Effector Mechanisms of Recombinant IgA Antibodies against Epidermal Growth Factor Receptor

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IgA is the most abundantly produced Ab isotype in humans, but its potential as immunotherapeutic reagent has hardly been explored. In this study, we describe anti-tumor mechanisms of mouse/human chimeric IgA Abs against the epidermal growth factor receptor (EGF-R). EGF-R Abs of IgG isotype are currently approved for the treatment of colon or head and neck cancers. As expected, the human IgG1, IgA1, and IgA2 variants of the 225 Ab demonstrated similar binding to EGF-R. Furthermore, IgA Abs were as effective as IgG in mediating direct effector mechanisms such as blockade of EGF binding, inhibition of EGF-R phosphorylation, and induction of growth inhibition. None of the three variants induced complement-mediated lysis. Human IgG1 effectively recruited MNC for ADCC, but activated PMN only weakly, whereas both IgA isoforms proved to be effective in triggering neutrophils. Interestingly, the IgA2 isoform was significantly superior to its IgA1 counterpart in recruiting PMN as effector cells. Because neutrophils constitute the most abundant effector cell population in human blood, this enhanced neutrophil recruitment lead to increased killing of EGF-R expressing tumor cells in whole blood assays. This killing was further enhanced when blood from G-CSF-primed donors was compared with healthy donor blood. Together, these data suggest EGF-R Abs of human IgA isotype to bear promise for therapeutic use in cancer. The Journal of Immunology, 2007, 179: 2936–2943.

The epidermal growth factor receptor (EGF-R) is a tyrosine kinase receptor with critical functions in the regulation of cell proliferation, differentiation and survival. Dysregulated function or expression of EGF-R is observed in common cancers such as lung, colon, head, and neck, and also on nonepithelial malignancies such as glioblastomas - often correlating with a poor prognosis for patients. Due to the documented involvement of constitutive EGF-R signaling in tumorigenesis, EGF-R constitutes a promising molecule for targeted therapy. So far, two EGF-R directed approaches have been successfully introduced into clinical practice: small molecule tyrosine kinase inhibitors (TKI), and EGF-R-directed mAbs. Prototypic TKI inhibitors (TKI), and EGF-R-directed mAbs (5). Prototypic TKI include gefitinib and erlotinib, which inhibit EGF-R tyrosine kinase activity by blocking the ATP-binding site of the receptor. Based on data from randomized phase III clinical trials in metastatic colon cancer patients, cetuximab and panitumumab were the first EGF-R directed mAbs receiving FDA approval. Other EGF-R Abs currently investigated in phase II or III clinical trials include nimotuzumab, matuzumab, and zalutumumab. All these Abs are of human IgG isotype. In contrast to TKI which block EGF-R signaling, EGF-R Abs recruit more diverse mechanisms of action. Conceptually, these effector mechanisms can be divided into direct mechanisms - mediated by the Abs’ variable domains - and indirect mechanisms, which are triggered by their constant domains. The former, include blockade of ligand binding, receptor modulation, induction of apoptosis, and inhibition of growth and survival. Indirect mechanisms include complement-dependent tumor cell lysis (CDC), effector cell-mediated tumor killing (ADCC), tumor cell phagocytosis, and potentially Ab-mediated Ag presentation. Effector cell-mediated mechanisms typically require interactions between the constant regions of Abs and their cellular receptors. In humans, three classes of leukocyte IgG receptors are distinguished: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), which contain different isoforms and harbor functionally relevant polymorphisms. The critical role of Fc receptors for the therapeutic efficacy of rituximab (anti-CD20) and herceptin (anti-HER-2/neu) was demonstrated by studies in FcγRI-chain knock-out mice, in which the signaling machinery of activating Fc receptors was disrupted. Similar studies, however, have not been reported for EGF-R Abs, and the knowledge about their relevant mechanisms of action in vivo is even more incomplete than for other therapeutic Abs. Recently, indirect evidence from animal models suggested that both direct and indirect mechanisms contribute. Interestingly, one of the clinically-approved EGF-R Abs, panitumumab, is of human IgG2 isotype, which interacts less efficiently with human FcγRs and does not activate human complement. However, most of the tumor-directed Abs used in clinical trials are of human IgG1 isotype. This decision is based on human IgG1’s capacity to effectively activate human complement and to recruit NK cells for ADCC. However, human IgG1 interacts with all FcγRIIb isoforms, including the inhibitory FcγRIIIb molecule.

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Received for publication May 1, 2007. Accepted for publication June 25, 2007.

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and the GPI-linked Fc-RIIIb, which does not trigger ADCC. Because human neutrophils express these FcR isoforms, this may explain why human IgG1 is suboptimal in triggering ADCC via polymorphonuclear cells (PMN). However, neutrophils are the most populous cytotoxic effector cell population and may significantly contribute to Ab efficacy in vivo (10). Another argument for the selection of IgG Abs for immunotherapy is their prolonged plasma half-life, which is caused by binding to the neonatal Fc receptor, thereby, protecting IgG from lysosomal degradation (11).

Experiments with PMN effector cells demonstrated that PMN were most effectively recruited for ADCC with bispecific Abs triggering the myeloid IgA receptor (FccRII, CD89) (12, 13). FccRI is constitutively expressed on monocytes/macrophages, PMN, some types of dendritic cells and Kupfer cells, but not on noncytotoxic cells. Functionally, FccRI mediates phagocytosis, oxidative burst, cytokine release, Ag presentation, and ADCC (14). Like other Ig receptors, FccRI requires association with the immuno-receptor tyrosine-based activation motif-containing Fcγ-chain for in vivo expression and function. The Fcγ-chain appears to interact more effectively with FccRI than with FcγRs (15). Human IgA Abs bind with medium affinity to FccRII (16), and were demonstrated to effectively trigger ADCC against solid and lymphoma tumor cells (17, 18). IgA represents the most abundantly produced Ab isotype in humans, and is critically involved in the host defense at mucosal surfaces (16). Two isoforms, IgA1 and IgA2, are distinguished in humans, with IgA2 containing a shorter hinge region and an increased resistance against enzymatic degradation by bacterial proteases. A small study with locally recombined secretory IgA against Streptococcus mutans suggested efficacy in preventing bacterial colonization of the oral cavity (19). However, the potential of IgA for cancer immunotherapy has not been thoroughly investigated (20).

In this study, we analyzed potential effector mechanisms of EGF-R directed Abs, and compared human IgA1 and IgA2 isoforms with their respective IgG1 counterpart. To address this issue, we generated mouse/human chimeric EGF-R Abs of IgG or IgA isoforms, containing the cetuximab variable regions, and analyzed their potential to kill EGF-R expressing tumor cells. Both isotypes proved similarly effective in binding to EGF-R and in mediating direct killing mechanisms. However, IgA2 was significantly more effective than IgG1 in recruiting neutrophils for ADCC, which resulted in improved tumor cell killing in whole blood assays.

Materials and Methods

Experiments reported here were approved by the Ethical Committee of the University of Schleswig-Holstein (Kiel, Germany) in accordance with the Declaration of Helsinki.

Generation of chimeric Abs

Genes for the Ab variable H and L chain regions were cloned as described in (18). Briefly, mRNA was isolated from the original 225 hybridoma (HB-8508; American Type Culture Collection), and cDNA was generated by standard methods. Variable regions were amplified by PCR using two polymerase (PepMAb) and a set of V gene-specific primers. Variable regions were inserted into pUC-HAVT20 vector to incorporate the HAVT20 leader sequence. After sequencing, HAVT20-V inserts were subcloned into pNUT vectors encoding human constant regions for k-L chain, and yL-, oL-, or o2-H chains, respectively (GenBank accession nos. AF237583, AY647978, and AY647979). To achieve higher production rates, resulting constructs were subsequently cloned into the glutamine synthetase-expression system (Lonza Biologics) under the control of the human CMV major immediate early promoter.

Transfection of CHO-K1 cells

CHO-K1 cells (Lonza) were cotransfected with H and L chain GS-expression vectors using Lipofectamine 2000 (In vitrogen Life Technologies) according to the manufacturer’s instructions. Selection was initiated by cell culture in glutamine free DMEM medium supplemented with 10% heat-inactivated dialysed FBS (both from Invitrogen Life Technologies), containing 50 μM methionine sulfoximine (Sigma-Aldrich). Single-cell clones were achieved by two steps of limiting dilution. Ab concentrations in supernatants of selected clones were analyzed by ELISA and indirect flow cytometry. High producing cell clones were further cultivated and adapted to grow nonadherently in serum free CD-CHO medium (In vitrogen Life Technologies).

Purification of chimeric Abs

Both IgA isoforms were chromatographically purified on thiolphilic agarose columns (Kem-En-Tec) as described (21), the chimeric IgG1 Ab on a protein A column (GE Healthcare). Separate columns were used for each Ab to avoid contamination. Concentrations of purified Abs were determined by semiquantitative indirect immunofluorescence, and by sandwich ELISA as described in (18). Mouse/human chimeric IgG1 Ab cetuximab (Merck), or human myeloma IgA1, or IgA2 Abs (both from BioDesign) were used as standards.

Target cells

Human epidermoid carcinoma cell line A431 (DSMZ) and human renal carcinoma cell line SK-RC-7 were kept in RMPI 1640, human glioblastoma cell line A1207 in DMEM. All target cell media were supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 U/ml streptomycin (all from Invitrogen Life Technologies). Ba/F3 cells (DSMZ) were cultured in RPMI 1640 additionally supplemented with 10% supernatant of WEHI-3B cells (DSMZ) as source of murine IL-3 (mIL-3). Human EGF-R transfected Ba/F3 cells (Matthias Peipp, manuscript in preparation) were cultured under selection pressure by adding 1 μg/ml genetin (Invitrogen Life Technologies).

For generation of FccRI (CD89)-transfected, CD89 containing plasmids were stably transfected into FcR-naïve BHK-21 cells (DSMZ) together with the common Fcγ-chain. Transfectants were cultured in RMPI 1640, selection pressure was maintained by adding 500 μg/ml genetin for FccRII, and 10 μM methotrexate (Sigma-Aldrich) for the Fcγ-chain.

Immunofluorescence analyses

For indirect immunofluorescence, 1 × 10⁶ target cells/sample were incubated for 30 min with purified Abs. After washing in PBS (Invitrogen Life Technologies) supplemented with 1% BSA (Sigma-Aldrich) and 0.1% sodium azide (Merck), cells were stained with monoclonal FITC-conjugated mouse anti-human k-L chain Ab (Caltag Laboratories) or polyclonal FITC-conjugated goat anti-human IgA or anti-human IgG F(ab′)₂-fragments (both from DakoCytomation), respectively. After a 30-min incubation in the dark, samples were washed again and analyzed on a flow cytometer (Coulter EPICS XL-MCL, Beckman Coulter). Relative fluorescence intensities were calculated as the ratio of mean fluorescence intensity of relevant to irrelevant isotype-matched Abs.

Isotypes were determined with FITC-conjugated Abs specific for human IgG1 (DakoCytomation), IgA1, or IgA2 (both from Beckman Coulter).

To compare Fc-mediated binding of IgA1 and IgA2 to FccRI, FccRII-transfected BHK-21 cells (see above) or freshly isolated PMN were incubated with IgA Abs at various concentrations for 30 min, washed and stained with polyclonal FITC-conjugated goat anti-human IgA. After washing again, cells were analyzed by flow cytometry.

For comparison of chimeric Abs’ capacity to block ligand binding, 1.5 × 10⁵ A431 cells were incubated with 2.5 μg/ml FITC-conjugated EGF (Invitrogen Life Technologies) and 200 μg/ml chimeric Abs for 30 min. After washing, cells were analyzed by flow cytometry. Blockade of ligand binding was calculated by the formula: percentage of inhibition of EGF-binding = (relative fluorescence intensity (RFI) without − RFI with antibody) × 100.

All experimental steps were performed at 4°C. An Ab against keyhole limpet hemocyanin (KLH; human IgG1; provided by Gennab) served as negative control for IgG1, human myeloma IgA1 or IgA2 Abs served as negative controls for IgA1 and IgA2, respectively.

Immunoblotting

Purified Ab preparations were resolved by SDS-PAGE with gradient gels (3 - 8%), using the X Cell Sure Lock System (Invitrogen Life Technologies) under reducing and nonreducing conditions. After transfer onto PVDF-membranes (GE Healthcare), membranes were washed with PBS and blocked for one hour in TBS-buffer (150 mM Tris, 100 mM NaCl, pH 7.5) containing 3.2% BSA and 1.2% nonfat dry milk. Immunoblots were incubated for one hour with polyclonal HRP-conjugated goat anti-human
IgA (Sigma-Aldrich; diluted at 1/10,000), goat anti-human κ-L chain (Bio- zol; diluted at 1/2,000) or goat anti-human IgG Ab (Caltag Laboratories; diluted at 1/5,000), respectively. After washing three times with TBS-buffer containing 0.4% Tween, blots were developed with enhanced chemo- luminescence reaction reagent (Pierce).

For EGF-R phosphorylation analyses, EGF-R transfected Ba/F3 cells were incubated with 2 μg/ml Abs three hours before treatment with human EGF (20 μg/ml; Becton Dickinson). Cells were lysed in a buffer containing 50 mM Tris (pH 7.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% NaDOC, 1% SDS, 50 mM NaF, 1 mM Na3VO4, and protease inhibitors. Proteins (50 μg) were electrophoresed on 3 - 8% SDS-polyacrylamide gels and transferred onto PVDF-membranes. Immunodetection was performed with the ECL system. Polyclonal rabbit Abs against pEGF-R (Y1068; Invitrogen Life Technologies), or total EGF-R (Santa Cruz Biotechnology) were used at a dilution of 1/1,000, monoclonal murine Ab against actin (Sigma-Aldrich) at 1/10,000. Secondary Abs (HRP-conjugated anti-rabbit or anti- mouse IgG, both DakoCytomation) were used at dilutions of 1/5,000 or 1/2,000, respectively.

**Determination of viable cell mass**

Growth inhibition of human EGF-R transfected Ba/F3 cells (Matthias Peipp, manuscript in preparation) was analyzed using the MTT assay (Hoffmann-La Roche). Cells were washed three times in culture medium lacking mL-3, diluted in RPMI 1640 medium containing human EGF (10 ng/ml) with or without mL-3, and seeded at 20,000 cells/well in 96-well plates at a final volume of 100 μl. Cells were treated with serial dilutions of relevant Abs or control Ab against KLH in culture medium for 72 h. Afterward, cells were washed and resuspended in 100 μl of culture medium. MTT assays were then performed according to the manufacturer’s instructions. All experimental points were set-up in triplicates, and experiments performed in triplicates at least three times. MTT in the presence of KLH Ab was used as a reference (100% cell growth), and values with EGF-R blocking antibody were calculated with the following formula: percentage of inhibition = (percentage of lysis without blocking antibody)/(percentage of lysis without blocking antibody) × 100.

**Isolation of mononuclear and neutrophilic effector cells**

After informed consent, upper 80 μl of peripheral blood was drawn from randomly selected healthy volunteers. Mononuclear (MNC) and polymorphonuclear cells were isolated as described in (18). Briefly, citrate anti-coagulated blood was layered over a discontinuous gradient consisting of 70 and 63% Percoll (Biochrom), respectively. After centrifugation, neutrophils were collected from the interface between the two Percoll layers, and MNC were collected from the plasma/Percoll interface. Remaining erythrocytes were removed by ice-cold hypotonic lysis. Purity of MNC and PMN preparations determined by cytoxin analyses >95%. Viability of cells tested by trypan blue exclusion was >95%.

**Antibody-dependent cellular cytotoxicity assays**

ADCC assays were performed as described (18). Briefly, target cells were labeled with 200 μCi (7.4 MBq) 51Cr for two hours. After washing, cells were adjusted to 107/ml. Whole blood, plasma, or isolated effector cells (50 μl), sensitizing Abs, and RPMI 1640 (10% PBS) were added to round-bottom microtiter plates (Nunc). Assays were started by adding target cells (50 μl), resulting in a final volume of 200 μl/well and an effector-to-target (E:T) cell ratio of 80:1 with isolated effector cells (unless otherwise indicated). After three hours at 37°C, plates were centrifuged and 51Cr release from supernatants was measured from triplicates as cpm. Percentage of cellular cytotoxicity was calculated with the following formula: percentage of specific lysis = (experimental cpm − basal cpm)/(maximal cpm − basal cpm) × 100, with maximal 51Cr release determined by adding perchloric acid (3% final concentration) to target cells, and basal release measured in the absence of sensitizing Abs and effector cells. Low levels (<5%) of Ab-independent cytotoxicity (effectors without target Abs) were observed in whole blood assays and with MNC, but not with PMN.

To analyze the involvement of FcRI in IgA-mediated cell killing, FcRII-blocking Ab My43 (murine IgM) was added at 10 μg/ml. Percent inhibition was calculated with the following formula: percentage of inhibition = (percentage of lysis without − percentage of lysis with blocking antibody)/(percentage of lysis without blocking antibody) × 100.

**Data processing and statistical analyses**

Data are displayed graphically and analyzed statistically using GraphPad Prism 4.0. Experimental curves were fitted using a four-parameter nonlinear regression model with a sigmoidal dose response (variable slope). Group data are reported as mean ± SEM. Differences between groups were analyzed by unpaired (or, when appropriate, paired) Student’s t test. Significance was accepted when p values were <0.05.

**Results**

**Production and characterization of chimeric Abs**

Chimeric isotype switch variants of the EGF-R Ab 225 were produced in CHO-K1 cells under serum-free suspension cell culture conditions, using a glutamine synthetase gene as selection marker. Selected clones demonstrated productivity rates of ~20 pg/cell/day for IgG1, 3 pg/cell/day for IgA1, and 5 pg/cell/day for IgA2. After purification on protein A, or thiophilic agarose columns, respectively, correct isotypes of the chimeric Abs were confirmed by indirect immunofluorescence analysis. Thus, EGF-R positive A431 cells were incubated with the EGF-R isotype variants at saturating concentrations and stained with isotype-specific secondary Abs (Table I). Next, isotype variants were characterized by Western blot analyses (Fig. 1) using anti-human α- or γ-H, or κ-L chain secondary Abs, respectively. Under nonreducing conditions, IgG1 and IgA1 variants demonstrated single bands of ~150–160 kDa, corresponding to monomeric Abs. With the IgA2 variant, major bands of ~120 kDa and 50 kDa were observed, which corresponded to H and L chain homodimers, respectively. This was a well described phenomenon of the IgA1m(I) allotype, in which disulfide bonds are typically formed between the two L and the two H chains (16), but not between H and L chains, as common for other Ab iso- and allotypes. Under reducing conditions, all variants demonstrated single bands of ~50–60 kDa for H chains, and 25 kDa for L chains, respectively. Under both reducing and nonreducing conditions, the H chains of the recombinant IgA1 and IgA2 Abs ran slower than the controls, most likely due to different glycosylation patterns. In conclusion, results from these experiments confirmed the expected Ab characteristics.

**Binding characteristics of chimeric Abs**

Ag specificity of the Ab panel was confirmed by indirect immunofluorescence on EGF-R transfected Ba/F3 cells. Using FITC-conjugated κ-L chain secondary Ab, all three variants demonstrated similar binding to EGF-R transfected cells at saturating concentrations (Fig. 2A, whereas no staining was observed on untransfected Ba/F3 cells (n = 3; data not shown). Next, we examined binding patterns of the Ab panel at variable concentrations using secondary anti-human κ-L chain Ab for staining of A431 cells (Fig. 2B). The clinically approved chimeric IgG1 variant of 225 (cetuximab) was used as standard. Half maximal binding concentrations proved to be nearly identical for cetuximab (9.9 μg/ml; 95% confidence interval (CI): 8.4 - 11.7 μg/ml), the chimeric IgG1 (10.4 μg/ml; 95% CI: 7.4 - 14.5 μg/ml), and the IgA1 and IgA2 versions (10.2 μg/ml; 95% CI: 8.2 - 12.8 μg/ml and 9.2 μg/ml; 95% CI: 6.4 - 13.2 μg/ml, respectively). These data indicated similar affinities of the isotype switch variants, but differences in K_{on} and K_{off} rates, which may be affected by Abs’ constant regions, cannot be excluded. The IgA2 Ab demonstrated lower maximal

| Table 1. Isotype determination of recombinant Abs | Anti-IgA1 | Anti-IgA2 | Anti-IgG1 |
|-----------------------------------------------|----------|----------|----------|
| IgA1                                         | 443.1 ± 40.8 | 4.7 ± 0.1 | 1.7 ± 0.4 |
| IgA2                                         | 4.9 ± 1.0  | 193.4 ± 11.2 | 3.0 ± 0.2 |
| IgG1                                         | 6.8 ± 1.2  | 2.9 ± 0.2  | 515.6 ± 55.3 |

*To confirm the isotypes of purified antibodies, A431 cells were incubated with saturating concentrations of the IgA1, IgA2, or IgG1 versions, and stained by FITC-conjugated anti-human IgA1, IgA2, or IgG1-specific antibodies. Data are presented as mean RFI ± SEM of three independent experiments.*
fluorescence intensities at saturating concentrations, which are probably explained by different reactivities with the secondary Ab. Fc-mediated binding of the two IgA isoforms to FcRRI was compared by indirect immunofluorescence. FcRRI-transfected BHK-21 cells were incubated with increasing concentrations of the EGF-R isotype variants and stained by FITC-labeled anti-human IgA Ab. Both IgA isoforms demonstrated binding to FcRRI-transfected (Fig. 2C), but not to untransfected (n = 3; data not shown) BHK-21 cells, whereas the IgG1 Ab did not bind to either (data not shown). Interestingly, our IgA2 Ab bound notably, albeit not significantly, stronger to FcRRI than its IgA1 version. Similar differences in binding to FcRRI were also observed with the IgA1, and IgA2, controls, and with freshly isolated PMN as alternative source of FcRRI (n = 3; data not shown).

**Direct effector mechanisms**

To investigate F(ab')-mediated mechanisms of the chimeric Abs, we first analyzed their capacity to block ligand binding on A431 cells. Coincubation of FITC-conjugated human EGF with cetuximab or the IgG1-, IgA1-, and IgA2-variants resulted in 98.6 ± 0.3%, 97.1 ± 0.1%, 96.2 ± 0.7%, or 93.4 ± 1.0% inhibition of EGF-binding, respectively, whereas a control Ab against KLH did not inhibit EGF-binding (Fig. 3A).

The EGF-R Ab cetuximab is known to inhibit ligand-induced receptor phosphorylation (4). Therefore, we compared the panel of Abs in their capacity to inhibit EGF induced phosphorylation of tyrosine 1068, using EGF-R-transfected Ba/F3 cells (Matthias Peipp, manuscript in preparation). As expected, all three EGF-R Ab variants demonstrated similar inhibition of EGF-induced tyrosine phosphorylation, whereas IgA myeloma proteins and an IgG1 control Ab were not effective (Fig. 3B).

Growth inhibition of EGF-R expressing cells is considered another mechanism of action for EGF-R Abs (4). To address this mechanism, we used an established model of EGF-dependent proliferation, in which EGF-R-transfected Ba/F3 cells proliferate in response to human EGF, or to murine IL-3 (Matthias Peipp, manuscript in preparation). Growth inhibition by EGF-R Abs of IgG1, IgA1, and IgA2 isotypes was compared in MTT assays. Importantly, all three Ab variants similarly inhibited EGF-induced cell proliferation.
growth (Fig. 3C), whereas IL-3-mediated proliferation was not affected (Fig. 3D). A control Ab against KLH did not inhibit cell proliferation via either stimulus. These data clearly demonstrated the observed growth inhibition to be EGF-R specific, and not caused by toxic effects of the Ab preparations.

**Indirect mechanisms**

Complement dependent cytotoxicity is considered an important mechanism of action for CD20 Abs, at least under certain clinical conditions (22). Therefore, we compared the panel of EGF-R Abs for their capacity to trigger CDC against three tumor cell lines expressing high EGF-R levels. However, neither the IgG1 nor the two IgA variants induced significant CDC, suggesting CDC not to be an important mechanism of Ab action for these Abs ($n = 4$; data not shown).

Next, we compared chimeric IgG1, IgA1, and IgA2 Abs in their capacity to trigger ADCC against EGF-R-expressing A431 tumor cells (Fig. 4). As effector cells, isolated MNC and PMN were compared using all three Ab variants. MNC triggered effective ADCC with the IgG1 version, with half maximal killing observed at 0.03 μg/ml (95% CI: 0.01 - 0.07 μg/ml) of the IgG1 Ab. However, MNC were not effective with the IgA1 or IgA2 isoforms, even at high Ab concentrations (Fig. 4A). Isolated PMN, in contrast, mediated significant tumor cell killing with both IgA Ab isoforms, but were not effective with the IgG1 Ab (Fig. 4B). Significant ADCC by IgA Abs was observed at Ab concentrations above 0.08 μg/ml, and half maximal killing was obtained at 0.27 μg/ml (95% CI: 0.11 - 0.69 μg/ml) and 0.21 μg/ml (95% CI: 0.12 - 0.37 μg/ml) with IgA1 and IgA2, respectively. Interestingly, the human IgA2 construct proved significantly more effective in triggering PMN-mediated ADCC than its IgA1 counterpart. Importantly, IgA2 triggered significant PMN-mediated ADCC already at E:T cell ratios as low as 5:1 (Fig. 4C). IgA-mediated tumor cell lysis by PMN was significantly blocked by the CD89 Ab My43 (Table II), indicating that the IgA Abs, indeed, killed via FcγRI.

To compare the Ab isotypes under more physiological conditions, human whole blood assays were used to assess the relative contributions of PMN and MNC for tumor cell killing. Under these assay conditions, human IgG1 and IgA1 triggered similarly low levels of A431 killing, whereas the human IgA2 isoform was significantly more effective (Fig. 5A). Similar results were obtained against A1207 tumor cells, which were generally more susceptible to ADCC than A431 cells ($n = 3$; data not shown), whereas no specific killing was observed against SK-RC 7 cells ($n = 3$; data not shown). To investigate the potential of myeloid growth factors to increase IgA-mediated ADCC, A431 carcinoma cells were incubated with the IgA2 Ab using whole blood from healthy donors, or from G-CSF-primed donors as effector source. Under these conditions, Ab mediated tumor cell killing reached significantly higher levels with G-CSF-primed, compared with healthy donor blood. This enhancement may be explained by higher PMN numbers in G-CSF-primed, compared with healthy donor blood (16.8 ± 3.1/nl vs 4.4 ± 0.6/nl).

**Discussion**

In this study, we describe recombinant human IgA Abs against EGF-R as potent reagents for the killing of EGF-R expressing tumor cells. These IgA Abs proved similarly effective as their human IgG1 counterparts in triggering direct effector mechanisms, but demonstrated significantly enhanced ADCC. This enhanced...

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**FIGURE 3.** F(ab’)-mediated direct mechanisms of Ab action. EGF-R Abs of IgG1, IgA1, or IgA2 isoforms were compared in their capacity to trigger direct mechanisms of action - using an Ab against KLH as nonbinding control. In A, we investigated the capacity of the three Ab variants to block FITC-conjugated EGF binding on A431 cells. Data are presented as mean ± SEM of percentage of inhibition of EGF-binding as calculated from three independent experiments. In B, EGF-induced signaling in EGF-R-transfected Ba/F3 cells was analyzed in the absence or presence of indicated Abs (2 μg/ml) by measuring pY1068 and total EGF-R levels in Western blots. Growth inhibition of EGF-R-transfected Ba/F3 cells was analyzed in the presence of increasing concentrations of EGF-R Abs, without (C) and with (D) addition of mIL-3. After 72 h with EGF, vital cell masses were measured by MTT assays. Data are presented as mean ± SEM of triplicates from four independent experiments, calculated in relation to proliferation in the presence of nonbinding Ab (see also Materials and Methods).
ADCC by IgA, compared with IgG1 Abs, was attributed to improved PMN recruitment. In vitro, Abs against EGF-R kill tumor cells by different mechanisms such as blockade of ligand binding, inhibition of phosphorylation, direct growth inhibition and ADCC. Which of these potential mechanisms operate in vivo is still uncertain, but Fc mediated killing appears to contribute significantly (23). For EGF-R Abs, this conclusion is supported by evidence from animal studies comparing whole Abs with their respective F(ab')2 fragments (24). Furthermore, the EGF-R Ab 2F8 demonstrated therapeutic efficacy in vivo at subsaturating concentrations, which effectively triggered ADCC, but which were too low to allow receptor blockade (8). Therefore, enhancing the ADCC capacity of therapeutic Abs is an important goal (25), which is pursued by several Ab engineering approaches (26). Our group aimed to improve ADCC by selecting appropriate target Ags (27), by combining therapeutic Abs with myeloid growth factors to enhance effector cell numbers and function (12) and by searching for optimal cytotoxic trigger molecules (12, 13). This later approach identified the myeloid IgA receptor (FcαRI, CD89) as a particularly potent cytotoxic receptor for triggering ADCC. Subsequently, several groups generated recombinant IgA Abs against tumor target Ags as natural ligands for FcαRI, which demonstrated significant tumor cell lysis in vitro (17, 18, 28). In this study, we describe human IgA isotype variants of the clinically approved EGF-R directed Ab cetuximab, which constitutes a validated target Ag on many common solid tumors (4, 5).

Today, human IgG1 is by far the most common Ab isotype in tumor therapy. An important reason for this selection is the extended plasma half-life ~3 wk for IgG Abs. This long half-life is conferred by binding to the neonatal Fc receptor, which protects IgG Abs from lysosomal degradation (11). Furthermore, IgG1 Abs were demonstrated to effectively trigger complement-mediated killing and ADCC by MNC (9). However, PMN were not investigated in these early studies, and solid tumor cells appear to be protected from CDC by high expression levels of complement regulatory proteins such as CD46, CD55, and CD59 (29). IgA, in contrast, proved more effective than IgG1 in recruiting PMN effector cells (18), and may be advantageous in reaching serosal surfaces, from which many common cancers originate. After binding to the polymeric Ig receptor, IgA dimers are actively transported to luminal surfaces, where IgA has an important function in the homeostasis of immune functions (16). Upon inflammatory stimuli, neutrophils gain rapid access to serosal surfaces, where they play a predominant role in the primary host defense against invading bacteria (30). Studies with cytokine-transfected tumor cells also suggested a significant contribution of neutrophils in tumor surveillance (31), but these observations await confirmation in more clinically relevant settings. Importantly, neutrophils’ potential function for Ab efficacy is underestimated when human IgG1 Abs are investigated. Human IgG1 binds effectively to all three human leukocyte FcγR classes (FcγRI, FcγRII, and FcγRIII), which can all be expressed by PMN. However, the most abundantly expressed FcγR is isofrom on PMN, FcγRIIb (CD16b), is a GPI-linked receptor, which does not trigger ADCC (6). Furthermore, PMN were reported to express the inhibitory FcγRIIb isofrom (32), although this issue is controversial (33). In our

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Table II. Inhibition of IgA-mediated ADCC by FcαRI (CD89)-directed antibody My43

| Specific lysis [%] | Without FcαRI-blockade | With FcαRI-blockade | Inhibition [%] |
|-------------------|-------------------------|---------------------|----------------|
| IgA1              | 32.2 ± 7.0              | 2.0 ± 2.6           | 93.7*          |
| IgA2              | 72.8 ± 17.4             | 5.2 ± 4.6           | 92.7*          |

*In order to demonstrate that IgA-mediated killing by PMN was triggered by CD89, FcαRI was blocked by antibody My43 (10 μg/ml). Killing levels of A431 cells with and without My43 were compared, and percentage of inhibition was calculated as described in Materials and Methods. Data from three independent experiments with PMN from different donors are presented as mean ± SEM, significant inhibition is indicated by *.

IgA antibodies were used at 2 μg/ml, E:T ratio was 80:1.
more difficult. As a consequence, human Fcγ transgenic mice demonstrated high levels of Fcγ under a myeloid cell specific promoter. In vitro, PMN of CD89- 

IgA1 and IgA2 to Fcγ no BIACORE data are available directly comparing affinities of forms were reported to be similar (14, 36), but to our knowledge IgA2 was clearly superior to IgA1 in recruiting PMN for ADCC, as the potential mechanism of IgA2's superior ADCC activity, we demonstrated by the requirement of lower Ab concentrations, lower E:T ratios and higher maximal killing levels. To investigate the potential mechanism of IgA2's superior ADCC activity, we examined the binding properties of the IgA Abs to FcγRI on both CD89 transfected cells and freshly isolated PMN. Under our assay conditions, IgA2 demonstrated stronger binding to FcγRI than IgA1 on both cell types. This difference was not statistically significant, but was similar for the recombinant EGF-R Abs and commercially obtained IgA controls. Affinities of FcγRI to both isoforms were reported to be similar (14, 36), but to our knowledge no BIACORE data are available directly comparing affinities of IgA1 and IgA2 to FcγRI. Additionally, the shorter hinge region of IgA2 compared with IgA1 may provide more rigid E:T cell bridging, thereby, leading to more efficient PMN recruitment. Comparable effects have been described for IgG Abs. For example, human IgG3 Abs, with their extended hinge region, appear to mediate FcγR-dependent effector-target bridging more readily than their shorter-hinged IgG1 equivalents (37). However, once bridging has occurred, the IgG1 Abs mediate ADCC more effectively (9). Further studies are required to investigate these hypotheses in detail.

Assessing the potential of human IgA Abs for tumor therapy is complicated by the lack of homology between the human and mouse IgA receptor systems. For example, mice do not express a human FcγRI (CD89) homologue, which makes in vivo models more difficult. As a consequence, human FcγRI transgenic mice have been generated (38, 39), some of which express the transgene under a myeloid cell specific promoter. In vitro, PMN of CD89-transgenic mice demonstrated high levels of FcγRI-mediated kill-
6. van de Winkel, J. G., and P. J. Capel. 1993. Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. Immunity 10:120–125.

7. Nimmerjahn, F., and J. V. Ravetch. 2006. Fc receptors: old friends and new family members. Immunity 24: 19–28.

8. Bleeker, W., J. F. M. van Assendelft, H. J. van Oijen, A. F. Gerritsen, M. Pleyte, M. Houtkamp, E. Halk, J. Goldstein, J. Schuurman, M. A. van Dijk, et al. 2004. Dual mode of action of a human anti-epidermal growth factor receptor monoclonal antibody for cancer therapy. J. Immunol. 173: 4699–4707.

9. Bruggemann, M., G. T. Williams, C. I. Bindon, M. R. Clark, M. R. Walker, R. Jefferis, H. Waldmann, and M. S. Neuberger. 1987. Comparison of the effector functions of human immunoglobulin A using a matched set of chimeric antibodies. J. Exp. Med. 166: 1351–1361.

10. Ghetie, V., and E. S. Ward. 2000. Multiple roles for the major histocompatibility complex class I-related receptor FcRn. Annu. Rev. Immunol. 18: 739–766.

11. Van de Winkel, J. G., and P. J. Capel. 1993. Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. Immunity 10: 120–125.

12. Valerius, T., B. Stockmeyer, A. B. van Spriel, R. F. Graziano, I. E. van den Herik-Oudijk, R. Repp, Y. M. D. van der Linden, J. L. D. van Kinkel, M. Gramatzki, and J. G. van de Winkel. 1997. FcRn (CD98) as a novel trigger molecule for hmonic antibody therapy. Blood 90: 4485–4492.

13. Stockmeyer, B., M. Dechant, M. van Egmond, A. L. Tutt, K. Sundarapandiyan, R. F. Graziano, R. Repp, J. R. Kalden, M. Gramatzki, M. J. Glennie, et al. 2000. Triggering Fc-receptor I (CD89) recruits neutrophils as effector cells for CD20-directed antibody therapy. J. Immunol. 165: 5954–5961.

14. Monteiro, R. C., and J. G. J. van de Winkel. 2003. IgA Fc receptors. Annu. Rev. Immunol. 21: 177–204.

15. Bakema, J. E., S. de Haaij, C. F. den Hartog-Jager, J. Bakker, G. Vidarsson, M. van Ooijen, M. J. van der Ende, and J. H. Leusen. 2006. Signaling through mutants of the IgA receptor CD89 and consequences for Fc receptor γ-chain interaction. J. Immunol. 176: 3630–3636.

16. Wood, J. M., and M. A. Kerr. 2006. The function of immunoglobulin A in immunity. J. Pathol. 208: 270–282.

17. Huls, G., A. J. Heijnen, E. Cuomo, J. van der Linden, B. Boel, J. G. van de Winkel, and T. Logtenberg. 1999. Antitumor immune effector mechanisms recruited by phage display-derived fully human IgG1 and IgA1 monoclonal antibodies. J. Immunol. 163: 601–606.

18. Dechant, M., G. Vidarsson, B. Stockmeyer, R. Repp, M. J. Glennie, M. Gramatzki, J. G. van de Winkel, and T. Valerius. 2002. Chimeric IgA monoclonal antibody and preventive immunotherapy in humans. Nat. Med. 5: 603–606.

19. Dechant, M., and T. Valerius. 2001. IgA antibodies for cancer therapy. Crit. Rev. Oncol. Hematol. 39: 69–77.

20. Porath, J., F. Maisano, and M. Belew. 1985. Thiophilic adsorption: a new method for protein fractionation. FERG Lett. 185: 306–310.

21. Weiner, G. J. 2003. Rituxinax: complementary mechanisms of action. Blood 101: 788.

22. Houghton, A. N., and D. A. Scheinberg. 2000. Monoclonal antibody therapies: a ‘constant’ threat to cancer. Nat. Med. 6: 373–374.

23. Fan, Z., H. Masui, I. Altas, and J. Mendelsohn. 1993. Blockade of epidermal growth factor receptor function by bivalent and monovalent fragments of 225 anti-epidermal growth factor receptor monoclonal antibodies. Cancer Res. 53: 4322–4328.

24. Wood, J. M. 2005. Immunology: tipping the scales toward more effective antibodies. Science 310: 1442–1443.

25. Carter, P. J. 2006. Potent antibody therapeutics by design. Nat. Rev. Immunol. 6: 343–357.

26. Wurfein, D., M. Dechant, B. Stockmeyer, A. L. Tutt, P. Hu, R. Repp, J. R. Kalden, J. G. van de Winkel, A. L. Epstein, T. Valerius, et al. 1998. Evaluating antibodies for their capacity to induce cell-mediated lysis of malignant B cells. Cancer Res. 58: 3051–3058.

27. Ingram, P. E., R. J. Owens, and J. M. Woof. 1997. Development of a human IgA antibody specific for the tumour antigen TAG-72. Biochem. Soc. Trans. 25: 330S.

28. Fishelson, Z., N. Donin, S. Zell, S. Schultz, and M. Kirschfink. 2003. Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. Mol. Immunol. 40: 109–123.

29. Pizzo, P. A. 1984. Granulocytopenia and cancer therapy: past problems, current solutions, future challenges. Cancer 54: 2649–2661.

30. Di Carlo, E., G. Forini, P. Lollini, M. P. Colombo, A. Modesti, and P. Musiani. 2001. The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood 97: 339–345.

31. van Mirre, E., W. B. Breunis, J. Geissler, C. E. Hack, M. de Boer, D. Roos, and T. W. Kuipers. 2006. Neutrophil responsiveness to IgG, as determined by fixed ratios of mRNA levels for activating and inhibitory FcγRII (CD32D), is stable over time and unaffected by cytokines. Blood 108: 584–590.

32. Veri, M. C., S. Giorlatos, H. Li, S. Burke, S. Johnson, J. Stavenhagen, K. E. Stein, E. Bonvini, and S. Koenig. 2007. Monoclonal antibodies capable of discriminating the human inhibitory Fcγ-receptor IIb (CD16B2) from the activating Fcγ-receptor IIa (CD16A2): biochemical, biological and functional characterization. Immunology 121: 392–404.

33. Tiroch, K., B. Stockmeyer, C. Frank, and T. Valerius. 2002. Intracellular domains of target antigens influence their capacity to trigger antibody-dependent cell-mediated cytotoxicity (ADCC). J. Immunol. 168: 3275–3282.

34. Fishelson, Z., N. Donin, S. Zell, S. Schultz, and M. Kirschfink. 2003. Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. Mol. Immunol. 40: 109–123.

35. Rogers, K. A., F. Scinicariello, and R. Attanasio. 2004. Identification and characterization of macrophage CD89 (immunoglobulin A Fc receptor). Immunology 113: 178–186.

36. van Egmond, M., A. J. van Vuuren, H. C. Morton, A. B. van Spriel, L. Shen, F. M. Holthuis, T. Saito, T. N. Mayadas, J. S. Verbeek, and J. G. van de Winkel. 1999. Human immunoglobulin A receptor (FcRn, CD89) function in transgenic mice requires both FcγR-chain and CR1 (CD11b/CD18). Blood 93: 4387–4394.

37. Mantelboim, O., P. Malik, D. M. Davis, C. H. Jo, E. Boyson, and J. L. Strominger. 1999. Human CD16 as a lysis receptor mediating direct natural killer cell cytotoxicity. Proc. Natl. Acad. Sci. USA 96: 5640–5644.

38. Morton, H. C., J. D. Atkin, R. J. Owens, and J. M. Woof. 1993. Purification and characterization of chimeric human IgA1 and IgA2 expressed in COS and Chinese hamster ovary cells. J. Immunol. 151: 4743–4752.

39. Walker, M. R., J. M. Woof, M. Bruggemann, R. Jefferis, and D. R. Burton. 1989. Interaction of human IgG chimeric antibodies with the human FcRI and FcRII receptors: requirements for antibody-mediated host cell-target cell interaction. Mol. Immunol. 26: 403–411.

40. Rogers, K. A., F. Scinicariello, and R. Attanasio. 2004. Identification and characterization of macrophage CD89 (immunoglobulin A Fc receptor). Immunology 113: 178–186.