Tumor Cell Killing Mechanisms of Epidermal Growth Factor Receptor (EGFR) Antibodies Are Not Affected by Lung Cancer-Associated EGFR Kinase Mutations

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The epidermal growth factor receptor (EGFR) serves as a molecular target for novel cancer therapeutics such as tyrosine kinase inhibitors (TKI) and EGFR Abs. Recently, specific mutations in the EGFR kinase domain of lung cancers were identified, which altered the signaling capacity of the receptor and which correlated with clinical response or resistance to TKI therapy. In the present study, we investigated the impact of such EGFR mutations on antitumor cell activity of EGFR Abs. Thus, an EGFR-responsive cell line model was established, in which cells with tumor-derived EGFR mutations (L858R, G719S, delE746-A750) were significantly more sensitive to TKI than wild-type EGFR-expressing cells. A clinically relevant secondary mutation (T790M) abolished TKI sensitivity. Significantly, antitumor effects of EGFR Abs, including signaling and growth inhibition and Ab-dependent cellular cytotoxicity, were not affected by any of these mutations. Somatic tumor-associated EGFR kinase mutations, which modulate growth inhibition by TKI, therefore do not impact the activity of therapeutic Abs in vitro. *The Journal of Immunology,* **2008, 180: 4338–4345.**

Monoclonal Abs constitute a rapidly growing class of cancer therapeutics (1), which recruit diverse mechanisms of action for tumor cell killing (2). Among the most intensively targeted tumor-related Ags is the epidermal growth factor receptor (EGFR)[4] (3, 4), because increased expression or dysregulated function of EGFR is found in various common cancers such as lung, colon, or head and neck, but also on nonepithelial malignancies such as glioblastomas (5). In this setting, EGFR Abs compete with small molecule tyrosine kinase inhibitors such as gefitinib (Iressa) and erlotinib (Tarceva), which demonstrated clinical activity in lung cancer patients (6, 7). Retrospective analyses revealed several clinical characteristics which correlated with response to tyrosine kinase inhibitor (TKI) therapy: adeno- or bronchoalveolar histology, never smoker, Asian origin, and female gender (8). Subsequently, several studies reported that EGFR gene copy number and protein expression by immunohistochemistry were important predictors for TKI response (9). Interestingly, sequencing the exons encoding the activation loop of EGFR in tumors from patients responding to TKI therapy demonstrated that somatic mutations in the intracellular domains of EGFR were enriched in these patient populations, compared with nonresponding patients (10–13). Competition experiments and molecular modeling suggested that these mutations affect binding of gefitinib or erlotinib to the ATP binding site of EGFR (10, 14). Functional studies with recombinant EGFR proteins (15), with different tumor cell lines carrying these mutations (12), or with cells transiently transfected with mutated EGFR (10, 11, 16) indicated that the mutated receptors were more sensitive to inhibition by gefitinib or erlotinib than wild-type (wt) EGFR. Importantly, tumors that initially responded to TKI therapy and then progressed on treatment, often expressed a secondary EGFR mutation (T790M), which rendered these cells TKI resistant (14, 17, 18).

Intracellular EGFR mutations were originally described as predictors for response to TKI treatment, but it soon became apparent that they also alter the biology of EGFR. Thus, non-small cell lung cancer (NSCLC) patients with intracellular EGFR mutations had a better prognosis irrespective of their treatment (19). Transgenic mice expressing inducible L858R or del19 EGFR mutants in pneumocytes developed lung cancers, which were responsive to EGFR inhibition, while wt EGFR-transgenic animals remained tumor free (20, 21). Furthermore, mutated EGFR selectively activated Akt and STAT signaling pathways, which promoted cell survival, but had no effect on proliferation (22). Importantly, EGFR-mutated NSCLC cell lines demonstrated markedly increased resistance to cell death signals induced by chemotherapeutic agents or Fas ligand (22).

Different mechanisms of action are discussed for the therapeutic activity of mAbs in vivo (2, 23). Besides Fc/FcR-mediated mechanisms (24, 25), interference with tumor cell signaling is considered as an important mechanism of action for therapeutic Abs (26). For example, lymphoma regression by anti-Id Abs correlated with their ability to induce signal transduction in tumor cells (27, 28). EGFR Abs in particular have been reported to influence intracellular signaling (29, 30). Our group previously reported that intracellular domains of target Ags can critically determine Ab efficacy (31). The aim of our present study was therefore to investigate the influence of lung cancer-derived

FIGURE 1. Ba/F3 transfectants express similar EGFR levels and respond to EGF. *a,* EGFR expression levels of Ba/F3 transfectants were analyzed. Indirect immunofluorescence data from one of six individual experiments are presented. Relative fluorescence intensity of EGFR-specific staining (■), compared with isotype control (□), was calculated for all six experiments and is presented in the table. *b,* Vital cell masses were analyzed by MTT assays after 72 h of incubation. All transfectants responded dose-dependently to recombinant human EGF ($p < 0.05$ indicated by *; two-tailed, unpaired Student's t test). In the absence of EGF, mutant EGFR-transfected Ba/F3 cells demonstrated enhanced survival (significance indicated by #) compared with wt or nontransfected cells. Data presented are means ± SEM of three independent experiments. *c,* In all transfectants, Y1068 EGFR phosphorylation was enhanced by EGF, but not by IL-3 stimulation. Mutated, but not wt EGFR-transfected, cells demonstrated low levels of autophosphorylation in the absence of EGF.
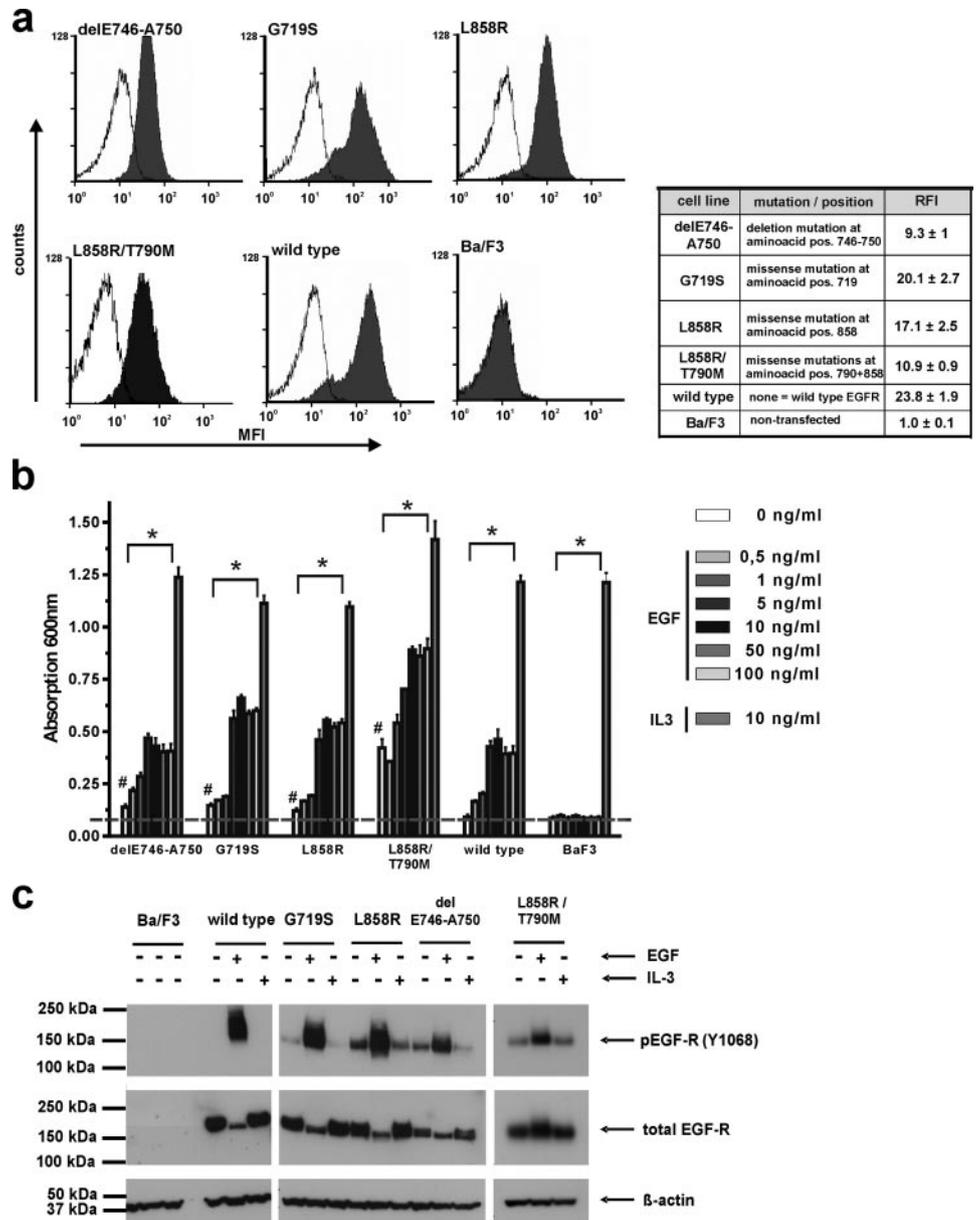
EGFR mutations on tumor cell killing by EGFR Abs. For this purpose, we set up an EGF-dependent cell line model in which the two prototypic TKI, gefitinib (Iressa) and erlotinib (Tarceva), demonstrated the expected activities. We then used this in vitro model to analyze killing mechanisms of two EGFR Abs, C225 (cetuximab, Erbitux) (32) and 2F8 (zalutumumab, HuMax-EGFr) (33). Importantly, both Fab and Fc-mediated effector mechanisms of EGFR Abs were not affected by primary or secondary EGFR mutations. However, growth inhibition by EGFR Abs was only observed when cells were growing EGF dependently, while Ab-dependent cellular cytotoxicity (ADCC) occurred also under ligand-independent assay conditions.

Materials and Methods
Blood donors

For effector cell isolation, 100 ml of blood was drawn from healthy volunteers after written informed consent was obtained. Experiments reported here were approved by the Ethical Committee of the Christian-Albrechts-University (Kiel, Germany) in accordance with the Declaration of Helsinki.

Abs and TKI

2F8 (HuMab-EGFr, zalutumumab) and a human IgG1 control Ab (HuMab-KLH) directed against keyhole limpet hemocyanin were obtained from Genmab. M225 (the murine parental Ab of C225) was purified from tissue culture supernatants of hybridoma 225 (LGC Promochem). Cetuximab (C225; Merck), gefitinib (Astra Zeneca), and erlotinib (Hoffmann-LaRoche) were purchased. TKI were solubilized in DMSO and further diluted in tissue culture medium. All EGFR inhibitors were used at clinically relevant concentrations.

Culture of eukaryotic cells

Ba/F3 cells (DSMZ, The German Resource Centre for Biological Material, Braunschweig, Germany) were cultured in RPMI 1640-Glutamax-I medium (Invitrogen Life Technologies) containing 10% FCS, penicillin, and streptomycin (R10[+]). Murine (m) IL-3 was either added as recombinant mIL-3 (R&D Systems) at 10 ng/ml or as supernatant from WEHI-3B cells (DSMZ; concentration 10% v/v). Medium for transfected Ba/F3 cells additionally contained 1 mg/ml geneticin (Invitrogen Life Technologies).

Construction of intracellular EGFR mutants

The intracellular EGFR mutations were generated by site-directed mutagenesis of the plasmid vector pUSE-EGFR (Upstate Biotechnology) harboring wt EGFR. Introduction of the respective mutations and the correctness of the EGFR coding region was confirmed by complete sequencing.

Stable transfection of Ba/F3 cells

Ba/F3 cells were stably transfected by nucleofection of 2 μg of plasmid DNA and 2×10^6 cells using the Amaxa transfection system according to

FIGURE 2. Sensitivity of primary and secondary EGFR mutations against TKI. Ba/F3 transfectants stimulated with 10 ng/ml EGF were incubated with increasing concentrations of gefitinib (*a*), gefitinib plus mIL-3 (*b*), erlotinib (*c*), and erlotinib plus mIL-3 (*d*), respectively. After 72 h, vital cell masses were measured by MTT assays. Data presented are means ± SEM of six (for gefitinib) and four (for erlotinib) experiments. Typical steady-state plasma concentrations of TKI (3, 5) are indicated by ↓. ▽, wt; ○, G719S; △, L858R; □, delE746-A750; and ◇, L858R/T790M. Individual data points lie above the curves, because a nonlinear regression model was used for curve generation (see *Materials and Methods*).
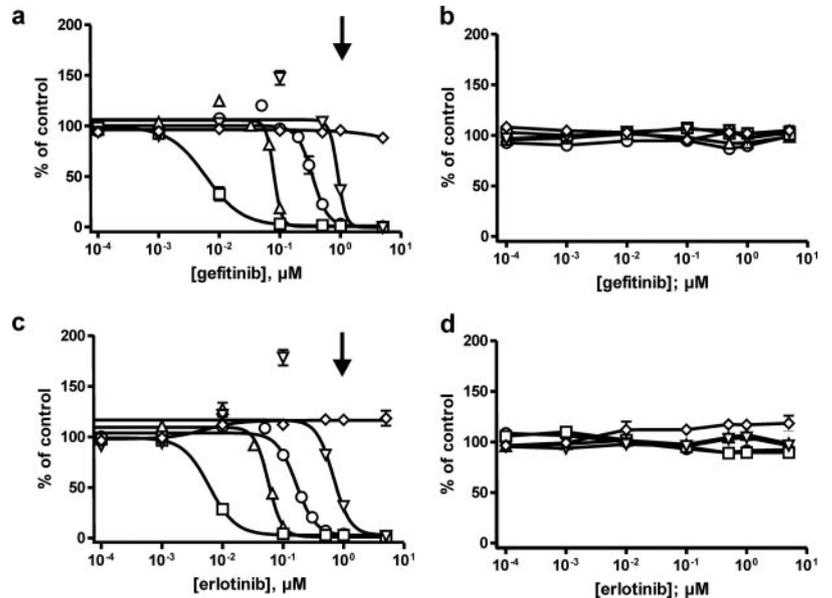
the manufacturer's instructions. Forty-eight hours after transfection, cells were put under selection by adding 1 mg/ml geneticin. After the completion of functional studies, mutated and wt EGFR sequences were again confirmed by sequencing exons 18–21. All cell populations were demonstrated to express the expected EGFR sequences (data not shown).

MACS sorting of transfectants

Twenty million transfected Ba/F3 cells were incubated with 4 ml of EGFR mAb m225 (mIgG1) at 20 μg/ml in PBS containing 0.5% BSA and 25% rabbit serum (to block nonspecific binding of the primary Ab). After 15 min on ice, cells were washed twice with PBS containing 0.5% BSA. The cell pellet was resuspended in 200 μl of PBS containing 0.5% BSA and 50% rabbit serum. Fifty microliters of anti-mouse IgG1 magnetic beads (Miltenyi Biotec) was added, and cells were incubated for another 10 min on ice. Cells were washed twice and separated on LD depletion columns according to the manufacturer's instructions (Miltenyi Biotec).

Flow cytometric analyses

For indirect immunofluorescence, 3×10^5 target cells were washed in PBS supplemented with 1% BSA (Sigma-Aldrich) and 0.1% sodium azide (PBA buffer). Cells were then incubated with Ab 2F8 or human control IgG1 at 40 μg/ml (diluted in PBA buffer/25% rabbit serum) for 30 min on ice. After washing with 500 μl of PBA buffer, cells were stained with FITC-labeled F(ab')$_2$ of polyclonal goat anti-mouse Abs (DakoCytomation). After a final wash, cells were analyzed on a flow cytometer (Coulter EPICS XL). Relative immunofluorescence intensity was calculated: relative immunofluorescence intensity = (mean fluorescence intensity-specific Ab)/(mean fluorescence intensity isotype control Ab).

Determination of viable cell mass (MTT assay)

Growth inhibition of Ba/F3 transfectants was analyzed by using the MTT assay (Roche Diagnostics). Cells were washed three times in culture medium lacking mIL3. Cells were then diluted in medium containing EGF and/or mIL-3 and seeded at 20,000 cells/well in 96-well plates in a final volume of 100 μl. Cells were treated with gefitinib, erlotinib, C225, or 2F8 by addition of 25 μl of 5-fold concentrated serial dilutions of the respective drug in culture medium lacking mIL-3. For gefitinib and erlotinib, DMSO concentrations at all dilution steps were kept constant at a final concentration of 0.05%. After 72 h, cells were washed twice and resuspended in 100 μl of culture medium. MTT assays were then performed according to the manufacturer's instructions.

Isolation of mononuclear effector cells (MNC)

Briefly, citrate-anticoagulated blood from healthy volunteers was layered over a discontinuous gradient consisting of 70 and 62% Percoll (Biochrom), respectively. After centrifugation, MNC were collected from the serum/Percoll interface. MNC typically contained ~60% CD3-positive T cells, 20% CD56-positive NK cells, and only 10% CD14-expressing mono-

cytes, as determined by immunofluorescence staining. Viability of cells tested by trypan blue exclusion was higher than 95%.

Immunoblotting

Ba/F3 cells were starved for 3 h before treatment with either EGF (20 ng/ml) or mIL-3. 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$_3$VO$_4$, and proteinase inhibitors. Eighty micrograms of protein was electrophoresed on 3–8% SDS-polyacrylamide gels (NuPage Acetat; Invitrogen Life Technologies) and transferred onto polyvinylidene difluoride membranes. Immunodetection was performed with the ECL system (Pierce). Polyclonal Abs against pEGFR (Y1068; BioSource International), total EGFR (Santa Cruz Biotechnology), and phospho-p44/p42 (Cell Signaling) were used at a dilution of 1/1000. mAbs against ERK-1 (BD Transduction Laboratories) and actin (Sigma-Aldrich) were used at dilutions of 1/5000 or 1/1000. Secondary Abs (HRP-conjugated anti-rabbit or anti-mouse IgG; DakoCytomation) were used at a dilution of 1/5000 or 1/2000.

ADCC assays

ADCC assays were performed as previously described (31). Briefly, target cells were labeled with 100 μCi of ^{51}Cr per 10^6 cells for 2 h. After washing three times with R10$^+$, cells were adjusted to 10^5 cells/ml. MNC in a total volume of 50 μl, sensitizing Abs at varying concentrations, and R10$^+$ were added to round-bottom microtiter plates. Assays were started by adding target cells (50 μl), resulting in a final volume of 200 μl and an E:T cell ratio of 80:1. After 3 h at 37°C, assays were stopped by centrifugation and ^{51}Cr release from triplicates was measured in cpm. Percentage of cellular cytotoxicity was calculated using the formula: percent 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 was measured in the absence of sensitizing Abs and effector cells.

Data processing and statistical analyses

Data are displayed graphically and statistically analyzed using GraphPad Prism 4.0. Experimental curves were fitted using a nonlinear regression model with a sigmoidal dose response (variable slope). For statistical analysis, the best-fit value of pairs of midpoints (log EC$_{50}$) for EGFR mutations was compared with wt using the F test. As null hypothesis, "log EC$_{50}$ is the same for data sets analyzed" was assumed; the alternative hypothesis was "log EC$_{50}$ is different for data sets analyzed." Values of p were calculated and the null hypothesis was rejected when $p < 0.05$.

Results

Establishing an EGF-dependent cellular in vitro model

To investigate the impact of the most common intracellular EGFR mutations on cell killing by TKI in comparison to mAbs, wt EGFR

Table I. *EC_{50} values for growth inhibition by TKI[a]*

Cell Line	EC_{50} Gefitinib (μM)	95% CI[b]	p	EC_{50} Erlotinib (μM)	95% CI	p
wt	0.90	0.80–1.03		0.68	0.52–0.88	
G719S	0.35	0.31–0.39	<0.0001	0.17	0.15–0.18	<0.0001
L858R	0.08	0.08–0.09	<0.0001	0.06	0.05–0.07	<0.0001
delE746-A750	0.006	0.005–0.008	<0.0001	0.006	0.005–0.008	<0.0001
L858R/T790M	Resistant			Resistant		

[a] Value of *p*: EC_{50} for wt EGFR was compared with EC_{50} values for mutant receptors. $p < 0.05$ = log EC_{50} value for mutant is significantly different from log EC_{50} for wt.
[b] CI, Confidence interval.

or EGFR variants carrying the primary mutations L858R, G719S, delE746-A750 deletion, or the secondary T790M mutation in combination with L858R were transfected into Ba/F3 cells. Ba/F3 is an IL-3-dependent murine pro-B cell line, which has been used before to study signaling by growth factor receptors (34). After transfec-

tion and selection, EGFR-expressing transfectants were enriched by MACS.

Ba/F3-wt, Ba/F3-G719S, and Ba/F3-L858R cells expressed similar levels of EGFR, whereas the EGFR expression levels of Ba/F3-delE746-A750 and Ba/F3-L858R/T790M cells were lower
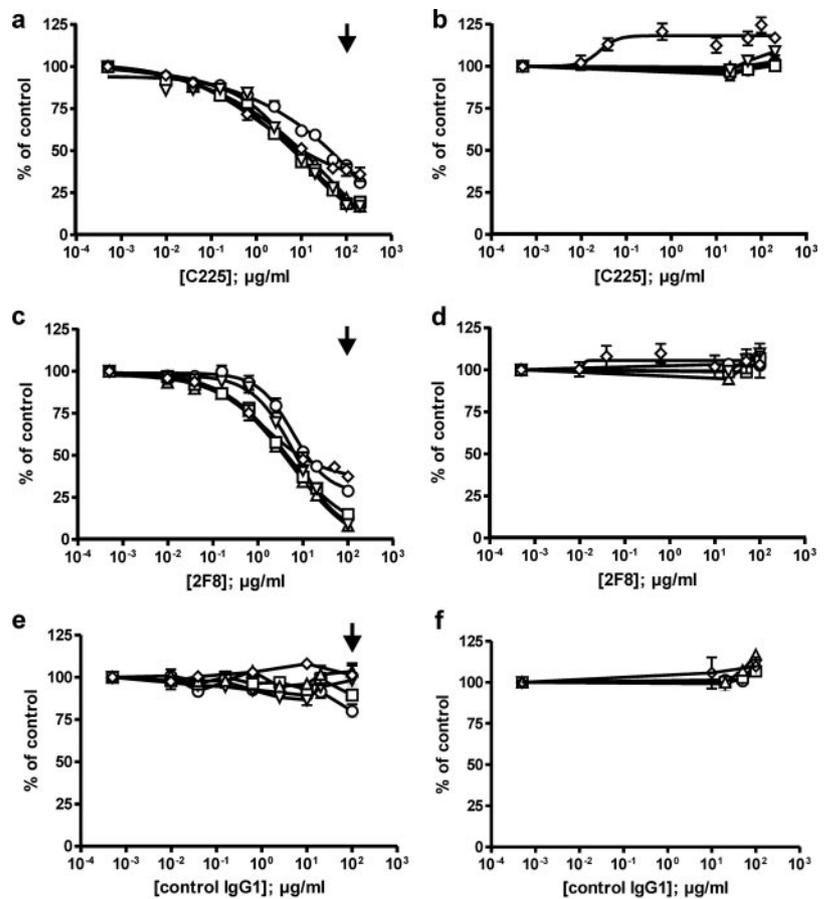
FIGURE 3. Inhibition of proliferation and signaling by EGFR Abs is not affected by primary and secondary EGFR mutations. Ba/F3 transfectants were incubated with increasing concentrations of C225 (*a*), C225 plus mIL-3 (*b*), 2F8 (*c*), 2F8 plus mIL-3 (*d*), control IgG1 (*e*), and control IgG1 plus mIL3 (*f*). Typical plasma concentrations of Abs (C225: (41); 2F8: Genmab; W. K. Bleeker and P. W. H. I. Parren, unpublished data) are indicated by ↓. After 72 h, vital cell masses were measured by MTT assays. Data presented are means ± SEM of five experiments for 2F8, six experiments for C225, and three experiments for control IgG1. *g*, EGF-induced signaling in the presence or absence of Abs was analyzed by measuring pY1068 EGFR and pERK levels in Western blots. EGFR-transfected Ba/F3 cells were incubated with 2 μg/ml Abs 3 h before treatment with human EGF (20 ng/ml; 20 min). ▽, wt; ○, G719S; △, L858R; □, delE746-A750; and ◇, L858R/T790M.
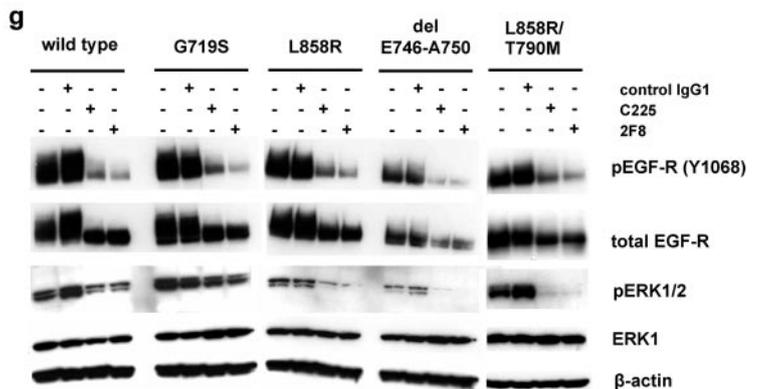
Table II. *EC_{50} values for growth inhibition by Abs[a]*

Cell Line	EC_{50} C225 (μg/ml)	95% CI[b]	p	EC_{50} 2F8 (μg/ml)	95% CI	p
wt	7.85	4.45–13.82		6.75	4.28–10.65	
G719S	n.a.[c]	n.a.	n.a.	6.01	3.65–9.89	0.7
L858R	n.a.	n.a.	n.a.	5.25	2.86–9.64	0.6
delE746-A750	10.23	2.682–39.03	0.7	4.12	1.97–8.64	0.3
L858R/T790M	1.48	0.47–4.64	0.1	1.65	0.72–3.78	0.1

[a] Value of *p*: EC_{50} for wt EGFR was compared with EC_{50} values for mutant receptors. $p < 0.05$ = log EC_{50} value for mutant is significantly different from log EC_{50} for wt.
[b] CI, Confidence interval.
[c] n.a., Not applicable; EC_{50} could not be determined, no plateau was reached.

(Fig. 1*a*). All transfectants demonstrated similar cell survival in the presence of mIL-3 and grew dose-dependently after adding EGF (Fig. 1*b*). Interestingly, the L858R/T790M transfectants grew notably faster than the other cells. Similarly, this mutation was more active in autophosphorylation assays (15), indicating that the L858R/T790M mutation alters receptor functions and growth characteristics more profoundly than the other mutations. In the absence of mIL-3 and EGF, mutant EGFR-transfected Ba/F3 cells demonstrated enhanced cell survival compared with wt-transfected or nontransfected cells (Fig. 1*b*). This is in accordance with previous data demonstrating that cellular transformation via overexpressed wt EGFR occurs only after receptor activation by EGF or TGF-α (35, 36). EGF, but not mIL-3, triggered Y1068 phosphorylation of transfected EGFR, while ligand-independent autophosphorylation of mutant, but not wt receptors was observed (Fig. 1*c*). Interestingly, EGF stimulation down-regulated EGFR expression levels of wt and mutated EGFR receptors carrying deletions or single-missense mutations, but not of the double-mutated L858R/T790M receptor (Fig. 1*c*). This is in accordance with another report that found impaired ubiquitinylation of the double-mutated receptor to reduce receptor degradation in tumor cell lines, thereby prolonging receptor signaling (37). Furthermore, mutant EGFR-transfected cells were capable of mIL-3-independent long- term cultivation, while wt transfectants only grew mIL-3 independent for short term (2–3 wk; data not shown), which is in accordance with published data (38). Thus, we set up this ligand-dependent cellular system which allowed comparative analyses of EGFR mutations in a defined cellular background.

Primary intracellular EGFR mutants are more sensitive to gefitinib or erlotinib than wt EGFR, whereas the T790M secondary mutation renders a primary L858R mutation TKI resistant

To examine the effects of TKI on primary and secondary EGFR mutations compared with wt EGFR and to test whether our cell line system reflects known characteristics of primary and secondary mutated EGFR, MTT assays were performed. Low concentrations of both gefitinib (Fig. 2*a*) and erlotinib (Fig. 2*c*) inhibited cell growth of Ba/F3 transfectants carrying primary EGFR mutations. In contrast, cells transfected with the double-mutated L858R/T790M receptor were resistant to TKI inhibition. Importantly, EGFR dependence of inhibition of primary mutations was demonstrated, since the inhibitory effects of TKI could be completely overcome by addition of mIL-3 (Fig. 2, *b* and *d*). Together, these data indicate that the observed growth inhibition was targeted specifically to EGFR and not caused by "nonspecific" toxicity of the TKI. Notably, Ba/F3 cells transfected with primary EGFR mutations proved significantly more sensitive to TKI treatment than wt EGFR-transfected cells. The order of sensitivity was delE746-A750≫L858R≫G719S>wt (for EC_{50} values and *p* values, see Table I).

Inhibition of signaling and proliferation by EGFR Abs is not affected by intracellular EGFR mutations

Therapeutic Abs mediate antitumor activity via both direct and indirect mechanisms of action (2). To investigate whether primary and secondary intracellular EGFR mutations affect growth inhibition by therapeutic EGFR Abs, the chimeric C225 Ab and the human

FIGURE 4. ADCC by EGFR Abs is not influenced by primary and secondary EGFR mutations. To analyze the impact of lung cancer-derived EGFR mutations on immune effector functions triggered by therapeutic Abs, Ba/F3 transfectants were analyzed in chromium release assays with MNC. C225 (*a*) and 2F8 (*b*). Control experiments against Ba/F3 transfectants were performed in the presence of increasing concentrations of a human IgG1 control Ab (*c*). ▽, wt; ○, G719S; △, L858R; □, delE746-A750; and ◇, L858R/T790M. Experiments with nontransfected Ba/F3 cells in the presence of increasing concentrations of C225 (□), 2F8 (▽), or a human IgG1 control Ab (○) were performed to analyze EGFR dependency of lysis (*d*). Data presented are means ± SEM of six (for 2F8), five (for C225), and three (control IgG1; nontransfected Ba/F3) experiments.
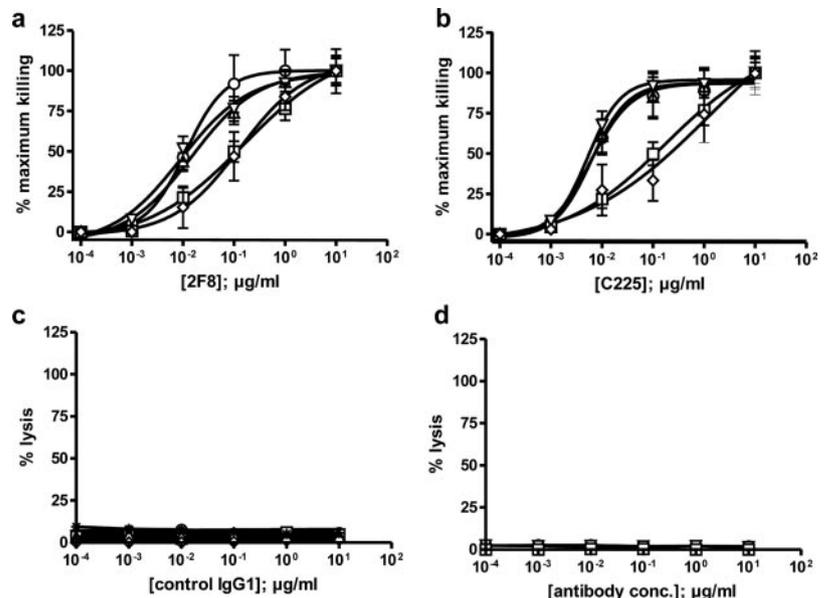
Table III. *EC$_{50}$ values for cell-mediated cytotoxicity by Abs[a]*

Cell Line	EC$_{50}$ C225 (μg/ml)	95% CI[b]	p	EC$_{50}$ 2F8 (μg/ml)	95% CI	p
wt	0.01	0.004–0.01		0.01	0.008–0.02	
G719S	0.01	0.005–0.03	0.5	0.01	0.006–0.02	0.8
L858R	0.01	0.004–0.02	0.5	0.02	0.011–0.03	0.3
delE746-A750	0.1	0.054–0.18	0.0001	0.11	0.061–0.18	<0.0001
L858R/T790M	n.a.[c]	n.a.	n.a.	0.13	0.033–0.49	0.04

[a] Value of p: EC$_{50}$ for wt EGFR was compared with EC$_{50}$ values for mutant receptors. $p < 0.05$ = log EC$_{50}$ value for mutant is significantly different from log EC$_{50}$ for wt.
[b] CI, Confidence interval.
[c] n.a., Not applicable; EC$_{50}$ could not be determined, no plateau was reached.

2F8 Ab were investigated for their capacity to inhibit signaling and growth of the Ba/F3 transfectant panel in the presence of EGF. Both Abs efficiently inhibited EGF-binding to EGFR (33) and inhibited cell growth of the Ba/F3 transfectants (Fig. 3, *a* and *c*). Inhibition by the C225 Ab was less efficient, and EC$_{50}$ values could not be determined for all mutants, because no lower plateau was reached. Importantly, growth inhibition by 2F8 was not significantly different for the primary EGFR mutations (EC$_{50}$ values; Table II), which was in clear contrast to inhibition by TKI. In addition, both Abs inhibited proliferation of cells carrying the double-mutated (L858R/T790M), TKI-resistant receptor (Fig. 3, *a* and *c*). EC$_{50}$ values were not significantly different from those observed with wt or single-mutated receptors (Table II). The demonstrated effects were not observed with an isotype-matched control Ab (Fig. 3*e*). Hypercrosslinking of 2F8 by anti-human IgG did not impact on the growth inhibition of wt EGFR-transfected cells, but diminished growth inhibition of cells carrying mutated EGFR (data not shown). Importantly, inhibitory effects of EGFR Abs could be overcome by adding mIL-3 (Fig. 3, *b*, *d*, and *f*), demonstrating that the proliferation inhibition was EGF/EGFR dependent. In conclusion, both TKI and EGFR Abs demonstrated growth inhibition only when cell proliferation was EGF driven, but not when another growth factor signal was obtained. This observation is in agreement with the concept of "oncogene addiction" (39).

Analyses of downstream signaling demonstrated that treatment with either of the two EGFR Abs reduced levels of EGFR-(Y1068) and ERK phosphorylation similarly in both wt and mutated EGFR transfectants (Fig. 3*g*). Together, these results demonstrate, for the first time in a defined cellular background, that inhibition of signaling and growth by therapeutic EGFR Abs is less affected by the three most commonly observed primary and secondary EGFR mutations in lung cancer.

Cellular cytotoxicity by therapeutic EGFR Abs is not affected by primary and secondary intracellular EGFR mutations

To analyze the impact of intracellular EGFR mutations on immune cell-mediated killing by EGFR Abs, both C225 and 2F8 were analyzed for their capacity to trigger ADCC. Both EGFR Abs triggered ADCC against the panel of Ba/F3 transfectants with MNC from healthy donors. Importantly, 2F8 and C225 also mediated significant lysis of TKI-resistant Ba/F3-L858R/T790M cells (Fig. 4, *a* and *b*). In ADCC experiments with nontransfected Ba/F3 cells (Fig. 4*d*) or with Ba/F3 transfectants and a human IgG1 control Ab (Fig. 4*c*), no lysis was observed, demonstrating cell killing to be EGFR dependent and specific. Killing of the delE746-A750 and L858R/T790M mutants was lower compared with the other transfectants. This may be attributable to the lower EGFR expression levels of these mutants, since Ag density was previously found to significantly affect ADCC. Indeed, in agreement with this, similar ADCC was observed for a Ba/F3-delE746-A750 mutant with a higher expression level comparable to the L858R and G719S EGFR transfectants, but which no longer responded to EGF (data not shown). However, no significant differences were observed in the EC$_{50}$ values for transfectants carrying wt, G719S, or L858R mutations, indicating that these common primary EGFR mutations in lung cancer do not affect ADCC (Table III).

Previous studies demonstrated that growth inhibition by TKI and EGFR Abs was overcome by adding mIL-3, which provided an alternative growth signal to EGFR-transfected Ba/F3 cells. Next, we wanted to analyze also whether ADCC is affected by the presence of mIL-3. Interestingly, both EGFR Abs triggered very similar levels of ADCC in the presence or absence of mIL-3, suggesting that ADCC operates also against non-oncogene-addicted tumor cells (Fig. 5, *a* and *b*). In contrast, ADCC was slightly, albeit not statistically significant, impaired in the presence of EGF. This is probably explained by ligand-induced receptor down-regulation. In summary, these experiments further demonstrated that, in contrast to TKI, mAbs' mechanisms of action were not affected by the kinase mutational status of EGFR, and that ADCC may kill tumor cells growing via alternative pathways.

Discussion

In this manuscript, the influence of lung cancer-associated EGFR mutations on killing mechanisms by EGFR-directed Abs was investigated. Growth inhibition and ADCC experiments demonstrated that

FIGURE 5. ADCC by EGFR Abs in the presence or absence of growth factor signals. To investigate the impact of EGF or mIL-3 on cell-mediated killing by EGFR Abs, wt EGFR-transfected BaF3 cells were analyzed in chromium release assays with MNC. *a*, C225 ($\square$), C225 plus EGF ($\bigcirc$), C225 plus mIL-3 ($\triangledown$; *b*) 2F8 ($\square$), 2F8 plus EGF ($\bigcirc$), and 2F8 plus mIL-3 ($\triangledown$). Data presented are means ± SEM of five experiments.
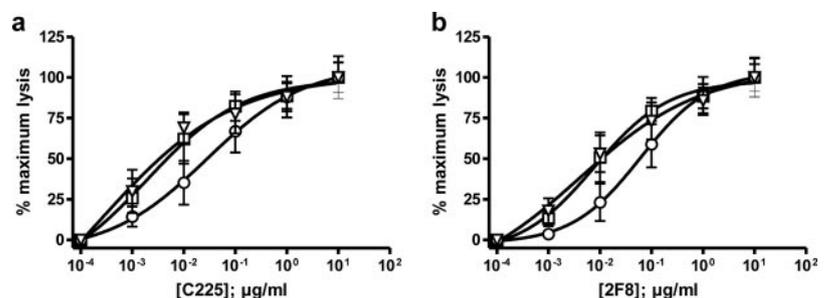
both primary and secondary intracellular EGFR mutations do not affect responses to EGFR-directed Abs in vitro. The presented Ba/F3 model system reflects several known characteristics of wt and mutated EGFR, e.g., response to EGF, differential sensitivity of primary mutated vs wt EGFR, and resistance of the double-mutated L858R/T90M variant to TKI inhibition (Fig. 2 and Ref. 14). In contrast to studies with patient-derived tumor cell lines (12, 40–42), the EGFR-transfected Ba/F3 model presented here has the important advantage of carrying defined EGFR mutations in a defined cellular background, probably without additional genetic changes, which are common in established tumor cell lines. Indeed, differences in the mutational status of EGFR, its expression level, as well as additional genetic alterations such as mutations of KRAS (41), may alter EGFR signaling. However, also in vivo studies with human tumor cell lines in immunodeficient mice indicated that C225 may be active against kinase-mutated EGFR (42, 43). Furthermore, C225 demonstrated in vivo efficacy against syngeneic kinase-mutated EGFR-driven tumors in conditional transgenic animals (21). In similar experiments, however, C225 did not inhibit the in vivo growth of tumors driven by EGFRvIII (44), an extracellularly mutated EGFR, which lacks the EGF-binding domain and which is constitutively activated.

Our experiments investigating direct growth inhibition (Fig. 3) and immune effector cell-mediated mechanisms of action of two EGFR-directed therapeutic Abs (Fig. 4) demonstrate that both primary and secondary EGFR kinase mutations do not affect responses to EGFR-directed Abs in vitro, in contrast to TKI treatment (Fig. 2). Thus far, clinical predictors of response to EGFR Abs are less well established than for TKI. For the CD20 Ab rituximab, animal data in FcγR-deficient mice as well as correlations between human FcγR polymorphisms and clinical outcomes of Ab therapy suggest an important role of FcR-mediated mechanisms for the therapeutic efficacy of this Ab (2). For EGFR Abs, data from a clinical trial with C225 in colorectal cancer patients also demonstrated correlations between FcR polymorphisms and clinical outcome (45). Furthermore, evidence from animal experiments suggested that both indirect, Fc-mediated, but also direct, Fab mediated effector mechanisms significantly contributed to EGFR Ab efficacy (33, 46). In support for the relevance of F(ab′)₂-mediated mechanisms, panitumumab, a human IgG2 Ab with limited immune effector functions, demonstrated clinical efficacy in colorectal cancer patients (47). In our assays, panitumumab was similarly effective in inhibiting cell growth of wt- and kinase-mutated EGFR-transfected Ba/F3 cells as C225 and 2F8, but did not effectively trigger ADCC (data not shown).

Importantly, intracellular EGFR mutations not only correlated with the response to TKI therapy, but also altered the sensitivity to chemotherapeutic agents and to Fas-ligand-induced apoptosis (22). Furthermore, these EGFR mutations affected intracellular signaling, which is considered relevant for the efficacy of many therapeutic mAbs (26). A retrospective analysis in lung cancer patients suggested that intracellular EGFR mutations did not correlate with the C225 response in vivo (48). However, no functional data were provided to support this observation, and the sample size of this clinical study was too small to draw definite conclusions. In this study, we demonstrate that three potential mechanisms of action of EGFR Abs, inhibition of signaling and proliferation and cell-mediated cytotoxicity, were not affected by the most common primary or secondary EGFR mutations in lung cancer patients. Recently, C225 was reported to down-regulate expression of intracellular lung cancer-associated EGFR mutants, another potential mechanism of action for EGFR Abs (42, 43).

EGFR-directed therapy has already become an important treatment option for many cancer patients. However, limited response rates and marginal survival benefits fuel the search for more ef-

fective reagents, both in the fields of TKI (4) and Abs (23). Because these novel therapies are expensive and also bear the risk of side effects, it has become increasingly relevant to identify patient populations with optimal benefit from individual approaches (49). If conclusions from the Ba/F3 model will translate into the therapeutic setting, our data suggest that responses to EGFR Abs will not be determined by the absence or presence of mutations in the ATP-binding region of EGFR. However, the data may also have another clinical implication: direct growth inhibition by either TKI or Abs is only achieved when cells proliferate via EGFR, but not when they receive survival signals via other growth factor receptors (in the presented experiments via the IL-3R; Figs. 2 and 3), an observation which is in agreement with Weinstein's hypothesis of "oncogene addiction" (39). Importantly, this hypothesis would predict that patients, who initially responded to TKI therapy, but progress because they acquire a secondary T790M mutation, may be candidates to respond to EGFR Abs. Their tumors should have been EGFR dependent, until they were rendered TKI resistant by acquiring the T790M mutation. These patients, which are expected to be more common in Asian populations (50), should have good chances to respond to second-line therapy with EGFR Abs, as our in vitro and recent in vivo (42) data would suggest that important killing mechanisms of EGFR Abs are not affected by the mutational status of EGFR.

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