

Enhancement of Antibody-Dependent Mechanisms of Tumor Cell Lysis by a Targeted Activator of Complement

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Abstract

Complement inhibitors expressed on tumor cells provide a hindrance to the therapeutic efficacy of some monoclonal antibodies (mAb). We investigated a novel strategy to overwhelm complement inhibitor activity and amplify complement activation on tumor cells. The C3-binding domain of human complement receptor 2 (CR2; CD21) was linked to the complement-activating Fc region of human IgG1 (CR2-Fc), and the ability of the construct to target and amplify complement deposition on tumor cells was investigated. CR2 binds C3 activation fragments, and CR2-Fc targeted tumor cells by binding to C3 initially deposited by a tumor-specific antibody. Complement deposition on Du145 cells (human prostate cancer cell line) and anti-MUC1 mAb-mediated complement-dependent lysis of Du145 cells were significantly enhanced by CR2-Fc. Anti-MUC1 antibody-dependent cell-mediated cytotoxicity of Du145 by human peripheral blood mononuclear cells was also significantly enhanced by CR2-Fc in both the presence and the absence of complement. Radiolabeled CR2-Fc targeted to s.c. Du145 tumors in nude mice treated with anti-MUC1 mAb, validating the targeting strategy *in vivo*. A metastatic model was used to investigate the effect of CR2-Fc in a therapeutic paradigm. Administration of CR2-Fc together with mAb therapy significantly improved long-term survival of nude mice challenged with an i.v. injection of EL4 cells. The data show that CR2-Fc enhances the therapeutic efficacy of antibody therapy, and the construct may provide particular benefits under conditions of limiting antibody concentration or low tumor antigen density. [Cancer Res 2007;67(19):9535–41]

Introduction

Many monoclonal antibodies (mAb) depend, at least in part, on their complement-activating properties for therapeutic efficacy in animal models of cancer and in patients (1–5). Activation of complement on a tumor cell surface results in the generation of C3 activation products that become covalently attached to the cell and act as opsonins for complement receptors expressed on phagocytes and natural killer cells. The engagement of leukocyte complement receptors can enhance antibody-dependent cell-mediated cytotoxicity (ADCC) and may also promote complement-dependent cell-mediated cytotoxicity (CDCC). Complement activation also results in the generation of chemotactic and inflammatory peptides (C3a and C5a) that may potentiate antitumor responses and ultimately in the formation of the cytolytic membrane attack complex (MAC;

see ref. 6 for review of complement-mediated effector mechanisms). However, tumor cells are protected from complement by the expression of membrane-bound complement inhibitory proteins that function to limit complement activation and C3 cleavage (CD55 and CD46) or inhibit MAC formation (CD59). Several studies have shown that complement inhibitors are expressed at increased levels on tumor cells, indicating that they play a role in tumor immune evasion (reviewed in ref. 7).

Antibody-mediated complement deposition on a tumor cell can be enhanced by down-regulating membrane complement inhibitors or blocking their function, but the application of such a strategy specifically for tumor cells *in vivo* is a technical challenge. One approach that has shown promise in a rat model of metastatic cancer is the use of a bispecific antibody construct that targets both a tumor antigen and a complement inhibitor, although inhibition of metastases was dependent on prophylactic administration (8). In this study, we investigate a strategy using a fusion protein to target and amplify complement deposition on a tumor cell without regard to modulating complement inhibitor expression. The fusion protein consists of a targeting domain, the C3-binding region of complement receptor 2 (CR2; CD21), linked to a complement-activating human IgG1 Fc domain.

CR2 is expressed on B cells and dendritic cells and is a member of the C3-binding protein family that binds inactivated C3 fragments (iC3b, C3dg, and C3d). Complement activation results in cleavage of serum C3 and the generation of C3b that becomes covalently attached to the activating surface and that participates in the amplification of the cascade. However, cell-bound C3b is rapidly converted to inactive iC3b, particularly when deposited on a cell surface containing regulators of complement activation (e.g., tumor cells). iC3b is subsequently digested to the membrane-bound fragments C3dg and then C3d by serum proteases, but this process is relatively slow. Thus, the C3 ligands for CR2 are relatively long lived at the site of complement deposition and may be present at high concentration on tumor cells that bind a complement-activating antitumor antibody. The complement-activating Fc domain of the fusion protein is expected to enhance complement deposition and also result in the further production of CR2 ligand. In addition to the cytotoxic effect of enhanced complement deposition, the increased Fc deposition may enhance Fc receptor-dependent mechanisms of cell cytotoxicity (ADCC). In the current study, we characterized CR2-Fc and antibody-dependent mechanisms of tumor cell lysis in both *in vitro* and *in vivo* model systems that involved the clinically relevant tumor-associated antigens MUC1 and GD2.

Materials and Methods

Cell lines and cDNA. The human prostate cancer cell line Du145 and the mouse lymphoma cell line EL4 were grown at 37°C in 5% CO₂ in RPMI 1640 with 10% heat-inactivated FCS (Gemini Bio-Product), 100 units/mL penicillin, and 100 µg/mL streptomycin. cDNAs encoding human CR2 and

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human IgG1 were provided by Dr. V.M. Holers (University of Colorado, Denver, CO) and Dr. S.L. Morrison (University of California at Los Angeles, Los Angeles, CA).

Antibodies and serum. Anti-human MUC1 mAb BCP8 and anti-GD2 mAb 14G2a (IgG2a) were provided by Dr. I.F. McKenzie (Austin Research Institute, Heidelberg, Victoria, Australia) and Dr. R.A. Reisfeld (Scripps Research Institute, La Jolla, CA), respectively. Goat anti-human and anti-mouse C3 IgG and all secondary antibodies were obtained from ICN Pharmaceuticals. Human IgG1 used as control was purchased from Sigma. C6- and C7-depleted human serum was purchased from Quidel, and normal human serum (NHS) was obtained from the blood of healthy volunteers in the laboratory under an Institutional Review Board-approved protocol. Normal mouse serum was prepared from BALB/c nude mice. All serum was stored in aliquots at -80°C until use.

Preparation of human CR2-Fc fusion protein. A cDNA construct for expression of the recombinant CR2-Fc fusion protein was prepared by joining the CR2 sequence encoding the four NH_2 -terminal short consensus repeat (SCR) units (residues 1–250 of mature protein) to sequences encoding Fc portion of human IgG1 constant region. The CR2 fragment (consisting of 1–4 NH_2 -terminal SCR units, 840 bp) and the human IgG1 Fc coding region (705 bp) were generated from the plasmids pBM-CR2 (9) and pED.253.Fc (kindly provided by G. Shaw, Wyeth Research, Cambridge, MA; ref. 10), respectively. The CR2 sequence was excised from pBM-CR2 vector using *Pst*I and *Not*I, and the sequence of IgG1 Fc region was excised from pED.253.Fc vector using *Not*I and *Eco*RI. The two fragments were ligated and cloned into the expression vector pHCMV1 (Genlantis). Sequence was verified. For expression, pHCMV1-huCR2Fc was transfected into Chinese hamster ovary (CHO) cells using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Stably transfected clones were selected by limiting dilution as described (11), and protein expression was quantified by SDS-PAGE and Western blot (anti-CR2 and anti-Fc). Recombinant CR2-Fc was purified from culture supernatant by protein A affinity chromatography.

Flow cytometry. To analyze binding of human CR2-Fc to C3-opsonized Du145 cells, the cells were incubated in 20 $\mu\text{g}/\text{mL}$ of anti-MUC1 mAb BCP8 for 30 min at 4°C , washed, and opsonized with C3 by incubation in 20% C6-depleted human serum for 45 min at 37°C . The C3-opsonized cells were then washed and incubated with 50 $\mu\text{g}/\text{mL}$ of CR2-Fc for 30 min at 4°C . After washing, cells were incubated with FITC-conjugated anti-human IgG Fc (1:100, 30 min at 4°C). Finally, cells were suspended in PBS containing 2 $\mu\text{g}/\text{mL}$ propidium iodide and analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems). For analysis of human C3 deposition on Du145 cells *in vitro*, cells (5×10^5) were resuspended in 20 μL PBS or anti-MUC1 mAb BCP8 at 50 $\mu\text{g}/\text{mL}$ and incubated for 30 min at 4°C . After washing, cells were incubated with or without the 50 $\mu\text{g}/\text{mL}$ of CR2-Fc in 30 μL of 25% C6-depleted human serum diluted in gelatin veronal buffered saline (GVB²⁺; Sigma) at 37°C for 30 min. Cells were then washed with GVB containing 20 mmol/L EDTA (to prevent further complement activation), incubated with FITC-conjugated goat anti-human C3 (30 min/ 4°C), and washed. Finally, cells were suspended in PBS containing 2 $\mu\text{g}/\text{mL}$ propidium iodide and analyzed by flow cytometry. Propidium iodide-positive (dead) cells were excluded when fluorescence intensity was calculated (always <5% of population). A similar procedure was followed for measuring mouse C3 deposition on EL4 murine T lymphoma cells. EL4 cells were incubated in PBS or anti-GD2 mAb 14G2a at 50 $\mu\text{g}/\text{mL}$ for 30 min at 4°C , washed, and then incubated in 30% fresh mouse serum for 30 min at 37°C . Complement activity was terminated by addition of GVB-EDTA, and the cells were washed and incubated with or without 50 $\mu\text{g}/\text{mL}$ of CR2-Fc in 30 μL of 30% fresh mouse serum diluted in GVB²⁺ at 37°C for 30 min. Cells were then analyzed for deposited C3 using FITC-conjugated anti-mouse C3.

Complement lysis assay. Complement-dependent cell lysis (CDC) was determined by ^{51}Cr release as described (12). Briefly, ^{51}Cr -loaded Du145 cells (1×10^5) were incubated in 20 μL PBS or sensitized with 20 μL of anti-MUC1 mAb BCP8 (50 $\mu\text{g}/\text{mL}$) for 30 min at 4°C . After centrifugation and removal of supernatant, cells were washed twice and incubated in 30 μL of 30% NHS in GVB²⁺ with or without CR2-Fc (50 $\mu\text{g}/\text{mL}$) for 60 min at 37°C .

Lytic activity was calculated using the means of replicate wells according to the following formula: % cell lysis = $[(a - c) / (b - c)] \times 100$, where a is cpm of the supernatant of each sample, b is cpm after lysis with 0.05% saponin, and c is cpm of the supernatant treated with GVB²⁺.

Cell cytotoxicity assays. Effector cells were peripheral blood mononuclear cells (PBMC) obtained from a healthy donor on the same day of the experiment using BD Vacutainer CPT Cell Preparation Tube with Sodium Heparin (BD Biosciences). For cytotoxicity assays, Du145 cells were preloaded with sodium ^{51}Cr as described (13) and seeded in round-bottomed 96-well plates (2×10^4 per well). Cells were then centrifuged and resuspended in RPMI 1640 with combinations of 50 $\mu\text{g}/\text{mL}$ BCP8, CR2-Fc, and 20% C7-depleted human serum (refer to figure) and effector cells (E:T, 100:1) dispensed into the wells. After incubation for 4 h at 37°C , plates were centrifuged and radioactivity in supernatants was determined. Lytic activity was calculated using the same formula above for CDC.

Biodistribution studies. The biodistribution of radiolabeled CR2-Fc was determined in nude BALB/c mice (National Cancer Institute, Frederick, MD) bearing s.c. Du145 tumors and treated with anti-MUC1 mAb. Human CR2-Fc and control human IgG1 were radioiodinated to 10 mCi/mg using the IODO-Beads method as described by the manufacturer (Pierce Biotechnology). S.c. tumors were established in nude BALB/c mice by s.c. inoculation of 5×10^5 Du145 cells. When the tumor size was $\sim 500 \text{ mm}^3$, 50 μg anti-MUC1 BCP8 mAb was given via tail vein injection followed 6 h later by 1.5 μg of ^{125}I -labeled CR2-Fc or control human IgG1. Forty-eight hours later, the mice were sacrificed, blood was removed, and the mice were perfused with PBS as described (14). Samples of skin, tumor, kidney, heart, lung, liver, intestine, and spleen were then removed, weighed, and counted for radioactive analysis as described (14). To correct for radioactive decay, aliquots of each labeling mAb were counted at the same time. The results were expressed as tissue to blood ratio.

Antibody therapy model. Normal BALB/c nude mice at 4 weeks of age were injected i.v. via the tail vein with EL4 cells (5×10^4) suspended in 0.1 mL PBS. Two days after tumor cell challenge, mice were injected i.v. either with 25 μg of anti-GD2 mAb 14G2a or with PBS followed 6 h later by injection of 50 μg CR2-Fc or PBS. End point was death or >20% weight loss. Group size was eight mice and all mice were housed in a clean room with food and water sterilized. All animal procedures were approved by the Medical University of South Carolina Animal Care and Use Committee.

Statistical analyses. Unpaired Welch's t tests were used to determine statistical differences. The log-rank test was used to compare differences on survival curves. Significance was accepted at the $P < 0.05$ level.

Results

Purification of CR2-Fc. Recombinant human CR2-Fc (4 NH_2 -terminal SCR units of CR2 linked to human IgG1 Fc region) was

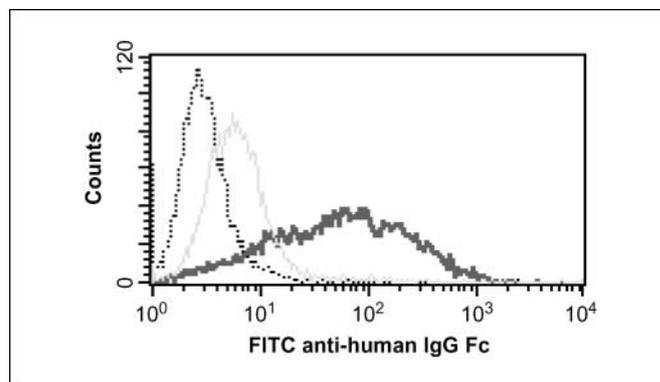


Figure 1. Binding of human CR2-Fc to C3-opsonized Du145 cells. Antibody-sensitized Du145 cells were sensitized with anti-MUC1 mAb BCP8 and incubated in fresh (dark gray trace) or heat-inactivated (light gray trace) C6-deficient serum. After washing the cells, CR2-Fc was added (20 $\mu\text{g}/\text{mL}$) and binding was detected by anti-human IgG1 Fc flow cytometry. Dotted trace, FITC control.

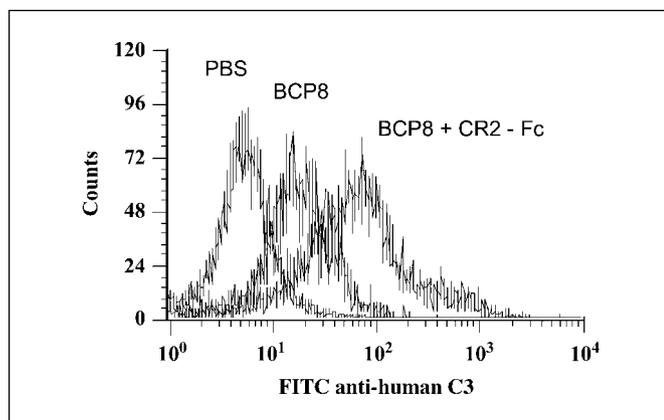


Figure 2. CR2-Fc enhances complement activation. Analysis of C3 deposition following incubation of Du145 cells with anti-MUC1 mAb BCP8 (50 $\mu\text{g}/\text{mL}$) and CR2-Fc (50 $\mu\text{g}/\text{mL}$) in 25% C6-depleted human serum.

purified by protein A affinity chromatography from the culture supernatant of stably transfected CHO cell clones. Analysis of CR2-Fc by SDS-PAGE and Western blot revealed a single band at 130 to 140 kDa under nonreducing conditions and 65 to 70 kDa under reducing conditions (data not shown).

Binding of CR2-Fc to complement-opsonized tumor cells. C3-binding activity by the CR2 domain in the CR2-Fc construct was determined by flow cytometry. Complement was activated on Du145 cells by incubation with anti-MUC1 mAb in C6-depleted human serum (to prevent MAC formation and cell lysis), and C3 deposition on the cell surface was confirmed by anti-C3 flow cytometry (data not shown). CR2-Fc bound to antibody-sensitized Du145 cells incubated in serum and opsonized with C3 but not to antibody-sensitized cells incubated in heat-inactivated serum (Fig. 1).

CR2-Fc enhancement of anti-MUC1-mediated complement deposition and cell lysis. To investigate whether CR2-Fc enhanced mAb-mediated C3 deposition *in vitro*, Du145 cells were coincubated with anti-MUC1 mAb and CR2-Fc in the presence of C6-depleted human serum. As expected, anti-MUC1 mAb activated complement and deposited C3 on Du145 cells, but C3 deposition was significantly enhanced (3.5-fold) by CR2-Fc (mean fluorescence, 21 versus 70; Fig. 2). Human tumor cells, including Du145, are relatively resistant to human complement-mediated lysis. Du145 cells (and most other human cancer cell lines) express not only CD55 and CD46 that control C3 deposition (15) but also CD59 that functions later in the pathway and inhibits complement-mediated cell lysis by controlling MAC formation. To investigate whether CR2-Fc also enhances antibody-mediated CDC, Du145 cells were coincubated with anti-MUC1 mAb and CR2-Fc in the presence of increasing concentrations of NHS. CR2-Fc enhanced cell lysis by ~40% compared with antibody alone (Fig. 3).

CR2-Fc enhancement of anti-MUC1-mediated ADCC. C3 deposited on a target cell can enhance ADCC (16–18). Thus, CR2-Fc has the potential to enhance ADCC via its ability to increase C3 deposition but may also enhance ADCC directly via engagement of its Fc domain by Fc γ receptors (Fc γ R). To investigate the effect of CR2-Fc on ADCC, *in vitro* cell-mediated cytotoxicity assays were done using human PBMC preparations and Du145 targets. Anti-MUC1 mAb (BCP8) triggered ADCC by human PBMC in the absence of complement (heat-inactivated serum), but the addition of CR2-Fc did not enhance cell lysis (Fig. 4). This is not surprising

because there would be no targeting ligand (C3) for CR2-Fc generated on the tumor cell in the absence of a complement source. Anti-MUC1-mediated ADCC was significantly enhanced when complement was added to the incubation (fresh C7-depleted human serum; $P = 0.002$), indicating complement-dependent enhancement of ADCC. Importantly, however, there was a further significant increase in ADCC when CR2-Fc was included in the incubation with anti-MUC1 mAb and a complement source ($P = 0.0118$; Fig. 4). These data show that CR2-Fc enhances ADCC of tumor cells and indicate that enhanced C3 deposition plays an important role in the enhanced cell lysis. There is appropriate cross-species activity in this assay system; human IgG1 (component of CR2-Fc) interacts with all human Fc γ Rs and mouse IgG2b (anti-MUC1 mAb BCP8) interacts with human Fc γ R2 and Fc γ R3 receptors (19). C7-depleted serum was used in these experiments to prevent MAC-mediated cell lysis.

Tumor targeting of CR2-Fc *in vivo*. A Du145 mouse xenograft model was used to investigate the targeting of CR2-Fc to tumor cells *in vivo*. It was first necessary to confirm that CR2-Fc binds mouse C3, and Fig. 5 shows that CR2-Fc binds Du145 cells opsonized with mouse C3 and also enhances cell lysis mediated by anti-MUC1 mAb and mouse complement *in vitro*. To investigate *in vivo* targeting, mice bearing s.c. Du145 tumors were injected i.v. with anti-MUC1 mAb followed 6 h later by an injection of ^{125}I -labeled CR2-Fc. The biodistribution of CR2-Fc to tumor and other tissues was determined at 24 and 48 h after ^{125}I -CR2-Fc injection. ^{125}I -CR2-Fc, but not control ^{125}I -IgG1, targeted to Du145 tumors in mice treated with anti-MUC1 mAb (Fig. 5C). There was also some CR2-Fc localization to kidneys, liver, and spleen, possibly reflecting sites of clearance (see Discussion).

Effect of CR2-Fc on mAb therapy. We investigated the ability of CR2-Fc to improve the outcome of mAb therapy in an EL4 lymphoma model of metastatic cancer. The model consisted of mouse EL4 cells (C57BL/6, GD2 positive) injected i.v. into nude mice and subsequent treatment with a single dose of anti-GD2 mAb. It is necessary to use immunodeficient mice because CR2-Fc

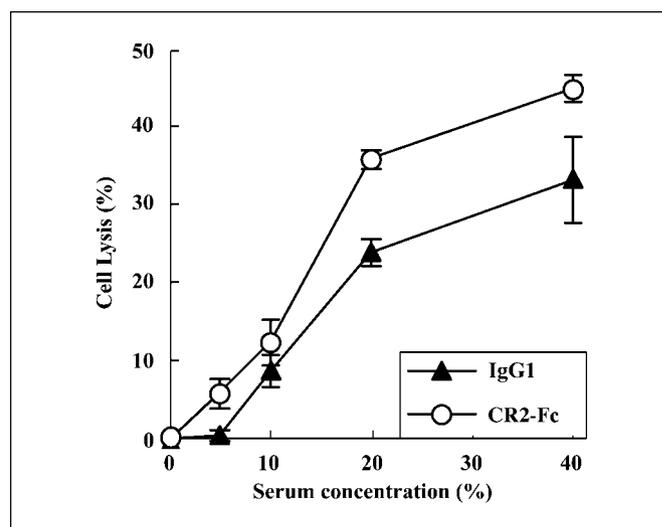


Figure 3. Complement-dependent lysis of anti-MUC1 mAb-sensitized Du145 cells in the presence and absence of CR2-Fc. Du145 cells were treated with anti-MUC1 BCP8 mAb (50 $\mu\text{g}/\text{mL}$) and CR2-Fc (50 $\mu\text{g}/\text{mL}$) in the presence of NHS at indicated concentration for 1 h at 37°C. Cell death was assayed by ^{51}Cr release assay. Points, mean ($n = 3$); bars, SD.

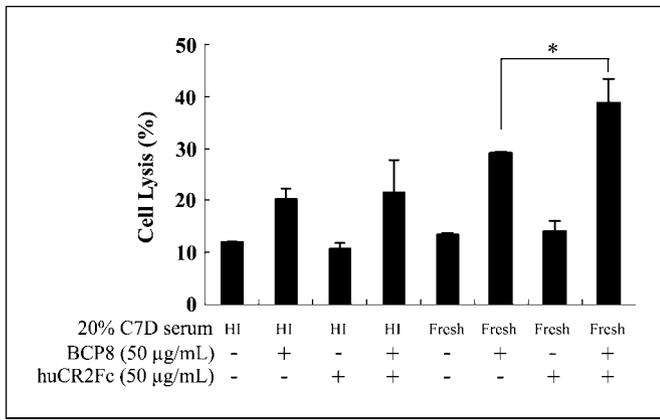


Figure 4. CR2-Fc enhanced cell-mediated cytotoxicity of Du145 cells. Du145 cells were incubated with PBMC (E:T, 100:1) under the indicated conditions. Cell-mediated lysis was determined by ⁵¹Cr release assay. C7D, C7-depleted human serum; HI, heat-inactivated C7-depleted serum; Fresh, fresh C7-depleted serum. Columns, mean (n = 3); bars, SD.

is recombinant human protein. We did not use a Du145 model because of the species selective activity of complement inhibitors. It has been shown that human DAF and CD59 [complement inhibitors expressed on Du145 (15)] have only limited activity against mouse complement (12, 20). Furthermore, whereas Du145 cells are completely resistant to human complement-mediated lysis in the absence of sensitizing antibody, Du145 cells display some

sensitivity to mouse complement lysis in the absence of antibody (data not shown). Thus, the use of a homologous system with regard to complement effector mechanism and target cell (in this case EL4) represents a more relevant model and provides a more rigorous test for investigating the potential of CR2-Fc to enhance mAb therapy. We have used the EL4 metastatic model previously to characterize anti-GD2 mAb therapy and have shown that both ADCC and complement-mediated enhancement of ADCC are operative therapeutic mechanisms of action (21). To validate the approach in the EL4 model, we first confirmed that CR2-Fc binds to mouse C3-opsonized EL4 cells (Fig. 6A) and enhances C3 deposition in the presence of anti-GD2 mAb *in vitro* (Fig. 6B). There was a limited amount of C3 deposition on EL4 cells following incubation in mouse serum in the absence of antibody, and this would account for the small further increase in C3 deposition when CR2-Fc is included in the incubation (Fig. 6B). As expected, in the presence of anti-GD2 mAb, there is a significant increase in C3 deposition, and there is a further significant increase in C3 deposition in the presence of CR2-Fc. In the therapeutic study, mice were treated with anti-GD2 mAb or PBS 48 h after challenge with EL4 cells followed 6 h later with an injection of CR2-Fc or PBS. At the mAb dose used (12.5 µg), there was 25% long-term survival of mice receiving mAb only, but there was a significant increase in long-term survival (~80%) of mice receiving both mAb and CR2-Fc (P < 0.05; Fig. 6C). Treatment with CR2-Fc alone had no significant effect on survival. All surviving mice were sacrificed on day 90 and all were tumor-free.

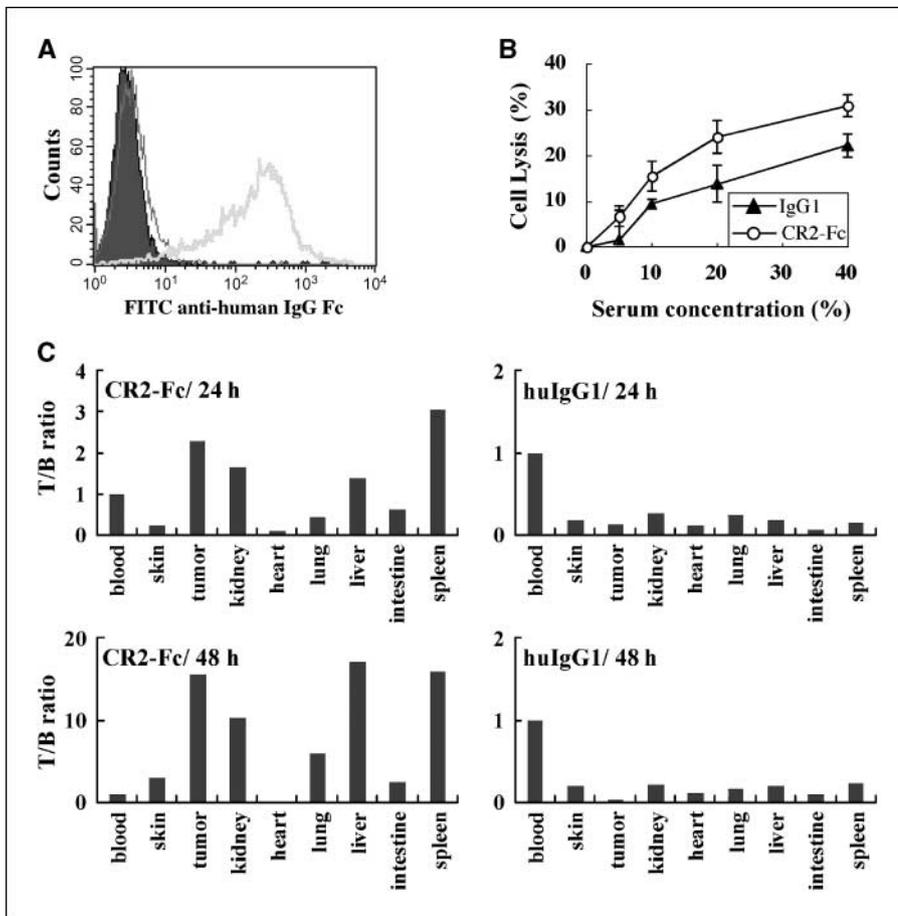
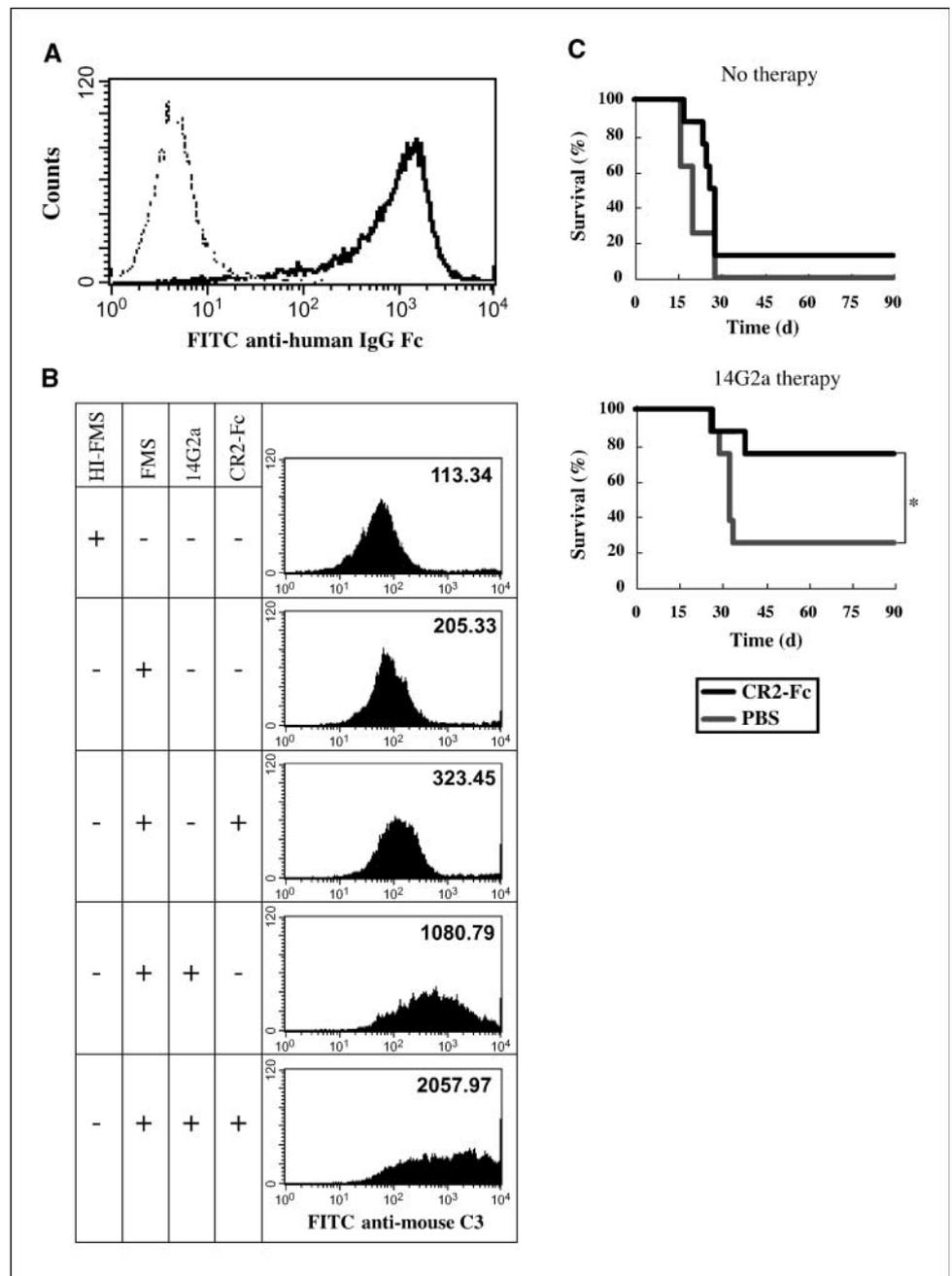


Figure 5. Interaction of CR2-Fc with mouse complement and *in vivo* targeting to Du145 tumors. A, binding of CR2-Fc to mouse C3-opsonized Du145 cells. Anti-MUC1-sensitized Du145 cells were incubated in 30% normal mouse serum, washed, and incubated with control human IgG1 (red trace) or CR2-Fc (green trace) at 20 µg/mL. B, enhancement of mouse complement-dependent lysis by CR2-Fc. Du145 cells were treated with anti-MUC1 mAb and CR2-Fc in the presence of normal mouse serum at indicated concentration for 1 h at 37°C. Anti-MUC1 mAb and CR2-Fc were both used at 50 µg/mL. Points, mean (n = 4); bars, SD. C, biodistribution of ¹²⁵I-CR2-Fc and control ¹²⁵I-IgG1 in Du145 tumor-bearing BALB/c nude mice. Radiolabeled proteins were injected into the tail vein 6 h after receiving anti-MUC1 mAb (50 µg), and biodistribution of radiolabel was determined after 24 and 48 h. Columns, average of two determinations. T/B ratio, tissue to blood ratio.

Figure 6. Characterization of CR2-Fc in an EL4 lymphoma model of cancer.

A. *in vitro* binding of CR2-Fc to mouse C3-opsonized EL4 cells. EL4 cells were opsonized with C3 by incubation in fresh mouse serum and anti-GD2 mAb 14G2a. Cells were then washed and incubated with control human IgG1 (light gray trace) or CR2-Fc (dark gray trace) at 20 $\mu\text{g/mL}$. Binding of CR2-Fc was detected by flow cytometry using FITC-labeled anti-human IgG Fc polyclonal antibody. Dashed line, FITC control. **B.** analysis of C3 deposition on EL4 cells *in vitro*. EL4 cells were incubated in 30% fresh mouse serum (FMS) or heat-inactivated serum (HI-FMS) in the presence or absence of anti-GD2 mAb 14G2a (50 $\mu\text{g/mL}$) and/or CR2-Fc (50 $\mu\text{g/mL}$) as indicated. After washing the cells, C3 deposition was detected by flow cytometry using FITC-labeled anti-mouse C3 polyclonal antibody. Number in the top right, mean fluorescence intensity. **C.** effect of CR2-Fc on anti-GD2 mAb therapy in EL4 model. CR2-Fc enhances survival of mice receiving anti-GD2 mAb therapy following challenge with EL4 cells. BALB/c nude mice were injected i.v. with 5×10^4 EL4 cells and the mice were treated 48 later with PBS (left) or 12.5 μg of anti-GD2 mAb (right). Six hours following mAb injection, mice were treated with 50 μg of CR2-Fc or PBS. $n = 8$ mice per group.



Discussion

Numerous studies have indicated that complement resistance and the expression of complement inhibitory proteins by tumor cells provide immune resistance and are a hindrance to the effectiveness of mAb therapy (see refs. 6, 7, 22–24 for reviews). Down-regulating complement inhibitor expression or blocking their function on tumor cells enhances antibody-mediated complement-dependent cytotoxicity *in vitro* and improves therapeutic outcome in animal models of cancer. Nevertheless, applying such approaches in a clinically relevant setting is problematic due to the widespread and abundant expression of complement inhibitors on normal tissue and the difficulty in appropriate targeting. In the strategy investigated herein, enhancement of tumor-specific complement deposition is achieved by amplifying

the complement activation signal (Fc opsonization). The targeting specificity is dependent on an antitumor mAb that generates the initial C3 targeting ligand for CR2-Fc on the tumor cell. Once bound, CR2-Fc will generate more of its own covalently bound ligand in close proximity to the original complement-activating signal. A high local density of C3 ligand will result in efficient cross-linking of Fc domains by C1q globular heads necessary for classic pathway complement activation.

In the current study, we show that an anti-MUC1 mAb activates and deposits C3 on a MUC1-positive cancer cell line *in vitro* and that, in the presence of CR2-Fc, there is enhanced C3 deposition with increased complement-mediated lysis and ADCC. In a previous study, Kennedy et al. (25) reported that an anti-iC3b mAb, 3E7, enhances C3 deposition on Raji cells in the presence of

rituximab (anti-CD20) and a complement source and enhances rituximab-mediated killing *in vitro*. The anti-iC3b mAb also targeted to B cells after *in vivo* infusion of rituximab in a cynomolgus monkey model. A subsequent study by Peng et al. (26) showed that a mouse/human chimeric mAb specific for C3b and iC3b enhanced rituximab-mediated killing of non-Hodgkin's lymphoma cell lines and cells isolated from non-Hodgkin's lymphoma and chronic lymphocytic leukemia patients. There is a significant potential advantage of CR2-Fc over anti-iC3b mAbs, as evidenced by its ability to significantly increase long-term survival of mice receiving mAb therapy; in the presence of serum, the initial covalently bound complement activation product C3b is very rapidly degraded to iC3b, which is in turn fairly rapidly degraded to the long-lived fragments C3dg and then C3d. CR2-Fc recognizes iC3b, C3dg, and C3d, whereas analysis of mAb 3E7 revealed specificity for only C3b/iC3b (27), the initial and relatively short-lived breakdown products of C3.

Our *in vitro* data show that anti-MUC1-dependent ADCC was enhanced by complement and further enhanced by cocubation with CR2-Fc. The complement-dependent enhanced lysis may be due to increased Fc opsonization as a result of CR2-Fc binding to deposited C3 and/or a direct effect of increased C3 deposition resulting from CR2-Fc binding; C3 can enhance ADCC via its engagement of complement receptors [principally complement receptor 3 (CR3)] expressed on effector cells (16–18). CR2-Fc also increased antibody-dependent direct complement-mediated lysis of tumor cells *in vitro* (both Du145 and EL4 with homologous serum). It is considered unlikely, however, that direct lysis is contributing to the therapeutic efficacy of CR2-Fc in the EL4 model used here. In a previous study investigating complement-mediated mechanisms of anti-GD2 mAb therapy in the same EL4 model, ADCC played a critical role in therapeutic activity, and complement was important for enhancing ADCC at limiting IgG concentrations (as used in the current study). Manipulation of CD59 expression (an inhibitor of the MAC) on EL4 cells modulated complement lysis *in vitro* but did not affect therapeutic outcome, suggesting direct complement-mediated lysis did not play a role in the therapeutic activity of the anti-GD2 mAb (21). We show that CR2 enhances anti-GD2 mAb-mediated deposition of mouse C3 *in vitro*, and increased C3 deposition is relevant to ADCC and CDCC effector mechanisms. We cannot, of course, directly extrapolate these mechanisms to human cancers, but the *in vitro* data presented using a human cell line show that the known complement-dependent mechanisms of tumor cell lysis are enhanced by CR2-Fc.

A biodistribution study showed that CR2-Fc targeted to MUC1-positive tumors in the presence of anti-MUC1 mAb in a xenograft model of human prostate cancer. There was also some targeting to the kidneys, liver, and spleen. The reason for localization to these other organs is not clear, but they may represent sites of clearance. Because of the localization of CR2-Fc in the kidney, it may be worthwhile in the future to determine whether there is any evidence of glomerulonephritis in CR2-Fc-treated animals. The anti-MUC1 mAb used is specific for human MUC1 (28), and it is therefore unlikely the mAb is binding directly to endogenously expressed protein in the mice. Because MUC1 is shed from tumor cells, one possibility is that MUC1 immune complexes are formed in the circulation. Immune complexes fix complement and would represent a target for CR2-Fc, and the liver and spleen are sites of immune complex clearance. Arguing against this possibility, however, is a previous report showing that a different CR2 fusion protein (CR2-Crry) also targets to the liver, spleen, and kidneys in

the absence of a given antibody (14). Regardless, the important point here is that the study shows the feasibility of CR2-Fc targeting to tumors in the presence of a tumor-specific/associated mAb. Different tumor targets and antibodies may yield different results with regard to C3 deposition and CR2-Fc targeting, and such questions are better addressed in more clinically relevant cancer models.

Various other approaches to overcome complement resistance of tumor cells to antibodies and complement have been investigated *in vitro*, and the subject is well reviewed (6, 7, 22, 23, 29). Two approaches to enhance antitumor complement effector mechanisms that have been investigated therapeutically in animal models of cancer are the use of bispecific antibodies and the use of β -glucan, a bioresponse modifier. In a rat model of metastatic colorectal cancer, a bispecific antibody directed against a tumor-associated antigen and Crry, a membrane complement inhibitory protein, significantly inhibited the outgrowth of lung tumors (8). The variable region recognizing Crry blocks its function, resulting in enhanced complement activation on the tumor cell surface. In the other approach, soluble β -glucan has been used as an adjuvant for mAb therapy, and it induces complement-dependent cellular cytotoxicity by enabling iC3b deposited on tumor cells to activate CR3 on immune effector cells (29–31). Several studies involving different models of cancer have shown that β -glucan can promote and enhance mAb therapy, including studies in which β -glucan was used in combination with anti-GD2 and anti-MUC1 mAb therapy (32). CR2-Fc-mediated enhancement of iC3b deposition on tumor cells may synergize with these therapeutic approaches when used in combination with mAb therapy. Furthermore, some cancers, including MUC1-positive cancers, are known to generate an antibody response (33, 34), and persistent complement activation on tumor cells from cancer patients has been reported (35). CR2-Fc may thus also enhance the tumoricidal effect of natural and elicited antitumor antibodies. In addition to enhancing immune effector mechanisms, complement activation can also enhance humoral (36) and T-cell responses (37–39), and CR2-Fc may therefore also potentiate the inductive phase of immunity.

In summary, there is increasing interest in the use of unconjugated mAbs to treat cancer; several have been approved for therapy and several more are under investigation in the laboratory and in the clinic. Their mechanisms of action seem to be variable, but evidence from animal models and from clinical studies indicates that ADCC and complement-dependent cytotoxicity play an important role in the efficacy of many mAbs. In this study, we show the potential of a strategy to enhance the effectiveness of mAb therapy by enhancing Fc opsonization and complement deposition. The approach may be additive to all mAb effector mechanisms, including those that do not have ADCC and complement cytotoxicity as a recognized component of their primary mechanism of action.

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