Dual Mode of Action of a Human Anti-Epidermal Growth Factor Receptor Monoclonal Antibody for Cancer Therapy


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Dual Mode of Action of a Human Anti-Epidermal Growth Factor Receptor Monoclonal Antibody for Cancer Therapy

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Epidermal growth factor receptor (EGF-R) overexpression is common in a large number of solid tumors and represents a negative prognostic indicator. Overexpression of EGF-R is strongly tumor associated, and this tyrosine kinase type receptor is considered an attractive target for Ab therapy. In this study, we describe the evaluation of mAb 2F8, a high avidity human mAb (IgG1κ) directed against EGF-R, developed using human Ig transgenic mice. mAb 2F8 effectively blocked binding of EGF and TGF-α to the EGF-R. At saturating concentrations, 2F8 completely blocked EGF-R signaling and inhibited the in vitro proliferation of EGF-R-overexpressing A431 cells. At much lower concentrations, associated with low receptor occupancy, 2F8 induced efficient Ab-dependent cell-mediated cytotoxicity (ADCC) in vitro. In vivo studies showed potent antitumor effects in models with A431 tumor xenografts in athymic mice. Ex vivo analysis of the EGF-R status in tumor xenografts in 2F8-treated mice revealed that there are two therapeutic mechanisms. First, blocking of EGF-R signaling, which is most effective at complete receptor saturation and therefore requires a relatively high Ab dose. Second, at very low 2F8 receptor occupancy, we observed potent antitumor effects in mice, which are likely based on the engagement of immune effector mechanisms, in particular ADCC. Taken together, our findings indicate that ADCC represents an important effector mechanism of this Ab, which is effective at relatively low dose. The Journal of Immunology, 2004, 173: 4699–4707.

The epidermal growth factor receptor (EGF-R) represents a 170-kDa type 1 transmembrane receptor overexpressed in a large number of human tumors, including carcinomas of the head and neck, breast, colon, prostate, lung, and ovaries. The degree of overexpression has been correlated with a poor clinical prognosis (1, 2). Expression of EGF-R is frequently accompanied by the production of EGF-R-ligands such as TGF-α, or EGF by tumor cells, suggesting that an autocrine loop participates in malignant transformation. Therefore, blocking the interaction between EGF-R and its ligands may affect tumor growth and possibly tumor cell survival (3). There is a growing interest in EGF-R-targeted anticancer strategies for treatment of various cancers, including nonsmall-cell lung cancer, head and neck cancer, colon cancer, and pancreas cancer. The two main classes of compounds that are currently tested in clinical trials are the small molecule tyrosine kinase inhibitors and mAb (4).

Different working mechanisms are supposed to operate in mAb-mediated immunotherapy of cancer. In the 1980s, most investigators accepted that opsonization of cancer cells and subsequent activation of immune effector mechanisms was the main mechanism leading to depletion of cancer cells. However, it is becoming increasingly clear that anti-cancer mAb are particularly effective when they interfere with, or alter, cellular signaling (5, 6). mAbs directed to the EGF-R ligand-binding domain might, potentially, block the interaction with EGF, TGF-α, and concomitantly modulate its signaling properties. A number of EGF-R-blocking mAb have been tested in vitro, and in murine xenograft models (7–9). Murine EGF-R-blocking mAb such as 225 and 528 could prevent tumor formation in athymic mice (10, 11) and coadministration of chemotherapeutic agents improved tumor eradication (12, 13), which established EGF-R as a promising target for Ab therapy. However, murine mAb do not constitute ideal therapeutic agents, as repeated treatment with these molecules frequently triggers allergic reactions and human anti-mouse Ab responses in patients (14, 15). This has led to the development of humanized mAb. One of these is IMC-C225, a chimeric version of the murine 225 mAb, in which the murine Ab variable regions were linked to human IgG constant regions (16). To minimize immunogenicity, ideally human Abs should be used for immunotherapy (17). Yang et al. (18) reported the development of mAb E7.6.3, a human IgG2 mAb generated using human transgenic mice. We used transgenic mice to generate fully human IgG1κ anti-EGF-R mAb. These are genetically engineered mice in which the gene clusters encoding murine Ab H and L chain genes have been inactivated, resulting in absence of murine Abs. Subsequently, DNA segments containing large parts of the human H and (κ) L chain gene clusters were introduced. The human DNA segments are fully functional, and undergo isotype switching and affinity maturation. These mice can be immunized with human target proteins and produce high avidity Abs in a way analogous to wild-type mice (19–21).

In this study, we present the in vitro and in vivo characterization of mAb 2F8 that was selected from a panel of anti-EGF-R Abs for further development based on performance in functional in vitro assays. The studies focus on the dose-effect relationships to dissect...
the importance of different potential therapeutic mechanisms, acting at different Ab concentrations.

Materials and Methods

Cell lines

A431 cells, an EGF-R overexpressing epidermoid cancer cell line, were from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany; cell line number ACC 91). BxPC-3 cells (pancreas cancer) and MDA-MB-468 cells (breast cancer) were from the American Type Culture Collection (ATCC, Manassas, VA). HN5 cells (head and neck cancer, LICR-LON-HNS) were a kind gift from Dr. C. J. Dean (Institute of Cancer Research, Sutton, Surrey, U.K.). SK-RC-29 cells (kidney) were a kind gift from Dr. E. Oosterwijk (University Hospital Nijmegen, Nijmegen, The Netherlands). Cells were cultured in RPMI 1640 medium (BioWhittaker, Verviers, Belgium), supplemented with 10% heat-inactivated FCS (Wisent, St-Bruno, Canada), 50 IU/ml penicillin, 50 μg/ml streptomycin, and, for BxPC-3, 2 mM L-glutamine. Cells were detached by treatment with trypsin-EDTA in PBS. For in vivo tumor studies, cells were always used in log phase, and tested for EGF-R expression and potential mycoplasma contamination before each experiment.

Antibodies

Human IgG1 anti-EGF-R mAb were generated by immunizing HuMAb mice (Medarex, Milpitas, CA) (20) with alternating A431 cells and purified EGF-R (Sigma-Aldrich, St. Louis, MO) administration. Of 46 anti-EGF-R mAbs, 6 were able to block the interaction between EGF-R and its ligands EGF and TGF-α. mAb 2F8 was selected for its potency to block the interaction between EGF-R and its ligands, EGF and TGF-α. The Abs were purified using protein A affinity chromatography, followed by size exclusion chromatography on an HR 200 column (Pharmacia, Peapack, NJ), and were formulated in PBS containing Tween 80 and mannitol. 2F8 Fab were made using papain digestion. M225, a murine IgG1 anti-EGF-R mAb, was produced using hybridoma cells obtained from ATCC (no. HB-8508). Polyclonal human IgG (hIgG), purified from single donor plasma, was used as control.

ELISA

2FS concentrations were determined using an ELISA in which purified EGF-R (Sigma-Aldrich) was coated to 96-well Microlon ELISA plates (Greiner Bioscience, Frickenhausen, Germany), 50 ng/well. After blocking plates with ELISA buffer (PBS supplemented with 0.05% Tween 20 and 2% chicken serum), samples, serially diluted in ELISA buffer, were added and incubated for 1 h at room temperature (RT). Plates were subsequently incubated with peroxidase-labeled goat anti-human IgG Fc-specific Ig (Jackson Immunoresearch Laboratories, West Grove, PA) and developed with ABTS (Roche, Mannheim, Germany). Absorbance was measured in a microplate reader (Bio-Tek Instruments, Winooski, VT) at 405 nm. For binding studies of 2F8 and 2F8-Fab, the same ELISA was used, with the exception of peroxidase-conjugated rabbit-anti-human κ chain (Dako-Cytomation, Glostrup, Denmark) as detecting Ab.

Flow cytometry

A431 cells were incubated with serial dilutions of mAb in FACS buffer (PBS supplemented with 0.05% BSA and 0.02% sodium azide), for 30 min at 4°C. Subsequently, cells were washed and incubated in the dark with FITC-conjugated F(ab)2, as secondary Abs: goat anti-human κ chain-FITC (BD Biosciences, Aalst, Belgium) or goat anti-mouse IgG-FITC (Protos/De Beer, Burlingame, CA), for human and mouse mAb, respectively. Samples were analyzed on a FACS Calibur (BD Biosciences).

Cell ELISA for assessing inhibition of ligand binding

A431 cells were cultured overnight in 96-well flat-bottom plates, 104 cells per well. The attached cells were gently washed with ice-cold culture medium and incubated for 30 min at RT with 3-fold serial dilutions of intact anti-EGF-R mAbs or with unlabeled EGF (Molecular Probes, Leiden, The Netherlands). Thereafter, 20 μl of biotin-conjugated EGF (Molecular Probes) was added at a final concentration of 25 ng/ml, and cells were further incubated for 1 h at RT. Plates were washed and fixed with 0.5% glutaraldehyde in PBS for 10 min at RT. Thereafter, wells were washed and blocked with PBS supplemented with 50 mM glycine, 1% BSA, and 0.05% Tween 20 in PBS, for 30 min at RT. Wells were incubated with HRP-conjugated streptavidin (DakoCytomation) for 1 h at RT. The assays were developed with freshly prepared ABTS solution for 30 min at RT in the dark. Absorbance was measured at 405 nm.

EGF-R phosphorylation

A431 cells were dispensed in 24-well plates (Greiner Bioscience), 5 × 104 cells/well, in culture medium. After 24 h, cells were replenished with starvation medium (culture medium containing only 0.5% FCS) and were further incubated overnight at 37°C and 5% CO2. Subsequently, serum-deprived subconfluent cells were preincubated for 30 min with various concentrations of Abs. Then, EGF (Molecular Probes) or TGF-α (PeproTech, Rocky Hill, NJ) was added to the wells at a concentration of 20 ng/ml, and cells were incubated for 15 min at 37°C, 5% CO2. Cells were put on ice to stop the ligand-induced stimulation and were washed twice with ice-cold PBS. Cells were dissolved in 1 × reducing SDS-PAGE sample buffer, 100°C, and cell lysates were collected in vials and stored at −20°C until further assessment for the presence of phosphorylated EGF-R and total EGF-R by immunoblotting.

Immunoblotting

Cell lysate samples were diluted in SDS-sample buffer and boiled for 5 min at 100°C and subjected to electrophoresis on a SDS-PAGE 4–15% acrylamide gel (Criterion Precast gels 4–15%; Bio-Rad, Hercules, CA). Proteins were transferred to the nitrocellulose membrane by blotting using a blot buffer containing 20% methanol. All conditions and buffers were as recommended by the manufacturer. Blots were processed for immunostaining with rabbit anti-phosphotyrosine (Upstate, Lake Placid, NY) and rabbit anti-EGF-R Abs (Cell Signaling Technology). In short, blots were incubated for 1 h at RT with blocking buffer (TBS, supplemented with 0.1% Tween 20 and 5% Top-Block; Fluka, Zwijndrecht, The Netherlands). Thereafter, blots were washed and incubated with primary Abs 1/1000 diluted in blocking buffer at 4°C overnight. Blots were washed and incubated for 1 h at RT with HRP-conjugated goat anti-rabbit IgG Ab (Cell Signaling Technology) 1/2000 diluted in blocking buffer. Blots were washed and incubated with 10 ml of chemiluminiscent detection substrate solution (Super Signal West Dura Extended Duration Substrate; Pierce, Rockford, IL) for 5 min. Thereafter, blots were placed between two sheet-protection sheets and exposed in GeneGnome Imager (Syngene, Frederick, MD).

Cell growth inhibition

Tumor cell growth in vitro was evaluated by measuring vital cell mass using a MTT assay. The MTT assay involves the intracellular reduction of the water soluble MTT to an insoluble formazan. Anti-EGF-R Ab dilutions were added to cell cultures in 96-well flat-bottom tissue culture plates (2000 cells/well). Plates were incubated at 37°C, and 10 μl of MTT-labeling solution 1 (Roche) was added either directly (t = 0 days) or after 96 h (t = 4 days) of incubation. Four hours after addition of MTT-labeling solution 1, the formed insoluble MTT was solubilized by adding 100 μl of MTT solubilization buffer (Roche). After overnight incubation at 37°C, the absorbance was measured at OD 562 nm, reference 630 nm in a microplate reader.

Complement-dependent cytotoxicity (CDC)

A431 cells were dispensed in 96-well V-bottom plates (Greiner Bioscience), 1 × 103 cells/well, in RPMI 1640, supplemented with 1% BSA and 0.02% azide. Cells were incubated with various concentrations of anti-EGF-R Abs at 4°C for 30 min. Subsequently, human serum was added and cells were incubated at 37°C for another 20 min. Cells were washed thrice with ice-cold FACS buffer and cells were incubated with 50 μl of FITC-conjugated rabbit anti-human C3c (DakoCytomation) in FACS buffer at 4°C in the dark for 30 min. Cells were washed thrice and resuspended in 300 μl of FACS buffer. Just before analysis on a FACSCalibur, 3 μl of To-PRO3 iodide (Molecular Probes, Leiden, The Netherlands), 1/4000 diluted in DMSO, was added for detection of cell death.

Isolation of PBMC from blood

BUFFY COATS FROM STANDARD BLOOD DONATIONS (SANQUIN BLOOD BANK, Utrecht, The Netherlands) were diluted by adding PBS and were carefully placed on top of 10 ml of Lymphocyte Separation Medium (BioWhittaker) in 50-ml tubes. Tubes were centrifuged at 800 × g for 20 min at RT. Thereafter, the PBMC were recovered from the plasma-medium interface and were washed several times with RPMI 1640 (BioWhittaker) until the supernatant was clear.

ADCC-dependent cell-mediated cytotoxicity (ADCC)

The capacity of mAb to induce effector cell-dependent lysis of tumor cells was evaluated in 3Cr-release assay. Target cells (2–5 × 105 cells) were labeled with 100 μCi Na235CrO4 (Amersham Biosciences, Uppsala, Sweden) under shaking conditions at 37°C for 1 h. Cells were washed thrice...
with PBS and were reseeded in culture medium \((1 \times 10^5 \text{cells/ml})\). Labeled cells were dispensed in 96-well plates \((5 \times 10^3, \text{in 50} \mu\text{l/well})\) and preincubated (RT, 30 min) with 50 \mu\text{l} of 10-fold serial dilutions of mAb 2F8 or M225 in culture medium, ranging from 20 ng/ml to 0.02 ng/ml (final concentrations). Culture medium was added instead of Ab to determine spontaneous \(^{51}\text{Cr} \text{release}\). Triton X-100 (1% final concentration) was added to determine the maximal \(^{51}\text{Cr} \text{release}\). Thereafter, PBMC were added to the wells \((5 \times 10^3/\text{well})\) and cells were incubated at 37°C overnight. The next day, supernatants were collected for measurement of the \(^{51}\text{Cr} \text{release}\) by determination of the cpm in a gamma counter. Percentage of cellular cytotoxicity was calculated using the following formula: 

\[
\text{specific lysis} = \frac{(\text{experimental release (cpm)} - \text{spontaneous release (cpm)})}{\text{(maximal release (cpm)} - \text{spontaneous release (cpm)})} \times 100
\]

For comparison, showed similar binding characteristics (Fig. 1).

**Immunohistochemistry**

Cryocut sections from frozen tumor xenograft biopsies were fixated with acetone. Endogenous peroxidase and endogenous biotin activities were blocked, as well as aspecific binding sites. Sections were stained for the presence of 2F8 using consecutive incubations with rabbit-anti-human IgG \(\gamma\)-chain (DakoCytomation). Staining for EGF-R was done with the same Ab after preincubation of the sections with a saturating concentration of 2F8 (10 \mu\text{g/ml}) and PBS (100 \mu\text{g/ml}) in PBS, 0.5% deoxycholate, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM NaPO\(_4\), 2 mM Na\(_3\)VO\(_4\), protease inhibitor mixture tablet (Roche), 1 mM PMSF added before use. EGF-R protein was immunoprecipitated from the precleared lysate with Sepharose 4B-conjugated 528 mAb, a mouse anti-EGF-R mAb, which does not compete with 2F8. Precipitated protein was solubilized in reducing sample buffer and run on SDS-PAGE as described above. Blots were stained for total EGF-R protein amount and phosphorylated EGF-R with monoclonal rabbit-anti-EGF-R and mouse-anti-phosphorylated tyrosine (P-Tyr-100; Cell Signaling Technology), respectively, and for human IgG with peroxidase-conjugated goat anti-human-IgG. Mouse-anti-rabbit-IgG and goat-anti-mouse-IgG Abs were used as secondary Abs. Chemiluminescent detection was performed as described above.

*Mouse tumor xenograft models*

Nude BALB/c mice \((nu/nu)\) were purchased either from Harlan (Horst, The Netherlands) or from Charles River Laboratories (Maastricht, The Netherlands) and all experiments were performed with 8–12 wk old female mice. Mice were housed in a barrier unit of the Central Laboratory Animal Facility \((

Utrecht, The Netherlands)\) and kept in filter-top cages with water and food provided ad libitum. All experiments were approved by the Utrecht University animal ethics committee.

Mice participating in experiments were checked thrice a week for signs of tumor growth including level of activity, skin abnormalities, diarrhea, and general appearance. We used a well-established s.c. tumor xenograft model using EGF-R-overexpressing human tumor cell lines \((9)\). Briefly, tumor cells were inoculated in the right flank of mice \((3–5 \times 10^6 \text{cells})\). Tumors grew uniformly and could easily be measured by Vernier calipers. Tumor volumes were scored as length \(\times\) width \(\times\) height \((\text{in } \text{mm}^3)\). mAbs were injected i.p. according to the study protocol. At the end of part of the experiments, tumors were excised for subsequent analysis of cells for EGF-R expression, EGF-R phosphorylation, and occupancy of EGF-R by 2F8. Part of the tumor was immersed in ice-cold FACS buffer, and cells were isolated by passage through a cell strainer, and stained with anti-IgG-ITC (with and without preincubation with saturating concentrations of 2F8) for subsequent analysis by flow cytometry. Other parts were snap frozen for immunoblotting or immersed in TissueTek and snap frozen in liquid nitrogen for subsequent immunohistochemical analysis. Furthermore, heparinized blood samples were drawn from the retro-orbital plexus for determination of plasma 2F8 concentrations.

**Immunoblotting**

For analysis of the EGF-R status of tumor xenografts, biopsies were first homogenized in lysis buffer containing: 100 mM NaCl, 10 mM Tris (pH 7.4), 150 mM NaCl, 0.5% SDS, 0.5% deoxycholate, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM NaPO\(_4\), 2 mM Na\(_3\)VO\(_4\), protease inhibitor mixture tablet \((\text{Roche})\), 1 mM PMSF added before use. EGF-R protein was immunoprecipitated from the precleared lysate with Sepharose 4B-conjugated 528 mAb, a mouse anti-EGF-R mAb, which does not compete with 2F8. Precipitated protein was solubilized in reducing sample buffer and run on SDS-PAGE as described above. Blots were stained for total EGF-R protein amount and phosphorylated EGF-R with monoclonal rabbit-anti-EGF-R and mouse-anti-phosphorylated tyrosine (P-Tyr-100; Cell Signaling Technology), respectively, and for human IgG with peroxidase-conjugated goat anti-human-IgG. Mouse-anti-rabbit-IgG and goat-anti-mouse-IgG Abs were used as secondary Abs. Chemiluminescent detection was performed as described above.

**Statistical analysis**

Data analysis was performed using GraphPad PRISM vs 3.02 \((\text{GraphPad, San Diego, CA})\), unless indicated otherwise. Binding curves were analyzed by fitting sigmoidal curves with variable slope using nonlinear regression. Group data were reported as mean ± SD or SEM. Differences between groups were analyzed using ANOVA. Levels of significance were indicated. Significance was accepted at the \(p < 0.05\) level.

**Results**

**Binding characteristics**

First, we investigated binding using flow cytometric analysis of EGF-R-overexpressing A431 cells. mAb 2F8 was found to bind to membrane-associated EGF-R with an EC\(_{50}\) of \(-1 \mu\text{g/ml} \text{(7 nM)}\). In comparison, we observed a similar avidity for mouse mAb M225, an EC\(_{50}\) of 0.5 \mu\text{g/ml} \text{(4 nM)}. Next, we evaluated whether mAb binding to the receptor leads to effective inhibition of ligand binding. To this end, subconfluent monolayers of A431 cells were preincubated with 2F8, 2F8 Fab, mouse anti-EGF-R mAb M225, control IgG, or unlabeled EGF, in concentrations ranging from 0.3 to 20 \mu\text{g/ml} for the Abs and 10 times lower for EGF. Fig. 1 shows the inhibition of binding of biotinylated EGF by 2F8. The concentration of half-maximal inhibition \((\text{IC}_{50})\) was calculated to be \(-2 \mu\text{g/ml} \text{for intact 2F8} \text{(14 nM)}\). Table I gives an overview of the binding parameters for 2F8, showing that the EC\(_{50}\) and IC\(_{50}\) values of intact 2F8 were about three times lower than that of Fab. mAb 2F8 may thus bind at least partly bivalently to the cells. The mouse IgG1 mAb M225 against EGF-R, which was evaluated for comparison, showed similar binding characteristics (Fig. 1).

**Table I. Summary of EC\(_{50}\) and IC\(_{50}\) values of mAb 2F8 from three distinct experiments**

<table>
<thead>
<tr>
<th>Assay</th>
<th>2F8 in Nanomolar Concentration (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2F8 binding to A431 cells (FACS)</td>
<td>EC(_{50}) 7 ± 2</td>
</tr>
<tr>
<td>2F8-Fab binding to A431 cells (FACS)</td>
<td>EC(_{50}) 20 ± 3</td>
</tr>
<tr>
<td>2F8 inhibition of EGF binding to A431 cells (ELISA)</td>
<td>IC(_{50}) 14 ± 1</td>
</tr>
<tr>
<td>2F8-Fab inhibition of EGF binding to A431 cells (ELISA)</td>
<td>IC(_{50}) 35 ± 7</td>
</tr>
</tbody>
</table>
Inhibition of EGF-R signaling and cell proliferation

Blockade of ligand binding is expected to prevent receptor activation, and therefore, we evaluated the ability of mAb 2F8 to block ligand-induced receptor phosphorylation using immunoblotting. When A431 cells were incubated with increasing concentrations of TGF-α, effective EGF-R phosphorylation was found to occur at TGF-α concentrations above 5 ng/ml, with maximum stimulation at 50 ng/ml. Preincubation of cells with 2F8 at a saturating concentration of 20 μg/ml prevented EGF-R-Tyr1068 phosphorylation induced by subsequent addition of TGF-α up to 50 ng/ml, without decreasing the total amount of EGF-R. Fig. 2 shows the concentration-effect relationship for inhibition of TGF-α or EGF-induced (20 ng/ml) phosphorylation. Inhibition was detectable at 0.2 μg/ml (1.3 x 10^-9 M), and maximum inhibition was reached at 20 μg/ml (1.3 x 10^-7 M). This closely corresponds to the concentrations needed for inhibition of ligand binding as shown in Fig. 1.

Stimulation of EGF-R by its natural ligands enhances cell proliferation, and this also occurs in EGF-R-overexpressing A431 cells under in vitro culture conditions. In the present study, the capacity of 2F8 to inhibit proliferation of A431 cells was determined in vitro using an MTT assay. A431 cells were cultured in the presence of various concentrations of 2F8 or M225. Measurement of the vital cell mass after 4 days of culture demonstrated that proliferation is inhibited in a concentration-dependent manner (Fig. 3), with a maximum inhibition of ~50%. For both Abs tested, the IC50 values for inhibition of cell proliferation were ~10^-8 M (1.5 μg/ml), which is similar to the IC50 values for inhibition of EGF binding to EGF-R-expressing A431 cells. We also assessed proliferation inhibition of other EGF-R-overexpressing cell lines. Proliferation of HN5, MDA-MB-468, and BxPC-3 cells was inhibited by 80, 60, and 15%, respectively, at a 2F8 concentration of 10 μg/ml (data not shown). The difference in inhibition efficiency observed between these cell lines likely reflects an underlying difference in autocrine EGF/TGF-α production, and/or the level of EGF-R overexpression.

Activation of immune effector mechanisms

Besides blockade of signaling, therapy by anti-EGF-R Abs may also operate by inducing immune effector-mediated killing of tumor cells. Therefore, we tested whether 2F8 can induce CDC and/or ADCC of EGF-R-expressing target cells.

To find out whether engagement of the complement system may contribute to antitumor effects of mAb 2F8, we studied C3 deposition on A431 cells in the presence of 5% human serum, using flow cytometry. C3c deposition after preincubation of A431 cells with 2F8 only slightly increased compared with that in the absence of 2F8. In this assay, cytotoxicity was detected using To-PRO3 iodide, which is not able to enter live cells but can do so in cells having leaky membranes where it becomes highly fluorescent after binding to DNA. It was observed that 2F8 induced a concentration-dependent increase in the number of To-PRO3 iodide-positive cells, even in the absence of serum. Although this indicated a cytotoxic effect, there was no evidence for a role of complement, because there was no increase after addition of serum and there was no correlation between To-PRO3 positivity and C3 deposition.

Next, we evaluated the capacity of 2F8 to induce ADCC of A431 cells. Freshly isolated human PBMC were used as effector cells and 51Cr release for assessing cytotoxicity. Interestingly, we observed no 51Cr release upon incubation without adding effector cells. Apparently, 2F8 alone induces cellular damage sufficient to permit To-PRO3 to enter the cells, but which does not lead to lysis and concomitant release of 51Cr, which is known to bind to intracellular proteins. In the presence of effector cells, it was consistently found that 2F8 is a very potent inducer of ADCC. Fig. 4 shows the results from a representative experiment. Table II summarizes the observed maximal specific lysis of A431 target cells, together with the established EC50 values, observed in three distinct experiments, performed with PBMC isolated from three different donors. The observed variation in maximal specific lysis (42–91%) between the three experiments can be explained by individual differences in PBMC activity. In addition, we assessed ADCC induction for BxPC-3 cells, which exhibited a 10-fold lower EGF-R expression level compared with A431 cells as determined by FACS analysis. In a comparative experiment, using the same effector cells, we observed equally high specific lysis (data not shown). Notably, for both cell lines, ADCC induction occurred at extremely low Ab concentrations; EC50 values were 0.007 and 0.011 μg/ml for BxPC-3 and A431, respectively. Thus, the EC50 of mAb 2F8 for ADCC was ~100-times lower than that...
for receptor binding on target cells. This means that very low receptor occupancy by 2F8 was already sufficient to induce ADCC and suggests that ADCC might play an important role in antitumor activity of this Ab in vivo. As was expected from literature (9, 22), the murine IgG1 Ab M225 was not capable to induce ADCC.

Antitumor activity in murine tumor xenograft models

The in vitro experiments described above demonstrated two potential mechanisms for depletion of tumor cells. We further evaluated their potential therapeutic roles using a mouse tumor model, which is more relevant to the clinical condition than the in vitro assays because it includes three-dimensional tumor growth, control by growth factors, tumor vascularization, distribution of the mAb in the body, and presence of immune effector systems. However, it is well known that in these tumor models minor differences in experimental conditions may affect the tumor growth. For unknown reasons, we observed marked differences in A431 tumor growth rates using BALB/c nu/nu mice from two different suppliers. In mice obtained from Harlan, s.c. A431 tumors showed a moderate growth rate, whereas very aggressive tumor growth was observed when using mice from Charles River Laboratories. Although it is not evident which model mimics best the tumor growth in patients, we took the opportunity to evaluate 2F8 in both models. In addition, we studied the antitumor activity of mAb 2F8 in inhibiting tumor growth of kidney cell (BxPC-3) and pancreas cell (BxPC-3) xenografts.

Preventive treatment of tumor growth

Using the A431 moderate growth model, we first evaluated the capacity of mAb 2F8 to prevent tumor formation. On days 1, 3, and 5 after tumor cell inoculation, we administered mAb 2F8 i.p. at three different doses: 3, 1.5, or 0.75 mg/kg on day 1, followed by three times lower doses on days 3 and 5. Although mice in the control huIgG group developed steadily growing tumors, reaching a volume of ~400 mm³ by day 30, all mice treated with mAb 2F8 were fully protected against tumor growth (data not shown). mAb 2F8 was also able to inhibit tumor growth of SK-RC-29 xenografts, which have a 15-fold lower expression level of EGF-R per cell (18). In this study, treatment of mice with a dose of 4 mg/kg loading dose on day 1, followed by 2 mg/kg doses twice per week, resulted in a strong reduction of tumor growth. Although mice in the control group developed tumors with a volume of ~1500 mm³ by day 30, the mice treated with mAb 2F8 grew tumors of only ~300 mm³ (data not shown, n = 6 per group).

Therapeutic treatment of tumor growth

Because the experiments with A431 xenograft showed mAb 2F8 to be capable of preventing tumor formation at a very low total dose, we evaluated the effect of mAb 2F8 on established tumors in this model (Fig. 5A). Mice with s.c. A431 tumors were randomly allocated to treatment groups (n = 6) and treatment with either mAb 2F8 or mouse mAb M225 started on day 12 when tumors reached a size of ~100 mm³. Two different dosing regimens were used: 1) A single-cycle treatment, which started with a loading dose of 3 mg/kg, followed by two doses of 1 mg/kg on days 14 and 16; and 2) A maintenance treatment, with the same loading dose but with continued 1 mg/kg doses twice per week until day 40. As illustrated in Fig. 5A, mAb 2F8 was capable of eradicating established A431 xenograft tumors at a total dose of only 5 mg/kg, in the single-cycle treatment. Single-cycle treatment with mAb M225 or treatment with irrelevant Abs did not result in tumor eradication, while maintenance treatment with mAb M225 had a minor but temporary effect.

In the aggressive tumor model, we also observed marked growth inhibition on treatment of established tumors, but no tumor eradication. In a first series, treatment was started on day 13 when tumors had reached an average volume of 100 mm³ by i.p. injection of a loading dose (12.5 mg/kg) of either mAb 2F8, mAb M225, or irrelevant human IgG1 at day 13, followed by subsequent i.p. injections of 5 mg/kg twice per week (days 16, 20, 23, 27, 30, 34, 37, 41, 44, and 48). On day 36, mice in the control group had tumor volumes of 1130 ± 85 mm³ (mean ± SEM, n = 6) and were sacrificed for ethical reasons. 2F8 treatment reduced tumor growth by 60% (450 ± 97 mm³ on day 36) and was about two times more effective than mouse mAb M225. Fig. 5B shows the results of a second series of experiments in the aggressive model. In this study, 2F8 was administered using different dosing schemes, 2 doses at a 2-wk interval, ranging from 10 to 100 mg/kg. Also, this experiment showed 2F8 to give marked growth reduction at a dose of 10 mg/kg, and that there is a clear dose-response relationship.

To evaluate the ability of mAb 2F8 to inhibit tumor growth in an aggressively growing tumor expressing lower levels of EGF-R per cell, we performed experiments on xenografts of the pancreas cell line BxPC-3 (10-fold lower EGF-R expression than A431 as measured by FACS analysis). Similar to A431, this cell line was highly sensitive to ADCC as discussed above. As described for A431, we observed significant reduction of tumor growth at a dose of 10 mg/kg mAb 2F8 (two doses given on days 15 and 42). Measured on day 50, control mice grew xenografts of ~1500 mm³, whereas...
mAb 2F8-treated mice showed a >50% reduction, with tumors of ~700 mm³ (data not shown, n = 6 per group).

**Effect of treatment on the EGF-R status in vivo**

To find out how the in vivo effect of mAb 2F8 treatment correlates with receptor blocking, we studied the EGF-R status in tumor xenografts during treatment for 2F8 receptor occupancy and for receptor phosphorylation. Eighteen mice with established A431 tumors, ranging in size from 300 to 700 mm³, were treated with various doses of mAb 2F8 (0, 3, 10, 30, or 100 mg/kg body weight). Two days after the single dose, blood samples were taken and the tumors were excised for analysis.

We observed that the plasma concentration was linearly related to the dose, with an apparent distribution volume of ~150 ml/kg, which is within the expected range (23). To examine whether the presence of the tumors affected the pharmacokinetics of mAb 2F8, we assessed Ab plasma concentrations in time in mice with or without A431 tumors. Both types of animals showed similar Ab half-lives of ~10 days (data not shown), which is the expected Ab half-life in nude mice, having low plasma IgG levels (24). Therefore, tumors of this size did not appreciably affect pharmacokinetics of the Ab in this model. Receptor occupancy was determined by flow cytometric measurement of 2F8 binding to cells isolated from the xenograft using a cell strainer. 2F8 binding after saturating all receptors by preincubation of isolated cells from the same sample at a 2F8 concentration of 20 g/ml, was taken as 100% saturation level. It was found that the occupancy was also linearly related to the dose. Conspicuously, 100% saturation in vivo was only observed after the highest dose, corresponding to a plasma concentration of 600 g/ml. This is well above the 2F8 concentration of 10 g/ml, which is sufficient for saturation in vitro. Receptor saturation was also determined by immunoblotting of immunoprecipitates of the receptor, and this yielded similar results (Fig. 6A). Concomitantly, EGF-R phosphorylation, which was 30–40% of maximum in tumors from untreated mice, was also only drastically reduced at the highest 2F8 dose. The results
were consistent with the immunohistochemical findings as shown in Fig. 6B. Staining tumor sections from mice from the different treatment groups for the presence of 2F8, using polyclonal anti-human-IgG Abs, showed a clearly darker staining after a dose of 100 mg/kg than after a dose of 10 mg/kg. Staining for EGF-R (anti-human-IgG after preincubation of the sections with 2F8) revealed that necrotic parts of the tumors were free of the receptor. The EGF-R-positive areas corresponded to the areas where also 2F8 binding was observed, indicating that in vivo 2F8 binds to all EGF-R-expressing cells in the tumors. Staining for EGF-R phosphorylation, using Abs specific for phosphotyrosine 1068, showed less staining at the highest dose, although it should be emphasized that this technique does not permit accurate quantification. More importantly, it showed that phosphorylation was rather homogeneous throughout the tumor, indicating that inhibition by 2F8 occurred evenly in all areas of the tumor.

Discussion

We evaluated a human IgG1κ mAb, 2F8, directed against human EGF-R, in several in vitro assays and in vivo in murine xenograft models. Our study showed that the EC50 of intact 2F8 for binding to cells was approximately three times lower than that of 2F8 Fab, which indicates that 2F8 binds, at least partly, in a bivalent way to EGF-R-expressing cells. Bivalent binding implies that 2F8 may...
dimerize EGF-R, an event that represents the basis of receptor activation after ligand binding. Because 2F8 binding did not induce EGF-R phosphorylation, it can be concluded that the mAb binds to the receptor in a way that does not lead to activation.

The 2F8 binding characteristics to cells were in accordance with the observed EGF-R-blocking effects in vitro. Half-maximal blocking of EGF binding occurred at a mAb concentration of \( \sim 2 \mu g/ml \). This again corresponded to the observed effect on ligand-induced receptor activation in vitro, as assessed by measuring EGF-R phosphorylation at high (20–50 ng/ml) ligand concentrations, i.e., leading to maximal phosphorylation. Half-maximal inhibition of phosphorylation was observed in the concentration range of 0.2–2 \( \mu g/ml \). Inhibition of A431 proliferation in vitro, in standard culture medium without addition of ligands, also showed a half-maximal effect at 1–2 \( \mu g/ml \).

Because engagement of immune-effector systems is one of the potential therapeutic mechanisms of anti-cancer Abs, we evaluated the induction of CDC and ADCC by 2F8. Because mAb 2F8 is an IgG1, we expected triggering of CDC. However, although mAb 2F8 was shown to have a functional C1q binding site, as evidenced by C1q binding to the Ab coated to ELISA plates (data not shown), we observed only little C3 deposition on 2F8-coated A431 cells in the presence of human serum. There was a cytotoxic effect, as detected by To-PRO3 staining, when A431 cells were incubated with saturating concentrations of 2F8, but this cytotoxicity appeared to be completely serum independent. Thus, 2F8 Abs on the A431 cells do not seem to trigger complement-mediated lysis, despite a high density of EGF-R target molecules. The explanation could be that 2F8 molecules bound to EGF-R on cells are not exposed in a proper way to efficiently interact with the complement system. Similarly, it has been observed that the induction of complement-mediated lysis of T cells is strongly dependent on Ag specificity (6, 25).

The lack of CDC is in sharp contrast with the potent induction of ADCC. In this assay, 2F8 alone did not induce \(^{51} \)Cr release, but it induced 40–90% lysis of A431 cells in the presence of PBMC from different human donors. Importantly, ADCC was achieved at very low Ab concentrations. Maximum specific lysis of target cells was already observed in the concentration range of 0.02–0.2 \( \mu g/ml \), which gives <5% receptor occupancy. As illustrated in Fig. 7, ADCC occurred at Ab concentrations, which are about two orders of magnitude lower than those needed for measurable receptor blocking.

Based on the EGF-R inhibitory activity and potent ADCC induction, we expected strong antitumor effects. To assess the in vivo efficacy, we used a murine xenograft model, which has also been extensively used to evaluate the antitumor effects of other anti-EGF-R mAb (8). We set up two models using athymic BALB/c mice from different suppliers, which displayed different tumor growth rates, and we took the opportunity to test 2F8 both in rather moderately growing and in aggressively growing tumor xenografts. mAb 2F8 was able to prevent the outgrowth of A431 tumors when administered at a total dose of only 1.25 mg/kg, divided over three injections in a 5-day period (data not shown). Although indicative of therapeutic potential, eradication of well-established tumors is more relevant. Excitingly, in a moderate model, mAb 2F8 induced complete tumor regression in mice bearing established tumors, at a total dose as low as 5 mg/kg (given over 6 days; Fig. 5A). Also in an aggressive model, 2F8 showed potent antitumor effects, albeit that much higher dosing was needed for growth arrest.

Interestingly, in our study the mouse mAb M225 was less effective in both models at a low dose, which is consistent with previous reports of other investigators about the effective dose of this Ab. High doses of >300 mg/kg of mAb M225 were needed to stop tumor growth of A431 xenografts in mice when applied as monotherapy (7, 12). Because the EGF-R-blocking activities in vitro were not stronger than those of M225, the higher efficacy of mAb 2F8 in inhibiting tumor growth in vivo must be linked to other molecular properties. We wondered whether it could be related to the IgG subclass differences, because mAb 2F8 represents a human IgG1 Ab, which can effectively interact with FcγRs, whereas mAb M225 is of the mouse IgG1 isotype, which interacts poorly (22, 26). This was evidenced in our study by the complete lack of ADCC induction by M225 in an in vitro assay using human PBMC. This seems not to be related to species differences, because it has been reported that M225 could also not recruit antitumor activity by mouse peritoneal macrophages in a tumor xenograft model, whereas the anti-EGF-R mAb 528, which is of the mouse IgG2a subclass, did (9). This suggests that M225 can only act via its receptor-blocking activity and is not able to recruit immune effector cells. This is consistent with a study of Goldstein (7), showing that at the same plasma level, M225 F(ab\(^\prime\))\(_2\), which completely lacks immune effector functions, were equally effective as the intact Ab in inducing tumor regression.

Because of the high doses needed for maximum antitumor effects, we wondered how this would relate to receptor saturation with mAb 2F8. In the analysis of the EGF-R status in the tumor xenografts, we observed that appreciable EGF-R saturation occurred only at plasma concentrations that were two orders of magnitude above the concentrations sufficient for maximum saturation in vitro. Furthermore, we observed in the in vivo studies that EGF-R phosphorylation was also only blocked at complete receptor saturation, which is consistent with the in vitro observations. This made clear that at the dose levels used in the treatment experiments (Fig. 5), EGF-R blockade could not have played a major role in antitumor effect of 2F8. In other words, mAb 2F8 was effective in vivo despite low receptor occupancy, which implies that other potent mechanisms were acting. As illustrated in Fig. 7, potent antitumor effects in vivo already occurred at minimal receptor occupancy, without evidence for receptor blocking. This corresponds to the difference between the concentration-effect relationships for ADCC and receptor blocking in vitro. Therefore, our experiments suggest a significant role for ADCC in the observed antitumor effects at low dose in vivo. The importance of ADCC is supported by observations on mAb C225, a chimeric human-mouse version of M225, which is of the human IgG1 subclass and consequently capable to interact with FcγR. IMC-C225 exhibited an improved antitumor activity in animal models with established A431 xenograft tumors, compared with M225 (7).

The question arises why such high plasma concentrations are required for saturation of the receptor. We speculated on several potential mechanisms, which are not mutually exclusive. First, it could be that ligands, present in high concentrations in the interstitial space of the tumors, compete with 2F8 for receptor binding. However, this is not likely to be the major reason because our analysis of the EGF-R status in the xenografts indicated <40% phosphorylation in untreated mice. Second, it could be that local interstitial concentrations are far below the plasma concentration. This could be due to a limited delivery by the poorly developed vascularization, which is a hallmark of tumor tissues. On itself, poor delivery is not expected to cause low local concentrations because the plasma half-life of intact IgG is also very long (27). However, it will do so when there is a sink due to uptake of Ab by cancer cells. It is known that EGF-R continuously recycles and is rapidly internalized after activation (28), and there are some indications that this may lead to concomitant uptake of anti-EGF-R Abs. In vitro, induction of receptor internalization after M225
binding to EGF-R-expressing cells has indeed been observed (29). In phase I clinical studies with mAb IMC-C225 (cetuximab), a chimeric version of M225, pharmacokinetics were found to be nonlinear, suggesting an additional clearance pathway that becomes saturated at higher dose levels (16). This saturable elimination pathway could well correspond to the mAb internalization after receptor binding. The need for high plasma levels for obtaining complete receptor saturation in tumor xenografts seems to be a general feature of anti-EGF-R Abs. Also for the human mAb E7.6.3 (ABX-EGF), it has been reported that a total dose of at least 60 mg/kg was needed for effective treatment of established A431 xenografts (30). Being of the human IgG2 subtype, E7.6.3 is not expected to engage immune effector mechanisms (26, 31). Therefore, like is the case for M225, its antitumor activity will entirely depend on receptor blocking, and thus on complete receptor saturation, which apparently only occurs at high plasma levels. If receptor internalization indeed plays a role, the plasma concentration needed for saturation is likely related to the receptor density and could be lower for tumor cells with lower expression.

It is clear from studies with small molecule tyrosine kinase inhibitors, like ZD1839 (Iressa; AstraZeneca, Wilmington, DE), that blocking EGF-R signaling represents a powerful mechanism in cancer treatment (3). Likewise, interference with EGF-R signaling may also be an important component of the therapeutic effects of anti-EGF-R mAbs. However, taken together, our experiments indicate that at suboptimal blocking conditions, engagement of immune effector mechanisms (26, 31). This points at a pivotal role for FcR-expressing cells for effective ADCC and complement-mediated lysis (CDC).

In conclusion, we showed that the fully human IgG1 mAb E7.6.3 (ABX-EGF), it has been reported that a total dose of at least 60 mg/kg was needed for effective treatment of established A431 xenografts (30). Being of the human IgG2 subtype, E7.6.3 is not expected to engage immune effector mechanisms (26, 31). Therefore, like is the case for M225, its antitumor activity will entirely depend on receptor blocking, and thus on complete receptor saturation, which apparently only occurs at high plasma levels. If receptor internalization indeed plays a role, the plasma concentration needed for saturation is likely related to the receptor density and could be lower for tumor cells with lower expression.

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