Biological Efficacy of a Chimeric Antibody to the Epidermal Growth Factor Receptor in a Human Tumor Xenograft Model

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ABSTRACT

The epidermal growth factor receptor (EGFR) is a protein tyrosine kinase expressed on many types of tumor cells, including breast, ovarian, bladder, head and neck, and prostatic carcinoma. There seems to be an association between up-regulation of the EGFR and poor clinical prognosis for a number of human cancers. The 225 antibody is a highly specific murine monoclonal antibody that binds specifically to the human EGFR with an affinity equal to its ligand, competes with the ligand for binding, and blocks activation of the receptor tyrosine kinase. In addition, 225 has been shown to inhibit the growth of human tumor xenografts in athymic nude mice.

The 225 antibody has recently been chimerized with human IgG1 in its constant region to increase its clinical utility by decreasing the potential for generation of human antimouse antibodies in recipients. This report compares the biological effects of 225 and its chimeric counterpart (designated C225) against established A431 tumor xenografts in nude mice. The results of these experiments indicated that C225 was more effective than 225 in inhibiting tumor growth in this model. In addition, many of the animals treated with C225 were tumor free at the end of each treatment protocol. It was determined that the dissociation constant of C225 was about 5-fold lower than 225. This suggested that the increased capacity of C225 to compete with ligand for binding to the EGFR was responsible for its enhanced in vivo antitumor effect.

INTRODUCTION

The EGFR2 is a protein tyrosine kinase encoded by the c-erb-B proto-oncogene and expressed on many normal and malignant cells (1, 2). Binding of one of its two ligands (EGF or TGF-α) to the EGFR can activate signal transduction pathways that regulate cell proliferation. A number of human epithelial cancers express high levels of the EGFR and may also produce TGF-α in an autocrine manner. Included in this group are tumors of the breast, lung, colon, prostate, kidney, bladder, head and neck, and ovary (1-4). For many of these cancers, there exists an association between up-regulation of receptor expression and poor clinical prognosis (4).

Because of the relationship between overexpression of the EGFR and clinically aggressive disease, a mAb directed against this receptor may prove to be a useful therapeutic reagent. The murine mAb 225 binds only to human EGFR, has a Κd similar to its ligand (1 nm), blocks activation of the EGFR by ligand, and induces internalization of the receptor. The antibody is able to inhibit the growth of cultured EGFR-expressing tumor lines and to repress the in vivo growth of these tumors when grown as xenografts in nude mice (5-9). More recently, a treatment regimen combining 225 plus doxorubicin or cis-platin was found to show therapeutic synergy against several well-established human xenograft models (10, 11).

An obvious problem with the use of a murine mAb in human clinical trials is the potential for the generation of human antimouse antibody responses (12, 13). Indeed, this was found to occur in Phase 1 clinical trials with 225 in patients with advanced squamous carcinoma of the lung. These trials established the feasibility of administering 225 at doses that produced receptor-saturating levels in the blood, without inducing toxicity (14). To avoid human antimouse antibody production, 225 was chimerized to the human IgG1 constant region. In this report, we compared the in vivo effects of 225 and C225 on established A431 xenografts in nude mice. A431 is a cell line that expresses very high levels of the EGFR (about 10⁹/cell) and is autocrine for the production of TGF-α (15, 16). In previous animal studies, A431 xenografts treated with 225 beginning on the day of tumor challenge, or within 5 days of tumor cell inoculation, were completely inhibited by the antibody alone, whereas 225 by itself had little or no effect on the growth of established tumors (6, 10, 11). The results of the present studies show that C225 has an enhanced biological effect on the growth of established A431 tumors in nude mice.

MATERIALS AND METHODS

Cell Lines and Media. A431 cells were routinely grown in a 1:1 mixture of DMEM and Ham’s F-12 medium supplemented with 10% fetal bovine serum, 2 mm L-glutamine, and antibiotics.

Preparation and Purification of Murine 225 and C225. The 225 antibody was grown as ascites in pristane-primed BALB/c mice. Ascites fluid was purified by high performance
The biological effect of a chimeric antibody of the ELISA and SPR data. Overnight at 37°C, cells were fixed with 3.7% neutral buffered cells (10^5/well) were grown in 96-well microtiter plates as per the ELISA protocol previously described (17). Briefly, A431 cells were grown to 90% confluence under asectic conditions. Purity was determined by SDS-PAGE. Be >95% pure by SDS-PAGE.

Relative Affinity Measurements Using ELISA. The relative binding affinity of the antibodies was determined using an ELISA protocol previously described (17). Briefly, A431 cells (10^6 or 10^5/well) were grown in 96-well microtiter plates overnight at 37°C. Cells were fixed with 3.7% neutral buffered formalin for 10 min at room temperature. After washing three times with PBS, wells were blocked with 1% BSA in HBSS for 2 h at room temperature. C225 or 225 was added to the wells at various concentrations (serial dilutions starting at 50 nM). After a 2-h incubation at 37°C, plates were extensively washed with PBS and either goat antihuman antibody:horseradish peroxidase (1:1000; Sigma Chemical Co., St. Louis, MO) or goat antiserum antibody:horseradish peroxidase (1:5000; Tago, Burlingame, CA) were added for 1 h at 37°C. After washing, the chromogen TMB (Kirkegaard and Perry, Gaithersburg, MD) was added for 30 min in the dark. The color reaction was stopped with 1 N sulfuric acid, and the plates were read at 450 nm in an ELISA reader. The relative binding affinity, defined as the concentration giving the half-maximal A, is an approximation of an antibody’s K_d.

Affinity Constants of 225 and C225 Using SPR Technology. The apparent binding affinities of murine 225 and C225 were also determined using the BIAcore™ (Pharmacia Biosensor, Piscataway NJ; manufacturer’s application note 301; Refs. 18–20). Briefly, soluble recombinant EGFR (a gift from Dr. Joseph Schlessinger, New York University, New York, NY) was immobilized on sensor chips via amino groups as described by the manufacturer. Real-time binding parameters of 225 and C225 to EGFR were established at various antibody concentrations, and the apparent K_d was calculated from the binding rate constants obtained by analyzing the data using BIAevaluation™ software.

In Vitro Inhibition of Cell Growth with 225 and C225. The in vitro inhibitory activity of 225 and C225 was determined as follows. A431 cells (10^4 cells/well) were plated in 96-well microtiter plates in complete growth medium. After adding C225 or 225 in various concentrations (four replicates/concentration, serial dilutions with a starting concentration of 5 μg/ml), plates were incubated for 48 h at 37°C, followed by a 24-h pulse with [3H]thymidine. Cells were harvested, collected on filter mats, and counted in a Wallac Microbeta scintillation counter. The percentage inhibition compares the decrease in [3H]thymidine incorporation of antibody-treated cells with cells grown in the absence of antibody or in the presence of an irrelevant human myeloma IgG1 antibody (Tago).

Phosphorylation Studies. Phosphorylation assays and subsequent Western blot analysis were done as previously described (21). Briefly, A431 cells were grown to 90% confluence in complete medium and then starved in RPMI and 0.5% BSA for 24 h prior to experimentation. Cells were stimulated with 10 ng/ml EGF in the presence of 10 μg/ml either 225 or C225 for 15 min at room temperature. Following stimulation, monolayers were washed with ice-cold PBS containing 1 mM sodium orthovanadate. Cells were lysed and subjected to SDS-PAGE followed by Western blot analysis. The phosphorylation patterns were determined by probing the blots with a mAb to phosphotyrosine (Upstate Biotechnology, Inc., Lake Placid, NY) followed by detection by the enhanced chemiluminescence method (Amersham, Arlington Heights, IL).

Animal Studies. Athymic nude mice (nu/nu; 6–8-week-old females) were obtained from Charles River Laboratories (Wilmington, MA) and maintained under clean conditions. Animals (7–10 mice/treatment group) were inoculated s.c. on the right flank with 10^7 A431 cells in 0.5 ml HBSS. Antibody therapy was begun when tumors reached an average volume >150 mm^3 (7–12 days). Treatments consisted of twice weekly i.p. injections of 225 or C225 (varying concentrations of antibody in 0.5 ml PBS) over 5 weeks. Control animals received PBS. Tumors were measured twice per week, and volumes were calculated using the following formula: π/6 × larger diameter × (smaller diameter)^2 (8). Animals were followed for at least 2 weeks after the final antibody treatment (i.e., 7 weeks after the start of therapy), at which time control and test animals with extremely large tumors were euthanized. Tumor-free mice and animals with small tumors were followed for an additional 2–3 months. Statistical analysis of tumor growth for each of the studies was determined by a Student’s t test using the computer program SigmaStat (Jandel, San Rafael, CA). P < 0.05 was considered significant.

In addition to demonstrating growth inhibitory effects of the antibodies, many animals were found to be in complete remission (i.e., tumor free). This biological effect was quantified as a RI and defined as the number of tumor free mice/total animals within a treatment group. Animals that died during treatment were considered treatment failures and were retained in the analysis. For example, one complete remission among 10 animals would equal a RI of 0.1.

### RESULTS

**In Vitro Properties of 225 and C225.** Initially, the biological effects of 225 and C225 were compared in a series of in vitro assays. The apparent K_d of the antibodies were found to be 0.1 and 0.201 nM for C225 and 1.17 and 0.868 nM for 225, using the ELISA and SPR methods, respectively (Table 1).

### Table 1 K_d for 225 and C225 as determined by various methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Receptor form</th>
<th>225 K_d (nM)</th>
<th>C225 K_d (nM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scatchard A431 lysates</td>
<td>1</td>
<td>nd&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Scatchard M24met cells</td>
<td>0.78</td>
<td>0.39</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>ELISA Fixed A431 cells</td>
<td>1.17</td>
<td>0.147</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPR Soluble receptor</td>
<td>0.868</td>
<td>0.201</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Scatchard results are expressed as K_d. SPR results as apparent K_d. The latter is a relative measure of the K_d. See "Materials and Methods" for description of the generation of the ELISA and SPR data.

<sup>b</sup> nd, not done.

**[Table 1](#) K_d for 225 and C225 as determined by various methods**

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These results (Table 1) were similar to published data for C225. The average cpm for cells grown in the absence of antibody was 82,674 ± 4,518. There was no inhibitory effect for either the human IgG1 or mouse IgG1 irrelevant control antibodies.

Inhibition of the growth of cultured A431 cells in the presence of 225 (M225) were inoculated with A431 cells, and treatments were begun in the absence of antibody. The average cpm for cells grown in the absence of antibody was 82,674 ± 4,518. There was no inhibitory effect for either the human IgG1 or mouse IgG1 irrelevant control antibodies.

In Fig. 1, inhibition of the growth of cultured A431 cells in the presence of 225 (M225) and C225. Data are presented as percentage inhibition. The average cpm for cells grown in the absence of antibody was 82,674 ± 4,518. There was no inhibitory effect for either the human IgG1 or mouse IgG1 irrelevant control antibodies.

In Vivo Properties of 225 and C225 against A431 Xenografts. The in vitro results indicated that chimerization of 225 did not affect the biological properties of the antibody and may have increased the relative binding affinity of C225 for the receptor. The capacity of the antibodies to inhibit the growth of established A431 xenografts in nude mice was then tested. Mice were inoculated with A431 cells, and treatments were begun when tumors reached an average volume >150 mm³. Animals were then randomly grouped and given injections of PBS and 225 (study 1) or PBS and C225 (studies 2 and 3). In study 1 and 2, animals received injections of 1 mg antibody (in 0.5 ml PBS) twice weekly over 5 weeks for a total dose of 10 mg antibody/animal. In study 3, animals received one of three possible doses: 1, 0.5, and 0.25 mg/injection for total doses of 10, 5, and 2.5 mg, respectively. At the end of a treatment protocol, tumor-free animals and those with small tumors continued to be monitored for an additional 2–3 months. In several of the studies, large SDs were observed in both the test and control groups. This resulted from the random selection of tumor-bearing animals for each treatment group. Randomization was an attempt to eliminate experimental bias that might have occurred from a preponderance of large or small tumors within a specific group.

![Fig. 1](image1.png)

**Fig. 1** Inhibition of the growth of cultured A431 cells in the presence of 225 (M225) and C225. Data are presented as percentage inhibition.

![Fig. 2](image2.png)

**Fig. 2** Inhibition of EGF-induced phosphorylation of the EGFR by 225 and C225. A431 cells were stimulated with EGF in the presence or absence of 225 or C225. Lysates were separated by SDS-PAGE and analyzed on Western blots using anti-phosphotyrosine to probe for phosphorylated proteins. A, serum free, no additions; B, EGF stimulated, no antibodies; C, EGF stimulated plus 225; D, EGF stimulated plus C225; E, 225 alone, no EGF; F, C225 alone, no EGF.

**Table 2** RIs for animals inoculated with A431 cells and treated with 225 or C225

<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>No. remissions/total</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>225</td>
<td>1/10</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>C225</td>
<td>4/10</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>C225, 1 mg</td>
<td>7/7</td>
<td>1.0</td>
</tr>
<tr>
<td>C225, 0.5 mg</td>
<td>4/7</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>C225, 0.25 mg</td>
<td>1/7</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>0/7</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

a A comparison of complete tumor remissions in athymic nude mice carrying established A431 tumors following treatment with PBS, 225, or C225 twice weekly for 5 weeks. Animals were treated with 1 mg antibody in 0.5 ml PBS by the i.p. route, except for experiment 4, which is a dose-response experiment in which mice were given 1, 0.5, or 0.25 mg/injection.

b Tumor-free animals/total number of animals. Animal mortality was treated as a treatment failure and included in the final analysis.

c The RI is defined as the fraction of mice that were tumor free on the day when PBS control mice and test animals with large tumors were euthanized. All animals showing complete remissions or small tumors were followed for an additional 2–3 months. Animals that died during the course of treatment were considered treatment failures and were retained in the analysis.

d The RI is defined as the fraction of mice that were tumor free on the day when PBS control mice and test animals with large tumors were euthanized. A complete remission at the 0.25-mg dose level showed a subsequent recurrence of tumor (day 47; see also Fig. 4B) and is not included in the analysis.
Fig. 3 Effect of 225 on the growth of established A431 xenografts in nude mice (study 1). Animals were treated with PBS or 1 mg 225 twice weekly for 5 weeks. Tumors were measured twice weekly and volumes calculated as described in "Materials and Methods." The average tumor volumes at the start of the study were 383 mm$^3$ for PBS and 513 mm$^3$ for 225. A, average tumor volume for each group over the course of the study; B, RI; C and D, effect of 225 or PBS on tumor growth in individual animals.

Fig. 3 shows the effect of 225 on the growth of A431 tumors in nude mice (study 1, treatments beginning on day 11). There was no difference in the average tumor volumes between the control and 225 groups over the course of the study (Fig. 3A) and only one complete tumor remission was observed (RI, 0.1; Fig. 3B and Table 2). When the antitumor responses of 225 were compared in individual animals, tumor growth was strongly inhibited by the antibody in 3 of 10 animals (including the one complete remission), and transient regressions were seen in 2 others (Fig. 3C). These results were similar to data that have been previously reported (6, 10, 11).

On the other hand, C225 alone was found to be extremely effective in inhibiting the growth of A431 tumors. As can be seen in Fig. 4A (study 2, treatments beginning on day 9), there was a significant antitumor effect of C225 beginning on day 33 ($P < 0.02$). The RI for the C225 group was 0.4, indicating that 4 of 10 animals were tumor free following the treatment regimen (Fig. 4B). Tumor-free animals were followed for an additional 3 months and remained in complete remission (data not shown). The antitumor response of C225 on individual mice was very dramatic, with tumor regressions observed in 9 of 10 animals, including 3 mice with tumor volumes $>2500$ mm$^3$ (Fig. 4C).

Because the 1-mg dose of C225 alone effectively inhibited the growth of A431 xenografts, a study was initiated to define the lowest dose of antibody retaining biological activity. Fig. 5 shows the results of a dose-response regimen on the growth of A431 tumors (study 3, treatments beginning on day 12). In this study the average tumor volume for the PBS group seemed to plateau at 5,000 mm$^3$ compared with the continued rapid growth of the control tumors in studies 1 and 2, in which volumes reached $>10,000$ mm$^3$. These differences resulted from the death of animals with actively growing tumors in the early stages of study 3 (Fig. 5B). In comparison to the PBS group, the
A431 xenografts were extremely sensitive to therapy with C225. At the 1-mg dose, all animals underwent complete remission by day 36 (Fig. 5, A, D, and F, and Table 2) and remained tumor free for more than 100 days following termination of the antibody injections. Significant tumor inhibition was seen beginning on day 18 (Fig. 5A). At the 0.5-mg dose level, the overall inhibition of tumor growth was not statistically significant from the controls. This resulted from the rapid growth of one tumor within this group (i.e., treatment failure), which skewed the statistical analysis (Fig. 5, B, F, and H). However, the RI was high for the 0.5-mg group (four of seven mice; RI, 0.57; Fig. 5D and Table 2), and two additional regressions were noted (Fig. 5G).

At the 0.25-mg dose, the average tumor growth seemed to be greater than the PBS control (Fig. 5C). This was due to the rapid growth of tumors in two animals, with a concomitant increase in the average tumor volume compared with the control (Fig. 5G). Overall, there was no significant difference between these groups, but it is important to note that four of seven mice at the 0.25-mg dose showed transient antitumor responses (Fig. 5G), and one animal was tumor free at the end of the study (RI, 0.14; Fig. 5D and Table 2). On day 47, there seemed to be a drop in the RI. At this time, a tumor reappeared in one mouse that had apparently undergone a complete remission. In this single case, the biological effect of C225 was transient, and this animal was not included as an RI in Table 2. As with the 1- and 0.5-mg-dose groups, no recurrences were observed in the one tumor-free animal in the 0.25-mg group.

In general, tumors that regressed but did not disappear began to grow once the antibody injections were stopped. How-
Fig. 5  Dose response of C225 on the growth of established A431 xenografts in nude mice (study 3). Animals were treated with PBS or C225 at either 1, 0.5, or 0.25 mg/animal twice weekly for 5 weeks as described in ″Materials and Methods." The average tumor volumes at the start of the study were 361 mm$^3$ for PBS; 183 mm$^3$ for C225, 1-mg dose; 160 mm$^3$ for C225, 0.5-mg dose; and 284 mm$^3$ for C225, 0.25-mg dose. A, average tumor volumes for the C225 1-mg-dose group versus PBS over the course of the study; B, average tumor volumes for the C225 0.5-mg-dose group versus PBS; C, average tumor volumes for the C225 0.25-mg-dose group versus PBS; D, RI (note: the drop in RI for the 0.25 mg dose group on day 47 resulted from the reappearance of a tumor in an apparent tumor-free animal; in this instance, the C225 had a transient biological effect); E–H, effect of C225 and PBS on the growth of tumors in individual animals. Statistical significance was determined by a Student’s t test and is shown by the asterisks. $P < 0.05$ was considered significant. Beginning on day 18, animals treated with 1 mg/dose C225 showed statistically significant biological effects compared with controls that continued throughout the course of the study. All animals within this group showed complete tumor remissions (RI, 1).
ever, posttreatment tumor progression in the C225-treated groups was characterized by an extended lag phase (i.e., stable disease) that was not seen in control animals (Figs. 4, C and D, and 5, E–H). It should also be noted that animals treated with either 225 or C225 showed greater survival characteristics compared with the PBS control group (data not shown). In all studies, >90% of the antibody-treated animals survived the therapeutic regimens, whereas only about 40% of the control animals were alive at the end of a study. These observations suggested that animal mortality in the control group was related to tumor progression.

**DISCUSSION**

In this report, we compared the *in vitro* and *in vivo* (i.e., tumor-inhibitory) properties of the murine anti-EGFR mAb 225 with C225, its chimeric counterpart. Because C225 was constructed using the Fv region of 225, it was expected that the biological characteristics of both antibodies would be similar. However, results of these studies have indicated that, although the *in vitro* characteristics of 225 and C225 were comparable, the *in vivo* effects of the antibodies differed considerably. Although we could not rule out the possibility that antibody isotype played a role in the differences seen between 225 and C225 (i.e., human IgG1 *versus* mouse IgG1), this did not seem to be the case. Recently it has been shown that neither 225 nor C225 induced complement-mediated lysis to any degree, and the antibody-dependent cellular cytotoxicity reactivity of these antibodies was species specific (22). These data suggested that, if inhibition of A431 xenografts was mediated through immune responses, 225 should be the more potent antibody because of its ability to activate the murine effector cells involved in antibody-dependent cellular cytotoxicity. As we have shown, the opposite is the case. In addition, there were differences in the way individual animals within a group responded to treatment with either 225 or C225. C225 alone was very effective in inducing complete tumor remission at the 1- and 0.5-mg doses, whereas 225 at the highest dose level showed marginal biological effects. Although C225 showed a significant antitumor effect on the growth of A431 xenografts in study 2, the R1 differed from the comparable group in study 3 (0.4 *versus* 1.0, respectively). We believe that this difference was due to tumor burden, because the initial tumor volume for the C225 treatment group in study 2 was 267 mm$^3$ compared with 183 mm$^3$ for the 1-mg dose level in study 3. The effect of tumor size on the biological efficacy of C225 is currently under investigation.

One possible explanation for the increased efficacy of C225 may be found in the higher affinity of the chimeric antibody for EGFR compared with its murine counterpart. C225 bound to EGFR with a relative affinity 5-fold higher than 225 (Table 1; Refs. 8 and 22). Although unexpected, the increased affinity of a recombinant antibody such as C225 is not novel. A humanized anti-CD33 mAb was shown to have a 10-fold higher binding avidity (authors' terminology) compared with both the original murine mAb and chimeric constructs (23). The authors speculated that changes in carbohydrate content at the antibody's variable region were responsible for the increased avidity of the humanized antibody. At present, the reasons for the lower $K_d$ of C225 are not known. However, TGF-α binds to the receptor with an affinity similar to 225 (1 nm), and it is likely that C225, with a 5-fold lower $K_d$, would have a competitive advantage over the ligand at saturating concentrations. Thus, C225 may inhibit the proliferation of A431 cells in nude mice by more efficiently blocking the autocrine loop responsible for the growth of these tumors *in vivo*.

The chimeric anti-EGFR antibody C225 has shown excellent biological activity *in vivo*. Phase I trials with C225 are currently underway, with the hope that the results from the preclinical animal models may translate into therapeutic efficacy in humans.

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