

## Antibody-Dependent Cellular Cytotoxicity Mediated by Cetuximab against Lung Cancer Cell Lines

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**Abstract Purpose:** Epidermal growth factor receptor (EGFR) is commonly overexpressed in lung cancer. Cetuximab is a chimeric mouse-human antibody targeted against EGFR. Compared with its inhibitory properties, its immunologic mechanisms have not been well studied. In this study, we investigated the antibody-dependent cellular cytotoxicity (ADCC) activity of cetuximab against lung cancer cell lines.

**Experimental Design:** We studied the correlation between EGFR expression in lung cancer cell lines and the ADCC activity of cetuximab as well as the influence of interleukin-2 and chemotherapy on the ADCC activity. EGFR expression was measured by a quantitative flow cytometric analysis and immunohistochemistry. The ADCC activity was assessed by a 4-h <sup>51</sup>Cr release assay. Peripheral blood mononuclear cells, purified T cells, natural killer (NK) cells, and monocytes from healthy donors or lung cancer patients were used as effector cells.

**Results:** Fresh peripheral blood mononuclear cells exhibited cetuximab-mediated ADCC activity against lung cancer cell lines at a low concentration of cetuximab (0.25 µg/mL). A logarithmic correlation was observed between the number of EGFRs and ADCC activity. Even low EGFR expression, which was weakly detectable by immunohistochemistry, was sufficient for maximum ADCC activity, and further increases in EGFR expression on the target cells had no further effect on the ADCC activity. In addition, ADCC activity was enhanced by interleukin-2 mainly through activation of NK cells and was less susceptible to immunosuppression by chemotherapy than NK activity in lung cancer patients.

**Conclusions:** These observations suggest the importance of ADCC activity as an immunologic mechanism of cetuximab in biological therapy for lung cancer patients.

Lung cancer is the leading cause of cancer death in the world. Non-small-cell lung cancer (NSCLC) comprises >80% of all lung cancer cases. The majority of NSCLC patients present with locally advanced or systemic disease and are inoperable. Systemic chemotherapy with cytotoxic agents is the current first-line treatment for such patients and improves their survival (1). However, complete response rates are still low and long-term survival remains poor, and more specific, effective, and less toxic therapies are being sought. An increased understanding of the molecular basis of lung cancer has led to novel

targeted strategies that inhibit specific key molecules in tumor growth and progression. Epidermal growth factor receptor (EGFR) is commonly overexpressed in NSCLC and its overexpression frequently correlates with a poor prognosis (2). Therefore, EGFR is an attractive molecular target, and agents to specifically target the EGFR are being developed.

There are two approaches for inhibiting EGFR signaling: to prevent ligand binding to the extracellular domain with a monoclonal antibody and to inhibit the intracellular tyrosine kinase activity with a small molecule. The latter approach has been applied clinically first. Gefitinib, a quinazolin derivative that specifically inhibits the activation of EGFR tyrosine kinase through competitive binding to the ATP-binding domain of the receptor, has received approval for patients with advanced NSCLC refractory to chemotherapy in Japan in July 2002 and subsequently has gained approval in over 30 countries, including the United States. However, recently, two large randomized studies (3, 4) revealed that the addition of gefitinib to conventional chemotherapy regimens did not improve any outcome variable. These disappointing results of EGFR tyrosine kinase inhibitors have led to increased interest in the monoclonal antibodies against EGFR because these two classes of agents may have substantially different mechanisms of action.

Cetuximab is a chimeric mouse-human antibody targeted against the extracellular domain of EGFR, thereby inhibiting the

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binding of activating ligands to the receptor. Consequently, cetuximab inhibits ligand-dependent activation of the EGFR and inhibits the downstream pathways, which cause cell cycle progression, cell growth, and angiogenesis. In addition, the binding of cetuximab initiates EGFR internalization and degradation, which leads to signal termination (5). Moreover, unlike EGFR tyrosine kinase inhibitors, cetuximab potentially can provoke immunologic antitumor effects, such as antibody-dependent cellular cytotoxicity (ADCC) activity, as it has a human IgG1 backbone (6). The ADCC activities of monoclonal antibodies for cancer have been well described in trastuzumab (Herceptin), an antibody for breast cancer (7), and rituximab (Rituxan), an antibody for B-cell lymphoma (8). These ADCC activities are thought to be crucial for their antitumor effects. However, about cetuximab, except for one study that showed its capacity to mediate ADCC activity against a cultured melanoma cell line (6), no published studies have focused on its ADCC activity, especially against lung cancer cell lines.

In the present study, we investigated the biological activity of cetuximab against a panel of lung cancer cells with respect to ADCC activity. In these lung cancer cells, we evaluated the relationship between EGFR expression as measured by quantitative fluorescence flow cytometry or by immunohistochemistry and cetuximab-mediated ADCC activity. Additionally, to assess the influence of chemotherapy on the ADCC activity of cetuximab, we compared the ADCC activity in peripheral blood mononuclear cells (PBMC) obtained from NSCLC patients before and after chemotherapy.

## Materials and Methods

**Cell lines and cell culture.** Eight lung cancer cell lines (LK-1, EBC-1, A549, LK87, Lu99, N417, Ms1, and LU65) and the epidermoid carcinoma cell line (A431) were used in this study. Ms1 lung cancer cell line was provided by Dr. T. Hirashima (Osaka Prefectural Medical Center for Respiratory and Allergic Disease, Osaka, Japan). A549 lung cancer cell line was obtained from the RIKEN Cell Bank (Tsukuba, Japan). LK-1, LU65, EBC-1, and Lu99 lung cancer cell lines and A431 epidermoid carcinoma cell line were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). LK87 lung cancer cell line was provided by Dr. S. Kobayashi (Miyagi Prefectural Semine Hospital, Semine, Japan) through the Cell Resource Center for Biomedical Research. N417 lung cancer cell line was provided by Dr. A.F. Gazdar and Dr. H. Oie (National Cancer Institute-Navy Medical Oncology Branch, NIH, Bethesda). EBC-1, A549, N417, and Ms1 were maintained in DMEM supplemented with 10% FCS, 50 units/mL penicillin, 50 units/mL streptomycin, and 4.0 mmol/l glutamine. The other lines were cultured in RPMI 1640 supplemented with 10% FCS, 50 units/mL penicillin, 50 units/mL streptomycin, and 2.05 mmol/l glutamine.

**Monoclonal antibody.** Cetuximab was obtained from Bristol-Myers Squibb (New York, NY). Rituximab, used as a control antibody, was obtained from Chugai Pharmaceutical (Tokyo, Japan). The monoclonal antibodies used for flow cytometry recognize the following surface molecules: antibodies to CD3, CD14, CD69, CD25, and CD16 were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany), and antibody to CD56 was obtained from Becton Dickinson (Franklin Lakes, NJ). Anti-EGFR antibody (clone 528) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-EGFR antibody (clone 31G7) for immunohistochemical analysis was obtained from Zymed (South San Francisco, CA). Murine IgG1 (Becton Dickinson), IgG2a (Sigma-Aldrich, St. Louis, MO), and IgG2b (Miltenyi Biotec) were used as isotype controls.

**Flow cytometric analysis.** For the analysis of the surface molecules of effector cells, isolated cells were incubated for 30 min at 4°C in PBS containing FITC-, phycoerythrin (PE)-, or allophycocyanin (APC)-conjugated monoclonal antibodies as indicated above. Cells were then washed and analyzed using a FACScan flow cytometer (Becton Dickinson). To determine the absolute number of EGFR molecules per cell, we carried out a quantitative flow cytometric analysis using DAKO QIFIKIT (DakoCytomation, Copenhagen, Denmark) and EGFR monoclonal antibody (clone 528). Briefly,  $10^4$  cells were incubated for 1 h at 4°C with 0.4 µg of the primary antibody or the isotype control IgG2a antibody in PBS containing 1% bovine serum albumin and 0.01% sodium azide. After washing thrice with this PBS, cells were incubated for 1 h with FITC-conjugated anti-mouse IgG (DakoCytomation) at 4°C. Similar to the samples labeled with FITC-conjugated anti-mouse IgG from this kit, standard beads coated with a known amount of mouse IgG molecules also were labeled with this secondary antibody. The labeled samples were washed thrice with the PBS and analyzed using flow cytometry (Becton Dickinson). The number of antibody binding sites per cell was calculated by comparing the mean fluorescent intensity value of the labeled cells with a calibration curve obtained by regression analysis of the mean fluorescent intensity values of the standard beads.

**Purification of effector cells and interleukin-2 treatment.** PBMCs were isolated from heparinized peripheral blood by lymphocyte separation medium (MP Biomedicals, Irvine, CA) density gradient centrifugation. Highly purified CD3<sup>+</sup> T cells, CD56<sup>+</sup> natural killer (NK) cells, and CD14<sup>+</sup> monocytes were obtained by magnetic cell sorting (MACS) using the system from Miltenyi Biotec according to the manufacturer's instructions. Freshly isolated PBMCs were incubated in the presence of microbead-conjugated anti-CD3 monoclonal antibody for 15 min at 4°C. After repeated washing, antibody-coated cells were passed through a MACS separator in a magnetic field. The positively selected CD3<sup>+</sup> T lymphocytes were washed repeatedly and flushed outside the magnetic field. The effluent was collected as CD3-negative lymphocytes and further separated into CD56<sup>+</sup> and CD56<sup>-</sup> lymphocytes using anti-CD56 monoclonal antibody-coated magnetic bead (Miltenyi Biotec). After positive selection of CD56<sup>+</sup> cells by MACS separator, the CD56-negative cells were used to successive selection of CD14<sup>+</sup> cells using anti-CD14 monoclonal antibody-coated magnetic bead (Miltenyi Biotec). The purity of CD3<sup>+</sup> T cells, CD3<sup>-</sup>CD56<sup>+</sup> NK cells, and CD3<sup>-</sup>CD56<sup>-</sup>CD14<sup>+</sup> monocytes were greater than 95%, 95%, and 91%, respectively, as determined by flow cytometry. To investigate the effect of interleukin-2 (IL-2; Sigma-Aldrich) on ADCC activity, PBMCs or purified subpopulations ( $10^6$  cells/mL) were preincubated at 37°C for up to 18 h in the presence of IL-2 (30 ng/mL; ref. 9). After incubation, the cells were washed once in RPMI 1640 and tested for their cytotoxic activity.

**Test for ADCC and NK activity.** After the target lung cancer cells were labeled with 100 µCi <sup>51</sup>Cr (Perkin-Elmer Life and Analytical Sciences, Boston, MA) for 60 min, target cells ( $10^4$  per well) and effector cells at various E:T ratios were coincubated in 200 µL DMEM or RPMI 1640 in a 96-well U-bottomed plate in triplicate for 4 h at 37°C with cetuximab or control antibody, rituximab. Next, the amount of radioactivity in the supernatant liquid was measured by a gamma counter. The percentage of specific cytolysis was calculated using the formula: percentage specific lysis = [(experimental counts per minute – spontaneous counts per minute) / (total counts per minute – spontaneous counts per minute)] × 100; ADCC activity = [(counts per minute with experimental antibody – counts per minute with control antibody) / (total counts per minute – counts per minute with control antibody)] × 100 (10); and NK activity = [(counts per minute of effector cells – spontaneous counts per minute) / (total counts per minute – spontaneous counts per minute)] × 100.

**Donors.** This study included 10 normal healthy medication-free donors (2 female, 8 male; mean age, 32.1 years; range 22-37 years) and 12 patients with histologically proven NSCLC. No patient had received prior chemotherapy. This study was approved by the ethics committee

of Tottori University, and informed consent for blood donation was obtained from all of the individuals.

**Immunohistochemical analysis.** Paraffin-embedded cell blocks were prepared from each lung cancer cell line, which were fixed in 4% paraformaldehyde. Tissue sections (3  $\mu$ m) were dewaxed with xylene, rehydrated in serial ethanol solutions, and then immersed in 0.6% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase. Heat pretreatment was used for antigen retrieval. The slides were preblocked with 10% normal rabbit serum at room temperature for 20 min and incubated at 4°C overnight with the primary antibody, anti-EGFR antibody (clone 31G7). The immunoreaction was visualized with 3,3'-diaminobenzidine. The staining results were measured semiquantitatively on a scale of 0, 1+, 2+, and 3+. We did the staining for the each cell line thrice, and the intensity was evaluated by two independent pathologists.

**Statistics.** The logarithmic correlation between ADCC activity and the expression levels of EGFR was analyzed by curvilinear regression using Microsoft Excel software (Microsoft, Redmond, WA).

## Results

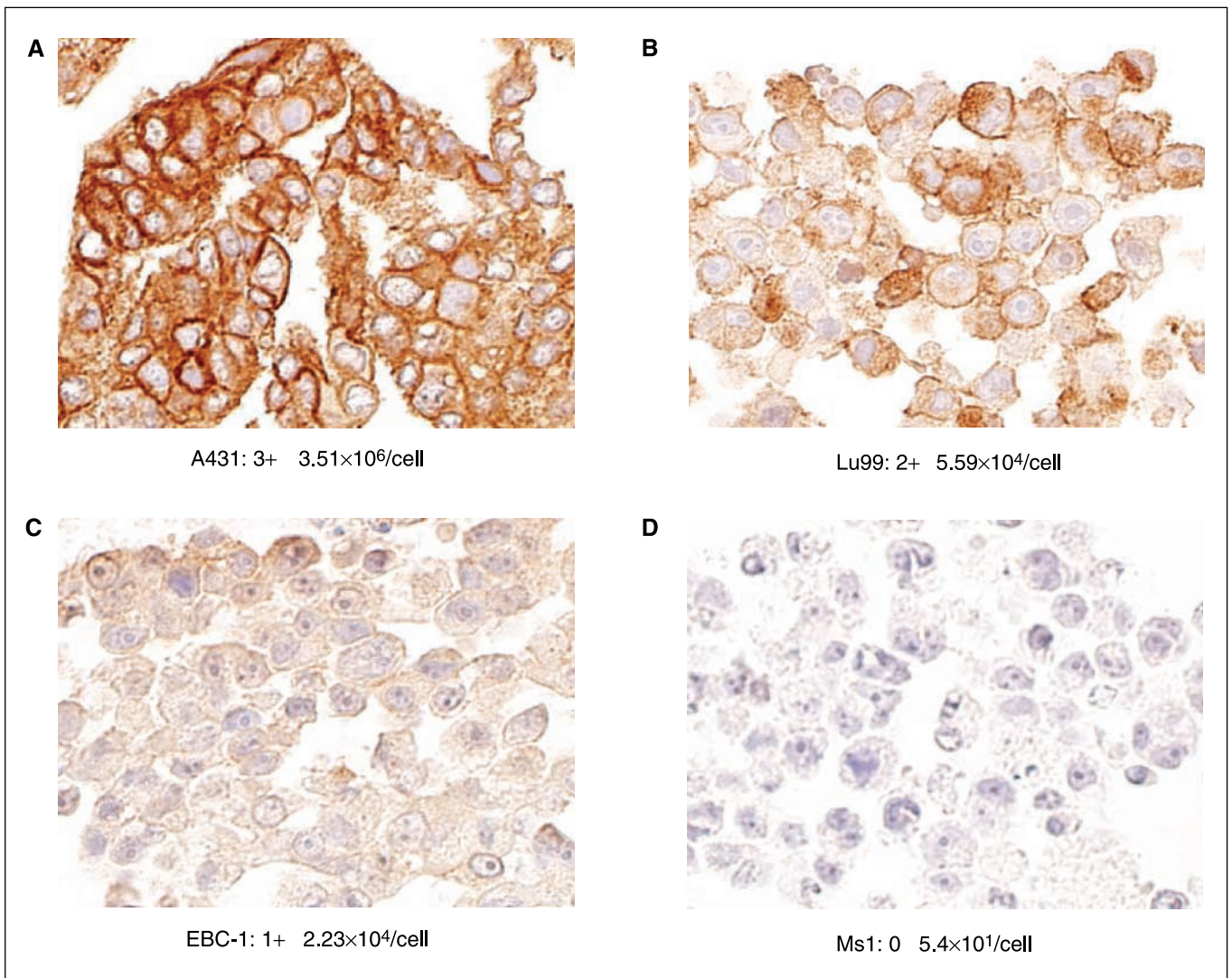
**Analysis of EGFR expression in lung cancer cell lines by flow cytometry and immunohistochemistry.** We first examined the expression of EGFR on eight lung cancer cell lines and an epidermoid carcinoma cell line, A431. A431 is used as a positive control for EGFR in most studies because it has been reported previously to highly express EGFR (11). We measured the number of EGFR on each cell line by quantitative flow cytometric analysis (DAKO QIFIKIT; ref. 12) and compared it with the evaluation by immunohistochemistry (scored semiquantitatively from 0 to 3+). Staining was scored as follows: 0, no membranous staining in any of the cells; 1+, weak intensity membranous and cytoplasmic staining of nearly equal intensity; 2+, moderate to strong intensity staining predominantly in the membranes; and 3+, strong intensity staining clearly localized to the cell membranes. Representative examples of 0, 1+, 2+, and 3+ immunohistochemistry staining for EGFR are shown in Fig. 1. As shown in Table 1, all types of lung cancer cell lines express EGFR to various degrees (ranging from  $5.4 \times 10^1$  to  $6.01 \times 10^4$  per cell), although the levels were considerably lower than that of the positive control cells, A431 ( $3.51 \times 10^6$  per cell). By immunohistochemistry, two cell lines (Ms1 and N417), which express little EGFR (ranging from  $5.4 \times 10^1$  to  $1.97 \times 10^2$  per cell), did not stain and were scored 0. The other seven cell lines that expressed between  $5.25 \times 10^3$  and  $3.51 \times 10^6$  EGFR molecules per cell scored from 1+ to 3+ in proportion to the number of binding sites per cell. These results indicated a good correlation between the number of EGFR molecules on the cells and their EGFR status estimated by immunohistochemistry.

**Cetuximab-mediated cytotoxicity against A549 cells by healthy human PBMCs.** To test whether cetuximab induces ADCC activity against lung cancer cell lines, we did a 4-h  $^{51}\text{Cr}$  release assay against A549 cells that weakly express EGFR using healthy human PBMCs ( $n = 5$ ) at various E:T ratios. As shown in Fig. 2A, at the higher E:T ratio of 40:1 and 20:1, cetuximab exhibited an enhanced cell lysis, about 55% and 40% lysis above background or NK activity levels, respectively. There was no significant ADCC activity by the control antibody, rituximab, against the A549 cells (data not shown). These data suggest that cetuximab was capable of activating ADCC activity efficiently even against lung cancer cells, which weakly express EGFR. Next, to identify the optimal cetuximab concentration

for ADCC activity, we did a 4-h  $^{51}\text{Cr}$  release assay with cetuximab concentrations ranging from 0.000025 to 1,000  $\mu\text{g}/\text{mL}$  using an E:T ratio of 20:1. As shown in Fig. 2B, cetuximab-mediated ADCC activity against A549 cells was already detectable at a concentration of 0.000025  $\mu\text{g}/\text{mL}$  and was saturated at 0.25  $\mu\text{g}/\text{mL}$ . These data indicate that a cetuximab concentration in excess of 0.25  $\mu\text{g}/\text{mL}$  was sufficient for maximum ADCC activity. We used this concentration of cetuximab for the subsequent assays.

To evaluate the cetuximab-mediated ADCC activity against lung cancer cell lines with various EGFR expression levels, we did a 4-h  $^{51}\text{Cr}$  release assay with healthy donors' PBMCs at an E:T ratio of 40:1 in the presence of the optimal dose of cetuximab (0.25  $\mu\text{g}/\text{mL}$ ) against four lung cancer cells. As shown in Fig. 2C, summarized data from five healthy donors show that cetuximab-induced cytotoxicity in A549, LK87, and Lu99 cells with different EGFR expression was about equal but was much higher than that in N417 cells with very little EGFR expression. To further explore this correlation, we expanded this assay to include lung cancer cell lines with various EGFR levels. As shown in Fig. 2D, the ADCC activity had no linear correlation with the number of EGFR molecules expressed on the cancer cell surface but had a logarithmic correlation ( $R^2 = 0.8957$ ): the maximum ADCC activity was observed in A549 cells, which have relatively small number of EGFR molecules per cell ( $5.25 \times 10^3$  per cell) and only scored 1+ by immunohistochemistry. The ADCC activity was not further enhanced in the other cells with higher EGFR expression. In addition, as IL-2 is known to activate PBMCs, we tested the effects of overnight treatment of PBMCs with IL-2 on cetuximab-mediated ADCC activity. ADCC activity was enhanced at any EGFR expression levels in the presence of low doses of IL-2 (Fig. 2C and D). These data suggest that the very weak EGFR expression of lung cancers is enough for ADCC activity and also suggest that this ADCC activity is enhanced by IL-2 even in the cells with very weak EGFR expression.

**NK cells are responsible for cetuximab-mediated ADCC activity and their augmentation by IL-2.** To determine which cell types were responsible for the ADCC activity observed, PBMCs were separated into  $\text{CD}3^+$  T cells,  $\text{CD}3^-\text{CD}56^+$  NK cells, and  $\text{CD}3^-\text{CD}56^-\text{CD}14^+$  monocytes by MACS system. After an overnight treatment of each cell subpopulations with IL-2, we assessed the early activation marker (CD69), IL-2 receptor (CD25), and IgG Fc receptor (CD16) by fluorescence-activated cell sorting analysis as described previously (13). As shown in Fig. 3A, the expression of CD69 markedly increased on NK cells and monocytes but less so on T cells after IL-2 treatment. CD25 and CD16 expressions were not changed on T cells and NK cells but were increased on monocytes. After the overnight culture, the percentage of each cell subpopulations was not changed (data not shown). These data suggest that NK cells and monocytes were activated but not expanded by the IL-2 treatment. Next, we tested these cell subpopulations as effector cells in ADCC assay. As shown in Fig. 3B, NK cells showed significant cytotoxicity against A549 lung cancer cell line in the absence and presence of cetuximab (Fig. 3B, left), and this cytotoxicity was enhanced by IL-2 (Fig. 3B, right). In contrast, T cells and monocytes did not show any cytotoxic activity under the standard ADCC conditions used here. These data suggest that  $\text{CD}3^-\text{CD}56^+$  NK cells were primarily responsible for the cetuximab-mediated ADCC activity and the augmentation by IL-2.



**Fig. 1.** Immunohistochemical staining for EGFR expression. *A* to *D*, representative EGFR immunohistochemistry scoring in four lung cancer cell lines. Immunohistochemical score and numbers of EGFR per cell. Magnification,  $\times 400$ .

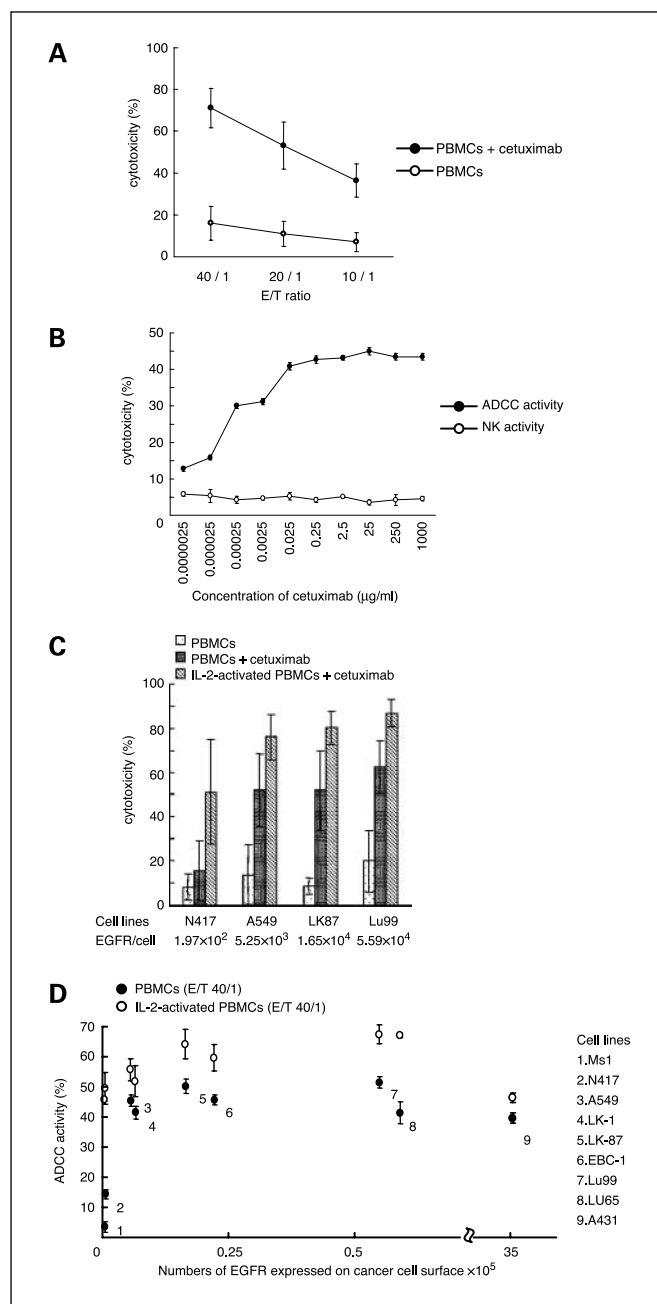
**Influence of chemotherapy on cetuximab-mediated ADCC activity against A549 lung cancer cells.** To evaluate the influence of chemotherapy on cetuximab-mediated ADCC activity, we compared the ADCC activity in the PBMCs of lung cancer patients before and after chemotherapy. We also evaluated the influence of chemotherapy on NK activity as a reference. This experiment included six patients (patients 1-6; Table 2). They received the weekly carboplatin and paclitaxel regimen, one of the representative regimens for advanced NSCLC (14), and cytotoxicities in the PBMCs were measured by a 4-h  $^{51}\text{Cr}$  release assay against A549, in the presence (ADCC activity) or absence (NK activity) of cetuximab, on day 1 (before chemotherapy) and day 28 (after chemotherapy). As shown in Fig. 4A, patients receiving chemotherapy showed a  $12.17 \pm 7.44\%$  (mean  $\pm$  SD) decrease in ADCC activity (Fig. 4A, left) and a  $29.26 \pm 17.52\%$  (mean  $\pm$  SD) decrease in NK activity (Fig. 4A, right). These results suggest that ADCC activity is less susceptible to immunosuppression by lung cancer chemotherapy than is NK activity.

Next, we tested responsiveness of cetuximab-mediated ADCC activity to IL-2 in lung cancer patients. In chemotherapy-naïve

patients, as with healthy donors, representative data showed that IL-2 primarily enhanced the cytotoxicity of NK cells in both the absence and presence of cetuximab (patient 7; Fig. 4B). The same result was obtained after chemotherapy in this patient

**Table 1.** EGFR expression analysis by quantitative flow cytometry and immunohistochemistry in lung cancer cell lines

Cell lines	EGFR expression (no. EGFR per cell)	Immunohistochemical score
Ms1	$5.4 \times 10^1$	0
N417	$1.97 \times 10^2$	0
A549	$5.25 \times 10^3$	1+
LK-1	$6.31 \times 10^3$	1+
LK87	$1.65 \times 10^4$	1+
EBC-1	$2.23 \times 10^4$	1+
Lu99	$5.59 \times 10^4$	2+
LU65	$6.01 \times 10^4$	2+
A431	$3.51 \times 10^6$	3+



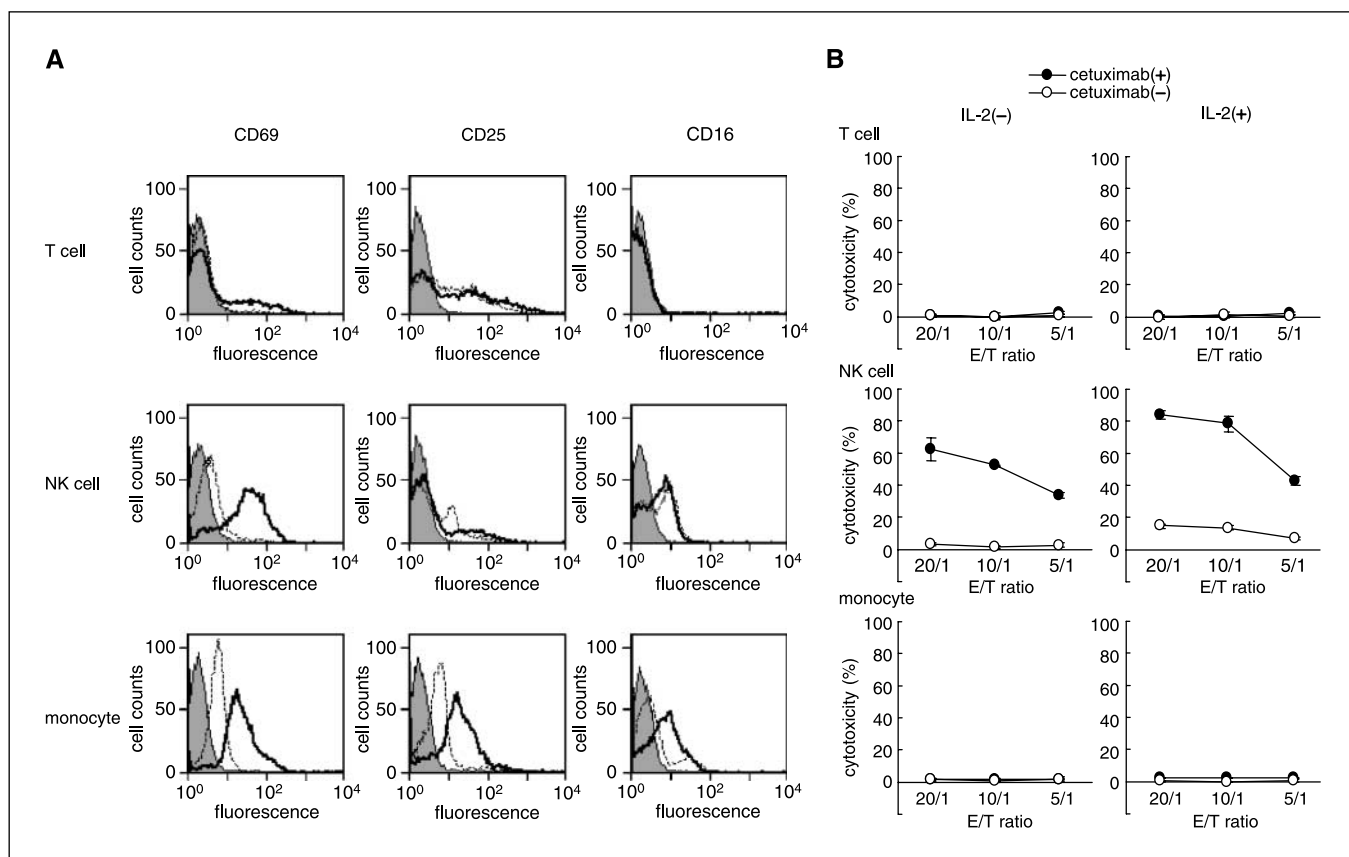
**Fig. 2.** Cytotoxicity against lung cancer cell lines mediated by cetuximab. **A**, cetuximab mediates cytotoxicity against the EGFR-expressing A549 lung cancer cell line. PBMCs from five healthy donors (two female and three male; mean age, 32.8 y; range, 22-37 y), using three different E:T ratios, were tested for cytotoxicity in the presence or absence of cetuximab (2.5 µg/mL). **B**, concentration-dependent curve of cetuximab-dependent ADCC activity and NK activity against A549 cells by healthy human PBMCs. A549 cells were incubated with PBMCs at an E:T ratio of 20:1 along with various concentrations of cetuximab (0.0000025-1,000 µg/mL, mean ± SD). Representative of five independent experiments. Points, mean of a triplicate experiment; bars, SD. **C**, PBMCs from five healthy donors (five male; mean age, 31.4 years; range, 28-37 years) were tested for the cytotoxicity against four different lung cancer cell lines in the absence and presence of cetuximab and tested the effect of IL-2 treatment (30 ng/mL for 18 h). Columns, mean; bars, SD. Numbers of EGFR per cell. **D**, correlation between the EGFR expression levels of target lung cancer cells and the cetuximab-mediated ADCC activity. X-axis, the number of EGFR molecules per cell expressed on the surface of the cancer cell; Y-axis, the ADCC activity as determined by a 4-h <sup>51</sup>Cr release assay. Healthy donors' PBMCs were incubated with and without IL-2 (30 ng/mL) for 18 h and tested the cetuximab-mediated ADCC activity against the various <sup>51</sup>Cr-labeled cell lines. Representative of five independent experiments. The lung cancer cell lines used are indicated. Points, mean of a triplicate experiment; bars, SD.

(data not shown). After chemotherapy, summarized data from five patients who underwent one course of conventional chemotherapy (patients 8-12) showed that ADCC (Fig. 4B, left) and NK (Fig. 4B, right) activity was enhanced by IL-2 treatment (Fig. 4C). These data suggest that the responsiveness of cetuximab-mediated ADCC activity in lung cancer patients to IL-2 was retained after chemotherapy.

### Discussion

In the present study, we evaluated cetuximab-mediated ADCC activity against lung cancer cell lines. There are several findings relevant to clinical therapeutic applications of cetuximab against lung cancer. First, EGFR-expressing lung cancer cell lines can be killed by cetuximab-mediated ADCC activity at clinically achievable concentrations. Furthermore, low EGFR expression levels on lung cancer cells, which are only weakly detectable by immunohistochemistry, are sufficient for maximum ADCC activity mediated by cetuximab. Further increases in EGFR expression levels have no further effect on ADCC activity. Second, IL-2 *ex vivo* treatment of PBMCs can enhance the cetuximab-mediated ADCC activity against lung cancer cell lines mainly through activation of CD3<sup>+</sup>CD56<sup>+</sup> NK cells. Third, cetuximab-mediated ADCC activity in lung cancer patients is less susceptible to chemotherapy-induced immunosuppression than NK activity and is retained responsiveness to IL-2 after chemotherapy. These data suggest the importance of ADCC activity as an anticancer mechanism of cetuximab.

Although many anticancer mechanisms that can account for the therapeutic effects of cetuximab have been proposed, the contribution of ADCC activity remains to be elucidated. A mouse-human chimeric antibody, cetuximab, was generated from a murine monoclonal anti-EGFR antibody (m225), which was developed to inhibit EGFR function by Kawamoto et al. (5). Originally, m225 was reported to have the following properties: binding competitively to the extracellular domain of EGFR, inhibiting the autophosphorylation of EGFR, and inducing its internalization and degradation (5). With these inhibitory properties against EGFR, m225 induces an anticancer effect by several mechanisms, such as blockade of cell cycle traversal by induction of increased levels of p27, increased levels of proapoptotic molecules, enhancement of the cytotoxic activities of chemotherapeutic agents, inhibition of angiogenesis, or inhibition of invasion and metastasis (15). Compared with these pharmacologic receptor blockade mechanisms, any immunologic action of m225, such as ADCC activity, was reported to be minimal because the antitumor activity against xenografts of human cancer cells is retained by the F(ab')<sub>2</sub> fragment of m225 (16). Accordingly, the activity of cetuximab, a mouse-human chimeric version of m225, has been believed to be primarily through its inhibition of EGFR-mediated signaling pathways. However, distinct from m225, cetuximab has a human IgG1 backbone, and chimeric IgG1 antibodies have been reported to efficiently induce ADCC activity of human effector cells, such as NK cells, macrophages, or monocytes (17). For two other therapeutic anticancer monoclonal antibodies, trastuzumab and rituximab, which have a human IgG1 backbone, studies have suggested that ADCC activity plays a predominant role in their *in vivo* antitumor effects (18). About cetuximab, to our knowledge, only one published study has addressed this issue. Naramura et al. (6)



**Fig. 3.** Phenotypical analysis of cell surface markers and ADCC activity of purified T cells, NK cells, and monocytes after IL-2 treatment. *A*, expression of CD69, CD25, and CD16 on human blood T cells, NK cells, and monocytes in response to IL-2. Freshly isolated PBMCs from healthy donors were separated into T cell (CD3<sup>+</sup> CD56<sup>+</sup>), NK cell (CD3<sup>-</sup> CD56<sup>+</sup> CD14<sup>-</sup>), and monocyte (CD3<sup>-</sup> CD56<sup>-</sup> CD14<sup>+</sup>) subpopulations using MACS. These effector cells were cultured with and without IL-2 (30 ng/mL) for 18 h. Cells were directly stained with anti-CD69 FITC/anti-CD25UPE/anti-CD16UAPC and phenotype was then analyzed by fluorescence-activated cell sorting. Solid histogram, matching isotype-negative controls; dotted line, untreated cells; thick line, IL-2-treated cells. Data are representative of three independent experiments. *B*, effect of IL-2 on ADCC activity of human peripheral T cells, NK cells, and monocytes. Each separated cell subpopulations were treated with and without IL-2 as above. These effector cells were tested for cytotoxicity against A549 cells in the presence or absence of cetuximab (0.25  $\mu$ g/mL) at the indicated E:T ratios. Representative of five independent experiments. Points, mean of a triplicate experiment; bars, SD.

reported that cetuximab has the capacity to mediate ADCC activity against cultured melanoma cell lines. These studies suggest that the immunologic effects of cetuximab should be investigated more thoroughly.

In our study, cetuximab-mediated ADCC activity was achieved at a very low antibody concentration. The maximum specific lysis of target cells was observed at a concentration of 0.25  $\mu$ g/mL. In a phase I study of cetuximab, at multiple

**Table 2.** Clinical characteristics of the 12 patients treated with chemotherapy

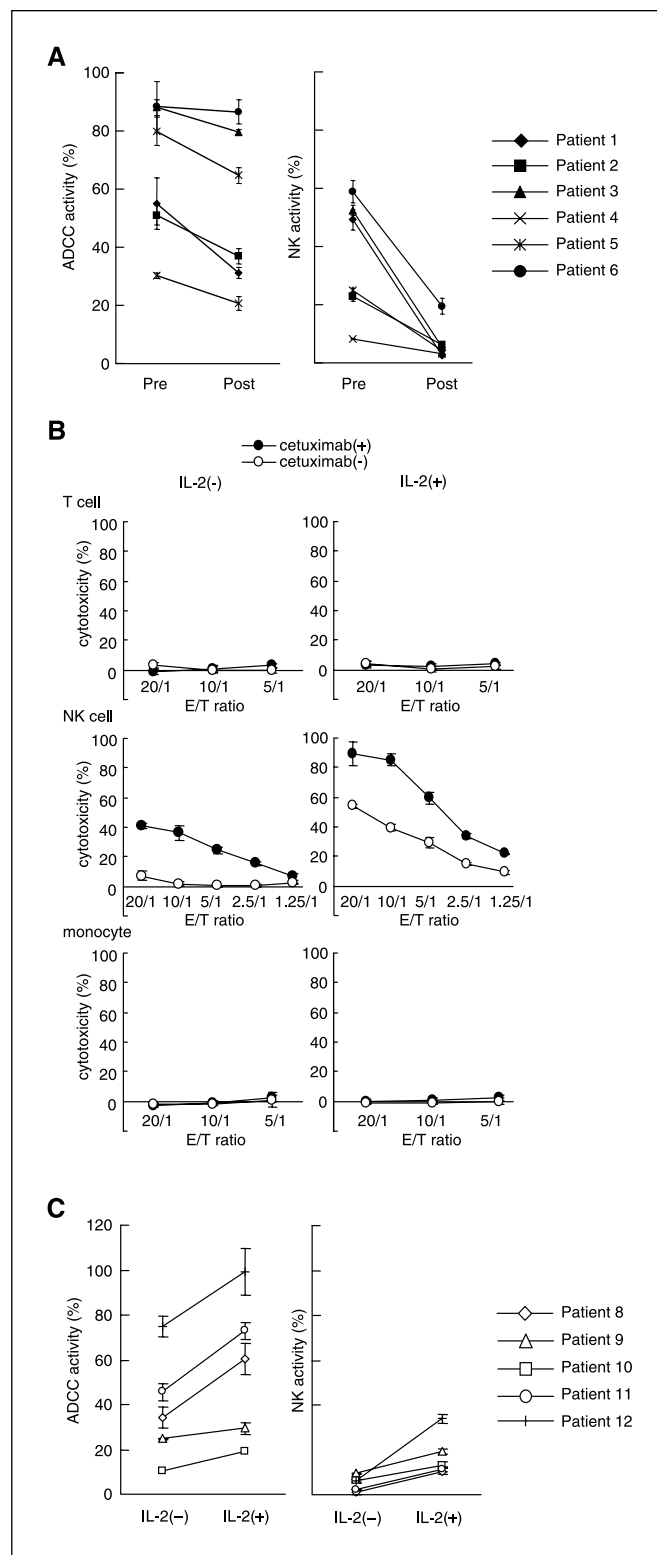
Patient	Age (y)	Gender	Performance status	Weight loss (kg/mo)	TNM classification	Pathologic subtype	Chemotherapy	Response to this chemotherapy
1	60	M	1	-3	cT2N3M1	NSCLC (unknown)	Weekly CBDCA+PTX	SD
2	65	M	0	-1	cT3N2M1	Adenocarcinoma	Weekly CBDCA+PTX	SD
3	39	M	0	-4	cT4N2M1	Adenocarcinoma	Weekly CBDCA+PTX	SD
4	51	M	0	-1	cT4N3M1	Large cell	Weekly CBDCA+PTX	PR
5	52	F	2	-1.5	cT4N3M1	Squamous cell	Weekly CBDCA+PTX	PD
6	49	M	0	$\pm$ 0	pT2N2M0	Large cell	Weekly CBDCA+PTX	NE
7	48	M	0	$\pm$ 0	cT4N3M1	Adenocarcinoma	Weekly CBDCA+PTX	SD
8	73	M	0	-1	cT4N2M0	Squamous cell	VNR+UFT	SD
9	76	F	0	$\pm$ 0	pT2N2M0	Adenocarcinoma	Weekly CBDCA+PTX	SD
10	68	M	0	-4	cT3N0M1	Adenocarcinoma	Weekly CBDCA+PTX	SD
11	70	M	0	$\pm$ 0	cT2N3M0	NSCLC (unknown)	CDDP+DOC	SD
12	62	M	1	-1	cT4N3M1	Squamous cell	CBDCA+PTX	PD

Abbreviations: TNM, tumor-node-metastasis; M, male; F, female; CBDCA, carboplatin; PTX, paclitaxel; VNR, vinorelbine; UFT, tegafur-uracil; CDDP, cisplatin; DOC, docetaxel; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable.

weekly doses of 400 mg/m<sup>2</sup>, the average plasma concentration at steady-state ( $C_{ss,avg}$ ) was estimated to be 56 μg/mL (19). In another phase I study using the schedule of 400 mg/m<sup>2</sup> loading dose followed by a 250 mg/m<sup>2</sup> weekly maintenance dose, the mean peak and trough plasma concentrations of cetuximab were estimated to be 192 and 75 μg/mL,

respectively, whereas the  $C_{ss,avg}$  was ~100 μg/mL (20). Based on these data, the  $C_{ss,avg}$  of cetuximab in cancer patients is estimated to be within the range of 56 and 100 μg/mL under current clinical dosing regimens (21). About the concentrations of cetuximab in tumors, the results of prior studies are variable, from four fold higher (22) to 50- to 100-fold lower (21) in tumor cells than in the serum. Even if one accepts the lowest value, a concentration of 0.25 μg/mL, which mediated maximum ADCC activity in our experiments, can be achieved in tumor tissue. In addition, the concentration of 0.25 μg/mL is considerably lower than that required to achieve the maximum inhibition of proliferation by pharmacologic receptor blockade mechanisms *in vitro*, which is ~1.25 μg/mL (23). This is consistent with an earlier report. Bleeker et al. (24) reported that fully human IgG1κ anti-EGFR monoclonal antibody, 2F8, can induce efficient ADCC activity at a very low concentration, although a relatively high antibody dose is needed to completely block EGFR signaling and inhibit cell proliferation. These data indicate that ADCC activity is effective at a relatively low dose and possibly represents an important clinical antitumor mechanism of cetuximab.

The correlation between antigen expression levels on target cells and ADCC activities of specific monoclonal antibodies have been studied in other systems, such as bovine serum albumin-coupled chicken erythrocytes (25), HER-2/*neu*-expressing cancer cells, and CD20-expressing lymphoma cells. In this study, we first explored the correlation between cetuximab-mediated ADCC activity against lung cancer cell lines and the expression levels of EGFR using quantitative flow cytometric analysis and immunohistochemistry. For ADCC activity, it has been reported that a certain minimum number of antigen molecules must be present on the target cells, and higher ADCC activity requires a higher antigen density on the target cells (25). With trastuzumab, a therapeutic monoclonal antibody against the HER-2 receptor, ADCC activity is reported to roughly correlate with HER-2 expression in breast (26) and uterine serous papillary (27) cancer cells as well glioblastoma multiforme cells using semiquantitative flow cytometric analysis for antigen estimation. With rituximab, a therapeutic monoclonal antibody against the B-cell-specific CD20 surface antigen, ADCC activity is reported to exhibit the same tendency



**Fig. 4.** ADCC/NK activity against the A549 lung cancer cell line in NSCLC patients before and after chemotherapy. The ADCC/NK activity in the patient blood samples was measured by examining the degree of lysis of a <sup>51</sup>Cr-labeled lung cancer target cell line (A549) mediated by PBMCs in the presence or absence of cetuximab. *A*, patient blood samples (patients 1-6) were collected before the commencement of chemotherapy (day 1) and after chemotherapy (day 28) with weekly carboplatin and paclitaxel regimen. Rituximab was used as a control monoclonal antibody. Data obtained at an E:T ratio of 40:1. PBMC at an E:T ratio of 10:1 yielded the same results (data not shown). ADCC activity of cetuximab (0.25 μg/mL) using PBMCs collected before (*Pre*, prechemotherapy) and after (*Post*, postchemotherapy) chemotherapy (*left*). NK activity of PBMCs collected before and after chemotherapy with the same regimen (*right*). *B*, effect of IL-2 on the ADCC activity of T cells, NK cells, and monocytes isolated from chemotherapy-naïve patients. Freshly isolated PBMCs were separated into T cell, NK cell, and monocyte subpopulations using MACS and treated with and without IL-2 (30 ng/mL) for 18 h. These effector cells were tested for cytotoxicity against A549 cells in the presence or absence of cetuximab (0.25 μg/mL) at the indicated E:T ratios (patient 7). Representative of three independent experiments. Points, mean of a triplicate experiment; bars, SD. *C*, augmentation of cetuximab-mediated ADCC activity by IL-2 treatment of patients' PBMCs after chemotherapy. PBMCs from lung cancer patients who have undergone one course of chemotherapy (patients 8-12) were incubated with and without IL-2 (30 ng/mL) for 18 h and tested for their ADCC activity (*left*) and NK activity (*right*) against A549 cells. Data obtained at an E:T ratio of 40:1.

in B-cell non-Hodgkin's lymphoma cell lines (28). In addition to these reports, previous studies used a quantitative flow cytometric analysis (12) to determine the absolute number of the antibody binding sites per cell and reported a more precise correlation. These studies found that the ADCC activity of trastuzumab (29) and anti-Ep-CAM-specific monoclonal antibody (30) weakly correlate with the logarithm of the number of their target cell surface antigens. Our results indicative that with cetuximab, low EGFR expression levels are sufficient for maximum ADCC activity and that further increases in the expression levels of EGFR have no obvious effect on ADCC activity are consistent with these previous findings and provide additional information about the ADCC activity of cetuximab.

In this study, we showed that maximum ADCC activity can be expected as long as target cells are stained by immunohistochemistry and is independent of the strength of staining. In this context, immunohistochemistry is an appropriate method to predict the effectiveness of cetuximab-mediated ADCC, although immunohistochemistry is semiquantitative and prone to interobserver scoring error. Cetuximab was approved by the Food and Drug Administration on December 2, 2004 for the treatment of only those metastatic colorectal adenocarcinomas, which express EGFR by immunohistochemistry, because the clinical trials exclusively enrolled only those patients whose tumors showed immunohistochemistry evidence of EGFR expression (31). However, the value of immunohistochemistry detection of EGFR in selecting patients for treatment with cetuximab remains controversial because accumulating data suggest that the response rate does not correlate with the extent of EGFR staining, such as the percentage of positive cells or the intensity of EGFR expression (31). There may be several reasons why immunohistochemistry staining for EGFR is a poor indicator of response to cetuximab, including improper tissue processing and handling, prolonged storage time of tissue samples, or timing and site of tissue collection (32). In this study, the positive correlation between the immunohistochemistry score and EGFR quantification by flow cytometry indicates that the immunohistochemistry method we adapted here is technically acceptable. The fact that the ADCC activity of cetuximab does not closely correlate with the expression levels of EGFR might be another biological factor to explain why the extent of EGFR staining does not correlate with the clinical effectiveness of cetuximab. Immunohistochemistry is superior to other methods for measuring EGFR in clinical specimens, such as a ligand binding assay (33) or quantitative flow cytometry (34), because it does not require cell disaggregation from fresh tissue or special equipment. Based on our data, immunohistochemistry might be useful if it is scored simply as negative or positive when assessing a tumor sample for the ADCC activity of cetuximab.

In this study, we used a mouse monoclonal anti-EGFR antibody for immunohistochemistry (clone 31G7), whereas the only kit approved by the Food and Drug Administration for EGFR testing of clinical specimens contains a different monoclonal antibody (clone 2-18C9; DakoCytomation). Both clone 31G7 and clone 2-18C9 were shown to be interchangeable for use in immunohistochemistry determinations in a published phase I and pharmacologic study of an EGFR antagonist (35). Moreover, the concordance between clone 31G7 antibody and DakoCytomation EGFR pharmDxTM kit

was confirmed by Chung et al. (36). Based on these studies and the positive correlation between our immunohistochemistry scoring and EGFR levels by flow cytometry in this study, the immunohistochemistry stain using clone 31G7 is likely to be comparable with that of clone 2-18C9.

We have shown that cetuximab-mediated ADCC activity against lung cancer cell lines is enhanced in response to IL-2. IL-2 is a lymphokine normally produced by T lymphocytes that affect the function of activated B cells, NK cells, T cells, and monocytes through the IL-2 receptor  $\alpha$ -chain (CD25) on their surface (37). CD3<sup>+</sup>CD56<sup>+</sup> T cells (NKT cells) included in the CD3<sup>+</sup> T cell subpopulations in our assay and NK cells are known to mediate non-MHC-restricted cytotoxicity, and monocytes and NK cells that have Fc receptors are effectors in ADCC (38). In the present study, we showed that only NK cells are major effectors of cetuximab-mediated ADCC activity augmented by IL-2, which is consistent with other reports using other monoclonal antibodies (39, 40). The lack of cytotoxicity in T-cell subpopulations might be due to the very small ratio of NKT cells among T cells (<5%; ref. 38) or shortness in duration of IL-2 treatment for their full activation. In monocytes, the lack of ADCC activity in spite of their phenotypically activated state after IL-2 treatment might be due to the nature of this antibody or to the mode of ADCC activity in monocytes (41). Further study using more sensitive conditions, such as longer incubation time of IL-2 treatment or cytotoxic assay, is necessary to clarify the role of T cells and monocytes in cetuximab-mediated ADCC.

The combination of IL-2 and a therapeutic monoclonal antibody has been explored extensively for rituximab (39) and trastuzumab (26) and has been reported to enhance ADCC activity *in vitro* (42) or *in vivo* mouse xenograft models (43). Based on these fundamental studies, several preclinical trials have been reported. Gluck et al. (44) carried out a phase I study evaluating combination therapy with rituximab and IL-2 in relapsed or refractory B-cell non-Hodgkin's lymphoma and found increased efficacy. In other studies, the coadministration of IL-2 with trastuzumab in patients with metastatic breast cancer that overexpressed HER-2 showed feasibility and an encouraging response (45). Compared with these promising pilot studies of the combination of IL-2 with therapeutic monoclonal antibodies, little is known about the combination of IL-2 with cetuximab. Our result that cetuximab-mediated ADCC activity against lung cancer cell lines is enhanced by IL-2 might lend support to the future concurrent use of cetuximab and IL-2 in patients with lung cancer.

Because ADCC activity may be an important anticancer mechanism of cetuximab, it is vital to assess the influence of conventional chemotherapy on the ADCC activity in cancer patients. Several basic studies have indicated synergism between cetuximab and several chemotherapeutic agents, including cisplatin and paclitaxel, in a wide variety of cell lines (46, 47). In addition, some preliminary clinical studies have shown the feasibility and efficacy of combining cetuximab with platinum-containing chemotherapy regimens (48). Thus, the combination of cetuximab with cytotoxic drugs will be more widely applied clinically. We and others have reported previously that these cytotoxic drugs deplete NK cell function to varying degrees (49). Because NK cells play a central role in ADCC activity, we tested ADCC activity before and after chemotherapy. We showed that both ADCC and NK activity



are impaired by chemotherapy but that cetuximab-mediated ADCC activity was less susceptible to immunosuppression than NK activity by cytotoxic chemotherapy. This is similar to the earlier report indicating that ADCC activity is less susceptible to a tumor-bearing state (50) than NK activity. In addition, we showed that ADCC activity responded to IL-2 treatment even after chemotherapy. These data might be the rationale for combination immunotherapy of cetuximab with cytokines, particularly IL-2, in patients who undergo conventional chemotherapy.

In conclusion, cetuximab has potential ADCC activity against EGFR-expressing lung cancer cell lines. Almost maximum ADCC activity was induced by very weak EGFR expression

levels, which are faintly detectable by immunohistochemistry. ADCC activity is enhanced by IL-2 and is less impaired by weekly chemotherapy with conventional cytotoxic drugs than is NK activity. These data provide new insight into the possible antitumor mechanism of cetuximab and may be helpful in directing future combination therapies for patients with lung cancer.

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