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Effector Mechanisms of Recombinant IgA Antibodies against Epidermal Growth Factor Receptor[1]

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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, IgA_1, and IgA_2 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 IgA_2 isoform was significantly superior to its IgA_1 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)[4] is a tyrosine kinase receptor with critical functions in the regulation of cell proliferation, differentiation and survival (1). 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 (2, 3), EGF-R constitutes a promising molecule for targeted therapy (4). 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 (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 regions - 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 (6, 7). 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 FcRγ-chain knock-out mice, in which the signaling machinery of activating Fc receptors was disrupted (7). 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 (8).

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 (9). However, human IgG1 interacts with all FcγR isoforms, including the inhibitory FcγRIIb molecule

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[4] Abbreviations used in this paper used in this paper: EGF-R, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; CDC, complement-dependent cell lysis; PMN, polymorphonuclear cell; ADCC, Ab-dependent cellular cytotoxicity; mIL-3, murine IL-3; MNC, mononuclear cells; RFI, relative fluorescence intensity; E:T, effector-to-target; CI, confidence interval.

and the GPI-linked FcγRIIIb, 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 (FcαRI, CD89) (12, 13). FcαRI is constitutively expressed on monocytes/macrophages, PMN, some types of dendritic cells and Kupffer cells, but not on noncytotoxic cells. Functionally, FcαRI mediates phagocytosis, oxidative burst, cytokine release, Ag presentation, and ADCC (14). Like other Ig receptors, FcαRI requires association with the immuno-receptor tyrosine-based activation motif-containing FcRγ-chain for in vivo expression and function. The FcRγ-chain appears to interact more effectively with FcαRI than with FcγRs (15). Human IgA Abs bind with medium affinity to FcαRI (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, IgA_1 and IgA_2, are distinguished in humans, with IgA_2 containing a shorter hinge region and an increased resistance against enzymatic degradation by bacterial proteases. A small study with locally applied recombinant 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 IgA_1 and IgA_2 isoforms with their respective IgG1 counterpart. To address this issue, we generated mouse/human chimeric EGF-R Abs of IgG or IgA isotypes, 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, IgA 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 Pwo polymerase (Peqlab) 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 κ-L chain, and γ1-, α1-, or α2-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 (Invitrogen 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 (Invitrogen Life Technologies).

Purification of chimeric Abs

Both IgA isoforms were chromatographically purified on thiophilic agarose columns (Kem-En-Tec) as described (21), the chimeric IgG1 Ab on protein A columns (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 IgA_1 or IgA_2 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 RPMI 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 mg/ml geneticin (Invitrogen Life Technologies).

For generation of FcαRI (CD89)-transfectants, CD89 containing plasmids were stably transfected into FcR-naive BHK-21 cells (DSMZ) together with the common FcRγ-chain. Transfectants were cultured in RPMI 1640, selection pressure was maintained by adding 500 μg/ml geneticin for FcαRI, and 10 μM methotrexate (Sigma-Aldrich) for the FcRγ-chain.

Immunofluorescence analyses

For indirect immunofluorescence, 1×10^5 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 κ-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), IgA_1, or IgA_2 (both from Beckman Coulter).

To compare Fc-mediated binding of IgA_1 and IgA_2 to FcαRI, FcαRI-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, 1.5×10^5 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)/(RFI without antibody) × 100.

All experimental steps were performed at 4° C. An Ab against keyhole limpet hemocyanin (KLH; human IgG1; provided by Genmab) served as negative control for IgG1, human myeloma IgA_1 or IgA_2 Abs served as negative controls for IgA_1 and IgA_2, 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 (Biozol; diluted at 1/2,000) or goat anti-human IgG Ab (Caltag Laboratoies; diluted at 1/3,000), respectively. After washing three times with TBS-buffer containing 0.4% Tween, blots were developed with enhanced chemoluminescent 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 ng/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 Na_3VO_4, and proteinase 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 mIL-3, diluted in RPMI 1640 medium containing human EGF (10 ng/ml) with or without mIL-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 were performed at least three times. MTT in the presence of KLH Ab was used as a reference (100% cell growth), and values with EGF-R directed Abs were calculated as percent of control according to the formula: absorption in the presence of EGF-R Ab/absorption in the presence of KLH Ab × 100.

Isolation of mononuclear and neutrophilic effector cells

After written informed consent, up to 80 ml 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 cytospin 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) ^{51}Cr for two hours. After washing, cells were adjusted to 10^5/ml. Whole blood, plasma, or isolated effector cells (50 μl), sensitizing Abs, and RPMI 1640 (10% FBS) 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 ^{51}Cr 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 ^{51}Cr 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 FcαRI in IgA-mediated cell killing, FcαRI-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 IgA_1, and 5 pg/cell/day for IgA_2. 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 IgA_1 variants demonstrated single bands of ~150–160 kDa, corresponding to monomeric Abs. With the IgA_2 variant, major bands of ~120 kDa and 50 kDa were observed, which corresponded to H and L chain homodimers, respectively. This is a well described phenomenon of the IgA_2m(1) 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 alloforms. 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 IgA_1 and IgA_2 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. 2*A*), 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. 2*B*). 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 IgA_1 and IgA_2 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 IgA_2 Ab demonstrated lower maximal

Table I. *Isotype determination of recombinant Abs*[a]

	Anti-IgA_1	Anti-IgA_2	Anti-IgG_1
IgA_1	443.1 ± 40.8	4.7 ± 0.1	1.7 ± 0.4
IgA_2	4.9 ± 1.0	193.4 ± 11.2	3.0 ± 0.2
IgG_1	6.8 ± 1.2	2.9 ± 0.2	515.6 ± 55.3

[a] To confirm the isotypes of purified antibodies, A431 cells were incubated with saturating concentrations of the IgA_1, IgA_2, or IgG_1 versions, and stained by FITC-conjugated anti-human IgA_1-, IgA_2-, or IgG_1-specific antibodies. Data are presented as mean RFI ± SEM of three independent experiments.

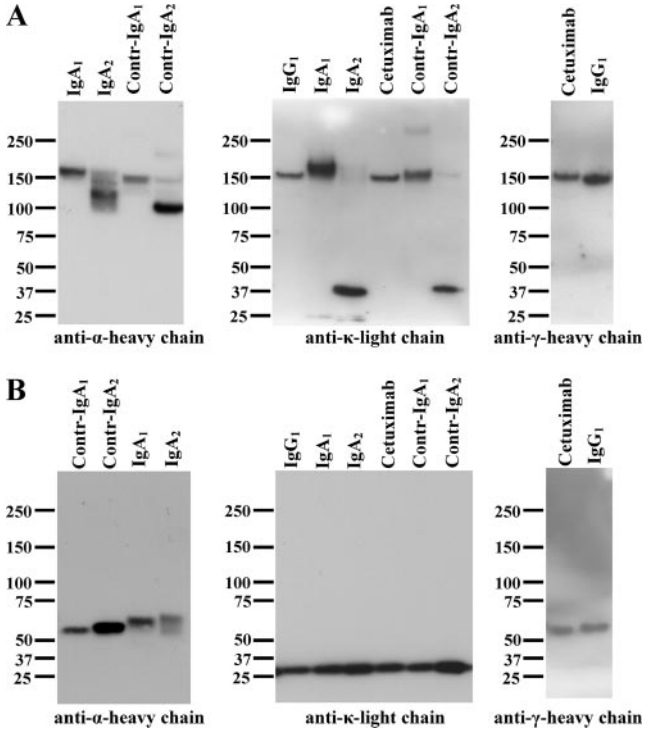

FIGURE 1. Western blot analyses of chimeric EGF-R Abs. Purified Abs of IgG1, IgA_1, or IgA_2 isotypes were separated by SDS-PAGE under nonreducing (*A*) or reducing (*B*) conditions. After transfer onto PVDF-membranes, Ab fractions were probed with anti-human α-, or γ-H, or κ-L chain Abs, as indicated. The positions of molecular mass markers (in kDa) are indicated. Under nonreducing conditions, a single band of ~150–160 kDa was seen with IgA_1 and IgG1 isotypes, corresponding to monomeric Abs. For IgA_2, additional bands of ~120 kDa and 50 kDa were detected, corresponding to H and L chain homodimers, respectively, which are typical for the IgA_2m(1) alloform. Under reducing conditions, all variants demonstrated single bands of ~50–60 kDa for H chains, and 25 kDa for light chains (one representive of at least four blots each is presented).

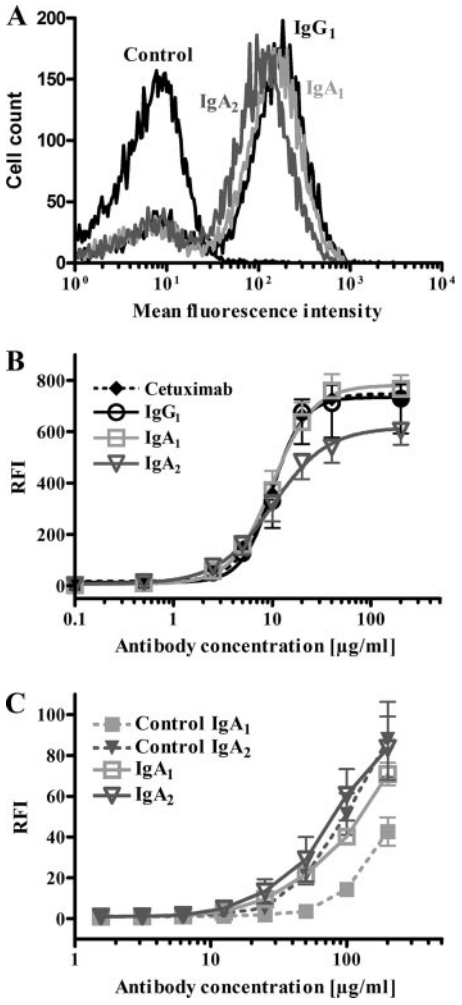

FIGURE 2. Binding to EGF-R and FcαRI (CD89). After staining with FITC-labeled anti-human κ-L chain Ab, saturating concentrations of chimeric IgA_1, IgA_2, or IgG1 variants demonstrated similar binding to EGF-R-transfected Ba/F3 cells (*A*), but did not bind to nontransfected cells (n = 3; data not shown). To estimate Ab affinity, serial dilutions of Abs were investigated for EGF-R binding on A431 cells, using cetuximab as reference and FITC-labeled anti-human κ-L chain Ab for staining (*B*). Relative fluorescence intensities are presented as mean ± SEM of at least four experiments. Cetuximab as well as the chimeric IgG1, IgA_1, and IgA_2 Abs demonstrated similar half-maximal binding at 9.9, 10.4, 10.2, or 9.2 μg/ml, respectively. In *C*, reverse binding of IgA_1 and IgA_2 Abs to FcαRI (CD89)-transfected BHK-21 cells was investigated at increasing concentrations to estimate antibodies' affinity for FcαRI. Human myeloma Abs of IgA_1 and IgA_2 isoforms served as controls. None of the Abs bound to untransfected BHK-21 cells (data not shown). Relative fluorescence intensities are presented as mean ± SEM of at least four 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 FcαRI was compared by indirect immunofluorescence. FcαRI-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 FcαRI-transfected (Fig. 2*C*), but not to untransfected (n = 4; data not shown) BHK-21 cells, whereas the IgG1 Ab did not bind to either (data not shown). Interestingly, our IgA_2 Ab bound notably, albeit not significantly, stronger to FcαRI than its IgA_1 version. Similar differences in binding to FcαRI were also observed with the IgA_1 and IgA_2 controls, and with freshly isolated PMN as alternative source of FcαRI (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-, IgA_1-, and IgA_2-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. 3*A*).

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. 3*B*).

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, IgA_1, and IgA_2 isotypes was compared in MTT assays. Importantly, all three Ab variants similarly inhibited EGF-induced cell

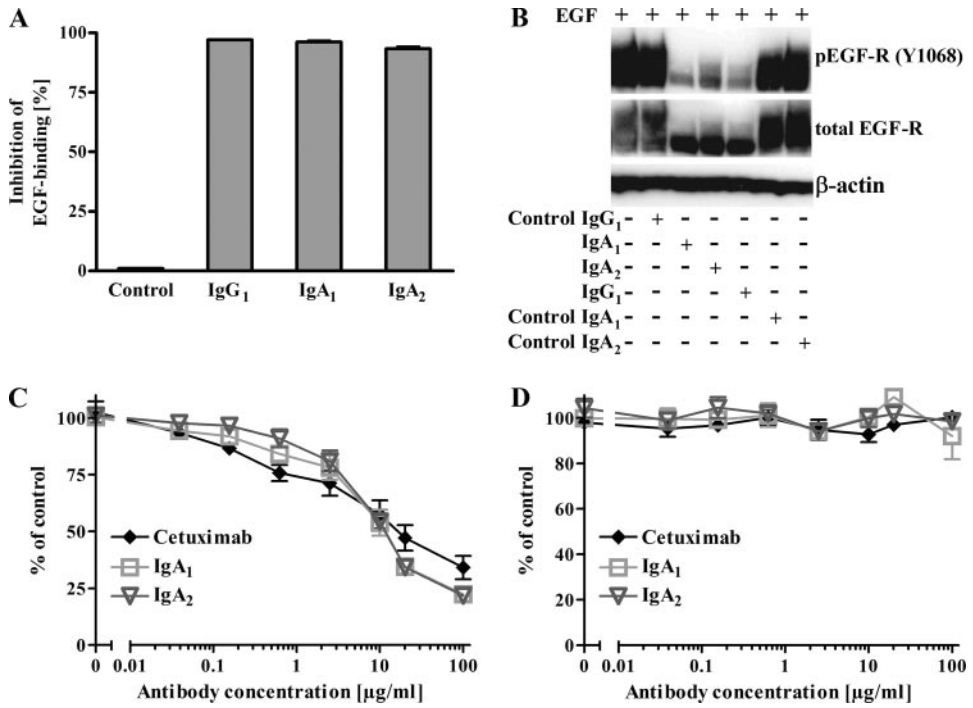

FIGURE 3. F(ab′)-mediated direct mechanisms of Ab action. EGF-R Abs of IgG1, IgA_1, or IgA_2 isotypes were compared in their capacity to trigger direct mechanisms of action - using an Ab against KLH as nonbinding control. In *A*, we investigated the capacity of the three Ab variants to block FITC-conjugated 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*).

growth (Fig. 3*C*), whereas IL-3-mediated proliferation was not affected (Fig. 3*D*). 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, IgA_1, and IgA_2 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 IgA_1 or IgA_2 isoforms, even at high Ab concentrations (Fig. 4*A*). Isolated PMN, in contrast, mediated significant tumor cell killing with both IgA Ab isoforms, but were not effective with the IgG1 Ab (Fig. 4*B*). 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 IgA_1 and IgA_2, respectively. Interestingly, the human IgA_2 construct proved significantly more effective in triggering PMN-mediated ADCC than its IgA_1 counterpart. Importantly, IgA_2 triggered significant PMN-mediated ADCC already at E:T cell ratios as low as 5:1 (Fig. 4*C*). 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 Fcα*R*I.

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 IgA_1 triggered similarly low levels of A431 killing, whereas the human IgA_2 isoform was significantly more effective (Fig. 5*A*). 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 IgA_2 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

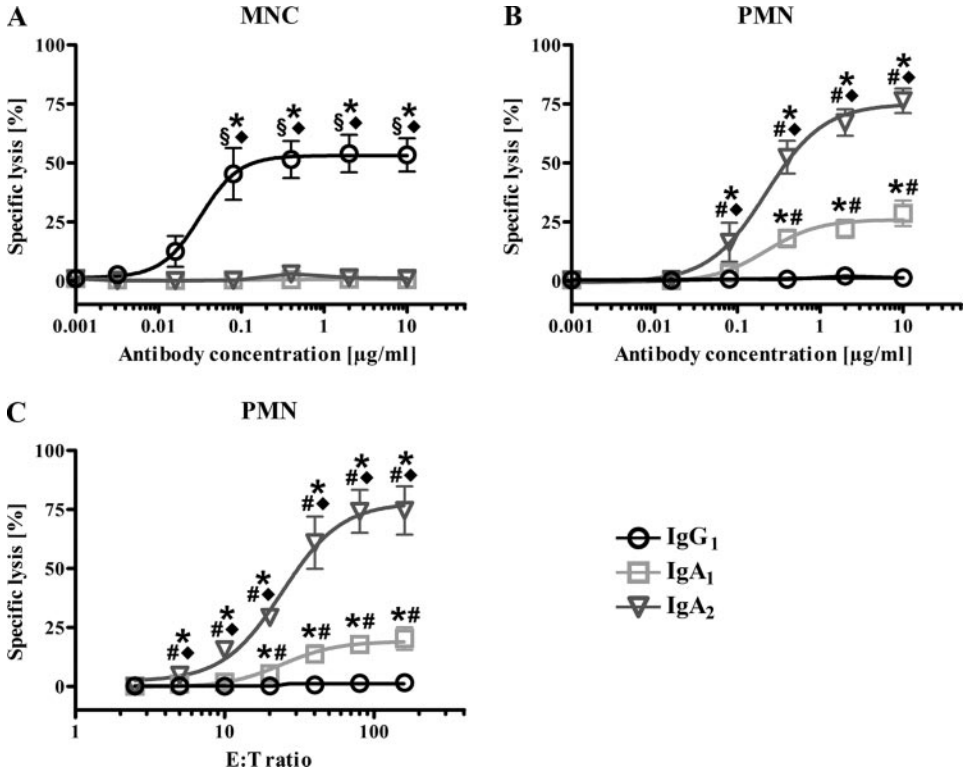

FIGURE 4. ADCC against A431 cells by EGF-R Abs of IgG1, IgA_1, and IgA_2 isotypes. To analyze the impact of EGF-R Ab isotypes on indirect immune effector functions, A431 cells were incubated with increasing concentrations of EGF-R Abs in 3-h chromium release assays. As effector source served either isolated MNC (*A*) or PMN (*B*) cells, which were used at E:T cell ratios of 80:1. To investigate the influence of E:T ratios on IgA-mediated ADCC by PMN (*C*), IgG1, IgA_1, or IgA_2 mediated killing of A431 cells was investigated at different E:T ratios using fixed Ab concentrations of 2 μg/ml. Data are presented as mean percentage of specific lysis ± SEM from at least three different donors. Significant ($p < 0.05$) Ab-mediated lysis is marked by asterisks (∗). Other symbols indicate significant differences of respective Abs compared with IgG1 (#), IgA_1 (♦), or IgA_2 (§), respectively.

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 ways of killing appear 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′) 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 isoform on PMN, FcγRIIIb (CD16b), is a GPI-linked receptor, which does not trigger ADCC (6). Furthermore, PMN were reported to express the inhibitory FcγRIIb isoform (32), although this issue is controversial (33). In our

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

	Specific lysis [%]		
	Without blockade	With FcaRI-blockade	Inhibition [%]
IgA_1	32.2 ± 7.0	2.0 ± 2.6	93.7*
IgA_2	72.8 ± 17.4	5.2 ± 4.6	92.7*

[a]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.

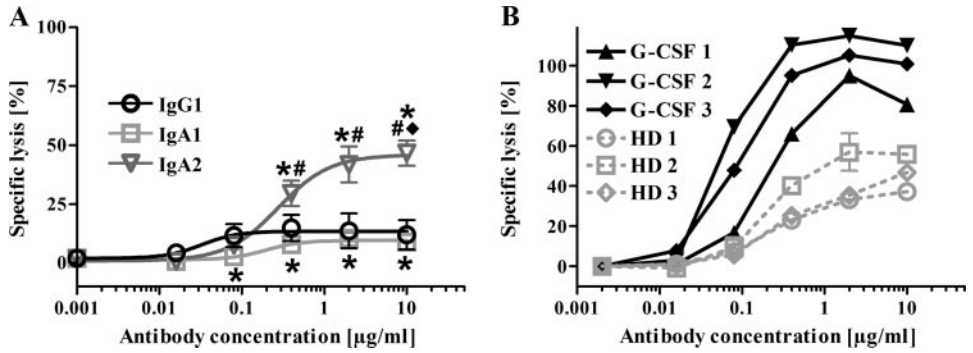

FIGURE 5. ADCC against A431 cells using unseparated blood as effector source. To address the relative contribution of PMN and MNC under more physiological conditions, human whole blood assays (25 vol %) were used to compare tumor cell killing by the three isotype variants (*A*). Data are presented as mean percentage of specific lysis ± SEM obtained from independent experiments with four different donors. Significant ($p < 0.05$) Ab-mediated lysis is marked by asterisks (*). Diamonds (♦) indicate significant difference compared with IgA_1, rhombs (#) compared with IgG1. In *B*, the potential of myeloid growth factors to increase IgA_2-mediated ADCC was investigated using unseparated blood (25 vol %) as effector source. Data from three healthy donors (HD, dashed lines) and three G-CSF-primed donors (G-CSF, solid lines) are presented as mean percentage of specific lysis ± SEM of triplicates.

hands, selection of appropriate target Ags appeared to be another critical issue for effective neutrophil recruitment (27), which may be related to the presence of intracellular signaling domains in tumor target Ags (34). Furthermore, neutrophils appear to require higher levels of target Ag expression (34), higher Ab concentrations (Ref. 34 and Fig. 4) and higher E:T cell ratios (35) than NK cells to become fully activated. However, neutrophils become the predominant effector cell type when tumor-directed Abs of human IgA isotype against appropriate target Ags were analyzed (Fig. 4 and Refs. 17, 18).

Two IgA isoforms are recognized in humans, which differ in their molecular characteristics and their presumed biological function (16). We did not observe differences between our IgA_1 and IgA_2 Abs in direct F(ab′)-mediated effector functions. However, IgA_2 was clearly superior to IgA_1 in recruiting PMN for ADCC, as demonstrated by the requirement of lower Ab concentrations, lower E:T ratios and higher maximal killing levels. To investigate the potential mechanism of IgA_2's superior ADCC activity, we examined the binding properties of the IgA Abs to FcαRI on both CD89 transfected cells and freshly isolated PMN. Under our assay conditions, IgA_2 demonstrated stronger binding to FcαRI than IgA_1 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 FcαRI to both isoforms were reported to be similar (14, 36), but to our knowledge no BIACORE data are available directly comparing affinities of IgA_1 and IgA_2 to FcαRI. Additionally, the shorter hinge region of IgA_2 compared with IgA_1 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 FcR-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 FcαRI (CD89) homologue, which makes in vivo models more difficult. As a consequence, human FcαRI 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 FcαRI-mediated killing, which was similar to human PMN (38). However, these transgenic mice proved difficult to cross into immunodeficient backgrounds. Therefore, recombinant IgA Abs against human tumor Ags have so far not been tested in mice. Recently, the CD89 genes from rhesus and cynomolgus monkeys were cloned, and derived amino acid sequences were highly homologous to their human counterpart (86.5 and 86.1%, respectively) (40). Until now, no data regarding the binding characteristics of human IgA to these FcαRI homologues have been published, but the grade of homology and earlier pharmacokinetic studies in nonhuman primates suggested binding properties comparable to the human situation (41). Thus, toxicity studies in primates appear to be feasible.

Another challenge is the large scale production and purification of IgA Abs. The requirement for chimerisation can be overcome by immunizing human cα transgenic mice (42), but also mice producing fully human IgA Abs have been generated (43). As demonstrated here, IgA Abs can be produced under serum-free suspension culture conditions using well established CHO-K1 cells, and a commonly used expression vector system. However, further optimization for large-scale production (e.g., in fermenters) is required. Furthermore, IgA glycosylation is more complex than for IgG (16), and may require optimization of production conditions to achieve well-characterized reagents. In addition, purification of IgA Abs is not well established, and no material is commercially available which allows single step high-grade purifications of IgA. These would certainly be important next steps to resolve for the successful development of human IgA Ab therapeutics for human disease.

Disclosures

The authors have no financial conflict of interest.

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