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ORIGINAL ARTICLE

Impact of Human IgA Antibodies on Complement-Dependent Cytotoxicity Mediated by Combinations of EGF-R-Directed Antibodies

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Abstract Dual combinations of non-crossblocking epidermal growth factor receptor (EGF-R)-directed monoclonal antibodies were demonstrated to effectively induce complement-dependent cytotoxicity (CDC) of tumor cells, whereas individual antibodies were ineffective. Here the modulating effects of different antibody isotypes on CDC were studied by adding them as a third antibody. Two different combinations of non-crossblocking EGF-R antibodies of human IgG1 isotype, 018/003 and 425/005, were investigated against the A431 and A1207 cell lines. As a third antibody, human IgG1, IgA1, and IgA2 isotype variants of the therapeutic EGF-R antibody 225 were employed that bind to an EGF-R epitope distinct from the other EGF-R antibodies. In this model, the human IgG1 antibody proved to further enhance CDC, whereas both IgA antibodies significantly blocked CDC. The IgG1 and IgA variants increased target opsonization at similar levels, but the isotypes differed in their effects on C1q fixation. Addition of IgG1 significantly enhanced complement factor binding on the target surface, whereas both IgA antibodies reduced complement binding. Control experiments revealed this blocking effect to be not specific to IgA antibodies, but to antibody constructs incapable of activating the complement system. Interestingly, the effects caused by the IgA2 isotype were consistently stronger than those by IgA1, which may be caused by stronger steric hindrance due to its reduced hinge flexibility. These results demonstrate that monoclonal IgA antibodies inhibit IgG-mediated complement activation in vitro and suggest that the appearance of IgA antibodies within a polyclonal immune response might inhibit complement activation in vivo.

Keywords IgA · Complement · CDC

Abbreviations

CDC	Complement-dependent cell lysis
EGF-R	Epidermal growth factor-receptor
Mabs	Monoclonal antibodies
ADCC	Antibody-dependent cellular cytotoxicity
MAC	Membrane attack complex

Introduction

Antibody-based immunotherapy has been implemented as part of standard therapy for an increasing number of patients (Reichert and Valge-Archer 2007). Today, most of the approved tumor-directed antibodies are of human IgG1 isotype as it effectively activates human complement for complement-dependent cytotoxicity (CDC) and recruits NK cells for antibody-dependent cellular cytotoxicity (ADCC). In recent years, IgA antibodies have attracted increasing attention as potential therapeutics for infectious and malignant diseases. IgA antibodies proved to effectively trigger respiratory burst and phagocytosis of various microbial pathogens (Monteiro and van de Winkel 2003; van Egmond et al. 2001; Wines and

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Hogarth 2006). Additionally, IgA antibodies were demonstrated to be exceptionally effective in recruiting polymorphonuclear cells (PMNs) for ADCC against tumor cells (Dechant et al. 2002, 2007; Huls et al. 1999; Otten et al. 2005). A recent study revealed IgA-induced crosslinking of FcαRI to trigger leukotriene B4 release and subsequent PMN migration in vitro and in vivo (van der Steen et al. 2009).

IgA is the most abundantly produced isotype, shares the common H_2L_2 heavy and light chain structure with other antibody isotypes, and is heavily glycosylated (Woof and Kerr 2006). In addition to its monomeric form, IgA antibodies can form dimers via controlled association with the plasma cell-produced joining (J) chain. The complex consisting of two IgA antibodies, J chain and the extracellular part of the poly-Ig receptor, the secretory component, is called secretory IgA (Mostov 1994). Two subclasses of IgA have been identified in humans, termed IgA1 and IgA2. They differ mainly in the length of their hinge regions, with IgA2 lacking a proline-rich sequence of 13 amino acids. Furthermore, there are two allotypic variants of IgA2, designated IgA2m(1) and IgA2m(2), differing in their heavy and light chain linkage (Corthesy 2002; Woof and Kerr 2006). In contrast to the Y-shaped IgG antibodies, both IgA1 and IgA2 were shown to form more compact T-shaped structures (Boehm et al. 1999; Furtado et al. 2004; Perkins and Bonner 2008). Notably, solution structure models suggested that IgA2m(1) is even tighter and less flexible than IgA1 due to its shorter hinge region (Furtado et al. 2004).

The contribution of complement to antibody efficacy in vivo has long been discussed [reviewed in (Maloney et al. 2002; Taylor 2004)]. In hematologic malignancies, effective antibody-mediated complement activation has been observed, for example by the human IgG1 antibodies rituximab (Di Gaetano et al. 2003) and alemtuzumab (Campath) (Alinari et al. 2007), whereas in solid tumors, complement killing has rarely been demonstrated. Evidence related to the role of IgA antibodies in the activation of the complement system is conflicting. Today, three different complement pathways have been identified (Walport 2001). The "classical" pathway is triggered by C1q binding to complexed IgG. Except for two reports (Burritt et al. 1977; Jarvis and Griffiss 1991), the classical pathway is consistently regarded to be not activated by IgA antibodies (Dechant and Valerius 2001; Kerr 1990; Woof and Kerr 2006). The "alternative" pathway is evolutionarily older and is started by direct cleavage of C3, and does not require antibodies. However, IgA antibodies were demonstrated to trigger the alternative pathway, at least under certain conditions (Hiemstra et al. 1987, 1988; Nikolova et al. 1994). The third pathway, the "mannan-binding lectin" (MBL)-pathway, is initiated by the binding of endogenous "mannan-binding protein" to certain sugar residues on pathogens' surfaces. This pathway was demonstrated to be activated by IgA antibodies (Roos et al. 2001) and to be involved in the pathophysiology of IgA nephropathy, the most common glomerulonephritis (Oortwijn et al. 2008). Complement activation through any of the three pathways may ultimately lead to the formation of the "membrane attack complex" (MAC), triggering lytic killing of bacteria and eukaryotic cells. Furthermore, complement components such as C5a and C3a are potent chemoattractants for immune effector cells, while other complement proteins, such as C3b and C3d, effectively enhance antigen presentation (Fearon 1998). On the other hand, complement activation is tightly regulated, for example by complement regulatory proteins such as CD46, CD55, and CD59 (Gorter and Meri 1999). In this context, IgA antibodies were also demonstrated to have modulating, complement-downregulating effects under certain circumstances (Chintalacharuvu and Morrison 1999; Jarvis and Griffiss 1991; Russell et al. 1989). Thus IgA's role in complement activation is unclear.

Among the most intensively targeted tumor-related antigens is the epidermal growth factor receptor (EGF-R) (Mendelsohn 2002). Today, two human monoclonal antibodies (mAbs) have obtained regulatory approval, cetuximab (225-IgG1) and panitumumab (E7.6.3). Additional EGF-R antibodies, such as zalutumumab (2F8), nimotuzumab (hR3), and matuzumab (425), are or have been investigated in clinical trials. Importantly, none of these antibodies was capable of triggering CDC individually. In contrast, certain dual combinations of human IgG1 mAbs recognizing non-overlapping epitopes of EGF-R were demonstrated to efficiently activate the classical pathway for CDC of tumor cells (Dechant et al. 2008). Antibody combinations have not yet received FDA approval, but are being investigated in clinical trials (Logtenberg 2007). Interestingly, compositions comprising at least three distinct EGF-R antibodies are also being tested (Pedersen et al. 2008).

The aim of this study was to investigate whether different isotypes of a third antibody, recognizing another non-overlapping epitope of EGF-R, would differently influence CDC induced by dual-antibody combinations. Interestingly, we observed that the human IgG1 isotype significantly increased CDC, whereas both IgA isotypes inhibited complement to different degrees: IgA2 more strongly than IgA1. Control experiments, however, showed that this blocking effect was not specific to IgA antibodies, but to antibody constructs that cannot activate the complement system. Together, these results demonstrate that our human IgA antibodies block CDC triggered by combinations of IgG1 antibodies.

Materials and Methods

The experiments reported here were approved by the Ethics Committee of the Christian-Albrechts-University, Kiel, Germany, in accordance with the Declaration of Helsinki.

Antibodies

The EGF-R-directed antibodies 225-IgG1 (cetuximab, chimeric IgG1) and 425 (matuzumab, humanized IgG1) were from Merck (Darmstadt, Germany) and antibodies 003 (LC1006-003), 005 (LC1006-005), and 018 (LC1006-018), all fully human IgG1, were from Genmab (Utrecht, The Netherlands). m225 (murine IgG1), parental to cetuximab, was purified from the original hybridoma (HB-8508; ATCC, Manassas, VA). Monomeric 225-IgA1 and 225-IgA2, both directed against EGF-R with variable regions of hybridoma m225, were recombinantly produced and purified as described in (Beyer et al. 2009). An EGF-R-specific scFv fragment in VL–VH orientation was generated by standard procedures (Krebber et al. 1997; Peipp et al. 2002) using the V regions that were isolated for the generation of EGF-R-specific IgA antibodies (Dechant et al. 2007; Lazar et al. 2006). The scFv was fused to wild-type IgG1-Fc and an engineered IgG1-Fc domain lacking C1q binding [(S239D/A330L/I332E, described in (Lazar et al. 2006)], resulting in the expression vectors pSEC-Tag-2-Hygro-225-Fc and pSEC-Tag2-Hygro-225-Fc(−/−). For mammalian expression, CHO-K1 cells were stably transfected with the Amaxa Nucleofection System (Lonza, Cologne, Germany) using transfection kit V according to the manufacturer's instructions. After 48 h, the medium was exchanged for culture medium containing 500 µg/ml hygromycin B. After 2 weeks of selective pressure, single-cell subclones were established by limiting dilution. High-producing cell clones were selected and supernatants were collected and stored at 4°C until use.

Recombinant proteins were purified by two-step affinity chromatography. In the first step the protein was captured on HiTrap protein A columns according to the manufacturer's conditions (GE Healthcare, Freiburg, Germany) using an Äkta purifier system. The elution fractions were pooled and applied to IMAC affinity chromatography using HisTrap HP columns (GE Healthcare) according to the manufacturer's instructions. The final elution peaks were concentrated and analyzed by SDS-PAGE and Western blot analysis. Capillary electrophoresis on an Experion System (Bio-Rad, Munich, Germany) was used to determine the concentration and purity of the recombinant proteins.

The human chimeric CD20 antibody rituximab was obtained from Hoffmann-La Roche (Basel, Switzerland). KLH (HuMab-KLH) (human IgG1, Genmab) and human myeloma IgA1 (control IgA1) and IgA2 (control IgA2) (both from Biodesign International, Saco, ME) served as isotype control antibodies. For direct immunofluorescence, the EGF-R antibodies were FITC-conjugated using the EZ-label kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Cell Lines

The human cell lines A431 (human epidermoid carcinoma) and Raji (Burkitt's lymphoma) (both from DSMZ, Braunschweig, Germany) were kept in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) and the human glioblastoma cell line A1207 (originally established by Dr. Aaronson, National Cancer Institute, NIH, Bethesda, MD, USA) in DMEM (Invitrogen). All media were supplemented with 10% fetal calf serum (Invitrogen) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (both from PAA, Pasching, Austria).

Complement-Dependent Cytotoxicity Assay

CDC assays were performed as described earlier (Dechant et al. 2008). Briefly, targets cells were labeled with 100 µCi (3.7 MBq) ^{51}Cr for 2 h. After washing three time, the cells were adjusted to 1×10^5 per ml. Fifty microlitre of freshly drawn human serum, sensitizing antibodies, and RPMI 1640 (10% FCS) were added to round-bottom microtiter plates (Nunc, Rochester, NY). The assays were started by adding 50 µl of tumor cells, resulting in a final volume of 200 µl/well and a final concentration of 25% serum. After 3 h of incubation at 37°C, the plates were centrifuged and 25 µl of supernatants was added to 150 µl of scintillator mixture (OptiPhase Scintillator Supermix, PerkinElmer, Boston, MA, USA) in 96-well sample plates (Wallac, Turku, Finland). Subsequently, the sample plates were sealed with a microplate Press-on adhesive sealing film (TopSeal-A, PerkinElmer) and incubated for 15 min on an orbital shaker. ^{51}Cr release was measured in triplicates of scintillation (sci) in a scintillation counter (MetaBase TriLux, PerkinElmer). The percent cytotoxicity was calculated with the formula:

$$\% \text{ Specific lysis} = (\text{experimental sci} - \text{basal sci})/ \times (\text{maximal sci} - \text{basal sci}) \times 100,$$

with maximal ^{51}Cr release determined by adding Triton X-100 (1% final concentration) to the target cells and basal release measured in the absence of sensitizing antibodies and serum. Antibody-independent cytotoxicity (serum without target antibodies) was not observed. Percent inhibition/stimulation of complement-dependent lysis was calculated by the formula:

$$(100 - ((100\% \times \%\ \text{specific lysis triple combination})/ \%\ \text{specific lysis double combination}))) \times (-1).$$

In select experiments, serum was incubated for 30 min at 56°C to completely inactivate the complement system.

Target Cell Sensitization

Target cell sensitization was determined by indirect immunofluorescence. Thus 1×10^5 target cells were incubated with EGF-R antibodies individually or in dual or triple combinations, each resulting in a final additive concentration of 10 μg/ml. After incubation for 1 h on ice, the cells were washed twice with PBS supplemented with 0.5% BSA and 0.01% sodium azide (both from Sigma-Aldrich, St. Louis, MO, USA). The cells were then stained with FITC-conjugated polyclonal goat anti-human kappa light chain antibody (Dako, Glostrup, Denmark) and incubated for another hour. After washing again, the cells were analyzed on a flow cytometer (Coulter EPICS XL-MCL). Relative immunofluorescence intensity (RFI) was calculated as the quotient of the experimental mean fluorescence intensity (MFI) and the MFI of the isotype control antibody KLH.

Crossblocking Experiments

Binding epitopes of EGF-R antibodies were analyzed by competitive immunofluorescence assays. 2×10^5 EGF-R-expressing target cells were incubated with the respective FITC-conjugated EGF-R antibodies at non-saturating concentrations in combination with a 200-fold excess of different unconjugated antibodies. After washing, the cells were analyzed by flow cytometry. The level of competition was calculated by the formula:

$$\%\,\text{Competition} = (\text{experimental MFI} - \text{background MFI})/(\text{maximal MFI} - \text{background MFI}) \times 100,$$

with the maximal MFI defined by the combination of FITC-conjugated EGF-R antibody with the isotype control antibody KLH.

Complement Deposition

Deposition of the complement protein C1q was analyzed by indirect immunofluorescence. 1×10^5 cells were incubated for 15 min at room temperature either with individual EGF-R antibodies or with antibody combinations at additive antibody concentrations of 10 μg/ml. After adding 1% (vol/vol) human serum, the cells were incubated for 10 min at 37°C and then put on ice. After washing three time, the cells were incubated for 1 h with FITC-conjugated C1q antibody (Dako). Stained cells were analyzed by flow cytometry. RFI was calculated as the experimental MFI of samples incubated with the respective EGF-R antibodies divided by the MFI of the control sample incubated with the irrelevant antibody KLH.

Data Calculation and Statistical Analyses

Data are displayed graphically and analyzed statistically using GraphPad Prism 4.0. Group data are reported as mean values ± SEM of at least three different experiments. Differences between groups were analyzed by the paired Student's *t* test and significance was accepted when p values were <0.05.

Results

It has been demonstrated that EGF-R antibodies of human IgG1 isotype do not activate complement as single agents, but combinations of two antibodies recognizing distinct non-overlapping EGF-R epitopes triggered CDC effectively (Dechant et al. 2008). Furthermore, these two antibodies were required to be of human IgG1 isotype. Here we investigated the role of human IgA antibodies in complement activation. For this purpose we used recombinantly generated monomeric antibodies of IgA1 and IgA2 isotype with variable regions identical to the clinically approved cetuximab (225-IgG1), as described in (Beyer et al. 2009). The IgA1 and IgA2 versions did not mediate CDC of A431 cells, neither individually nor in dual combination with human IgG1 antibody 425. In contrast, the human IgG1 225-IgG1 proved to be effective in the same combination ($57.9 \pm 2.8\%$, $n = 3$; Fig. 1), as expected.

Next we investigated the monomeric IgA1 and IgA2 antibodies in triple combinations. First we verified in immunofluorescence-based experiments that 225-IgA antibodies' crossblocking properties are identical to 225-IgG1, as expected (Fig. 2). In addition, these experiments revealed that antibodies 003, 005, 018, 425, and 225-IgG1 and -IgA all recognized distinct non-overlapping epitopes of EGF-R. Thus two different dual combinations of human IgG1 antibodies not crossblocking each other or the 225 binding epitope were identified: 018/003 and 425/005 (Fig. 2). Both combinations were further used to study the effect of supplementing the 225-derived antibody constructs 225-IgG1, 225-IgA1, and 225-IgA2, which only differ in their isotype. Therefore, ^{51}Cr-release CDC assays were performed with combinations 018/003 and 425/005, both at an additive antibody concentration of 10 μg/ml. As

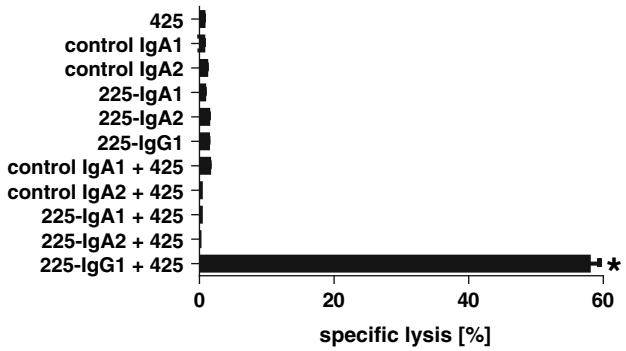

Fig. 1 IgA antibodies did not mediate complement-dependent killing. The CDC of A1207 glioblastoma tumor cells was analyzed in 3-h chromium release assays in the presence of individual EGF-R antibodies or in the presence of antibody combinations (final antibody concentration: 10 μg/ml). Individual EGF-R antibodies did not trigger CDC. The combination of 425 (human IgG1) with the human IgG1 version of 225 (225-IgG1) led to significant complement-mediated killing (significance indicated by an *asterisk*), whereas combinations of 425 with 225-IgA1 or -IgA2 were not effective. Data are presented as the mean ± SEM of three independent experiments

targets we used A431 and A1207 tumor cell lines, which are similar in their surface expression of EGF-R, but differ in complement-regulating proteins such as CD46, CD55, and CD59 (A431 > A1207) (Dechant et al. 2008). At saturating doses, specific lysis of A431 mediated by 018/003 proved to be 24.6 ± 10.7% and by 425/005 45.9 ± 4.9%. Lysis of A1207 was 43.2 ± 8.7 and 51.0 ± 4.5%, mediated by 018/003 and 425/005, respectively ($n = 4$ each, data not shown). The third antibody was titrated in with increasing concentrations and the percentage of stimulation/inhibition was calculated (Fig. 3). Interestingly, the addition of 225-IgG1 proved to stimulate CDC dose dependently, whereas the addition of the respective EGF-R-specific IgA antibodies resulted in significant blockade of CDC. The effects proved to be more pronounced with cell line A431 (Fig. 3a, b) than with A1207 (Fig. 3c, d). Moreover, the IgA2 construct was found to be significantly more effective than its IgA1 counterpart. The isotype control antibody KLH and the control IgA1 and IgA2 did not influence CDC.

To verify that the observed lysis was complement dependent, we heat-inactivated serum for 30 min at 56°C, which completely abrogated killing (Fig. 4a). To test whether the observed effects were EGF-R specific, we additionally studied the CDC of the CD20-positive cell line Raji mediated via the CD20-directed antibody rituximab, which is known to be effective in mediating CDC as a single agent. In this system, the addition of 225-IgG1 and 225-IgA1 or 225-IgA2 did not influence CDC (Fig. 4b).

To investigate whether the observed blockade was IgA specific, we additionally examined the influence of the parental murine IgG1 antibody 225, which does not

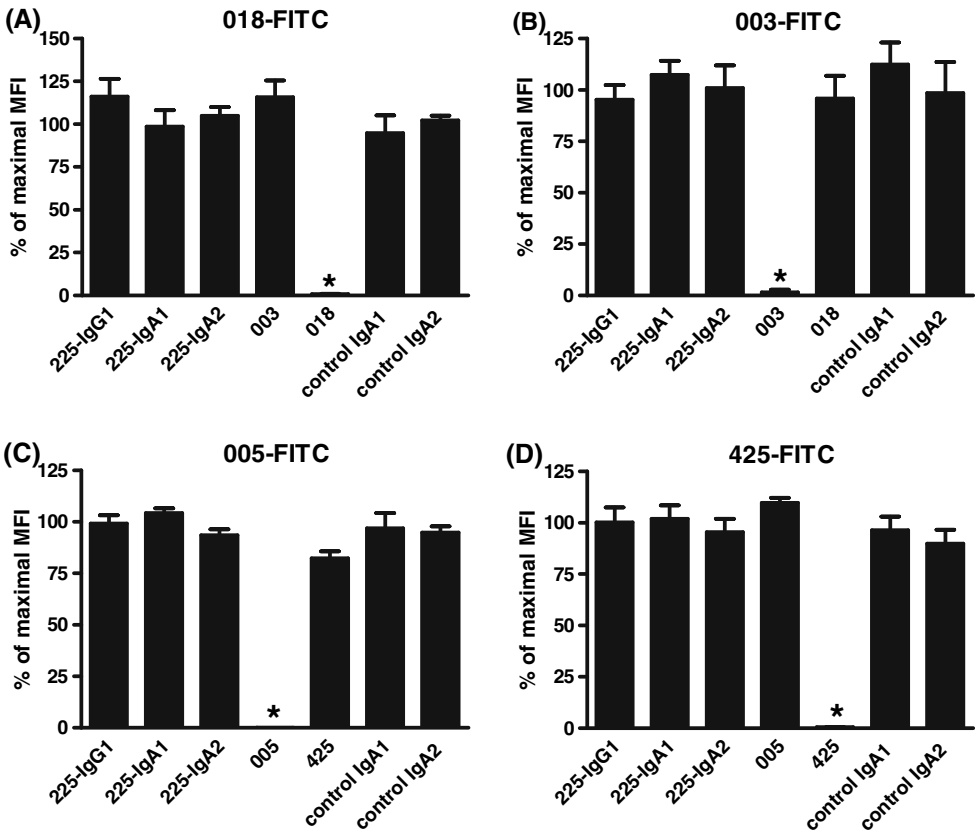

Fig. 2 Epitope mapping by crossblocking experiments. A1207 cells were stained with non-saturating concentrations of FITC-conjugated EGF-R antibodies in the presence of a 200-fold excess of the indicated unlabeled antibodies. Immunofluorescence in the presence of an irrelevant control antibody determined the maximum fluorescence. "% of maximal MFI" was calculated as described in "Materials and Methods". Data are presented as the mean ± SEM of three independent experiments; an *asterisk* indicates significant inhibition. Results presented here demonstrated that antibodies 018 (**a**), 003 (**b**), 005 (**c**) and 425 (**d**) bind to epitopes different from 225-IgG1 and its IgA counterparts. In addition, the combinations 018/003 and 425/005 proved to be non-crossblocking. As controls, nonbinding IgA1 and IgA2 antibodies were used

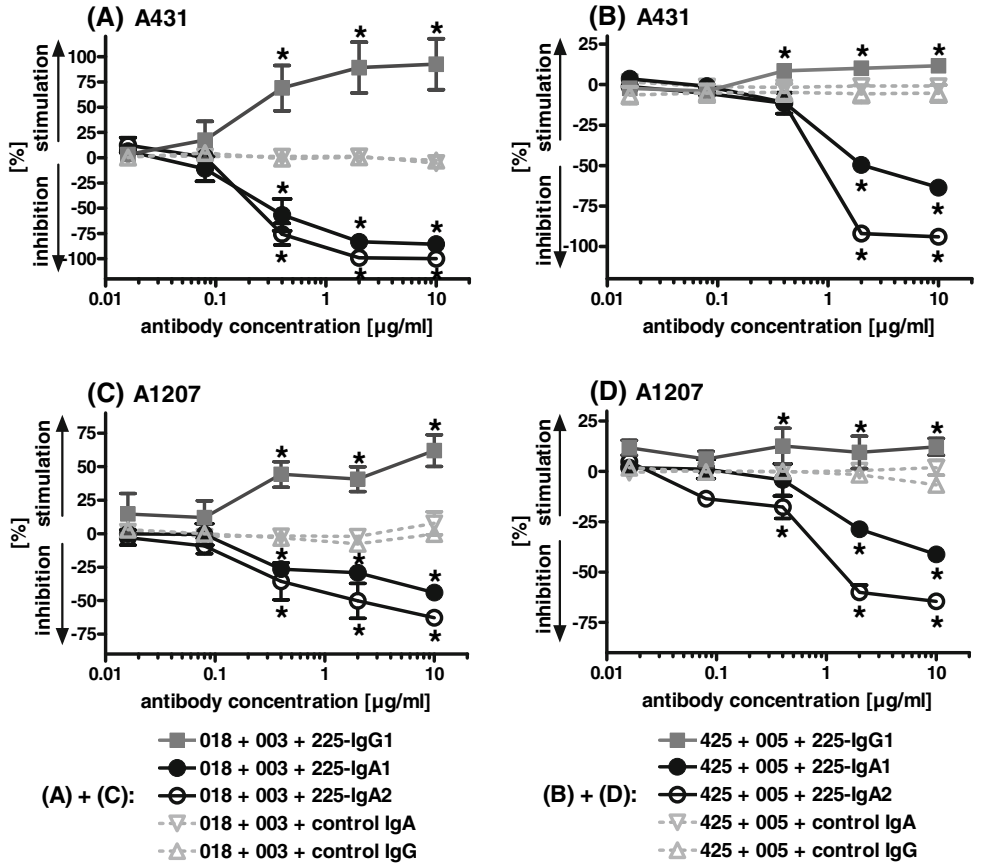

Fig. 3 Effect of adding a third antibody to dual combinations of EGF-R mAbs on CDC. Complement-dependent killing of A431 (**a**, **b**) or A1207 (**c**, **d**) target cells was investigated in 3-h chromium release assays. In **a** and **c**, specific lysis induced by the combination of 018 and 003 is displayed, in **b** and **d** the corresponding lysis by 425 and 005, both at the fixed concentration of 10 µg/ml. A third non-crossblocking antibody was titrated into the respective combination as indicated: 225-IgG1 (*closed squares*, *dark grey line*), 225-IgA1 (*closed circles*, *black line*), 225-IgA2 (*open circles*, *black line*), control IgG (human IgG1 KLH; *closed upward triangles*, *grey dotted line*), and control IgA (unspecific human IgA; *closed downward triangles*, *grey dotted line*). "% inhibition/stimulation" was calculated as described in "Materials and Methods". Data are reported as the mean ± SEM of four independent experiments; an *asterisk* indicates significant inhibition or stimulation, respectively. Against both cell lines, CDC was significantly stimulated by the human IgG1 version of 225, but inhibited by its human IgA counterparts, whereas control IgG and IgA did not influence CDC

activate human complement, and a scFv-Fc fusion construct with a protein-engineered Fc incapable of binding C1q [225-Fc(−/−)] (Lazar et al. 2006). As a control we also tested an analogous 225 scFv-Fc fusion construct with an intact C1q binding site (225-Fc). Importantly, these experiments revealed the blockade of CDC to be not IgA specific, but common to other constructs incapable of activating the complement system themselves (Fig. 4c, d). Interestingly, the degree of the observed effect differed among these constructs: murine IgG1 m225 and 225-Fc neither stimulated nor blocked, 225-IgA1 blocked approximately 50%, and both 225-Fc(−/−) and 225-IgA2 blocked CDC almost completely.

To identify the underlying molecular mechanisms, we next compared target opsonization by indirect immunofluorescence. Addition of 225-IgG1, 225-IgA1, and -IgA2 to both antibody combinations increased target opsonization similarly (Fig. 5a, b). Subsequently, we analyzed complement deposition on target cells by direct immunofluorescence. Addition of 225-IgG1 to both 018/003 and 425/005 combinations significantly increased C1q deposition on A431 targets, whereas addition of both 225 IgA monomers significantly, but only to a minor degree, decreased C1q deposition (Fig. 5c, d).

Discussion

In this study we investigated the influence of EGF-R-directed human IgA antibodies on CDC. IgA antibodies' role in complement activation is still unclear. Whereas it is widely accepted that IgA antibodies do not activate the classical pathway, some groups reported that IgA antibodies trigger the alternative pathway, at least under

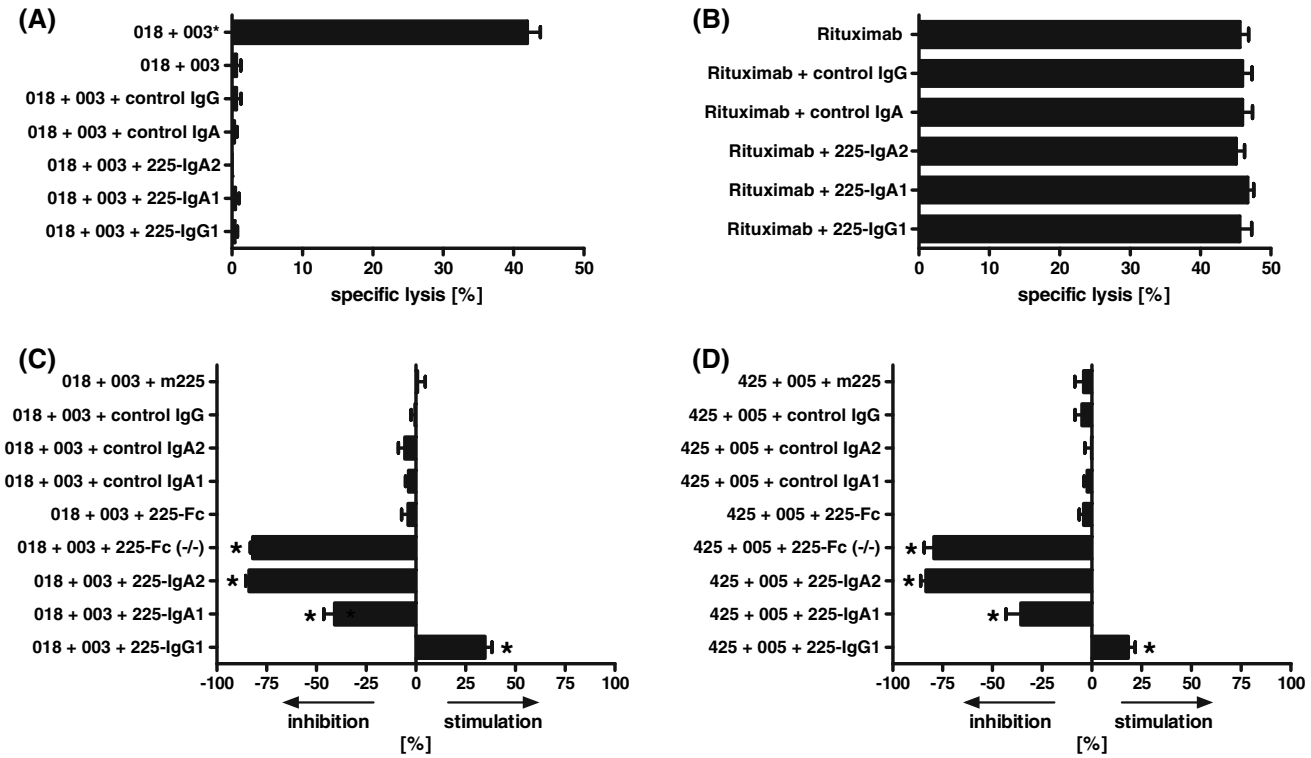

Fig. 4 Killing is complement mediated and antigen specific, but the inhibitory effect of IgA antibodies is not specific to IgA. **a** Heat inactivation of human serum (56°C for 30 min) proved that the killing by EGF-R antibody combinations is complement dependent, since only not heat-inactivated serum (indicated with 018 + 003*) induced CDC. **b** Next, the impact of EGF-R antibodies was investigated in rituximab-triggered CDC assays against EGF-R-negative CD20-expressing Raji cells. In these assays, addition of EGF-R-specific or control antibodies had no effect. Data in **a** and **b** are presented as the mean ± SEM of three independent experiments. To test if CDC stimulated by the dual IgG combinations of antibodies 018/003 **c** and 425/005 **d** was specifically inhibited by the EGF-R-directed IgA antibodies, we investigated the effects of parental murine IgG1 antibody 225 (m225), a 225 scFv-Fc construct with a mutated IgG1-Fc domain unable to trigger CDC [225-Fc(−/−)] and a control 225 scFv-Fc construct with an intact C1q binding site (225-Fc). Results from these experiments revealed the complement-inhibitory effects to be not specific to the IgA isotype, because also the mutated IgG1-Fc inhibited CDC. Data in **c** and **d** are presented as the mean ± SEM of three independent experiments; an *asterisk* indicates significant inhibition or stimulation, respectively

certain conditions (Hiemstra et al. 1987, 1988; Nikolova et al. 1994). Additionally, at least one group demonstrated that the MBL-pathway is activated by IgA antibodies (Roos et al. 2001). In contrast, other groups reported a specific modulating, complement downregulating effect of IgA antibodies under certain circumstances (Chintalacharuvu and Morrison 1999; Jarvis and Griffiss 1991; Russell et al. 1989). In these experiments, IgA antibodies were required to bind to the target cells, but epitope crossblocking could be excluded as a simple reason. Furthermore, it was demonstrated that these effects were restricted to IgA's $F(ab)_2$ fragments, as cleavage of the Fc parts did not abrogate blockade (Jarvis and Griffiss 1991; Russell et al. 1989). The aim of our study was to further clarify IgA antibodies' properties with respect to the complement system.

As a model system we examined CDC of tumor cells via EGF-R-directed antibodies, which, as single agents, were known not to activate the complement system efficiently enough to mediate CDC. Dual combinations of human IgG1 EGF-R antibodies binding to non-overlapping EGF-R epitopes, however, proved to be effective in triggering CDC (Dechant et al. 2008). We chose two different dual combinations of human IgG1 EGF-R antibodies which were both capable of mediating CDC of human A431 and A1207 tumor cells and which both bind to epitopes distinct from and non-overlapping with that of prototypic EGF-R antibody 225. In this model we were able to specifically test the influence of different derivates of 225 with identical variable regions differing only in their Fc parts and complement binding properties: human IgG1 225-IgG1, human IgA counterparts 225-IgA1 and 225-IgA2, a 225 scFv-Fc fusion with eliminated C1q binding site, a control fusion protein with intact C1q binding site, and the parental 225 antibody of murine IgG1 isotype.

First we demonstrated that IgA antibodies do not mediate CDC of tumor cells, neither as single agents nor in any dual or triple combination. Accordingly, none of the

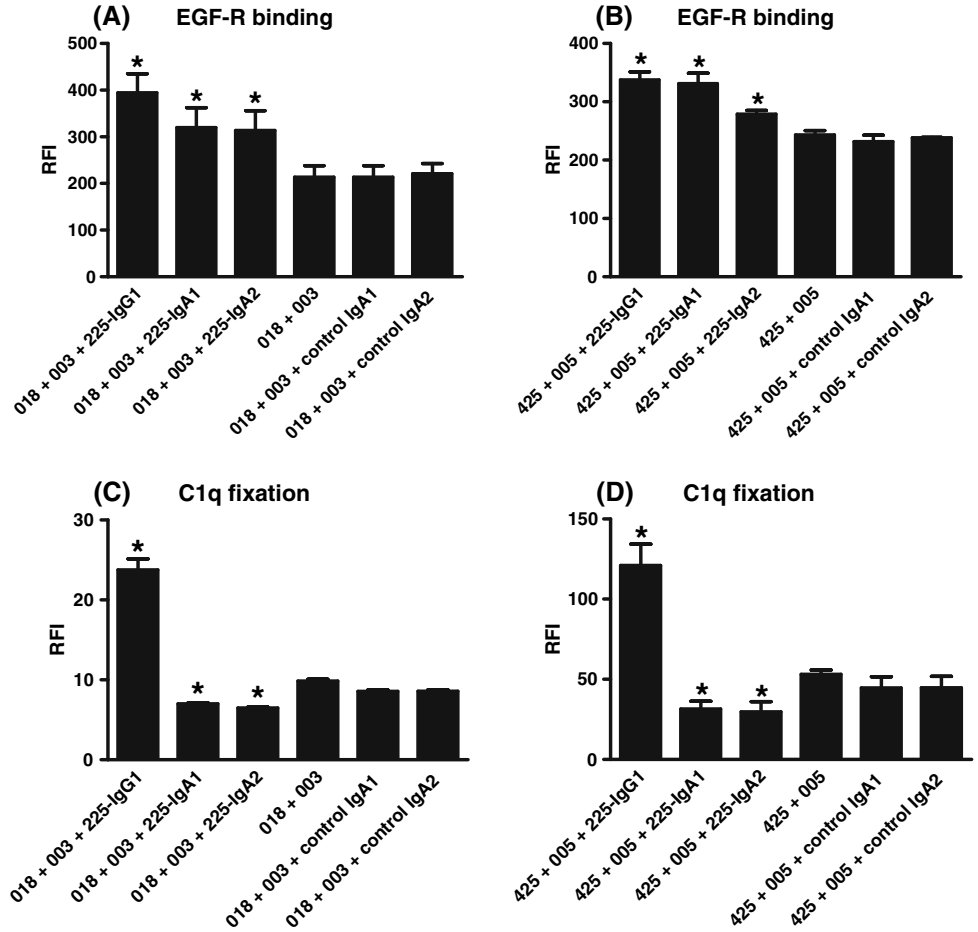

Fig. 5 225-IgG1 and -IgA antibodies enhanced sensitization, but IgA1 and IgA2 inhibited C1q deposition. The capacity of the indicated third antibodies to enhance sensitization of A431 cells by the EGF-R antibody combinations 018/003 **a** and 425/005 **b** was analyzed by indirect immunofluorescence using FITC-labeled anti-human kappa light chain antibody. Individual antibodies or their combinations were used at a 10 μg/ml final concentration. Addition of human IgG1 mAb 225-IgG1 as well as both IgA counterparts led to a significant enhancement of sensitization. The effect of adding the indicated third antibodies to the antibody combinations 018/003 **c** and 425/005 **d** on C1q deposition on A431 cells was analyzed by indirect immunofluorescence using a FITC-labeled C1q-specific antibody. In both combinations, 225-IgG1 significantly enhanced, while its IgA1 or IgA2 counterparts significantly reduced C1q deposition. RFI was calculated as described in "Materials and Methods". Data are presented as the mean RFI ± SEM of three (**b**), four (**a**, **c**), or five (**d**) independent experiments. An *asterisk* indicates significant differences in experimental RFI compared with the respective dual combinations (018/003 in **a** and **c** or 425/005 in **b** and **d**)

three complement pathways appeared to be efficiently activated by IgA antibodies. Furthermore, IgA antibodies were revealed to block complement killing mediated by the other employed IgG1 antibodies, in agreement with former reports (Chintalacharuvu and Morrison 1999; Jarvis and Griffiss 1991; Russell et al. 1989). The antigen specificity of the observed blockade was verified in a CD20 lymphoma control system. However, to prove that these effects were indeed IgA specific, we also tested other 225-derived constructs. The addition of human IgG1 225-IgG1, capable of binding C1q, even stimulated CDC, whereas the murine IgG1 parental antibody 225 and the control 225 scFv-Fc fusion protein neither stimulated nor blocked CDC. In contrast, the protein-engineered 225 scFv-Fc fusion protein 225-Fc(−/−), which is not able to fix C1q and thus does not activate complement via the classical pathway, proved to block CDC comparably to IgA. Interestingly, the human IgG1 and IgA versions of 225 were similarly effective in enhancing target sensitization, excluding different target-antigen binding as an explanation for the observed isotype differences. In contrast, the human IgG1 and IgA's differed in cell surface C1q fixation: IgG1 significantly enhanced and the IgA variants reduced fixation of complement factors.

In conclusion, our IgA antibodies did not activate complement efficiently enough to result in CDC, but even blocked CDC triggered by other antibodies binding to the same target antigen. In contrast to former reports

(Chintalacharuvu and Morrison 1999; Jarvis and Griffiss 1991; Russell et al. 1989), however, this blocking property of IgA appeared to be not specific to IgA. Thus blockade appears to be rather an effect of a steric interference with complement components. This interpretation of our results was supported by the observation that the degree of the observed effects differed among the constructs: murine IgG1 m225 and 225-Fc neither stimulated nor blocked, 225-IgA1 intermediately blocked, and both 225-Fc(−/−) and 225-IgA2 completely blocked CDC. In parallel, murine IgG1 is supposed to be the most flexible construct, whereas both IgA antibodies consist of a more compact T-shaped structure. Furthermore, IgA2 was demonstrated to be even more compact and rigid than IgA1 due to its shorter hinge region (Furtado et al. 2004). The divergent influence of 225-Fc and its conventional 225-IgG1 counterpart on CDC may also, in addition to affinity differences, most likely be due to the constructs' different flexibilities.

Further studies are required to determine whether these results from an experimental in vitro model with mAbs have biological relevance. For example, the appearance of IgA antibodies in a polyclonal immune response may dampen complement activation by IgG1 antibodies. Furthermore, the presence of non-complement-activating antibodies may impact graft survival, for example in xenotransplantation, where complement-mediated cytotoxicity is a major barrier to successful transplantation (Cooper 2003).

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