Complement-Dependent Tumor Cell Lysis Triggered by Combinations of Epidermal Growth Factor Receptor Antibodies

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Abstract

Therapeutic monoclonal antibodies against the epidermal growth factor receptor (EGFR) have advanced the treatment of colon and head and neck cancer, and show great promise for the development of treatments for other solid cancers. Antibodies against EGFR have been shown to act via inhibition of receptor signaling and induction of antibody-dependent cellular cytoxicity. However, complement-dependent cytotoxicity, which is considered one of the most powerful cell killing mechanisms of antibodies, seems inactive for such antibodies. Here, we show a remarkable synergy for EGFR antibodies. Combinations of antibodies against EGFR were identified, which resulted in potent complement activation via the classic pathway and effective lysis of tumor cells. Studies on a large panel of antibodies indicated that the observed synergy is a general mechanism, which can be activated by combining human IgG1 antibodies recognizing different, nonoverlapping epitopes. Our findings show an unexpected quality of therapeutic EGFR antibodies, which may be exploited to develop novel and more effective treatments for solid cancers. [Cancer Res 2008;68(13):4998–5003]

Introduction

The epidermal growth factor (EGF) receptor (EGFR) is a tyrosine kinase receptor, which serves as a target for cancer therapy (1, 2). Today, both specific tyrosine kinase inhibitors (TKI) and monoclonal antibodies against EGFR are being developed—with cetuximab (C225) and panitumumab (E7.6.3) being the first EGFR antibodies to receive Food and Drug Administration (FDA) approval. A potential advantage of antibodies compared with TKI is their broader recruitment of effector mechanisms, which are intensively investigated for therapeutic antibodies (3). For EGFR antibodies, in vitro and in vivo studies have suggested inhibition of signaling, receptor down modulation and antibody-dependent cellular cytoxicity to contribute to therapeutic efficacy (1, 4, 5). Attempts to show complement-dependent cytotoxicity (CDC) by EGFR antibodies in contrast have failed (4, 6, 7), and complement depletion studies in mice suggested CDC not to be a significant mechanism of action for EGFR antibodies in vivo (8). However, complement is one of the most potent cell killing systems (9), and the potential of enhancing anti-EGFR activity against tumors by engaging CDC is therefore of considerable interest. We aimed to achieve this goal by combining EGFR antibodies. By this approach, we identified promising combinations of EGFR antibodies, which bound to distinct epitopes of the receptor, and which potently deposited complement components C1q and C4c on tumor cells—leading to effective complement-mediated cell killing. Although only few antibody combinations have thus far been investigated clinically (10), this approach seems attractive to further improve antibody efficacy in patients.

Materials and Methods

Experiments reported here were approved by the Ethical Committee of the Christian-Albrechts-University, Kiel, Germany, in accordance with the Declaration of Helsinki. Normal human serum (NHS) or plasma was freshly drawn from randomly selected healthy donors, who gave written informed consent before analyses.

Tumor cell targets. Human epidermoid carcinoma cell line A431 (DSMZ) was kept in RPMI 1640, human glioblastoma cell line A1207 (originally established by Dr. Aaronson, National Cancer Institute, NIH, Bethesda, MD) in DMEM. Additionally used human cell lines were as follows: Caco-2 (colon cancer), SCC-25 (squamous tongue cell carcinoma), SH-SY5Y (neuroblastoma), Hep 3B and Hep G2 (both hepatocellular carcinomas), H1975, and H2030 [both non–small cell lung cancer; all from American Type Culture Collection (ATCC)], SK-RC 7, SK-RC 12, SK-RC 14, and SK-RC 24 (all renal cell carcinomas). Medium were supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 U/mL streptomycin, and 4 mmol/L L-glutamine (all from Invitrogen). Cell viability was tested by trypan blue exclusion.

Cell lines were characterized for quantitative surface expression of EGFR and complement regulatory proteins CD46, CD55, and CD59 using antibodies 225 (EGFR; ATCC), J4-48 (CD46; Immunotech), IA10 (CD55), or p282 (CD59; both from BD PharMingen), respectively. After washing, cells were stained with FITC-conjugated polyclonal goat anti-mouse immunoglobulin and analyzed by flow cytometry (Coulter EPICS XL-MCL; Beckman Coulter). For calculation of the surface expression of antigens, the Qifikit (DAKO) was used according to the manufacturer's instructions. All experimental steps were performed at 4°C for 30 min.

EGFR antibodies. The EGFR-directed antibodies used were as follows: 225 (HB-8508, murine IgG1; ATCC), cetuximab (C225, chimeric IgG1), matuzumab (h425, humanized IgG1; both from Merck), and panitumumab (E7.6.3, human IgG2; Amgen). Additional EGFR antibodies zalutumumab (2F8), 003 (LC1006-003), 005 (LC1006-005), 008 (LC1006-008), 011 (LC1006-011), and 018 (LC1006-018)—all of human IgG1 isotype—were generated

from human immunoglobulin transgenic mice as described (11). As human IgG1 isotype control served an antibody against keyhole limpet hemocyanin (KLH; from Genmab). 225 F(ab')$_2$ fragments were produced by ficin digestion of antibody 225 using the ImmunoPure kit (Pierce) according to the manufacturer's instructions. A mouse/human chimeric IgA$_1$ variant of 225 was generated and produced as described (12).

Target cell opsonization was investigated by indirect immunofluorescence. A431 cells (1×10^5) were incubated with EGFR antibodies alone or in combinations at antibody concentrations of 10 μg/mL. After washing, cells were stained with FITC-conjugated polyclonal goat anti-human κ light chain (DAKO) and analyzed by flow cytometry. Relative fluorescence intensities (RFI) were calculated using the formula: experimental mean fluroescence intensity (MFI) / MFI with isotype control antibody KLH.

For direct immunfluorescence, EGFR antibodies were FITC-conjugated using the EZ-label-kit (Pierce) according to the manufacturer's instructions.

To compare antibody affinities, 1×10^5 A431 cells were incubated with EGFR antibodies at various concentrations, washed, and stained with FITC-conjugated polyclonal goat anti-mouse immunoglobulin (DAKO). After washing again, cells were analyzed by flow cytometry. EC$_{50}$ values were determined by GraphPad-Prism 4.0 using a four-variable nonlinear regression model with a sigmoidal dose response (variable slope).

Crossblocking studies. EGFR-binding epitopes of different EGFR antibodies were analyzed by competitive immunofluorescence binding assays. EGFR-expressing target cells (2×10^5) were incubated with FITC-conjugated EGFR antibodies at nonsaturating concentrations in combination with 200-fold excess of different unconjugated antibodies. After washing, samples were analyzed by flow cytometry. Level of competition was calculated by the formula: % competition = (experimental MFI − background MFI) / (maximal MFI − background MFI) × 100, with maximal MFI defined by the combination of FITC-conjugated EGFR antibody with isotype control antibody KLH.

Inhibition of EGF-binding. For comparison of the capacity to block ligand binding of EGFR antibodies, 1.5×10^5 A431 cells were coincubated with 2.5 μg/mL FITC-conjugated EGF (Invitrogen) and 200 μg/mL antibodies for 30 min. After washing, cells were analyzed by flow cytometry. Blockade of ligand binding was calculated by the formula: % inhibition of

EGF − binding = (RFI without − RFI with antibody) / (RFI without antibody) × 100.

**Complement deposition.** Complement deposition was determined by incubating 1×10^5 target cells for 15 min at room temperature either with individual EGFR antibodies or with antibody combinations at additive antibody concentrations of 10 μg/mL, followed by addition of 1% (vol/vol) NHS and incubation at 37°C for 10 min. After washing, samples were stained with polyclonal FITC-conjugated C1q or C4c antibodies (both from DAKO) and analyzed by flow cytometry.

Complement-dependent cytotoxicity assay. Complement-dependent cytotoxicity (CDC) assays were performed as described in (12). Briefly, target cells were labeled with 200 μCi (7.4 MBq) ^{51}Cr for 2 h. After washing, cells were adjusted to 10^5/mL. Freshly drawn human serum (50 μL), sensitizing antibodies, and RPMI 1640 (10% FCS) were added to round-bottomed microtiter plates. Assays were started by adding target cells (50 μL), resulting in a final volume of 200 μL per well and a final concentration of 25% serum. Pilot experiments showed this serum concentration to provide plateau killing levels. After 3 h at 37°C, ^{51}Cr release from the supernatants was measured in triplicates as cpm. Percentage of cytotoxicity was calculated with the formula: % specific lysis = (experimental cpm − basal cpm) / (maximal cpm - basal cpm) × 100, with maximal ^{51}Cr release determined by adding perchloric acid (3% final concentration) to target cells, and basal release measured in the absence of sensitizing antibodies and serum. Antibody-independent cytotoxicity (serum without target antibodies) was not observed.

To determine the contribution of different complement activation pathways, final concentrations of 5 mmol/L MgCl$_2$ and 10 mmol/L EGTA were added to selectively inhibit the classic pathway, or 10 mmol/L EDTA (both from Roth) for complete blockade of complement activation, respectively. Furthermore, for some experiments, serum was heated to 50°C for 15 min to selectively inactivate the alternative pathway, or to 56°C for 30 min for complete heat inactivation of the complement system.

Data processing and statistical analyses. Data are displayed graphically and analyzed statistically using GraphPad Prism 4.0. Experimental curves were fitted using a four-variable nonlinear regression model with a sigmoidal dose response (variable slope). Group data are reported as

Figure 1. Complement deposition and complement-dependent killing by EGFR antibodies. C1q (*A*) and C4c (*B*) deposition on A431 cells was analyzed in the presence of individual EGFR antibodies or in the presence of antibody combinations (final antibody concentration 10 μg/mL). Although individual EGFR antibodies did not trigger C1q or C4c deposition, the combination of cetuximab and matuzumab led to significant complement deposition (significance indicated by *). *Columns,* mean of four independent experiments; *bars,* SE. Cetuximab (○), matuzumab (X), or their noncrossblocking combination (□) were analyzed for their capacity to dose-dependently trigger CDC of A431 (*C*) or A1207 (*D*) target cells, which differed mainly in their surface expression of complement regulatory proteins (see Supplementary Table S1). *Columns,* mean of "% specific lysis" from four independent experiments; *bars,* SE (*C* and *D*). *, significant lysis.
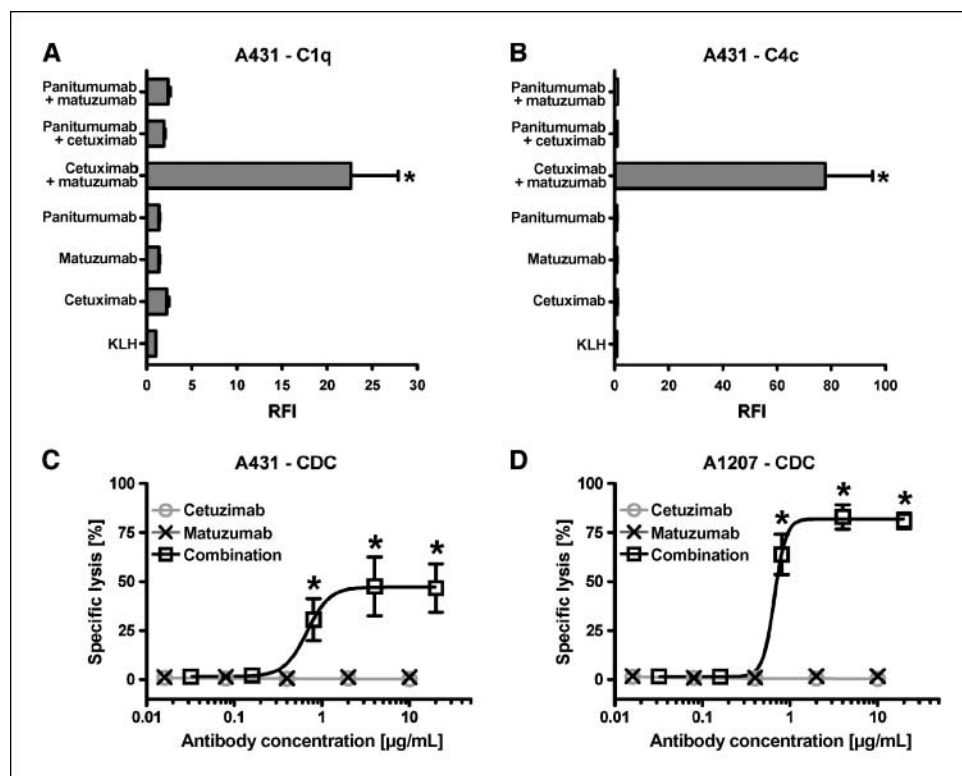
mean ± SE, unless otherwise indicated. Differences between groups were analyzed by unpaired (or, when appropriate, paired) Student's *t* tests. Significance was accepted when *P* values were <0.05.

For assessment of synergistic CDC by combinations of antibodies, the combination index (CI) was determined by dividing the sum of specific lysis induced by single-agent treatments with specific lysis induced by the combination treatment. CI values of 1 were considered additive, values of <1 were considered synergistic.

Results and Discussion

As individual EGFR antibodies do not trigger CDC, we investigated whether antibody combinations might provide benefit. Interestingly, we found that a combination of two antibodies, cetuximab and matuzumab, led to an increase in C1q and C4c fixation (Fig. 1*A* and *B*), which was not observed for combinations of these antibodies with panitumumab. Furthermore, strong activation of CDC was observed for the cetuximab/matuzumab combination. Whereas both individual antibodies did not induce CDC of A431 squamous cell carcinoma or A1207 glioblastoma cells, we observed 50% to 80% lysis of both cell lines for the combination (Fig. 1*C* and *D*). A CI of 0.02 for A431 cells and 0.01 for A1207 cells (at 10 μg/mL) was calculated, indicating very strong synergism. This cell lysis occurred in the presence of high expression levels of complement regulatory proteins CD46, CD55, and CD59 (Supplementary Table S1). Anti-EGFR combinations, therefore, could overcome strong target cell complement defense. Experiments with Ca^{2+} and Mg^{2+} depletion and heat inactivation of serum indicated the classic complement pathway to be responsible for CDC under these conditions (Supplementary Fig. S1).

Emerging evidence suggests EGFR antibodies to affect EGF receptor clustering (13), which may significantly effect C1q binding and subsequent complement activation. Antibody-induced clustering, however, should not be affected by antibody isotype. Thus, in an attempt to better understand the mechanism underlying the enhanced CDC by the antibody combination, the contribution of individual Fc portions of EGFR antibodies was investigated. For this purpose, matuzumab (human IgG1) was combined with cetuximab (human IgG1), its murine parental antibody 225 (mouse IgG1), respective F(ab')$_2$ fragments, or a recombinant monomeric IgA1 isotype variant of cetuximab (12). Interestingly, significant CDC was observed only in the presence of two human IgG1 antibodies (Fig. 2*A*). These results suggested altered EGFR clustering alone did not represent a relevant mechanism underlying the engagement of CDC by EGFR combinations.

Results presented thus far may have suggested panitumumab combinations fail to trigger CDC because panitumumab is of human IgG2 isotype (14). To investigate the molecular requirements in more detail, we investigated target cell opsonization by individual antibodies and antibody combinations. Immunostaining of the cetuximab and matuzumab combination indeed increased by 49.6% (*P* = 0.03; *n* = 3), whereas combinations with panitumumab did not increase binding levels, compared with individual antibodies (Fig. 2*B*). These data suggested nonoverlapping binding epitopes to be critical for CDC induced by EGFR antibody combinations.

To test for this hypothesis, we investigated six novel EGFR antibodies of human IgG1 isotype (zalutumumab, 003, 005, 008, 011, and 018), which were generated from human immunoglobulin transgenic mice (11). These antibodies were first characterized for their apparent binding affinities (Supplementary Table S2), for their activity to block EGF binding (Supplementary Fig. S2) and for their
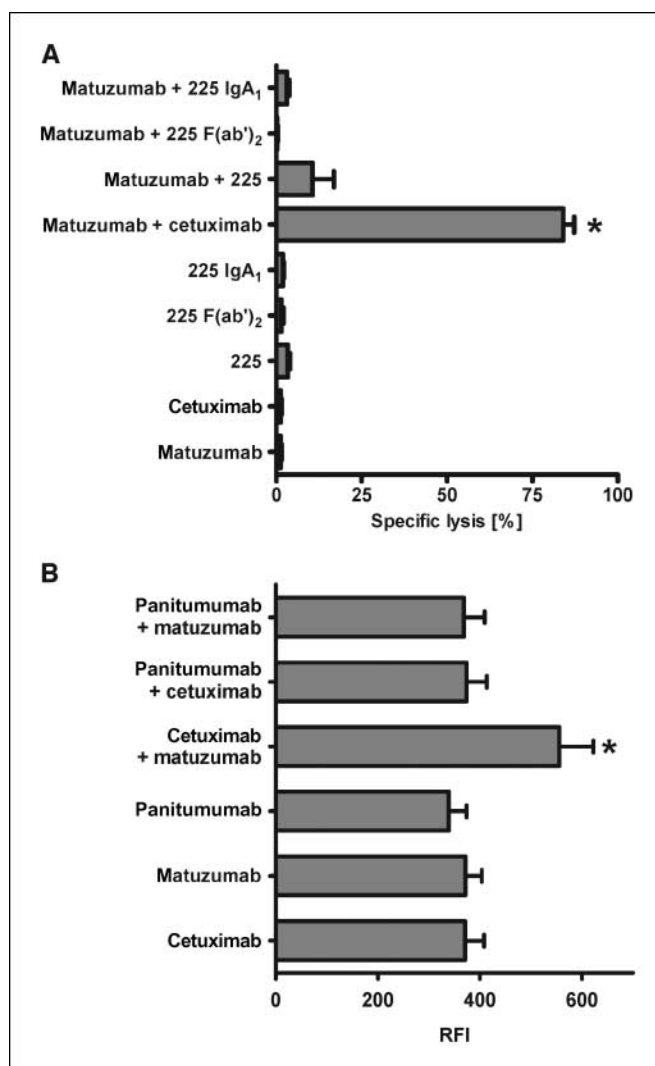

Figure 2. Molecular requirements of CDC mediated by EGFR antibody combinations. *A,* combinations of matuzumab (IgG1) with cetuximab (IgG1), 225 (mIgG1), 225 F(ab')$_2$ fragments, and 225 IgA1 showed that CDC was only observed in the presence of two human IgG1 Fc fragments (*, significant lysis). A1207 cell line served as targets. *Columns,* mean of % specific lysis from four independent experiments; *bars,* SE. *B,* EGFR antibody combinations were analyzed by indirect immunofluorescence with anti-human κ-FITC for their capacity to enhance opsonization of A431 cells. Individual antibodies or their combinations were used at 10 μg/mL final concentration. RFI was calculated as described in Materials and Methods. *Columns,* mean of four independent experiments; *bars,* SE. *, significant increase in target cell opsonization.

capacity to crossblock each other (Fig. 3). Crosscompetition studies indeed indicated cetuximab and matuzumab to compete with panitumumab for binding but did not compete with each other. Results from these experiments further identified two clusters of binding epitopes. Cluster 1 antibodies consisted of cetuximab, matuzumab, panitumumab, zalutumumab, and 018, which, with the exception of 018, strongly inhibited EGF binding. Cluster 2 antibodies only weakly blocked EGF binding and consisted of antibodies 003, 005, 008, and 011. For cluster 1, antibody combinations of cetuximab/matuzumab, cetuximab/018, and panitumumab/018 were noncrossblocking, whereas the remaining combinations competed with each other for binding. For cluster 2, all combinations were crossblocking. Importantly, none of the combinations of cluster 1 and cluster 2 antibodies was crossblocking.

Results from EGF and crossblocking experiments are displayed schematically in Fig. 4A.

Next, all possible dual combinations of the eight available IgG1 antibodies were investigated for complement deposition (Supplementary Fig. S3), and CDC induction was compared using A1207 cells (Fig. 4B). Strong synergism was observed for complement-mediated cell lysis triggered by all noncrossblocking antibody combinations, whereas none of the crossblocking combinations triggered CDC. Significantly, none of the individual antibodies induced significant CDC (data not shown), whereas almost complete lysis was obtained for all noncompeting antibody combinations. To exclude that these observations were restricted to A1207 cells, similar experiments were performed with A431 target cells. Again, noncrossblocking antibody combinations triggered significant CDC, whereas individual antibodies or blocking antibody combinations were not effective (Supplementary Fig. S4).

The contribution of complement to antibody efficacy in vivo has long been the subject of discussion. Two of the most effective antibodies against malignant B cells—rituximab and campath—both activate complement. For solid tumors, complement killing has rarely been shown in homologous systems, and the relevance of heterologous assays in vitro and in vivo is arguable (15). Notably, also Her-2/neu antibody combinations triggered CDC, but these experiments were performed with murine antibodies and complement against a human cell line (16). However, the resistance of solid tumor cells to CDC seems relative, rather than absolute. Thus, it can be overcome by inhibiting complement regulatory proteins (15) or by using select antibody combinations (ref. 17 and this article). Although antibody combinations against EGFR have been analyzed before, none of these studies addressed complement activation (18, 19). Eventually, complement efficacy may be further enhanced by using antibodies with improved
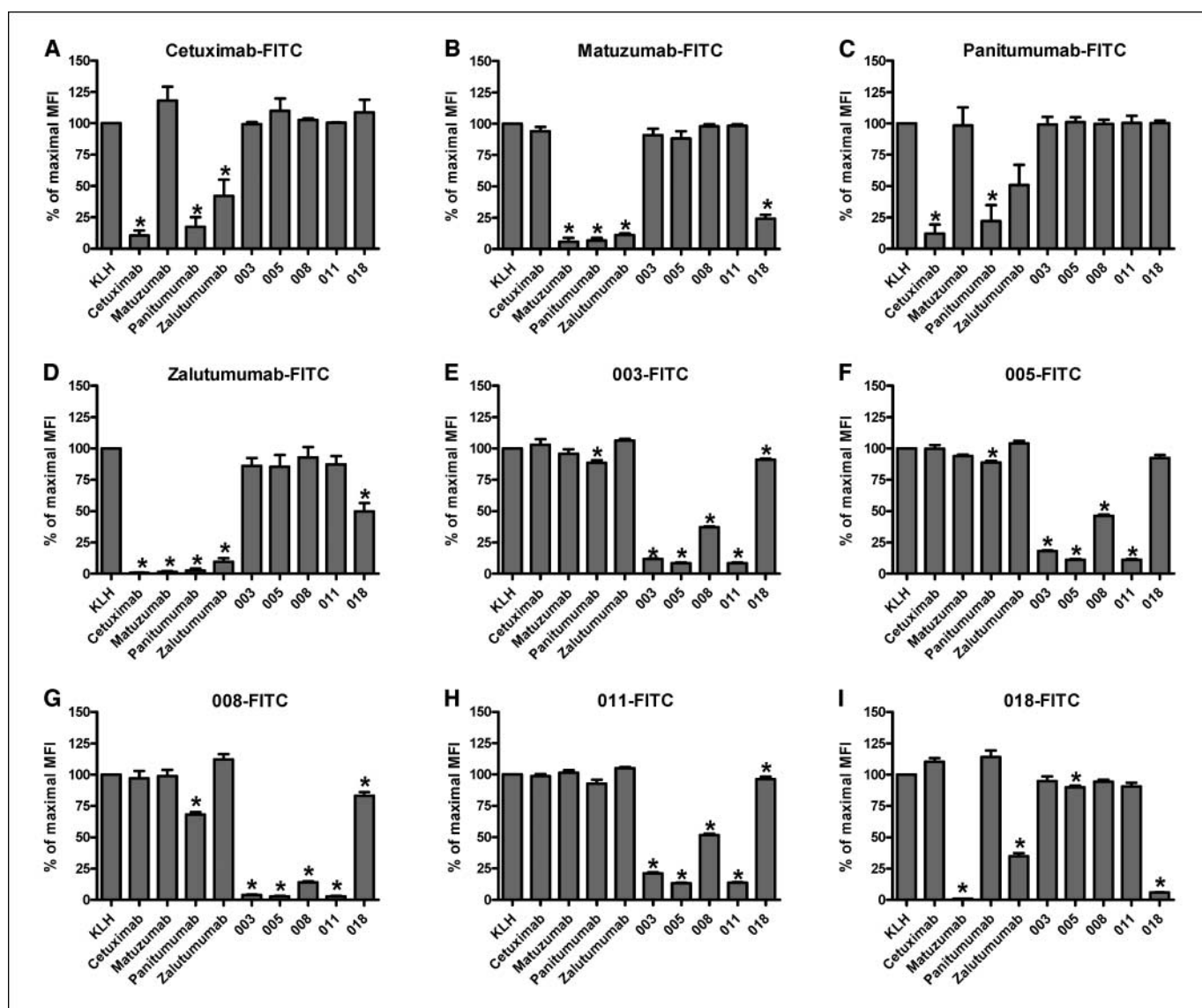

Figure 3. Crossblocking experiments. A431 cells were stained with nonsaturating concentrations of FITC-conjugated EGFR antibodies in the presence of 200-fold excess of the indicated unlabeled antibodies. Immunofluorescence in the presence of an irrelevant control antibody (anti-KLH; human IgG1) determined the maximum fluorescence. Percentage of maximal MFI was calculated as described in Materials and Methods. Columns, mean of at least three independent experiments; bars, SE. *, significant inhibition.
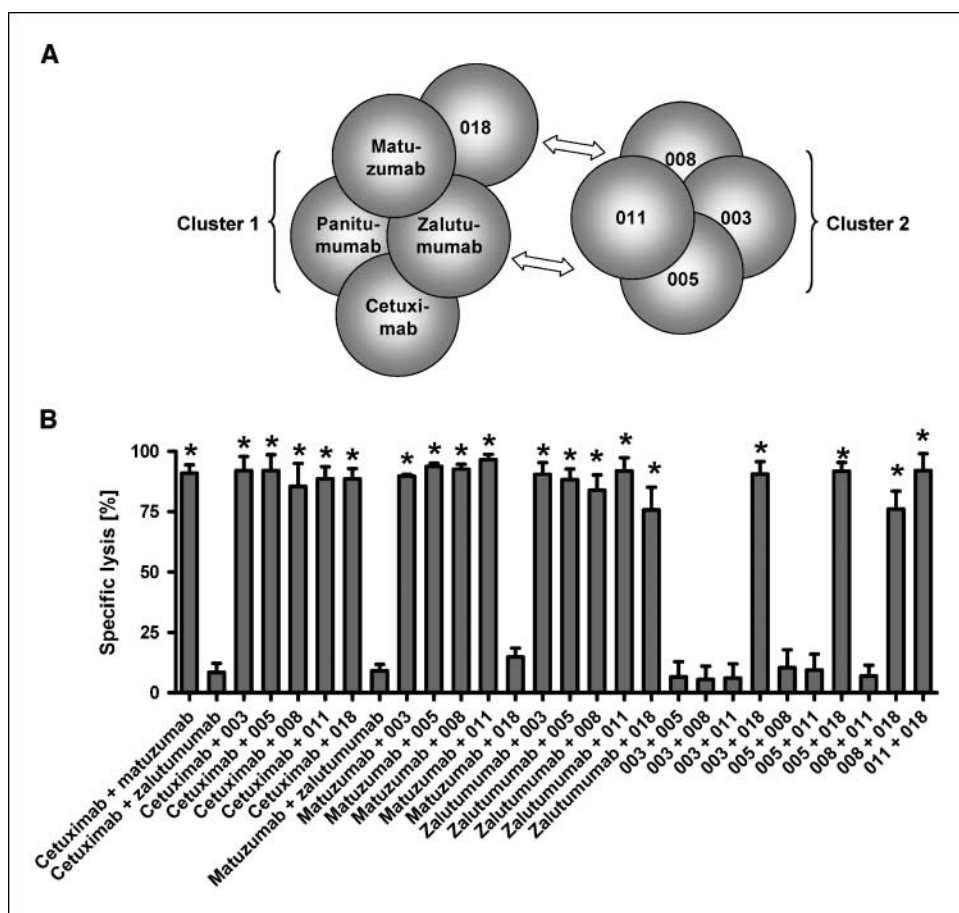
Figure 4. Epitope mapping and induction of CDC by EGFR antibodies. *A,* data from Fig. 3 are displayed schematically: significant inhibition is indicated by overlapping diagrams, whereas nonoverlapping diagrams indicate that the respective antibodies did not significantly crossblock each other. The degree of overlap represents the degree of inhibition. *B,* crossblocking and noncrossblocking combinations of EGFR antibodies were analyzed for their capacity to trigger CDC of A1207 cells. Whereas none of the crossblocking combinations triggered CDC, all noncrossblocking combinations led to significant CDC (*). *Columns,* mean of % specific lysis from at least three independent experiments; *bars,* SE.

C1q-binding. For example, K326W and E333S mutations in the Fc region of human IgG1 have been shown to increase C1q binding and CDC (20). Thus far, we observed CDC only against target cell lines with high EGFR expression levels (Supplementary Fig. S5). However, this requirement for high EGFR expression may also limit the risk of complement-mediated side effects because normal tissue expresses EGFR at much lower levels than malignant cells (21).

Considering the limited homology between human and mouse EGFR, as well as complement and complement defense systems, animal models may be difficult to interpret with respect to efficacy and toxicity of EGFR antibody combinations (15). Accordingly, preliminary studies with EGFR antibody combinations against A431 cells in severe combined immunodeficient mice were not successful (data not shown). However, individual EGFR antibodies analyzed here have been approved for human use or are currently in phase III clinical trials. Thus, phase I/II studies may be a next step to determine whether there is a therapeutic window for EGFR antibody combinations in the clinic. Today, antibody combinations have not received FDA approval but are being investigated in clinical trials (10). For example, a phase I study with a combination of 25 RhD antibodies for the treatment of idiopathic thrombocytopenia (22) has recently completed accrual. Of note, concentrations required for half maximal CDC in our experiments were significantly lower than those required for effective receptor blockade—another important mechanism of action for EGFR antibodies (4). Our study shows that the antibody isotype and cognate epitope are important attributes of EGFR antibodies,

which can be exploited to generate potent antibody combinations that synergize and induce complement-dependent tumor lysis, thereby overcoming complement defense. This insight may lead to the development of improved immunotherapeutic regimens with EGFR antibodies.

Addendum

Recently, crystallographic mapping of the matuzumab-binding epitope was described, which is distinct from the cetuximab epitope (23). These novel structural data complement our functional observation that cetuximab and matuzumab have synergistic activity.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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