constructed as described (20). Sp2/0 hybridoma cell lines secreting the chimeric and humanized M195 were grown *in vitro*, and the mAbs were purified on PA-Sepharose (Pharmacia, Piscataway, NJ) by affinity chromatography using sequential pH step elutions (4). Purity was determined on SDS-polyacrylamide gels stained with Coomassie brilliant blue.

Received 3/10/92; accepted 10/7/92.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by ROICA55349-01 and the Lucille P. Markey charitable trust. D. A. S. is a Lucille P. Markey scholar.

² To whom requests for reprints should be addressed.

³ The abbreviations used are: IgG, immunoglobulin G; IgM, immunoglobulin M; mAb, monoclonal antibody; AML, acute myelogenous leukemia; HAMA, human anti-mouse antibody; IL-2, interleukin 2; SDS, sodium dodecyl sulfate; PBMC, peripheral blood mononuclear cells; CMC, complement-mediated cytotoxicity; MTT, dimethylthiazol diphenyltetrazolium bromide; thiazolyl blue; ADCC, antibody-dependent cellular cytotoxicity; CDR, complementarity-determining region.

Cells and Cell Lines. Hematopoietic cell lines, including SKLy16, RAJI, HL60, and U937 cells, were obtained from the human tumor banks of the Cancer Immunology Laboratory at Sloan-Kettering Institute. Hematopoietic cell lines were grown in RPMI 1640/5% newborn calf serum/10% serum-plus (Hazelton Biologics, Inc., Lenexa, KS). Mouse NIH-3T3 fibroblasts transfected with the *ras* gene to enhance growth rate and AL67 cells, which were NIH3T3 cells transfected with the *ras* and *CD33* genes and expressing cell surface CD33 (21), were the generous gift of Dr. Look, Dr. Ashman, and Dr. Peiper (St. Jude's Children Research Hospital, Memphis, TN). Fibroblasts were grown in RPMI 1640/10% serum-plus/10% fetal calf serum with 2 mM glutamine.

Heparinized peripheral blood samples were obtained from healthy volunteers and patients on the Leukemia Service at Memorial Hospital on institutional review board-approved protocols. PBMC were separated on Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient centrifugation.

Direct and indirect flow cytometry was conducted as described (5).

Radioiodination and Radioimmunoassay. Purified mAbs were labeled with Na-¹²⁵I (New England Nuclear, Boston, MA) using chloramine-T to start and sodium metabisulfite to stop the reaction. MABs were labeled to 2 to 10 μ Ci/ μ g of protein. Immunoreactivity was determined by incubating 3 to 5 ng of radiolabeled mAb with an excess of antigen (5×10^6) HL60 cells for 1 h at 4°C. Immunoreactivity of the four radiolabeled constructs and the original M195 mAb was determined to be about 50%. Specific binding in these radiobinding assays was determined by subtracting the amount of radiolabeled mAb bound in the presence of an excess of unlabeled mAb as previously described (4).

Competition Radioimmunoassay. Comparison of antigen-antibody avidity between different M195 constructs was done in competition with ¹²⁵I-M195 and ¹²⁵I-HuG1. Increasing amounts of cold mAb were incubated with 2×10^5 HL60 cells and 50 ng of ¹²⁵I-mAb in 200 μ l of RPMI for 1 h at 0°C. Cells were washed 3 times in RPMI and counted. To avoid nonspecific and Fc-receptor binding, competition assays were done in the presence of human serum. Samples were run in duplicate, and the avidity of binding was measured by comparing the concentrations of unlabeled competitor where 50% reduction of binding was achieved as described (18). Experiments were repeated 2 to 3 times each, and arithmetic means of the data are presented.

Modulation of Cell Surface Antigen. Internalization of the cell surface antigen was measured by incubating 0.5 μ g/ml of radiolabeled mAb with 5×10^5 HL60 cells over time at 0° and 37°C. Cell pellets were washed twice in RPMI, and then surface-bound M195 was stripped with 1 ml of 50 mM glycine/150 mM NaCl, pH 2.8, at 24°C for 10 min. Total cell-associated radioactivity and acid-resistant (internalized) radioactivity were determined.

CMC. Twenty-five μ l of HL60 cells (5×10^6 cells/ml) or fresh leukemia samples were incubated with 25 μ l of diluted rabbit or human complement and 25 μ l of serial dilutions of mAb at 37°C for 60 min. MAb M31 (IgM anti-CD15) was used as a positive control. Live and dead cells were enumerated using trypan blue or propidium iodide exclusion.

Rabbit serum was purchased from PelFreeze (Rogers, AK); human sera were obtained from volunteers. All complement sources were stored at -70°C until use and not reused. Complement was used at the

maximum concentrations not showing nonspecific lysis of the target cells, usually from 1:6 to 1:10 final dilution.

Alternately, CMC was determined using the MTT (Sigma Co., St. Louis, MO) assay as described previously (22). MTT measures cell viability and correlates well with the trypan blue method. AL67 fibroblasts are incubated with MTT together with mAb and human complement for 4 h, and the metabolized MTT (blue formazan precipitate) is solubilized with 0.04 M HCl in 2-propanol. The assay is then quantified using an enzyme-linked immunosorbent assay at 570 nm (BT2000 Microkinetics Reader, Fisher Biotech, Springfield, NJ).

Measurement of CD33 Density on AL67 Cells. The antigen density of CD33 on HL60 cells versus AL67 fibroblasts was estimated by determining antigen levels and dividing by cell surface area. Cell diameter was measured both by forward light scatter on flow cytometry and by direct microscopy. The surface area of nonadherent AL67 fibroblast cells was calculated to be 1.6 times that of HL60 cells. Radiobinding experiments showed that the number of M195 antigen sites was 42 times greater for AL67 fibroblasts than HL60 cells (420,000 versus 10,000). The relative increase in the number of sites was confirmed by measuring mean peak fluorescence using a flow cytometer. Assuming a spherical shape for AL67 cells when in suspension, a calculation of the surface area yielded an average antigen density of CD33 epitopes that was 27-fold greater for AL67 fibroblasts than HL60 cells.

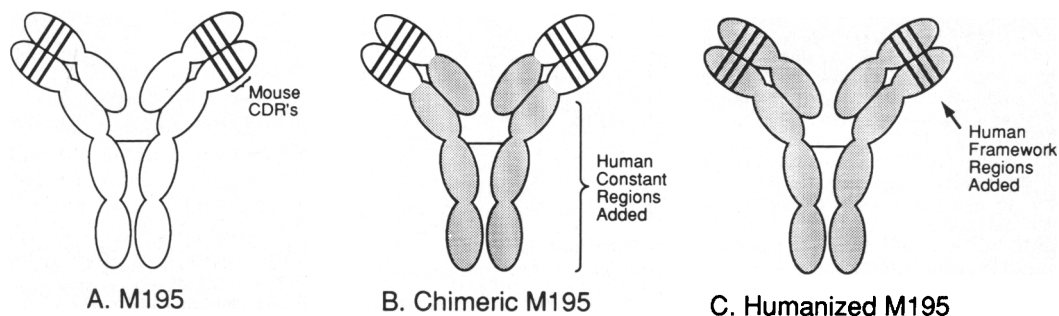
Enzymatic Cleavage of CD33 from the Cell Surface. CD33+ AL67 fibroblasts were incubated with 0.35 units of proteinase K (Bethesda Research Labs, Gaithersburg, MD) at 37°C. At various time intervals, an aliquot was washed twice with medium and split in half, and cells were tested as a target for CMC with human complement and HuG1 as described above. The number of antigen binding sites was quantified using direct radioimmunobinding and also by indirect flow cytometry on the second aliquot.

ADCC. Chromium release assays to determine if chimeric or humanized M195 mAbs were capable of mediating ADCC were conducted as described previously (4). PBMCs from human volunteers were used as effector cells, and either HL60 cells or CD33+ AL67 fibroblasts were used as positive targets. Assays were conducted at 37°C for 5 h and harvested using a Skatron press, and released ⁵¹Cr was counted in a Packard gamma counter. Detergent lysed cells were used as a 100% control. Effector cell only and mAb only treated target cells were used as negative controls. CD33-negative RAJI and NIH-3T3 cells and a control HuG1 mAb (Fd 79) (19) were used as controls. Samples were done in quadruplicate, and the mean was presented. Experiments were repeated 3 to 5 times. Standard deviations were always less than 10% of the mean value.

RESULTS

Specificity. Four new M195 mAb constructs were made (Fig. 1) as described (20). Specificity was first confirmed against a panel of CD33+ and CD33- cell lines by radioimmunoassay and then against fresh hematopoietic samples from 47 patients using a direct fluorescein conjugate of HuG1 M195. The specificity for myeloid leukemia cells and monocytes in direct comparison with other anti-CD33 antibodies and the absence of

Fig. 1. Diagram of chimeric and humanized M195 constructs. A, parental IgG2a murine mAb. In B, chimeric IgG1 (ChG1) and IgG3 (ChG3) were made by combining mouse M195 variable regions with human constant regions. In C, humanized IgG1 (HuG1) and IgG3 (HuG3) are entirely human except for CDR-grafted murine-derived antigen binding sites and other selected amino acids. ○, sequences of mouse origin; ●, sequences of human origin.



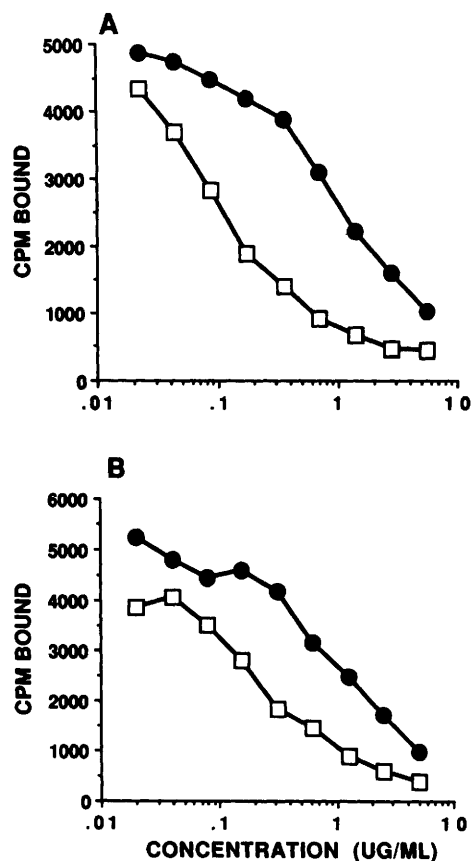


Fig. 2. Competition binding assays with murine M195 and HuG1. HL60 cells (2×10^5) were incubated with increasing amounts of cold murine M195 (●) and HuG1 (□) and 50 ng of mouse ^{125}I -M195 (A) or ^{125}I -HuG1 (B) for 1 h at 4°C in the presence of 2% human AB serum. Cells were then washed, and cell-associated radioactivity was measured. The HuG1 showed an increased binding avidity to HL60 cells compared to the parental murine mAb. Regression analysis showed the linear portions of the two curves to be significantly different ($P < 0.0001$ for both figures).

reactivity with cells of other lineages were confirmed in these assays. The analysis included blasts, lymphocytes, monocytes, and myeloid cells, separately gated. Immunohistochemistry confirmed lack of reactivity with normal tissues as well.

Competition Radiobinding Assays. Initial binding curves had suggested that the CDR-grafted constructs had similar or perhaps improved binding avidity as compared to the mouse M195 (20). To accurately compare the avidity of binding of the human constructs to mouse M195, unlabeled mouse M195 and constructs were allowed to compete with radiolabeled murine M195 for antigen sites on HL60 cells. Human serum was added to prevent potential artifacts attributable to nonspecific and human Fc receptor binding. Murine M195 has an avidity of $2.2 \times 10^9 \text{ M}^{-1}$ (20). This value was fixed, and the avidity of the new constructs was computed relative to it. These experiments showed that the binding avidity of ChG1 was comparable to that of the mouse M195 ($K_a = 2.2 \times 10^9 \text{ M}^{-1}$), while ChG3 showed slightly less binding avidity than the original mAb ($K_a = 1.5 \times 10^9 \text{ M}^{-1}$). Similar competition experiments using unlabeled constructs in competition with radioiodinated mouse M195 or unlabeled constructs in competition with radioiodinated HuG1 showed a statistically significant 8.6- ($K_a = 1.9 \times 10^{10} \text{ M}^{-1}$) and 5- ($K_a = 1.1 \times 10^{10} \text{ M}^{-1}$) fold, respectively, greater calculated avidity of binding for the HuG1 as compared to the mouse M195 (Fig. 2). HuG3 also showed a 4-fold greater avidity than the murine M195 ($K_a = 8.8 \times 10^9 \text{ M}^{-1}$). The increased avidities of the humanized mAbs as measured by

these more accurate competition experiments were consistent with, but greater in magnitude than, the increased avidities suggested by Scatchard analysis (20).

Internalization of Bound M195. Studies *in vitro* and in humans *in vivo* have shown that M195 is capable of rapidly internalizing into target cells upon binding to antigen (4, 9). Direct radioimmunobinding studies at 37°C showed that the radiolabeled mouse mAb became associated with HL60 cells over time in an acid-resistant compartment, *i.e.*, not on the cell surface (Fig. 3A). Both HuG1 (Fig. 3B) and HuG3 (not shown) behaved with similar kinetics. At 0°C , no radioactivity entered the cell above a baseline level less than 10% of the total cpm. However, at 37°C the amount of radiolabel inside the cell steadily increased. By 4 h, 25% of the cell-associated radiolabel was internalized into the cell.

CMC. Assays were performed to determine whether M195 constructs were capable of killing cells in the presence of rabbit and human complement. All four mAbs were capable of CMC of HL60 cells with rabbit complement (Fig. 4). Murine M195,

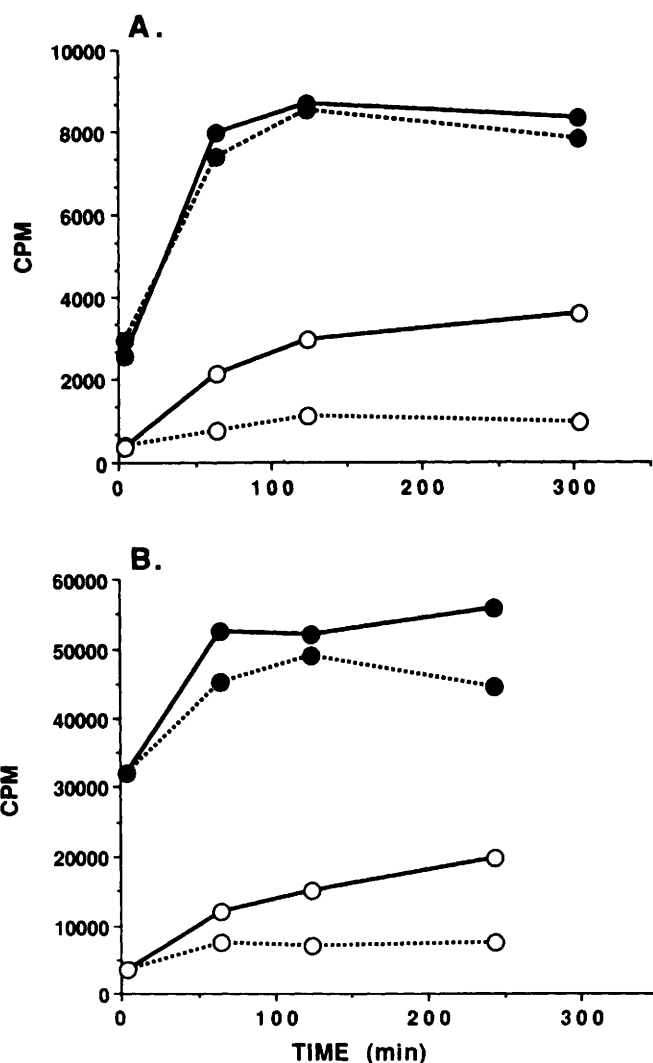


Fig. 3. Internalization of mouse or humanized ^{125}I -M195 over time. HL60 cells were incubated with murine ^{125}I -M195 (specific activity, $1.2 \times 10^3 \text{ cpm/ng}$) (A) or ^{125}I -HuG1 M195 (specific activity, $8 \times 10^3 \text{ cpm/ng}$) (B) for the times shown along the *abscissa*. Total cell-associated cpm were determined at 0°C (●—●) and 37°C (○—○). Cell surface-bound IgG was then stripped using 50 mM glycine-HCl, 150 mM NaCl, pH 2.8, and internalized cpm were measured at 0°C (○—○) and 37°C (○—○). At 37°C , both the mouse and humanized mAb showed that 25% of the label entered the cell by 4 h.

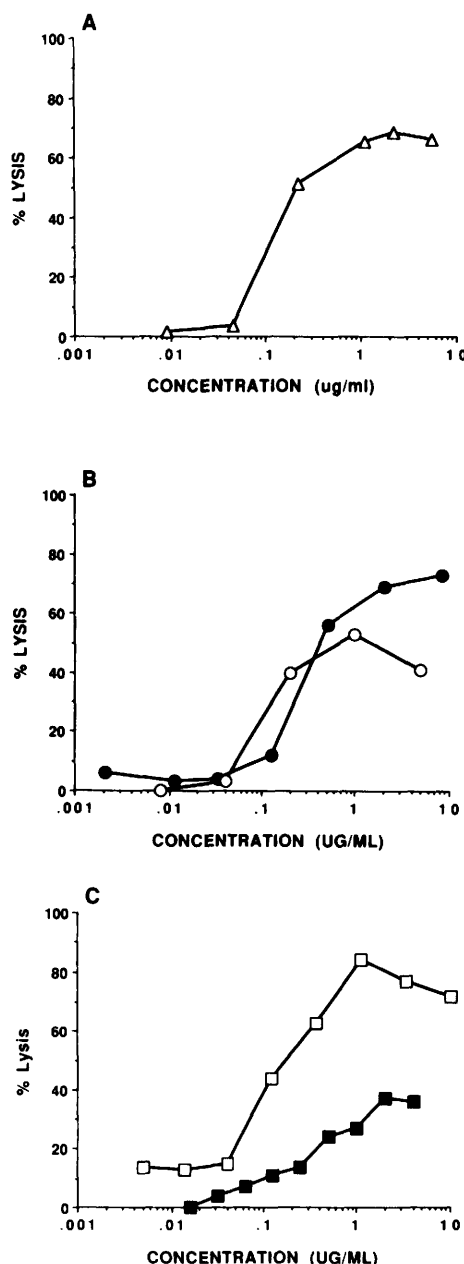


Fig. 4. CMC on HL60 cells with M195 constructs using rabbit complement. Twenty five μ l of HL60 cells (5×10^5 cells/ml) were incubated for 1 h at 37°C with equal volumes of diluted rabbit complement and serial dilutions of mAb. Trypan blue exclusion or propidium iodide was used as an indicator. Murine M195 (Δ) (A), ChG1 ($\circ$) and ChG3 ($\bullet$) (B), and HuG1 ($\square$) and HuG3 ($\blacksquare$) (C) were all effective at lysing the HL60 cells with rabbit complement. All constructs showed a prozone effect at the highest concentrations of mAb.

ChG1, ChG3, and HuG1 yielded 50% cell lysis at a concentration of 0.1 to 0.5 $\mu\text{g}/\text{ml}$. However, HuG3 was consistently less efficient at CMC with rabbit complement, achieving less than 50% maximum cell killing. All four constructs showed a prozone effect, with a decline in activity at the highest concentrations of mAb. None of the mAbs was effective in lysing HL60 cells when human serum from several normal individuals at varying concentrations was used as a source of complement.

AL67 fibroblasts, which are mouse NIH3T3 cells that express high levels of human CD33⁺ antigen, were studied to determine whether an increased antigen density would permit M195 and its constructs to kill by activation of the complement cascade using human complement. Assayed by trypan blue exclusion, M195 and all four constructs were cytotoxic to the

AL67 fibroblasts with both rabbit and human complement. Control NIH3T3 fibroblasts did not bind mAb and were not killed by M195 or the constructs in the presence of either complement. To quantitate the titer of the constructs for human CMC against the adherent AL67 fibroblasts, an MTT assay, which measures cellular metabolism, was used (Fig. 5). Human CMC was antibody concentration dependent. Murine M195, ChG1, and HuG1 demonstrated cytotoxicity curves with 50% cell lysis seen at 1 to 3 $\mu\text{g}/\text{ml}$ (Fig. 5). HuG3 was also similar to murine M195, while ChG3 was less effective at cell killing with human complement, even at high concentrations of antibody (results not shown).

Correlation of CD33 Antigen Density and CMC. To determine a relationship between the number of antigen sites and CMC activity using the HuG1 and human complement, AL67 fibroblasts were incubated with proteinase K at varying time intervals to cleave CD33 sites. The number of remaining CD33 sites correlated with the ability of HuG1 to cause cell killing using human complement. As shown in Fig. 6, a critical number of CD33 sites was needed for CMC to occur. When fewer than 150,000 sites were present, CMC was minimal. Above this critical level, the amount of CMC linearly increased with the number of CD33 antigen sites.

Antibody-dependent Cellular Cytotoxicity. The capabilities of M195 constructs at mediating ADCC were determined in chromium release assays. Mouse M195 did not show evidence of ADCC using PBMCs as effector cells. In contrast, experiments with ChG1 and ChG3, as well as HuG1 and HuG3, demonstrated a dose and effector cell-dependent activity against HL60 targets. ChG1 and ChG3 showed a maximum killing of 20 to 30% in 5-h assays at high effector:target ratios (100:1) and increasing antibody concentrations (1 to 10 $\mu\text{g}/\text{ml}$) (results not shown). The CDR-grafted HuG1 showed slightly lower levels of ADCC, with a maximum killing of 10 to 20% at 5 h of incubation with HL60 target cells as shown in a representative experiment (Fig. 7A). HuG3 also showed similar levels of ADCC. No additional killing was seen with any construct at concentrations above 10 $\mu\text{g}/\text{ml}$ and, in some cases, reduced effects were seen with increasing antibody, possibly due to increased modulation. When CD33⁺ AL67 fibroblasts were used as target cells, HuG1 showed high levels of killing (40 to 70%)

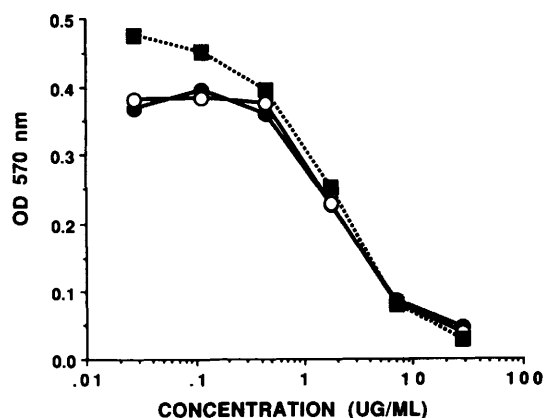


Fig. 5. CMC on CD33⁺ AL67 fibroblasts with M195 constructs using human complement. An MTT assay (see text) was used to quantitate the titer of mAb for human CMC on AL67 fibroblasts that have been transfected with the *ras* gene and express high levels of human CD33 antigen. Cells are incubated with human complement and then MTT for 4 h. The assay is then quantified using an ELISA at 570 nm. M195 ($\bullet$), ChG1 ($\blacksquare$), and HuG1 ($\circ$), as well as HuG3 (results not shown), all demonstrated a similar dose-dependent cytotoxicity curve. The ability of M195 mAbs to perform human CMC with CD33⁺ fibroblasts may be explained by their higher antigen density compared to HL60 cells.

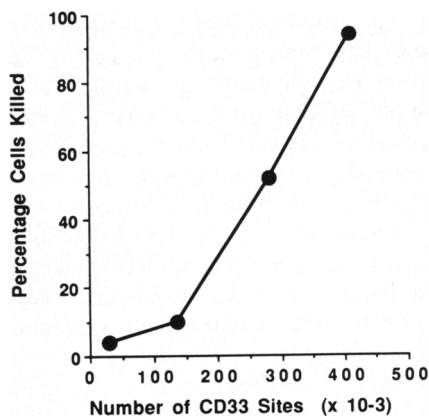


Fig. 6. Correlation of CD33 antigen density and CMC using HuG1 and human complement. CD33⁺ AL67 fibroblasts were incubated with 0.35 units of proteinase K at varying time intervals. The number of sites was determined by direct radioimmunoassay and was plotted against the percentage of cells killed after CMC in the same aliquot. Above a critical number of CD33 sites, the ability of HuG1 to mediate CMC using human complement linearly increased with the antigen density.

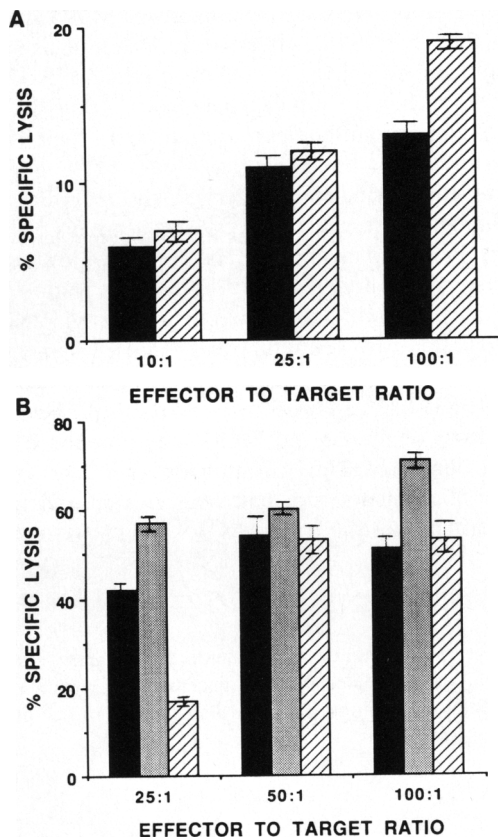


Fig. 7. ADCC of humanized M195 as a function of concentration and effector:target ratio at 5 h of incubation. Chromium release assays were conducted as described in "Materials and Methods" using either HL60 or CD33⁺ AL67 fibroblasts as target cells and PBMCs as effector cells. A, HuG1 at 1 µg/ml (■) and 10 µg/ml (▨) against HL60 cells; B, HuG1 at 0.1 µg/ml (■), 0.33 µg/ml (▨), and 3.3 µg/ml (▩) against AL67 fibroblasts. HuG1 demonstrated dose-dependent and effector cell-dependent increases in ADCC against HL60 cells. When tested against CD33⁺ AL67 fibroblasts, HuG1 showed higher levels of killing than against HL60 cells at a log lower concentration. Columns, mean; base, SD.

at a log lower concentration. This high level of killing was independent of effector:target ratio (Fig. 7B).

These results for ADCC varied, depending on the source of effector cells, largely because of variations in nonspecific effector cell killing of targets in the absence of mAb. No killing was

seen with a control HuG1 mAb (FD79) (19) and HL60 cells or with HuG1 M195 and CD33-negative RAJI cells or NIH3T3 cells. In other experiments (not shown), killing was seen at mAb concentrations ranging from 1 to 100 µg/ml and at effector:target ratios of 10 to 100:1, but not at 5:1.

DISCUSSION

Techniques are now available to genetically graft the mouse IgG CDR regions onto human IgG framework regions, resulting in new humanized IgG that are entirely human except for the hypervariable regions involved in antigen binding (12–14). The two major aims in developing a humanized M195 mAb are to take advantage of natural immune effector functions to destroy leukemic cells and to avoid neutralizing HAMA responses. In this way, an unmodified M195 may be used rather than relying on conjugation to radiolabels or toxins. Newly genetically engineered chimeric and humanized versions of M195, a mouse IgG2a mAb reactive with the CD33 antigen of early myeloid cells, show improvements over the murine mAb in both avidity and recruitment of host effector cells.

ChG1 and ChG3 bound to HL60 cells with similar or slightly less avidity than that of the original murine mAb. Other chimeric mAbs have shown similar avidities to the mouse immunoglobulin from which they were derived (23–25). In contrast, the complete reengineering of the humanized IgG did modify the avidity, in this case toward increased avidity. The HuG1 and HuG3 showed a markedly higher avidity, respectively, than the original mouse M195, in competition with radiolabeled M195 for binding to HL60 cells. Since competition experiments for HuG1 were done using either ¹²⁵I-HuG1 or ¹²⁵I-M195, it is unclear which result is more accurate and may relate to radioiodination damage. Since radioiodination would decrease avidity, the higher avidity is the more accurate description. These results corroborated the suggested higher binding avidities of the human mAb, compared to the murine and chimeric M195, as calculated from the Scatchard plots (20).

Direct radiobinding experiments to determine avidities as are done for Scatchard analysis may be affected by artifacts generated in the radioiodination of the immunoglobulin either due to radioiodination in the binding site or denaturation during the process. There are five tyrosine residues in the heavy chain CDR and one tyrosine residue in the light chain CDR of M195 that could be attacked in the radioiodination procedure.

This is the first reported instance of a newly constructed humanized mAb having a higher avidity than the parental murine mAb. Other humanized mAbs, anti-Tac-H (18) and mAbs against herpes simplex virus gB and gD glycoproteins (19), showed comparable to slightly lower, but not improved, avidities compared to the original mouse mAb in competition binding experiments. Comparisons of the variable region sequences of M195 (20) have revealed a loss of a carbohydrate binding site in the variable region from the CDR-grafted M195 antibodies. Thus, the mechanism for the increased avidity involves a change in a carbohydrate content in the humanized V region relative to the mouse V region. This hypothesis has now been confirmed by construction and analysis of new variable regions with and without the carbohydrate site.⁴

The ability to internalize and carry radioactivity into target cells has been important in the *in vivo* therapeutic effects of M195 (9, 10), or for the use of toxins to purge bone marrows of

⁴ Manuscript in preparation.

AML cells *in vitro* (26). Here we show that the HuG1 and HuG3 were also capable of internalizing into HL60 cells over time at 37°C. Antigenic modulation may pose specific problems for future mAb trials. Although a rapid decrease of mAb from the cell surface allows for radiolabel and toxins to enter the cell and cause killing, a disadvantage of this process would be limited access of the mAb for CMC and ADCC (14, 27).

One of the advantages of chimeric and human mAbs over murine mAbs is their ability to mediate ADCC (18, 28, 29) with human PBMC. It is surprising that the humanized M195 mAbs were slightly less effective at ADCC than the chimeric mAbs. One possible explanation is the change in a carbohydrate moiety attachment site on the IgG framework region that occurred during reconstruction increased avidity and rates of modulation (not shown). This alteration may be responsible for the decreased activity.

Isotype is of importance in determining the level of ADCC. Junghans *et al.* (18) reported that only the human G1 isotype (chimeric and humanized variants) of anti-Tac-H promoted ADCC. Brüggenmann *et al.* (30) also found that chimeric IgG1 was the most effective isotype in mediating ADCC against a hapten-derivatized human T-cell line. Among the CAMPATH-1 family of mAb, only rat IgG2b was active in ADCC (31). Others have found higher levels of macrophage ADCC when IgG mAbs of different isotypes and recognizing different antigens were used in combination against lymphoma targets (32).

Cytokines may be necessary to optimize ADCC activity by increasing various effector cell functions and numbers. IL-2 has been shown to potentiate ADCC of human effector cells with mouse anti-melanoma mAb (33, 34), against malignant B-cell lines using the murine Lym-1 mAb (35), and against the IL-2 receptor using chimeric and humanized anti-Tac mAb (18). In addition, γ -interferon (36) and granulocyte-macrophage (37) and macrophage colony-stimulating factor (38) have also been shown to augment ADCC against human tumor cells using human effector cells. Combining IL-2 and PBMCs as effector cells to augment chimeric and humanized M195-mediated ADCC showed that similar enhancement occurred against HL60 target cells (data not shown).

Murine, ChG1, ChG3, HuG1, and HuG3 all killed HL60 cells at low concentrations in the presence of rabbit complement. This shows that M195 is capable of binding rabbit C1q and initiating the complement cascade. Although HuG3 was the least effective mAb in this regard, this cannot be explained by differences in isotype alone, since ChG3 was as efficient at rabbit CMC. The ChG3 showed decreased avidity, as well as increased instability in solution over time (months), as evidenced by progressive precipitation and breakdown on SDS-gels. This subtle instability may result from the long hinge region of the IgG3 and may account for the reduced activities seen (30).

None of the M195 constructs showed any killing of HL60 cells with human complement. The ability to kill AL67 cells with M195 constructs was a function of CD33 antigen density, since effective killing of AL67 fibroblasts, but not HL60 cells with a 27-fold lower antigen density, was seen with dilute human serum as a complement source. The lack of killing of HL60 cells could not be solely attributed to homologous restriction factors on HL60 cells (39), since reduction of the number of CD33 sites on AL67 cells by enzymatic cleavage also rendered them insusceptible to CMC. Others have found that the tumoricidal effects of an IgG2a mAb correlated with anti-

body density on the tumor cells (40, 41). Because more efficient killing of AL67 cells was seen, this suggested, as with CMC, that ADCC was also dependent on antigen density. Marked killing (80%) was seen against AL67 targets at low effector:target ratios and low humanized M195 concentrations. We are currently investigating drugs to up-regulate the CD33 antigen density on HL60 cells. This might permit adequate C1q binding and, hence, enhancement of CMC with human complement using M195 constructs. MABs which have potent CMC *in vitro* have shown tumoricidal effects *in vivo*, but whether lack of these effects *in vitro* will correlate with a lack of effect in humans *in vivo* is not known (14).

The mouse M195 alone has shown no immunological effector functions in humans in human trials (9). Thus, the evidence that the engineered M195 constructs demonstrate ADCC and the ability to fix human complement *in vitro* is encouraging. Although not directly cytotoxic via human CMC with the expected numbers of CD33 sites for a typical leukemia cell, opsonization via complement receptors may be possible. It is therefore possible that immunological functions *in vivo* will also be enhanced. Clinical trials using humanized M195 are planned to see whether they will be effective antileukemic agents.

Although chimeric mAbs are the most effective mediators of ADCC *in vitro*, they are still capable of generating HAMA and human antichimeric antibodies that might limit their usefulness *in vivo* (14). Hence, a humanized version of M195 may still be favored over the murine and chimeric mAbs, regardless of their biological functions, especially as carriers of toxins or radioisotopes. The increased avidity of HuG1 may allow us to use significantly smaller doses *in vivo* to achieve saturation of target cells. We have already shown therapeutic activity with ^{131}I -M195 in patients with refractory and relapsed leukemias, but retreatments have been limited by the development of HAMA (10). ^{131}I -HuG1 or ^{131}I -HuG3 may avoid this problem. Indeed, repeated doses of humanized M195 may potentially be given with the avoidance of HAMA responses, which have previously resulted in shortened serum half lives *in vivo* and in limited treatment efficacy of the mouse M195 mAb (10).

ACKNOWLEDGMENTS

The authors wish to thank A. Agregado, K. McGraw, and K. Class for their technical assistance and M. Czuczman, J. Gaynor, and L. Old for their helpful suggestions. They are also grateful to L. Forte for her secretarial assistance.

REFERENCES

1. Andrews, R. G., Torok-Storb, B., and Bernstein, I. D. Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. *Blood*, 62: 124-132, 1983.
2. Griffin, J. D., Linch, D., Sabbath, K., Larcom, P., and Schlossman, S. F. A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. *Leuk. Res.*, 8: 521-534, 1984.
3. Andrews, R. G., Takahashi, M., Segal, G. M., Powell, J. S., Bernstein, I. D., and Singer, J. W. The L4F3 antigen is expressed by unipotent and multipotent colony-forming cells but not by their precursors. *Blood*, 68: 1030-1035, 1986.
4. Tanimoto, M., Scheinberg, D. A., Cordon-Cardo, C., Huie, D., Clarkson, B. D., and Old, L. J. Restricted expression of an early myeloid and monocytic cell surface antigen defined by monoclonal antibody M195. *Leukemia* (Baltimore), 3: 339-348, 1989.
5. Scheinberg, D. A., Tanimoto, M., McKenzie, S., Strife, A., Old, L. J., and Clarkson, B. D. Monoclonal antibody M195: a diagnostic marker for acute myelogenous leukemia. *Leukemia* (Baltimore), 3: 440-445, 1989.
6. Robertson, M. J., Soiffer, R. J., Freedman, A. S., Rabinowe, S. L., Anderson, K. C., Ervin, T. J., Murray, C., Dear, K., Griffin, J. D., Nadler, L. M., and

- Ritz, J. Human bone marrow depleted of CD33-positive cells mediates delayed but durable reconstitution of hematopoiesis: clinical trial of MY9 monoclonal antibody-purged autografts for the treatment of acute myeloid leukemia. *Blood*, 9: 2229–2236, 1992.
7. Stiff, P. J., Schulz, W. C., Bishop, M., and Marks, L. Anti-CD33 monoclonal antibody and etoposide/cytosine arabinoside combinations for the *ex vivo* purification of bone marrow in acute nonlymphocytic leukemia. *Blood*, 77: 355–362, 1991.
 8. Lemoli, R. M., Gasparetto, C., Scheinberg, D. A., Moore, M. A. S., Clarkson, B. D., and Gulati, S. C. Autologous bone marrow transplantation in acute myelogenous leukemia: *in vitro* treatment with myeloid-specific monoclonal antibodies and drugs in combination. *Blood*, 77: 1829–1836, 1991.
 9. Scheinberg, D. A., Lovett, D., Divgi, C. R., Graham, M. C., Berman, E., Pentlow, K., Feirt, N., Finn, R. D., Clarkson, B. D., Gee, T. S., Larson, S. M., Oettgen, H. F., and Old, L. J. A Phase I trial of monoclonal antibody M195 in acute myelogenous leukemia: specific bone marrow targeting and internalization of radionuclide. *J. Clin. Oncol.*, 9: 478–490, 1991.
 10. Schwartz, M. A., Lovett, D. R., Redner, A., Divgi, C. R., Graham, M. C., Finn, R., Gee, T. S., Andreiff, M., Oettgen, H. F., Larson, S. M., Old, L. J., and Scheinberg, D. A. Leukemia cytoreduction and marrow ablation after therapy with ¹³¹I-labeled monoclonal antibody M195 for acute myelogenous leukemia (AML). *J. Clin. Oncol.*, in press, 1993.
 11. Brown, P. S., Jr., Parenteau, G. L., Dirbas, F. M., Garsia, R. J., Goldman, C. K., Bukowski, M. A., Junghans, R. P., Queen, C., Hakimi, J., Benjamin, W. R., Clark, R. E., and Waldmann, T. A. Anti-Tac-H, a humanized antibody to the interleukin 2 receptor, prolongs primate cardiac allograft survival. *Proc. Natl. Acad. Sci. USA*, 88: 2663–2667, 1991.
 12. Winter, G., and Milstein, C. Man-made antibodies. *Nature (Lond.)*, 349: 293–299, 1991.
 13. Waldmann, T. A. Monoclonal antibodies in diagnosis and therapy. *Science (Washington, DC)*, 252: 1657–1662, 1991.
 14. Chapman, P. B., Scheinberg, D. A., DiMaggio, J. J., and Houghton, A. N. Unconjugated monoclonal antibodies as anticancer agents. *In: H. F. Oettgen (ed.), Immunology and Allergy Clinics of North America II*, pp. 257–275. Philadelphia, PA: W. B. Saunders, 1991.
 15. Hale, G., Clark, M. R., Marcus, R., Winter, G., Dyer, M. J. S., Phillips, J. M., Riechmann, L., and Waldmann, H. Remission induction in non-Hodgkin's lymphoma with reshaped human monoclonal antibody CAMPATH-1H. *Lancet*, 2: 1394–1399, 1988.
 16. Mathieson, P. W., Cobbold, S. P., Hale, G., Clark, M. R., Oliveira, D. B. G., Lockwood, C. M., and Waldmann, H. Monoclonal antibody therapy in systemic vasculitis. *N. Engl. J. Med.*, 323: 250–254, 1990.
 17. Queen, C., Schneider, W. P., Selick, H. E., Payne, P. W., Landolfi, N. F., Duncan, J. F., Avdalovic, N. M., Levitt, M., Junghans, R. P., and Waldmann, T. A. A humanized antibody that binds to the interleukin 2 receptor. *Proc. Natl. Acad. Sci. USA*, 86: 10029–10033, 1989.
 18. Junghans, R. P., Waldmann, T. A., Landolfi, N. F., Avdalovic, N. M., Schneider, W. P., and Queen, C. Anti-Tac-H, a humanized antibody to the interleukin 2 receptor with new features for immunotherapy in malignant and immune disorders. *Cancer Res.*, 50: 1495–1502, 1990.
 19. Co, M. S., Deschamps, M., Whitley, R. J., and Queen, C. Humanized antibodies for antiviral therapy. *Proc. Natl. Acad. Sci. USA*, 88: 2869–2874, 1991.
 20. Co, M. S., Avdalovic, N. M., Caron, P. C., Avdalovic, M. V., Scheinberg, D. A., and Queen, C. Chimeric and humanized antibodies with specificity for the CD33 antigen. *J. Immunol.*, 148: 1149–1154, 1992.
 21. Peiper, S. C., Ashmun, R. A., and Look, A. T. Molecular cloning, expression, and chromosomal localization of a human gene encoding the CD33 myeloid differentiation antigen. *Blood*, 72: 314–321, 1988.
 22. Mosmann, T. Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65: 55–63, 1983.
 23. Brown, B. A., Davis, G. L., Saltzgaber-Muller, J., Simon, P., Ho, M.-K., Shaw, P. S., Stone, B. A., Sands, H., and Moore, G. P. Tumor-specific genetically engineered murine/human chimeric monoclonal antibody. *Cancer Res.*, 47: 3577–3583, 1987.
 24. Barker, E., Mueller, B. M., Handgretinger, R., Herter, M., Yu, A. L., and Reisfeld, R. A. Effect of a chimeric anti-ganglioside GD₂ antibody on cell-mediated lysis of human neuroblastoma cells. *Cancer Res.*, 51: 144–149, 1991.
 25. Colcher, D., Milenic, D., Roselli, M., Raubitschek, A., Yarranton, G., King, D., Adair, J., Whittle, N., Bodmer, M., and Schlom, J. Characterization and biodistribution of recombinant and recombinant/chimeric constructs of monoclonal antibody B72.3. *Cancer Res.*, 49: 1738–1745, 1989.
 26. Roy, D. C., Griffin, J. D., Belvin, M., Blättler, W. A., Lambert, J. M., and Ritz, J. Anti-MY-9-blocked-ricin: an immunotoxin for selective targeting of acute myeloid leukemia cells. *Blood*, 77: 2404–2412, 1991.
 27. Shawler, D. L., Miceli, M. C., Wormsley, S. B., Royston, I., and Dillman, R. O. Induction of *in vitro* and *in vivo* antigenic modulation by the anti-human T-cell monoclonal antibody T101. *Cancer Res.*, 44: 5921–5927, 1984.
 28. Stevenson, F. K., Bell, A. J., Cusack, R., Hamblin, T. J., Slade, C. J., Spellberger, M. B., and Stevenson, G. T. Preliminary studies for an immunotherapeutic approach to the treatment of human myeloma using chimeric anti-CD38 antibody. *Blood*, 77: 1071–1079, 1991.
 29. Liu, A. Y., Robinson, R. R., Murray, E. D., Jr., Ledbetter, J. A., Hellström, I., and Hellström, K. E. Production of a mouse-human chimeric monoclonal antibody to CD20 with potent Fc-dependent biologic activity. *J. Immunol.*, 139: 3521–3526, 1987.
 30. Brüggemann, M., Williams, G. T., Bindon, C. I., Clark, M. R., Walker, M. R., Jefferis, R., Waldmann, H., and Neuberger, M. S. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. *J. Exp. Med.*, 166: 1351–1361, 1987.
 31. Dyer, M. J. S., Hale, G., Hayhoe, F. G. J., and Waldmann, H. Effects of CAMPATH-1 antibodies *in vivo* in patients with lymphoid malignancies: influence of antibody isotype. *Blood*, 73: 1431–1439, 1989.
 32. Ralph, P., and Nakiwiz, I. Cooperation of IgG monoclonal antibodies in macrophage antibody-dependent cellular cytotoxicity (ADCC) to tumor targets. *J. Leukocyte Biol.*, 35: 131–139, 1984.
 33. Ortaldo, J. R., Woodhouse, C., Morgan, A. C., Herberman, R. B., Cheresch, D. A., and Reisfeld, R. Analysis of effector cells in human antibody-dependent cellular cytotoxicity with murine monoclonal antibodies. *J. Immunol.*, 138: 3566–3572, 1987.
 34. Munn, D. H., and Cheung, N. K. V. Interleukin-2 enhancement of monoclonal antibody-mediated cellular cytotoxicity against human melanoma. *Cancer Res.*, 47: 6600–6605, 1987.
 35. Biddle, W. C., Pancook, J., Goldrosen, M., Han, T., Foon, K. A., and Vaickus, L. Antibody-dependent, cell-mediated cytotoxicity by an anti-Class II murine monoclonal antibody: effect of recombinant interleukin 2 on human effector cell lysis of human B-cell tumors. *Cancer Res.*, 50: 2991–2996, 1990.
 36. Vaickus, L., Biddle, W., Cemerlic, D., and Foon, K. A. Interferon gamma augments Lym-1-dependent, granulocyte-mediated tumor cell lysis. *Blood*, 75: 2408–2416, 1990.
 37. Kushner, B. A., and Cheung, N. K. V. GM-CSF enhances 3F8 monoclonal antibody-dependent cellular cytotoxicity against human melanoma and neuroblastoma. *Blood*, 73: 1936–1941, 1989.
 38. Munn, D. H., and Cheung, N. K. V. Phagocytosis of tumor cells by human monocytes cultured in recombinant macrophage colony-stimulating factor. *J. Exp. Med.*, 172: 231–237, 1990.
 39. Shin, M. L., Hansch, G., Hu, V. W., and Nicholson-Weller, A. Membrane factors responsible for homologous species restriction of complement-mediated lysis: evidence for a factor other than DAF operating at the stage of C8 and C9. *J. Immunol.*, 136: 1777–1782, 1986.
 40. Herlyn, D., Powe, J., Ross, A. H., Herlyn, M., and Koprowski, H. Inhibition of human tumor growth by IgG2A monoclonal antibodies correlates with antibody density on tumor cells. *J. Immunol.*, 134: 1300–1304, 1985.
 41. Welt, S., Carswell, E. A., Vogel, C. W., Oettgen, H. F., and Old, L. J. Immune and non-immune effector functions of IgG3 mouse monoclonal antibody R24 detecting the disialoganglioside GD₂ on the surface of melanoma cells. *Clin. Immunol. Immunopathol.*, 45: 214–229, 1987.