Anti-CD3 × Anti-Epidermal Growth Factor Receptor (EGFR) Bispecific Antibody Redirects T-Cell Cytolytic Activity to EGFR-Positive Cancers \textit{In vitro} and in an Animal Model

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Abstract

Purpose: Targeting epidermal growth factor receptor (EGFR) overexpressed by many epithelial-derived cancer cells with anti-EGFR monoclonal antibodies (mAb) inhibits their growth. A limited number of clinical responses in patients treated with the anti-EGFR mAb, (cetuximab), may reflect variability in EGFR type or signaling in neoplastic cells. This study combines EGFR-targeting with the non-MHC–restricted cytotoxicity of anti-CD3 activated T cells (ATC) to enhance receptor-directed cytotoxicity.

Experimental Design: ATC from normal and patient donors were expanded \textit{ex vivo}. Specific cytolytic activity of ATC armed with anti-CD3 × anti-EGFR (EGFRBi) against EGFR-expressing cancer cells derived from lung, pancreas, colon, prostate, brain, skin, or EGFR-negative breast cancer cells was evaluated in 51Cr release assays. \textit{In vivo} studies comparing tumor growth delay induced by EGFRBi-armed ATCs or cetuximab were done in severe combined immunodeficient/Beige mice (SCID-Beige) bearing COLO 356/FG pancreatic and LS174T colorectal tumors.

Results: At effector/target ratios from 3.125 to 50, both EGFRBi-armed normal and patient ATC were significantly more cytotoxic, by 23% to 79%, against EGFR-positive cells over ATC, cetuximab, anti-CD3 alone, or ATC armed with irrelevant BiAb directed at CD20. EGFRBi-armed ATC also secreted significantly higher levels of some TH1/TH2 cytokines compared with ATC alone. In mice, i.v. infusions of EGFRBi-armed ATC (0.001 mg equivalent/infusion) were equally effective as cetuximab (1 mg/infusion) alone for significantly delaying the non-MHC restricted cytotoxicity of anti-CD3 activated T cells (ATC) to enhance receptor-directed cytotoxicity.

Conclusions: Combining EGFR antibody targeting with T cell–mediated cytotoxicity may overcome some limitations associated with EGFR-targeting when using cetuximab alone.

Since its identification as the cellular homologue of the avian erythroblastosis virus oncogene known to mediate malignant transformation (1, 2), epidermal growth factor receptor (EGFR) has served as a target for various antitumor therapies (3). EGFR belongs to the erbB receptor family of growth factors, EGFR homodimerizes or heterodimerizes with a second EGFR or another member of the erbB receptor family, respectively, initiating a signaling cascade through mitogen-activated protein kinases and other transcription activators leading to proliferation, differentiation, and repair (4, 5). In malignant cells, EGFR is often overexpressed and/or mutated with its constitutive activation leading to proliferation, angiogenesis, invasion, metastasis, and inhibition of apoptosis (6–9).

The role of EGFR in tumorigenesis makes it an ideal target for strategies to treat cancers of the lung, brain, colon, pancreas, kidney, and prostate. Accordingly, monoclonal antibodies (mAb) that target either the extracellular ligand-binding domain or the intracellular tyrosine kinase signaling cascade of EGFR have shown efficacy as antitumor agents (10). A humanized mAb to EGFR, cetuximab (Erbitux), which competitively binds the extracellular domain of EGFR to inhibit ligand activation of the receptor (7), was approved by the Food and Drug Administration in 2004 for the treatment of metastatic colon cancer in combination with the topoisomerase inhibitor irinotecan. Despite early implications that cetuximab may be effective at circumventing EGFR-associated

Abstract
mechanisms of chemoresistance, results from the study arm that included cetuximab monotherapy proved disappointing (11). Variability in cetuximab antitumor activity despite over-expression of EGFR on tumors may reflect EGFR-independent cell cycle progression similar to what has been found when using some EGFR kinase inhibitors (12) or may be caused by the expression of mutant EGFR for which activation is independent of ligand binding (13).

Combining anti-EGFR with other biologics that provide an independent mechanism of killing is one approach to circumvent receptor type or receptor activation status that may limit EGFR-directed mAb therapy. For example, EGFR has been used extensively as a target for delivery of chimeric tumor toxins. Although potent as antitumor agents, nonspecific toxicities mediated by the toxin moieties against normal host tissues have prevented the administration of effective doses in clinical trials (14–17). On the other hand, using a patient’s own antitumor immunity may offer a safer approach with a wider therapeutic margin. Arming ex vivo expanded activated T cells (ATC) from patients with a bispecific antibody (BiAb), which is comprised of an anti-CD3 mAb heteroconjugated to another mAb directed at a selected tumor-associated antigen (TAA) makes every T cell a TAA–specific CTL. Once infused back into the patient and upon engagement with the TAA, the ATC moity of the drug mediates non-MHC–restricted, perforin/granzyme B–mediated cytosis of tumor cells (18).

In this study, we have exploited the high specificity and binding affinity (K_{d} = 0.39 nmol/L) of cetuximab for EGFR and have heteroconjugated it to anti-CD3 to produce EGFRBi. We report that arming ATCs with EGFRBi leads to enhanced tumor specific cytotoxicity and achieves, at a considerably lower dose, comparable tumor growth delay to cetuximab alone against human pancreatic tumors in an immunodeficient animal model.

Materials and Methods

**BiAbs.** Anti-CD3 (OKT3, Orthoclone; Orthobiotech, Bridgewater, NJ) was reacted with 5- to 10-fold molar excess of Truant’s reagent (2-iminothiolane HCl; Pierce, Rockford, IL) and anti-EGFR (Eritux, cetuximab; Bristol Meyer Squibb, New York, NY), anti-CD20 (Rituxan, rituximab; Genentech, South San Francisco, CA) was reacted with 4-fold molar excess of sulfoinoscinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate at room temperature for 1 hour (19). Antibodies were then purified on Econo-Pac 10 DG chromatography columns (Bio-Rad, Hercules, CA) in PBS to remove unbound crosslinker. The cross-linked mAbs were mixed immediately at equimolar ratios and heteroconjugated at 4°C overnight. The products of the heteroconjugation were resolved by SDS-PAGE (4-15% gradient) and stained with Gelcode Blue (Pierce). By densiometric quantitation, EGFRBi resolved as 63.4% monomer, 29.0% dimer (active), and 7.6% multimer (active) fractions (data not shown). The Food and Drug Administration does not require purification of active products. Additionally, our testing of purified dimers of Her2Bi (anti-CD3 × anti-HER-2/new) in cytotoxicity assays has shown no significant increase in activity over the mixture of dimers, multimers, and monomers in vitro. With a dimer to monomer ratio of 0.46 and a dimer to multimer ratio of 3.87, the lot of EGFRBi as well as the lots of CD20Bi used for these studies all fell within acceptable criteria that we have established for clinical release of clinical trial BiAbs.

**Cell lines.** The following human cell lines were cultured in RPMI 1640 (Cambrex, Walkersville, MD): A549, a lung carcinoma [American Type Culture Collection (ATCC), Manassas, VA]; IMR-90, normal lung fibroblast (ATCC); MIA PaCa-2, pancreatic carcinoma (ATCC); COLO 356/FG (a generous gift from M.P. Vezederis, Roger Williams Medical Center, Providence, RI), a highly metastatic subclone (20) derived from the pancreatic adenocarcinoma COLO 356 (ATCC); LS174T, colon adenocarcinoma (ATCC); HCT-8, colon adenocarcinoma (ATCC); PC-3, an androgen-insensitive prostate adenocarcinoma (ATCC), and A-431, epithelial carcinoma (ATCC). The following human cell lines were cultured in MEM (Life Technologies, Inc., Grand Island, NY): Calu-6, anaplastic epithelial carcinoma (ATCC); UI-373 MG, glioblastoma (ATCC); U-87 MG, glioblastoma (ATCC); SK-N-MC, an EGFR-negative neuroblastoma (ATCC); LNCaP, an androgen-sensitive prostate carcinoma (ATCC); and DU-145, an androgen-insensitive prostate carcinoma. SCC-25, a human squamous cell carcinoma (ATCC) was cultured in DMEM (Life Technologies). The EGFR-negative breast carcinoma, MDA-MB-453 (ATCC), was cultured in Iscove’s modified Dulbecco’s medium (Life Technologies). Media was supplemented with 10% FCS (Valley Biomedical, Inc., Winchester, VA), 1% l-glutamine (Cambrex), and 2% Pen/Strep (Cambrex).

**Activated T Lymphocytes (ATC).** Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood of normal healthy donors or were prepared from leukapheresis products of patients who had previously undergone chemotherapy (>1 month before leukapheresis) for hormone-refractory prostate cancer (HRPC), metastatic breast cancer, or lung cancer. Anti-CD3–activated ATC were expanded in culture from peripheral blood mononuclear cells as previously described (21, 22). Following 6 to 14 days of culture, ATC expansion products of donors averaged 89.0 ± 7.5 CD3+ cells (42.8% ± 17.3 CD4 and 46.7% ± 13.2 CD8). No correlation has been found between cytotoxicity of armed ATC from donors and the %CD4 or %CD8 cells comprising each expansion product (23). Blood collection and use of human blood products for research were conducted under Institutional Review Board approved protocols at Roger Williams Medical Center, and signed consents were obtained from normal and patient donors. ATC were generated in RPMI and cryopreserved.

**ATC arming and in vitro cytotoxicity.** Cryopreserved ATC were thawed, washed, counted, and armed with the indicated doses of BiAb per 10^6 cells for 15 minutes at room temperature. Armed cells were washed to remove unbound antibody and were resuspended in culture medium. Specific lysis of tumor targets by BiAb-armed ATC was determined in [3]Cr-release assays as previously described (19). Because the reported mechanisms of action for cetuximab suggest cytostatic, apoptotic, or antiangiogenic activity rather than cytolytic activity (24), we compared cetuximab activity to EGFRBi-armed ATC activity in vitro in a cell proliferation assay. COLO 356/FG cells were seeded (10^5 per well) into six-well plates and allowed to adhere overnight. The following day, the medium was removed, and fresh medium alone or containing the unconjugated mAbs (500 ng/well), unarmed ATC (10^6/mL), EGFRBi-armed ATC (10^6/well armed with 50 ng EGFRBi/10^6 ATC), or a combination of the mAbs with unarmed ATC was added to wells in triplicates. Cultures were incubated for 72 hours, after which time COLO 356/FG cells were harvested from plates by treating with trypsin-EDTA and counted with a Coulter Particle Counter (Coulter Electronics, Hialeah, FL) gated to exclude ATC.

**Flow cytometry.** Binding of the anti-CD3 moiety following incubation of BiAbs with T cells has been previously shown (19). Binding of EGFRBi to target cells was evaluated using goat anti-mouse IgG2a-phycocerythrin for detection of the anti-CD3 moiety of the BiAb. A goat IgG-phycocerythrin isotype control was used to determine nonspecific staining. Target cells (10^5) were incubated with EGFRBi (1 µg/mL) for 30 minutes at room temperature. The cells were washed thrice and then analyzed by flow cytometry on a FACS caliber System (BD Biosciences, San Jose, CA) using “CELLquest” software (BD Biosciences). Cell populations for analysis were gated to exclude dead cells based on forward scatter versus side scatter plots.

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Measurement of cytokine secretion. Tumor target cells (3 × 10^5) were seeded and allowed to adhere overnight at 37°C in flat-bottomed microtiter wells. Unarmed or armed ATCs were then added to tumor targets at an effector/target (E/T) of 10:1 and were allowed to aggregate overnight. Supernatants were collected from cocultures and analyzed for cytokines using the TNF-α/TNF-β human cytokine multiplex kit [interleukin-2 (IL-2), IL-4, IL-5, IL-10, IL-12, IL-13, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ, and tumor necrosis factor-α (TNF-α)] and the Bioplex Protein Array system (Bio-Rad) according to instructions of the manufacturer. This is a novel, multiplexed, particle-based, flow cytometric assay that is able to quantify multiple analytes from a single sample. The limit of detection for these assays is <10 pg/mL based on detectable signal of ≥2 SD above background (Bio-Rad). Cytokine concentration was automatically calculated by the Bioplex Manager Software (Bio-Rad), which uses a standard curve derived from recombinant cytokine standards. The specific mean concentrations (±SD) of cytokines produced by EGFReBi-armed ATCs were determined by subtracting the nonspecific cytokine concentrations produced by unarmed ATCs exposed to the target cells. Results are reported only for those cytokines in which a significant increase in concentration was observed for EGFReBi-armed ATC over unarmed ATC.

Tumor growth delay studies. In vivo studies to evaluate antitumor activity were done in 8- to 10-week-old, male severe combined immunodeficient/Beige mice (SCID-Beige, Taconic, Germantown, NY). This strain carries a double mutation that results in a lack of T cells and B cells as well as impaired natural killer cell function. Mice were maintained by the Roger Williams Medical Center Animal Care Facility, and all treatments were preapproved and administered in accordance with Institutional Animal Care and Use Committee guidelines. COLO356/FG and LS174T tumor cells were expanded in culture, harvested by treatment with trypsin-EDTA, centrifuged at 400 × g, washed twice, and then resuspended in fresh, unsupplemented medium at 5 × 10^7 cells/mL. The cell suspension was then implanted s.c. into the right rear flanks of the mice (0.1 mL/mouse). Seven days later, when tumors reached ~60 mm^2, groups of five mice received one of the following treatments via tail vein injection once per week for 6 weeks: (a) RPMI + IL-2 (3,000 IU); (b) cetuximab (1 mg); (c) ATC (2 × 10^5) + IL-2 (3,000 IU); or (d) EGFReBi-armed ATC (2 × 10^5) + IL-2 (3,000 IU). Progress of tumors was determined twice weekly by external caliper measurements, and tumor volumes were calculated using a standard hemiellipsoid formula: (length × width^2)/2.

Statistical analysis. All statistical analyses were done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Results from cytotoxicity and cytokine assays were compared by unpaired t test. Comparisons of tumor growth curves as a function of treatment in tumor growth delay studies were made with the nonparametric Kruskal-Wallis test followed by Dunn’s multiple-comparisons test. Body weight and postmortem organ weights of mice treatment groups were compared using one-way ANOVA followed by Bonferroni’s multiple comparison tests.

Results

EGFRBi binding studies. Binding of the anti-CD3 moiety to ATC following heterocugugation of BiAbs has been previously shown (19). To evaluate binding of the anti-EGFR moiety of the EGFReBi construct to target tumor cells, we used the EGFReBi-overexpressing cell line, A431, and an EGFReBi-negative cell line, MDA-MB-453. EGFReBi binding was evaluated using phycoerythrin-labeled goat anti-mouse IgG2a to detect the anti-CD3 moiety of the BiAb. By flow cytometry, positive staining was detected in 99.9% of the A431 population with a mean fluorescent intensity of 2,516 (Fig. 1); in contrast, EGFReBi did not bind MDA-MB-453 cells. Other cell lines were evaluated for EGFReBi binding and showed various intermediate values (Table 1).

Arming titration. We armed ATC from normal donors with EGFReBi (0-500 ng/10^6 ATC) to determine an effective arming dose that would result in significantly enhanced cytotoxicity over unarmed ATC at all E/T (6.25:1 to 50:1) against representative brain (U-87 MG), colon (LS174T), and pancreatic (COLO 356/FG) carcinoma cell lines. For these studies, unarmed or EGFReBi-armed ATC, or ATC armed with an irrelevant BiAb (CD20Bi) were incubated with 51Cr-labeled target cells at 37°C overnight and % specific cell lysis was quantitated based upon 51Cr release. Significant (P < 0.001 to P < 0.05) increases in cell lysis compared with unarmed ATC were observed beginning at an arming dose of 25 ng/10^6 ATC (data not shown). Cytotoxicity directed at all three cell lines by EGFReBi-armed ATC increased in a dose-dependent fashion up to 25 to 50 ng/million ATC. CD20Bi-armed ATC cytotoxicity was negligible in all cases and did not differ significantly from unarmed ATC killing. For all subsequent evaluations, we selected an arming dose of 50 ng EGFReBi/10^6 ATC.

Cytotoxicity. To evaluate the cytotoxicity of EGFReBi-armed ATC against EGFReBi-expressing tumor cells of various origins, normal donor ATC (n = 3-7) were armed with EGFReBi (50 ng/10^6 cells), and % specific lysis was again determined in 51Cr-release assays. Arming normal ATC with EGFReBi significantly enhanced cytotoxicity directed at U-87 MG, U-373 MG, COLO 356/FG, MIA-PaCa2, DU-145, LNCaP, PC-3, HCT-8, LS174T, IMR-90, Calu-6, A549, and A431 at E/T between 6.25 and 50. In contrast, no enhancement of killing was observed in the EGFReBi-negative cell lines, SK-N-MC (P = 0.12) or MDA-MB-453 (P = 0.40; Fig. 2).

No correlation was found between the level of binding of EGFReBi to target cell lines and the E/T dose of EGFReBi-armed ATC required for lysis of 50% of target cells (ED50, Table 1). Cytotoxicity mediated by antigen-specific binding, however, was shown by the ability of free anti-EGFR (50 µg/mL) to significantly block EGFReBi-armed ATC killing in representative cell lines: COLO 356/FG (P = 0.0005), A549 (P = 0.004), LS174T (P = 0.007), and U-87 MG (P = 0.02; Fig. 2).
Additionally, in cell proliferation assays in which ATC alone, EGFRBi-armed ATC (E/T of 10:1), or equivalent concentrations of unconjugated mAbs (anti-CD3 and/or anti-EGFR) were cocultured with COLO 356/FG, EGFRBi-armed ATC showed superior inhibition of target cells compared with control conditions or cetuximab alone (Fig. 3).

Because of the higher likelihood of variability in immune cell function of cancer patients due to T-cell anergy and/or previous chemotherapy, we evaluated the ability of EGFRBi to enhance the cytotoxicity of ATC acquired from patients previously treated with chemotherapy and/or radiation. EGFRBi-armed ATC from two HRPC patients showed significantly enhanced lysis of the HRPC cancer cell lines, PC-3 and DU-145 (Fig. 4), compared with their unarmed ATC. Cytotoxicity of EGFRBi-armed ATC from these patients at E/T between 6.25 and 50 did not differ significantly from EGFRBi-armed ATC from normal subjects against DU-145 ($P = 0.50$) or PC-3 ($P = 0.64$). Similarly, EGFRBi arming also enhanced patient ATC killing against other target cell lines (Table 2).

**Cytokine production.** Cell culture supernatants were analyzed for Th1 (IL-2, IL-5, IL-12, GM-CSF, IFN-γ, and TNF-α) and Th2 (IL-4, IL-10, and IL-13) cytokines. Significant increases were predominantly observed for IFN-γ, GM-CSF, and IL-13 in the supernatants from patient and/or normal EGFRBi-armed ATC over their unarmed ATC counterparts when added to target cells A549, Calu-6, IMR-90, U-87 MG, U-373, PC-3, LS174T, and COLO 356/FG (Fig. 5).

**EGFRBi-armed ATC delay COLO 356/FG tumor growth in SCID-Beige mice.** In concurrent studies, we evaluated the ability of EGFRBi-armed ATC from the same normal donor to delay the growth of established (60 mm$^3$) COLO 356/FG pancreatic or LS174T colorectal tumors. When COLO 356/FG

### Table 1. EGFRBi binding and EGFRBi-armed ATC cytotoxicity against tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Binding (% gated x MFI)</th>
<th>ED_{50} (E/T)</th>
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<tbody>
<tr>
<td>A431 (epidermoid)</td>
<td>2,513</td>
<td>18</td>
</tr>
<tr>
<td>A549 (lung)</td>
<td>290.8</td>
<td>&gt;50</td>
</tr>
<tr>
<td>PC-3 (prostate)</td>
<td>257.7</td>
<td>18</td>
</tr>
<tr>
<td>IMR-90 (normal lung)</td>
<td>105.3</td>
<td>17</td>
</tr>
<tr>
<td>COLO 356/FG (pancreatic)</td>
<td>86.6</td>
<td>6.25</td>
</tr>
<tr>
<td>HCT-8 (colorectal)</td>
<td>19.8</td>
<td>5</td>
</tr>
<tr>
<td>U87-MG (brain)</td>
<td>11.6</td>
<td>10</td>
</tr>
<tr>
<td>LS174T (colorectal)</td>
<td>11.4</td>
<td>10</td>
</tr>
</tbody>
</table>

$r^2 = 0.010$ ($P = 0.82$)

Abbreviation: MFI, mean fluorescent intensity.

Fig. 2. Cytotoxicity profiles of EGFRBi-armed ATC against various target cell lines compared with unarmed ATC and in the presence of excess cetuximab. Normal donor ATC were armed with 50 ng EGFRBi/10^6 ATC, and cytotoxicity assays were done in the absence or presence of free, unconjugated cetuximab (50 μg/mL). Points, mean cytotoxicity of armed or unarmed ATC from normal donors ($n = 3–7$) at the indicated E/T; bars, SE.
specific cytotoxicity (cytolytic activity; chemotherapy and radiation, was evaluated. Points, mean %
cytolytic activity of ATC obtained from HRPC patients, previously treated with
cytolytic activity against HRPC cell lines. Effect of EGFRBi arming on the
Fig. 4.
mediated significant tumor growth delay compared with ATC
ATC (0.001 mg EGFRBi) administered i.v. weekly for 6 weeks
were grown as s.c. xenografts in SCID-Beige mice, cetuximab
unarmed ATC (10⁷/well), EGFRBi-armed ATCs (10⁷/well armed with 50 ng EGFRBi/10⁶
were counted after 72 hours. Results from three independent experiments are
presented as the number of cells for each treatment as a percentage of the cells
cultured with medium only. After 72 hours, the cells in the control wells had gone
through 1.5 doublings. Bars, SD.

were grown as s.c. xenografts in SCID-Beige mice, cetuximab
(1 mg) alone or ATC (2 × 10⁷) armed with 50 ng EGFRBi/10⁶
ATC. (0.001 mg EGFRBi) administered i.v. weekly for 6 weeks
mediated significant tumor growth delay compared with ATC
alone or vehicle administered on the same schedule (P < 0.001;
Fig. 6A). In contrast, EGFRBi-armed ATC at this same dose and
schedule were ineffective for delaying LS174T tumor growth
compared with cetuximab (Fig. 6B), and mice in the LS174T
groups were euthanized on day 20 after only 3 weeks of
treatments because tumors in three of the four groups
approached limits of acceptable tumor burden.

No gross toxicities were observed in any of the treatment
groups. Body weights of mice over the course of treatments
were significantly lower (6-8%) in the unarmed ATC treatment
groups (P < 0.001) but did not differ significantly between the
other three treatment groups. Postmortem necropsy of vital
organs showed significant (P < 0.01-0.05) 23% to 30% and 22%
to 32% increases in liver and kidney weights, respectively, in
mice treated with cetuximab compared with the other three
treatment groups.

BiAbs created to redirect CD3-, CD16-, or CD64-positive
effector cells to numerous tumor-associated antigens (25–40),
including EGFR (41, 42), have shown promising antitumor
effects in both preclinical and clinical studies (reviewed in
ref. 18). Most of these studies, however, are based on the
strategy of injecting BiAbs alone into patients, which holds an
increased risk for inducing dose-limiting toxicities associated
with massive cytokine release (i.e., "cytokine storm"; refs. 30,
43). In contrast, we arm ex vivo expanded, autologous ATC
with BiAbs, such as anti-CD3 × anti-Her2 (Her2Bi) or anti-CD3 ×
anti-CD20 (CD20Bi), before reinfusing them back into the
patient. Using this alternative strategy, we have safely infused
up to 1.6 × 10¹¹ armed ATC into patients without encountering
dose-limiting toxicities (44).

In this study, we have characterized a new BiAb (EGFRBi) for
improving targeting to EGFR-positive tumors. Arming T cells with highly specific EGFRBi redirects and facilitates
non-MHC–restricted, T cell–mediated cytolyis against EGFR-
positive tumor cells independent of EGFR signaling pathways.
Specific cytolyis was observed for all the EGFR-positive tumor
cell lines tested, regardless of the tissue of origin. A lack of
correlation between EGFRBi binding by flow cytometry analyses
and the ED₅₀ dose of EGFRBi-armed ATC, however, may be
explained by variable levels of low-affinity receptors on the cell
lines tested. EGFRBi bound with low affinity may be lost in the
preparation for flow cytometry but still may be sufficient for
engagement and induction of targeted cell lysis in vitro.

In some clinical trials, impaired T-cell responses observed
in glioblastoma patients (45, 46) may have presented
limitations to clinical efficacy of a murine anti-EGFR mAb
for which activity of the mAb was dependent upon the
presence of lymphocytes and monocytes to induce comple-
ment activation and antibody-dependent cellular cytotoxicity
(47). Although it is common to find impaired or depressed
T-cell functions in patients with various cancers, reduced
cytotoxic activity was found only in EGFRBi-armed ATC from
patients directed at U-87 MG targets. More importantly,
EGFRBi-armed ATC from patients killed U-87 MG signifi-
cantly better (P = 0.007) than their unarmed counterparts.
Even patients’ T cells with depressed or inhibited functions
due to tumor-mediated suppression or previous chemother-
apy, therefore, may benefit from infusions of autologous ATC
armed with EGFRBi.

The reversal of tumor cell tolerance by arming T cells with a
BiAb may be achieved, in part, by the ability of BiAb-armed
ATC to modulate cytokine and chemokine regulatory networks

Fig. 3. Antiproliferative effects of EGFRBi-armed ATC compared with mAbs or
unarmed ATC against pancreatic cancer cells. Adherent COLO 356/FG cells
were treated with unconjugated OKT3 (anti-CD3) or cetuximab (500 ng/well),
unarmed ATC (10⁷/well), EGFRBi-armed ATCs (10⁷/well armed with 50 ng EGFRBi/10⁶
ATC), or a combination of the mAbs with unarmed ATC. COLO 356/FG cells
were counted after 72 hours. Results from three independent experiments are
presented as the number of cells for each treatment as a percentage of the cells
cultured with medium only. After 72 hours, the cells in the control wells had gone
through 1.5 doublings. Bars, SD.

Fig. 4. ATC acquired from HRPC patients and armed with EGFRBi mediate
cytolytic activity against HRPC cell lines. Effect of EGFRBi arming on the
cytolytic activity of ATC obtained from HRPC patients, previously treated with
chemotherapy and radiation, was evaluated. Points, mean %
specific cytotoxicity (cytolytic activity; n = 2); bars, SD.
involved in host immune surveillance (reviewed in ref. 18). IFN-γ and GM-CSF are not only known to be directly tumoricidal but also serve to modulate immune networks to induce local and/or systemic immune responses to tumors. In fact, we have consistently detected Th1 and Th2 cytokines in the serum of immunotherapy patients after armed ATC infusions (44). Polarization of patients’ in vivo immune responses toward type 1 immunity is consistent with concomitantly increasing levels of cytotoxicity directed at tumor target cells mediated by their peripheral blood mononuclear cells. BiAb-armed ATC, therefore, may induce innate and/or adaptive immune responses that lead to the secretion of TGF-β, IL-4, and IL-10 (48, 49).

Table 2. Cytotoxicity of patient ATC armed with EGFRBi compared with normal armed ATC and unarmed patient ATC

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% Specific cytotoxicity range</th>
<th>n</th>
<th>P (patient armed ATC vs unarmed ATC)</th>
<th>P (patient armed ATC vs normal armed ATC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87-MG</td>
<td>15.9 ± 9.0 to 36.4 ± 13.8</td>
<td>3</td>
<td>0.007</td>
<td>0.018</td>
</tr>
<tr>
<td>U373-MG</td>
<td>17.7 ± 12.2 to 70.9 ± 18.4</td>
<td>2</td>
<td>0.016</td>
<td>0.56</td>
</tr>
<tr>
<td>SK-N-MC</td>
<td>41.5 ± 16.2 to 78.1 ± 61</td>
<td>3</td>
<td>0.025</td>
<td>0.16</td>
</tr>
<tr>
<td>LS174T</td>
<td>26.8 ± 5.3 to 74.7 ± 4.3</td>
<td>4</td>
<td>0.006</td>
<td>0.85</td>
</tr>
<tr>
<td>A549</td>
<td>11.05 ± 4.5 to 37.3 ± 9.2</td>
<td>6</td>
<td>0.009</td>
<td>0.28</td>
</tr>
<tr>
<td>Calu 6</td>
<td>32.3 ± 10.8 to 56.8 ± 11.4</td>
<td>5</td>
<td>0.002</td>
<td>0.86</td>
</tr>
<tr>
<td>IMR-90</td>
<td>42.1 ± 1.3 to 58.7 ± 5.2</td>
<td>3</td>
<td>0.005</td>
<td>0.61</td>
</tr>
<tr>
<td>SCC25</td>
<td>15.4 ± 1.6 to 46.1 ± 15.6</td>
<td>2</td>
<td>0.032</td>
<td>0.49</td>
</tr>
<tr>
<td>COLO 356/FG</td>
<td>22.8 ± 5.4 to 53.5 ± 4.8</td>
<td>4</td>
<td>0.002</td>
<td>0.25</td>
</tr>
<tr>
<td>LNCaP</td>
<td>7.1 ± 5.0 to 40.0 ± 12.7</td>
<td>2</td>
<td>0.03</td>
<td>0.30</td>
</tr>
</tbody>
</table>

+E/T range: 3.12:1 to 25:1.
The antiproliferative effect of cetuximab due to its ability to induce cell cycle arrest has been shown against colon adenocarcinoma (50), prostate carcinoma (51), and glioblastoma (52). Against the latter, cytotoxic effects of cetuximab seemed confined to tumor cell lines in which there was both amplification and overexpression of EGFR (52). This suggests that despite high expression of EGFR, cells with a slower EGFR turnover rate may not be as susceptible to targeting with cetuximab. In our studies, cetuximab failed to inhibit proliferation of the COLO 356/FG pancreatic cell line in vitro, whereas EGFRBi-armed ATC induced significant killing of these same targets. Conversely, cetuximab alone significantly delayed growth of established COLO 356/FG tumors in vivo, suggesting that its primary mechanism of action against this line may occur indirectly through antiangiogenesis (53). This mechanism of action may also explain, in part, the report of antitumor activity of cetuximab in patients with EGFR-negative colorectal tumors (54).

Although the total dose of EGFRBi on $2 \times 10^{12}$ armed ATC was 0.001 mg/wk for 6 weeks compared with a total dose of 1 mg/wk for cetuximab, the therapeutic efficacy was the same. EGFRBi-armed ATC, therefore, mediated potent antitumor activity against COLO 356/FG at a 1,000-fold lower dose than cetuximab alone. Because <2% of EGFRBi product contains active heteroconjugates, the final arming dose of EGFRBi on ATC is ~5,000-fold less than the effective cetuximab dose used in this study. Additionally, although immunodeficient animal models provide a means to evaluate any indirect, antiangiogenic effects of cetuximab (53), they may grossly underestimate EGFRBi/ATC indirect antitumor activity because there are no endogenous immune cells in this mouse strain to be modulated by cytokine networking.

EGFRBi-armed ATC were not as effective as cetuximab, however, against LS174T tumors despite their potent cytotoxicity directed at these targets in vitro. Interestingly, by flow cytometry (Table 1), EGFRBi binding to LS174T was nearly one log lower compared with COLO 356/FG cells, suggesting that binding analysis may serve as a better predictor for in vitro activity of EGFRBi. Higher doses of EGFRBi-armed ATC may, therefore, be necessary to provide antitumor efficacy against LS174T and possibly HCT-8 and U-87 MG, to which EGFRBi also bound poorly. The EGFRBi-armed ATC total dose used in these mice would be equivalent to a $3.2 \times 10^{10}$ total dose in humans, but significantly higher doses of BiAb-armed ATC have been attained in humans. In phase I testing, we have infused HER2Bi-armed ATC at total doses of up to $1.6 \times 10^{11}$ armed ATC without encountering any cardiac or other dose-limiting toxicities (44). Moreover, expansion of ATC achieving total doses of up to $3.8 \times 10^{11}$ is technically feasible and their administration did not result in dose-limiting toxicities in previous trials (23). Although the EGFRBi-armed ATC cytotoxicity and binding data for the normal, human lung fibroblast IMR-90 would seem to suggest a risk for normal lung tissue toxicities that may limit dose escalation when using this targeting strategy, in previous preclinical as well as clinical studies exploring toxicities of i.v. administered EGFR-targeted fusion toxin (DAB153EGF), normal lung was not identified as a target organ (17, 55). In these former studies, kidney and liver, instead, seemed to be the primary normal tissues at risk for EGFR-directed normal tissue toxicities, and this is consistent with our own findings of significantly increased kidney and liver weights of mice treated with cetuximab alone. Taken together with the absence of normal tissue toxicities in mice treated with the EGFRBi-armed ATC, this would suggest that the IMR-90 cell line may not accurately represent in vivo expression levels of EGFR and/or accurately predict EGFRBi-armed ATC-induced normal tissue toxicities.

In summary, our results suggest that EGFRBi-armed ATC induce cytolytic activity against EGFR-positive tumor cells independent of EGFR-mediated signaling pathways and thus may provide a viable alternative to targeting cetuximab-refractory tumors. Both the in vitro cytotoxicity and cytokine production studies as well as the in vivo SCID-Beige mice studies provide a strong rationale for the initiation of phase I/II clinical trials in patients with EGFR-positive tumors of the lung, colon, pancreas, prostate, and other EGFR-overexpressing tumors after surgical resection or debulking. Building on our experience with ATC and BiAb armed ATC in phase I/II trials, this approach may provide enhanced antitumor activity coupled with a low toxicity profile when used as an adjuvant to surgery or in addition to standard adjuvant chemotherapy.

**Acknowledgments**

We thank Nicola M. Kouttab, Ph.D., Fernando Sawicki, and Denise Avila of the Roger Williams Medical Center Flow Cytometry Laboratory for technical assistance; Leslie P. Cousins, Ph.D. for critical reading and helpful suggestions on the article; and Debra DeVito for assistance with preparation of the article.


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Anti-CD3 × Anti-Epidermal Growth Factor Receptor (EGFR) Bispecific Antibody Redirects T-Cell Cytolytic Activity to EGFR-Positive Cancers In vitro and in an Animal Model

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