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Recombinant Dimeric IgA Antibodies against the Epidermal Growth Factor Receptor Mediate Effective Tumor Cell Killing

Stefan Lohse,* Stefanie Derer,* Thomas Beyer,† Katja Klausz,* Matthias Peipp,* Jeanette H. W. Leusen,‡ Jan G. J. van de Winkel,§,¶ Michael Dechant,† and Thomas Valerius*

Dimeric IgA Abs contribute significantly to the humoral part of the mucosal immune system. However, their potential as immunotherapeutic agent has hardly been explored. In this article, we describe the production, purification, and functional evaluation of recombinant dimeric IgA against the epidermal growth factor receptor. Human joining chain-containing IgA was produced by non-adherent Chinese hamster ovarian (CHO)-K1 cells under serum-free conditions. Purification by anti-human κ and anti-His-tag affinity, as well as size exclusion chromatography, resulted in a homogenous preparation of highly pure IgA dimers. Functional studies demonstrated dimeric IgA to be at least as effective as monomeric IgA in triggering Ab-dependent cellular cytotoxicity by isolated monocytes or polymorphonuclear cell and in human whole-blood assays. Importantly, dimeric IgA was more effective in F(ab)-mediated killing mechanisms, such as inhibition of ligand binding, receptor downmodulation, and growth inhibition. Furthermore, only dimeric but not monomeric IgA or IgG was directionally transported by the polymeric Ig receptor through an epithelial cell monolayer. Together, these studies demonstrate that recombinant dimeric IgA Abs recruit a distinct repertoire of effector functions compared with monomeric IgA or IgG1 Abs. The Journal of Immunology, 2011, 186: 3770–3778.

Antibodies constitute an integral part of the adaptive immune system (1), in which IgA Abs as part of the mucosal system protect large serosal surface areas from invasion by pathogens and toxins (2). In humans, two IgA isotypes, IgA1 and IgA2, are distinguished, which differ, for example, in their glycosylation profiles, the length of their hinge regions, F(ab) arm orientation, H and L chain linkage, and functional activity (3, 4). In contrast with IgG, IgA Abs bind to distinct cellular receptors (5) and exist as monomeric, dimeric, and secretory isomers. Although monomeric IgA is predominantly produced by plasma cells in the bone marrow, plasma cells in the lamina propria mainly secrete dimeric IgA. This dimer formation requires the presence of the so-called tailpiece, an 18 aa C-terminal extension of the IgA Fc part (6). Penultimate cysteines of two IgA monomers covalently bind to cysteines in the 15-kDa joining (J) chain to form dimeric IgA. Incorporation of the J chain into dimeric IgA is required for its transport onto mucosal surfaces, because the presence of J chain is essential for IgA binding to the polymeric Ig receptor (plgR) on the basolateral surface of epithelial cells (7). After endocytotic internalization and transcytosis, plgR is cleaved at the luminal surface, releasing secretory IgA, which consists of dimeric IgA covalently bound to the extracellular domain of plgR (8).

Effector functions of IgA Abs include pathogen neutralization, receptor blockade, oxidative burst, phagocytosis, and Ab-dependent cellular cytotoxicity (ADCC). Although many of these functions are similar to those of IgG Abs, significant differences have also been described. Although IgG1 Abs efficiently activate the classical complement pathway, IgA Abs do not bind C1q. Furthermore, IgA Abs bind to a different set of cellular FcRs compared with IgG Abs. Among these heterogeneous IgA-binding molecules (5), the myeloid receptor for IgA (FcgR1, CD89) is probably the best characterized receptor, which is expressed by monocytes/macrophages and polymorphonuclear cells (PMN). Interestingly, phagocyte activation by IgA is often stronger than by IgG Abs (9), although both receptor systems use the same ITAM-dependent intracellular signaling cascades (10). In addition, dimeric and secretory IgA are tetravalent for their respective Ags, which distinguishes them from bivalent IgG Abs. Increased Ab valency has been demonstrated to improve the efficacy of artificially dimerized or engineered IgG Abs (11, 12).

IgA is the second most prevalent Ab isotype in blood and has been estimated to be the most abundantly produced Ig in humans (3). Generation of IgA responses has been recognized as an important aim in vaccine studies (13). However, passive immunotherapy with IgA Abs has hardly been investigated (14), whereas targeted therapy with IgG Abs is a rapidly evolving field (15).
One of the most widely investigated target Ags is the epidermal growth factor receptor (EGFR), which is a signaling membrane tyrosine kinase (16). Today, two IgG Abs against the EGFR are FDA approved (17), and 14 others are tested in clinical trials. With the exception of panitumumab, which is of human IgG2 isotype, all these EGFR Abs are of human IgG1 isotype. Human IgG1 has been the preferred isotype for tumor therapy, because IgG1 effectively recruits NK cells for ADCC and may trigger complement-dependent cytoxicity (CDC) (18). However, human IgG1 was significantly less effective than monomeric IgG in recruiting human neutrophilic phagocytes for tumor cell killing (19–22). Phagocytes were demonstrated to constitute an important effector cell population for Ab therapy in syngenic animal models (23), and the contribution of PMN for tumor surveillance is actively investigated (24).

In this article, we describe the production, purification, and biochemical, as well as functional, characterization of recombinant dimeric IgA Abs against EGFR. Thus, J chain-containing dimeric IgA proved more effective in F(ab')-mediated killing mechanisms compared with monomeric Abs. Dimeric and monomeric IgA were similarly effective in triggering ADCC by monocytes and PMN. Interestingly, dimeric but not monomeric IgA or IgG was actively transported through an epithelial monolayer, suggesting therapeutic dimeric IgA may reach serosal surfaces. Thus, dimeric IgA may constitute a promising Ab isotype for cancer immunotherapy, which displays enhanced tumor cell killing by different modes of action and altered pharmacokinetics compared with IgG1 Abs.

Materials and Methods

Experiments reported in this article were approved by the Ethical Committee of Christian-Albrechts-University (Kiel, Germany) in accordance with the Declaration of Helsinki.

Cell lines

Human epidermoid carcinoma cell line A431 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), baby hamster kidney 21 (BHK-21) cells cotransfected with FcR (CD89) and Fcγ chain (20), and murine BaF3 cells transfected with human EGFR were all kept in RPMI 1640; Madin-Darby canine kidney (MDCK) cells (European Cell Culture Collection, Salisbury, U.K.) in MEM supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen). Selection pressure for BHK transfectants was maintained by adding 1 mg/ml genetin (PAA, Pasching, Austria) for FcR, and 20 μM methotrexate (Sigma, St. Louis, MO) for Fcγ chain, for human plgR transfected MDCK cells by adding 1 mg/ml hygromycin B (PAA); and for EGFR transfected BaF3 cells by adding 1 mg/ml genetin and 5% (v/v) supernatant of WEHI-3B (Germany Collection of Microorganisms and Cell Cultures) cells to supply IL-3.

225-IgA production and purification

225-IgA1 was produced from the variable regions of the 225 (cetuximab) Ab as described previously (25). DNA coding for the human J chain (26) was first subcloned into the pcDNA6His vector (Invitrogen) to add an epitope entry site of the encephalomyocarditis virus to enhance J chain incorporation. The vector was first subcloned into the pcDNA6His vector (Invitrogen) to add an epitope entry site of the encephalomyocarditis virus to enhance J chain incorporation. It was subsequently, gel filtration with a Superdex 200 26/600 column was performed to separate dimeric from monomeric and polymeric IgA. For analytical size exclusion chromatography, the Superdex 200 26/600 column was used. All purification steps were run on an ÄKTAprime liquid chromatography system (GE Healthcare).

UV absorbance at 280 nm, pH, and conductivity of the effluent stream were continuously recorded and analyzed using Unicorn 4.11 software (GE Healthcare). Determination of Ab concentrations and specific production rates was done as described earlier (25).

Gel electrophoresis

Purified Ab preparations were resolved by SDS-PAGE as described earlier (25). For detection of the hexa-histidine-tagged J chain, monoclonal mouse anti-Penta-His Ab was used according to manufacturer’s instructions (Qiagen). Molecular mass and purity of Ab preparations were analyzed by silver staining of proteins separated under denaturing, non-reducing conditions on 3–8% Tris-acetate gels using Roti-Black-P Kit (Roth, Karlsruhe, Germany) or by Coomassie staining of proteins separated under native conditions on 4–16% Bis-Tris gels using Native PAGE Kit (Invitrogen), both according to manufacturer’s instructions.

Flow cytometry, inhibition of epidermal growth factor binding, and EGFR downmodulation

To measure EGFR and FcγRI binding, flow cytometric analyses were done as described earlier (25). For ligand-blocking experiments, A431 cells were incubated with epidermal growth factor-Alexa Fluor 488-biotin-streptavidin complex (Invitrogen) and increasing concentrations of 225-IgA or control Abs. Downmodulation of EGFR was analyzed on murine BaF3 cells transfected with human EGFR (25), which were incubated with increasing concentrations of 225-IgA and control IgA for 4 h. Residual surface EGFR was detected with Alexa Fluor 488-labeled 425 Ab, using the Dylight Fluor Ab Labeling Kit (Pierce, Rockford, IL). Results were calculated using relative fluorescence intensities (RFI) as follows: % EGFR downmodulation = 100 × (RFI m425-FITC/RFI sample) × 100. All samples were analyzed on a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA), collecting 1 × 10⁴ events for each experiment. Data were analyzed using XL-System II V3.0 software.

Growth inhibition and induction of apoptosis assays

Growth inhibition of DiFi colon carcinoma cells was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay. Cells were seeded at 4 × 10⁴ cells/ml, and treated with serial dilutions of EGFR or control Abs. After 72 h, MTS substrate was added, and absorption at 490 nm was measured after 24 h. All experimental points were set up in triplicates, and experiments were performed at least three times. Viable cell mass in the presence of control Ab served as reference (100% cell growth) to calculate growth inhibition by EGFR Abs according to the formula: absorption (EGFR Ab)/absorption (control) × 100. To measure apoptosis induction, we seeded 5 × 10⁴ cells in 2 ml culture media in six-well plates. Control Abs were added at final concentrations of 20 μg/ml. After incubation for up to 72 h, total protein preparations of cells were collected and analyzed by bicinchoninic acid assay (Bio-Rad). A total of 15 μg proteins was resolved under denaturing reducing conditions on 4–12% Bis-Tris gels (Invitrogen). After gel electrophoresis, proteins were blotted onto polyvinylidene difluoride membranes (GE Healthcare). Poly(ADP-ribose) polymerase (PARP) cleavage was detected using a monoclonal rabbit Ab specific for full-length and cleaved human PARP, and a peroxidase-labeled polyclonal goat anti-rabbit IgG Ab (both Cell Signaling Technology, Boston, MA).

Transcytosis assay

MDCK cells were transfected with cDNA coding for the human plgR gene, which was kindly provided by Dr. Charlotte Kaetzel (University of Kentucky, Lexington, KY) (27). Expression of plgR was confirmed by immunofluorescence using mouse anti-human-plgR Ab (SPM217; GenTex, Irvine, CA). For transcytosis assays, cells were trypsinized and seeded onto 0.4-μm porous filters (Costar Corning Transwell-Clear-Filters; Corning Life Sciences, Lowell, MA). After 3 d (7 for Calu3 cells), confluence of

225-IgA was affinity-purified as described previously (25) using Capture Select Fab x chromatography media (Capture Select, Naarden, The Netherlands) and prepacked Superdex 200 26/600 columns (GE Healthcare). To separate IgA associated with the hexa-histidine-tagged J chain from IgA without J chain, we performed Ni²⁺ immobilized metal ion affinity chromatography using HisTrap HP columns (GE Healthcare) according to manufacturer’s instructions. Subsequently, gel filtration with a Superdex 200 26/600 column was performed to separate dimeric from monomeric and polymeric IgA. For analytical size exclusion chromatography, the Superdex 200 26/600 column was used. All purification steps were run on an ÄKTAprime liquid chromatography system (GE Healthcare).
monolayers was determined by light microscopy and transepithelial resistance measurement using the Millipore ERS system (Millipore, Billerica, MA). Monolayers were used for transfection experiments when transepithelial resistance was $>400 \Omega$ (1.5 kΩ for Calu3 cells) (28). Monomeric or dimeric 225-IgA or control IgA (50 μg each) was added into basolateral compartments. 225-IgG1 served as leakage control in all basolateral media. In control experiments, dimeric IgA was added into the apical medium to confirm the direction of transcytosis. Untransfected MDCK cells served as negative control. After 24 h of incubation, supernatants of both apical and basolateral compartments were collected and subjected to immunoblot analyses. Thus, 5 μl supernatants and 200 ng control proteins were loaded onto denaturing 3–8% Tris-Acetate gels (Invitrogen). After gel electrophoresis, proteins were blotted onto polyvinylidene difluoride membranes (GE Healthcare). Membranes were probed with HRP-conjugated monoclonal mouse anti-human IgA, IgG, or secretory component (SC) Abs (all Sigma), respectively.

**ADCC assays**

ADCC was measured using a $^{51}$Cr release assay as described previously (21). In brief, citrate-anticoagulated blood from healthy volunteers was layered over a discontinuous Percoll (Biochrom, Berlin, Germany) gradient consisting of 70 and 63% Percoll. After centrifugation, mononuclear cells were collected from the plasma/Percoll interface and PMN from the interface between the two Percoll layers. Flow cytometry was used to verify that monocytes were further isolated from mononuclear cells by CD14$^+$ selection using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Whole blood or isolated effectors and sensitizing Abs at various concentrations were added to round-bottom microtiter plates (Wallac, Turku, Finland). Assays were performed at 37˚C (3 h for whole blood and PMN assays, 12 h for monocytes) and in contrast with IgA2m(1), which forms covalently bound homodimers of H or L chains, respectively. These biochemical characteristics allow IgA1 Abs to be correctly analyzed also under denaturing conditions, for example, in SDS-PAGE.

**Purification and biochemical characterization of dimeric 225-IgA**

For purification of J chain-containing dimeric 225-IgA, two different affinity and one size exclusion chromatography were combined. First, a human κ-L chain-specific affinity chromatography was used to isolate human IgA Abs from serum-free cell culture supernatants. Next, immobilized metal ion affinity chromatography separated His-tagged J chain-containing molecules from monomeric IgA or from spontaneous IgA dimers without incorporated J chain. Finally, size exclusion chromatography with a preparative Superdex column served to select dimeric 225-IgA (elution volume, 128–150 ml) from higher polymeric forms (elution volume, 110–128 ml). In Supplemental Fig. 1, eluates of the different purification steps were analyzed by gel filtration and Western blots, using anti-human α-chain Abs for detection. Thus, anti-κ chromatography resulted in three fractions (Supplemental Fig. 1A), which represented polymeric, dimeric, and monomeric IgA (fraction 1), monomeric IgA (fraction 2), and α-H and L chain heterodimers (fraction 3; Supplemental Fig. 1B). After His-tag–directed affinity chromatography of fraction 1, two fractions were obtained (Supplemental Fig. 1C), which contained polymeric, dimeric, and monomeric IgA (fraction 1), and predominantly dimeric IgA (fraction 2), respectively (Supplemental Fig. 1D). When fraction 2 was reanalyzed by size exclusion chromatography (Supplemental Fig. 1E), a distinct peak was detected, which was shown to represent predominantly dimeric IgA (Supplemental Fig. 1F, lane 1). Purified monomeric IgA is shown for comparison (Supplemental Fig. 1E, lane 2).

Molecular mass and purity of J chain-containing dimeric 225-IgA were analyzed by gel electrophoresis under denaturing and native conditions using silver stain or Coomassie blue for protein detection, respectively (Fig. 1A, 1B). Both methods demonstrated the purified 225-IgA preparation to consist predominantly of a molecule of the expected molecular mass of ∼320 kDa (Fig. 1A, lane 5, 1B, lane 4). Association with the human J chain was demonstrated by staining for the introduced His-tag. Thus, Western blots and indirect immunofluorescence with EGFR-expressing A431 cells stained positive with His-tag Abs in the presence of dimeric 225-IgA (Fig. 1C, lane 4, 1D, second panel), but not in the presence of control Ab preparations. Western blots with anti-human α-H or κ-L chain Abs confirmed the correct assembly of 225-IgA (Fig. 1E, lane 4).

**Functional characterization of dimeric 225-IgA**

F(ab)–mediated effector functions of dimeric 225-IgA were analyzed. Thus, purified recombinant dimeric 225-IgA was compared with monomeric 225-IgA for binding to EGFR-expressing A431 cells (Fig. 2A). In these experiments, dimeric IgA displayed a higher avidity (Supplemental Fig. 2A, 2B) and a significantly lower EC₅₀ compared with monomeric IgA (EC₅₀: 76.16 ± 6.77 versus 335.2 ± 2.01 nM; p < 0.001), respectively. Correspondingly, dimeric 225-IgA was significantly more effective than monomeric 225-IgA in inhibiting binding of FITC-labeled epidermal growth factor to A431 cells (EC₅₀: 14.99 ± 2.64 versus 124.5 ± 19.01 nM; p < 0.01; Fig. 2B). Furthermore, dimeric 225-IgA was more potent in mediating EGFR downmodulation compared with monomeric 225-IgA (EC₅₀: 6.47 ± 2.62 versus 146.1 ± 53.13 nM; p < 0.01; Fig. 2C). In addition, growth of EGFR-expressing DiFi colon carcinoma cells was inhibited at significantly lower concentrations by dimeric than by monomeric 225-IgA (EC₅₀: 6.21 ± 0.89 versus 49.77 ± 0.44 nM; p < 0.01;
In classical transcytosis assays (Fig. 4A), as expected, only J chain-containing dimeric 225-IgA demonstrated concentration-dependent binding with an EC_{50} of 5.9 µg/ml, whereas monomeric 225-IgA and a control IgA did not bind to pIgR. A dimeric IgA preparation, which was separated by size exclusion chromatography from a non-J chain-expressing transfectedoma, did not bind to pIgR, confirming the requirement of J chain for pIgR binding.

In classical transcytosis assays (Fig. 4B), media from the basolateral and apical compartments were analyzed by Western blot after the indicated time periods for the absence or presence of SC, IgG, or IgA. In Fig. 4B, the left panel represents the staining controls, whereas the right panel was obtained from non-transfected MDCK cells. 225-IgG was added to the basolateral compartment, whereas the right panel was obtained from non-transfected MDCK cells. 225-IgG was added to the basolateral compartment.
After 24 h (t24), dimeric IgA was quantitatively transported from the basolateral to the apical compartment (↑), whereas no transport in the other direction (↓) was observed. Interestingly, transported dimeric 225-IgA demonstrated the expected shift in molecular mass and stained positive for SC. Furthermore, secretory 225-IgA recovered from the apical compartment was capable to mediate growth inhibition of DiFi cells (Fig. 4C). As expected, monomeric IgA was not transcytosed.

In addition, we performed transcytosis assays with human Calu3 cells (Supplemental Fig. 3A), which endogenously express human pIgR and low levels of EGFR (Supplemental Fig. 3B). Also in this model, selective and time-dependent transport of dimeric 225-IgA was observed from the basolateral to the apical compartment (↑), whereas no transport in the other direction (↓) was observed. Interestingly, transported dimeric 225-IgA demonstrated the expected shift in molecular mass and stained positive for SC. Furthermore, secretory 225-IgA recovered from the apical compartment was capable to mediate growth inhibition of DiFi cells (Fig. 4C). As expected, monomeric IgA was not transcytosed.

Discussion

IgA Abs for immunotherapy

In this article, we describe effector functions of a recombinant J chain-containing dimeric IgA Ab against EGFR and compare its efficacy with monomeric IgA and IgG1 Abs. Dimeric and secretory IgA have been demonstrated to play an important role in the protection of serosal surfaces (2), and generation of an IgA-mediated immune response has been recognized as an important aim for many vaccine studies (13). However, passive immunotherapy with IgA Abs has hardly been explored, although Ab therapy with IgG Abs is clinically successful in many different diseases (15). Several potential reasons can be identified to explain the slow progress of clinical development of IgA Abs: production and purification are still considered more difficult for IgA than for...
and monomeric 225-IgA are marked by number signs. Indirect immunofluorescence. Recombinant dimeric 225-IgA and a commercial IgA standard demonstrated similar binding to Fc receptors. Differences between relevant and control Abs (225-IgG in monomeric 225-IgA, but not with control IgA or 225-IgG. Data represent means of five independent experiments. C, Isolated human PMN were investigated as effector cells in ADCC assays against A431 tumor cells using varying Ab concentrations. Monomeric and dimeric 225-IgA were significantly more effective in recruiting PMN than 225-IgG. D, Unseparated human whole blood served as effector source in whole-blood ADCC assays. Significant ADCC was observed in the presence of increasing concentrations of dimeric and monomeric 225-IgA, but not with control IgA or 225-IgG. Data represent means ± SEM of three independent experiments. Asterisks indicate significant differences between relevant and control Abs (225-IgG in A and control IgA in B–D, respectively). Significant differences in EC50 between dimeric 225-IgA and monomeric 225-IgA are marked by number signs.

IgG Abs, although the generation of recombinant monomeric and dimeric IgA has been described (26, 29, 30). In addition, IgA Abs are heavily glycosylated (31), which poses challenges on a reproducible and stable production technology. Because of the lack of FcRn binding, IgA has a shorter serum half-life compared with IgG. Serum t1/2 was critical for the efficacy of IgG Abs (32) but may be less relevant for IgA Abs, which display altered pharmacokinetics and may reach tumors via alternative routes. Furthermore, mice do not express an FcαRI homolog (5), which makes the establishment of relevant small animal models more complicated. Consequently, no IgA Ab has obtained regulatory approval, adding to the business risks of their development. Recently, we described the generation of recombinant monomeric IgA, using production and purification technologies that are similar to those that are well established for IgG Abs (26). In this article, we extend these studies to the production of recombinant human J chain-containing IgA dimers. As target Ag we selected the EGFR, one of the most extensively studied target Ags in oncology against which an IgG1 and an IgG2 Ab have obtained FDA approval (15, 16). However, additional studies are required to determine the characteristics of an optimized IgA Ab for clinical application (e.g., monomeric versus dimeric IgA, selection of IgA1 or one of three IgA2 isoforms, impact of Ab glycosylation and protease resistance).

Role of the Ab valency: F(ab)- and Fc-mediated effects of dimeric IgA

Dimeric IgA consists of two monomeric IgA molecules connected by the J chain, forming a near-planar structured molecule (33). The resulting dimer consists of four F(ab) and two Fc domains, and therefore has four potential Ag binding sites. Thus, dimeric IgA is expected to cross-link tumor target Ags more effectively than monomeric IgA or IgG Abs. Consequently, dimeric IgA was more effective than monomeric IgA in EGFR binding, receptor blockade, receptor downmodulation, and growth inhibition (Fig. 2A–D), particularly at lower Ab concentrations. At saturating concentrations, both Ab preparations were similarly effective, which is probably explained by steric hindrance leading to bivalent or monovalent binding of dimeric IgA. This later mechanism may also explain similar efficacy of dimeric and monomeric IgA in PARP cleavage (Fig. 2E), because this assay was performed at saturating Ab concentrations. Results from our experiments with dimeric IgA are in agreement with studies in which artificially dimerized IgG Abs or tetravalent human CD22 Abs demonstrated enhanced tumor cell killing compared with wild-type Abs (11, 12).

In ADCC assays with isolated monocytes or PMN, both dimeric and monomeric 225-IgAs were similarly effective, indicating that the presence of human J chain does not interfere with IgA binding to FcαRI. These functional studies are supported by structural data demonstrating that J chain does not interfere with the FcαRI binding site (34). Unexpectedly, dimeric IgA appeared to be more effective than monomeric IgA in whole-blood assays. Interestingly, IgA was also reported to mediate inhibitory activity (35), which we did not observe under our assay conditions.

Increased valency for FcRs may explain the enhanced ADCC activity of IgA compared with IgG Abs; in contrast with IgG Abs, where one Ab Fc part binds to one FcγR, monomeric IgA interacts

FIGURE 3. Fc-mediated effector functions. A, Binding of the indicated Abs to FcαRI/Fcγ-AP-transfected BHK-21 cells was determined by indirect immunofluorescence. Recombinant dimeric 225-IgA and a commercial IgA standard demonstrated similar binding to FcαRI, whereas binding of monomeric 225-IgA was less effective and 225-IgG failed to bind. Results of three experiments are displayed as mean ± SEM of RFI. B, Isolated human monocytes served as effector cells in ADCC assays against EGFR-expressing A431 tumor cells. Monomeric and dimeric 225-IgA and 225-IgG Abs mediated all similar tumor cell killing, whereas a control IgA1 Ab was not effective. Results are shown as mean ± SEM of “% specific lysis” obtained from five independent experiments. C, Isolated human PMN were investigated as effector cells in ADCC assays against A431 tumor cells using varying Ab concentrations. Monomeric and dimeric 225-IgA were significantly more effective in recruiting PMN than 225-IgG. D, Unseparated human whole blood served as effector source in whole-blood ADCC assays. Significant ADCC was observed in the presence of increasing concentrations of dimeric and monomeric 225-IgA, but not with control IgA or 225-IgG. Data represent means ± SEM of three independent experiments. Asterisks indicate significant differences between relevant and control Abs (225-IgG in A and control IgA in B–D, respectively). Significant differences in EC50 between dimeric 225-IgA and monomeric 225-IgA are marked by number signs.

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Increased valency for FcRs may explain the enhanced ADCC activity of IgA compared with IgG Abs; in contrast with IgG Abs, where one Ab Fc part binds to one FcγR, monomeric IgA interacts
FIGURE 4. Transcytosis of dimeric IgA. A, Binding of monomeric (mIgA) and dimeric 225-IgA (dIgA) to pIgR+ MDCK cells was analyzed by indirect immunofluorescence using an anti-human κ-λ chain-specific FITC-labeled Ab for detection. Only J chain-containing dimeric (225-IgA+J), but not monomeric or dimeric IgA without J chain (225-IgA-J), demonstrated concentration-dependent binding to pIgR-expressing MDCK cells. Results are presented as mean RFI ± SEM obtained from three independent experiments. B, Transcytosis of mIgA and dIgA through a confluent monolayer of human pIgR-transfected MDCK cells (MDCK-plgR+) was analyzed by Western blot experiments using SC, IgG-, or IgA-specific Abs for detection. 225-IgG, added to the basolateral compartment, served as leakage control and did not enter the apical compartment. As demonstrated, mIgA was retained in the basolateral compartment, whereas dIgA was transcytosed from the basolateral to the apical compartment within 24 h. This transport was unidirectional because dIgA was not transported from the apical to the basolateral compartment. Untransfected MDCK cells did not transport dIgA. After transcytosis, dIgA stained positive for SC and demonstrated a higher molecular mass compared with dIgA, demonstrating in vitro generation of secretory 225-IgA. C, Supernatants from apical wells of transcytosis assays from B were used in growth inhibition assays with DiFi cells. Only dimeric IgA transported by pIgR-transfected MDCK cells (dIgA ↑ plgR+) mediated growth inhibition of DiFi cells, whereas apical supernatants from wells with monomeric 225-IgA (mIgA ↑ plgR−), dIgA in the absence of plgR (dIgA1 ↑ plgR−), or MDCK medium alone (medium control) did not mediate growth inhibition.

with FcαRI in a 1:2 stoichiometry (36). Thus, dimeric IgA could theoretically bind to four FcαRI molecules simultaneously, thereby activating and stimulating effector cells more efficiently than monomeric IgA. This tetravalency could be a possible advantage for dimeric compared with monomeric IgA or IgG Abs.

Contribution of ADCC to the effector mechanisms of therapeutic Abs

Functional studies revealed that monomeric IgA Abs recruit a different spectrum of effector mechanisms compared with IgG Abs (21). In addition to direct effector mechanisms such as blockade of growth factor-induced signaling and induction of growth inhibition or apoptosis (37), ADCC is increasingly recognized as an important mechanism of action for therapeutic cancer Abs (38). For example, tumor-directed Abs lost their therapeutic efficacy in animals with disrupted FcαRI signaling (39). Because human IgG1 has been demonstrated to optimally recruit NK cells as effector cells for ADCC (17), this isotype has been chosen for most cancer Abs. However, also, human IgG2 Abs, such as panitumumab, trigger ADCC, although not by NK cells but by myeloid cells such as monocytes and PMN (40). Currently, it is unclear which effector cell type is most relevant for the therapeutic efficacy of Abs. Because most preclinical investigations used human IgG1 Abs, which preferentially interact with NK cell-expressed FcγRIIa, results from these studies were in favor of NK cells (41). These observations triggered substantial efforts to enhance IgG binding to NK cell-expressed FcγRIIa (42). However, syngenic animal models also demonstrated a significant contribution of phagocytic myeloid cells (22), including monocytes and tissue macrophages (43), as well as granulocytes (24). Clinical evidence for the potential relevance of myeloid cells is derived from the repeated observation that the functionally relevant 131H/R polymorphism of the myeloid cell-expressed FcγRIIa receptor significantly affects response to therapeutic Abs against different target Ags such as CD20 (44), human EGFR 2 (45), and EGFR (46). As demonstrated in Fig. 3, 225-IgA was as effective in recruiting monocytes and significantly more effective in recruiting PMN than human IgG1, suggesting that IgG Abs may be therapeutic in vivo.

Pharmacokinetics of IgA versus IgG Abs

In addition to preferentially recruiting myeloid instead of NK cells, IgA Abs display different pharmacokinetics compared with IgG Abs. IgG Abs bind to FcRn, which prevents lysosomal degradation, and thereby extends the serum t1/2 of IgG (47). Mono- meric IgA does not bind to FcRn and, therefore, has a reduced serum t1/2 of approximately 7 d (47). In contrast, dimeric IgA, but not monomeric IgA or IgG, binds to the plgR, which transports dimeric IgA from the basolateral to the apical compartment of epithelia (8), thereby dimeric IgA is transported into secretions where it exerts important immunological functions (2). Whether epithelial cancers can be effectively attacked from their luminal side requires further investigation, but our results (Fig. 4C) demonstrate that transported dimeric IgA is biologically active.

Monomeric IgA and IgG1 Abs reach their target Ags via the tumor vasculature. Physiologically, dimeric IgA is produced by B cells in the lamina propria, which is in close proximity to the basolateral side of epithelial cells (48). After J chain-mediated binding to plgR, dimeric IgA is then transported through the epithelial cells to the luminal surface (7). Murine models were used to investigate whether systemically administered dimeric IgA was able to cross the epithelial barrier. For example, i.v. applied or
backpack hybridoma-produced dimeric IgA was detected on mucosal surfaces and neutralized their respective target Ags (49–51). Whether i.v. applied dimeric IgA would also reach basolateral epithelial sites in humans and then be transported to the mucosal surface is an unresolved question. As demonstrated in Fig. 4 and previously by others (7), transcytosis is an efficient process. Thus, it can be expected that dimeric IgA reaching the basolateral compartment will be quantitatively transported to the luminal side.

In conclusion, monomeric IgA has been demonstrated to effectively recruit phagocytic cells for tumor cell killing (20–22). In this article, we demonstrate that IgA-containing dimeric IgA is as least as effective in triggering ADCC as monomeric IgA. Importantly, dimeric IgA is more effective than monomeric Abs in direct effector mechanisms, such as EGFR blockade, EGFR down-modulation, and growth inhibition. Furthermore, dimeric IgA is actively transported through an epithelial monolayer. Together, these results demonstrate that recombinant dimeric IgA triggers important biological functions and constitutes an interesting Ab format for tumor therapy.

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