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TARGETING HUMAN CYTOTOXIC T LYMPHOCYTES TO KILL HETEROLOGOUS EPIDERMAL GROWTH FACTOR RECEPTOR-BEARING TUMOR CELLS

Tumor-Infiltrating Lymphocyte/Hormone Receptor/Recombinant Antibody

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A genetically engineered conjugate between an anti-CD3 antibody and epidermal growth factor (EGF) was tested for its ability to mediate the lysis of receptor-bearing cells by human CTL. This construct was made by fusing an EGF coding sequence to the 3′ end of the human γ-1 H chain gene sequence and expressing the modified gene in transfected cells together with the V regions of a mouse antibody specific for the human T cell marker, CD3. The resulting conjugate was able to compete with EGF for its receptor and, at extremely low concentrations, was able to mediate the lysis of receptor-bearing tumor targets by a tumor-infiltrating lymphocyte line or by a CTL line established from peripheral blood. The construction of such conjugates by genetic engineering represents a general approach to the direct expression of highly specific hetero-bifunctional reagents without the necessity of further in vitro manipulations.

Antibodies have been shown to mediate the lysis of tumor cells in vitro by bridging the FcR on the cytotoxic effector cell and the antigenic site on the target cell (1). The binding is mediated by the V regions of the H and L chains of the anti-tumor cell antibody and the FcR binding site on the C region of the Ig H chain. In an analogous manner, CTL have been targeted to cells for which they have no specificity through the use of cross-linking reagents. These include several hetero-bifunctional reagents that share the same mechanism; they bridge a specific marker on the tumor cell surface to a component of the TCR and in this way activate the lytic program of the CTL (2–4). The most widely used anti-T cell specificity is that directed against CD3, a closely associated component of the TCR (5), although anti-TCR (6) antibodies have also proven useful.

Among the approaches used to combine anti-T cell and anti-tumor cell specificities is the conjugation of a peptide hormone, an analog of α-melanocyte-stimulating hormone, to an anti-CD3 antibody by chemical means (7). This conjugate was found to mediate the specific lysis of MSH-bearing melanoma cells by CTL. This approach has some advantages over hetero-bispecific antibodies that have been produced by the fusion of two antibody-producing cells. First because the random association of the multiple H and L chains (only a fraction of which are active) is avoided and results in a more homogeneous preparation. Second, the targeting of hormone receptors, relative to other tumor-associated Ag, may lead to the preferential killing of those cells that overexpress the hormone receptor and thus, are the most rapidly growing and in some cases the most tumorigenic. In fact, the concentration of hormone receptor on the surface of an epidermal carcinoma cell line (in this case EGF[2]) was directly correlated to its tumorigenicity in nude mice (8).

We have been studying the genetic approach to the construction of Ig/hormone conjugates using the EGF/EGFR system as a model. Several different types of tumors exhibit enhanced EGFR expression that is often associated with increased tumorigenicity but that may also serve to discriminate them from their normal cell counterparts (9–12). Our approach was to fuse the EGF sequence to the carboxyl-terminal end of the H chain gene of a chimeric anti-CD3 Ig. The resulting conjugate should be perfectly configured as a cross-linking reagent with two Ag binding sites at one end and two EGF molecules (one on each H chain) at the other. The expression of this construct in hybridoma cell transfectants has made it possible to make a homogeneous preparation of this conjugate and study its ability to mediate the T cell lysis of EGFR-bearing tumor cells.

MATERIALS AND METHODS

Plasmid constructions. An EGF gene fragment was synthesized from the known protein sequence (13) and ligated to an engineered *Sma*I site at the 3′ end of the human Cγ1 gene (Fig. 1). An *Xho*I site was placed to the 3′ side of the EGF coding sequence for ligation to a fragment containing the 3′ untranslated region and poly A addition signal from the mouse Ig Cκ gene.

V region cassettes encoding the H and L chain V regions of the mouse anti-CD3 antibody, OKT3 (ATCC no. CRL 8001), were constructed from cloned cDNA as described (14) and inserted into the chimeric antibody expression vector pdHL2 to give pdHL2-CD3. The modified H chain, to which EGF was fused, was inserted into the pdHL2-CD3 plasmid as a *Hin*dIII to *Eco*RI fragment. A second construct was made by replacing the *Hin*dIII to *Nsi*I fragment of the Cγ1 gene with the corresponding fragment from the human Cγ4 gene. The *Nsi*I site (not shown in Fig. 1) is located 51 bp upstream of the *Sma*I site. In both cases the lysine residue, normally found at the carboxy terminus of Ig H chains, was omitted from the fusion proteins (Fig. 1).

Cell culture and transfection. Mouse hybridoma cells (Sp2/0 Ag14) were maintained in DMEM, transfected, and then analyzed

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[2] Abbreviations used in this paper: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TIL, tumor-infiltrating lymphocyte.

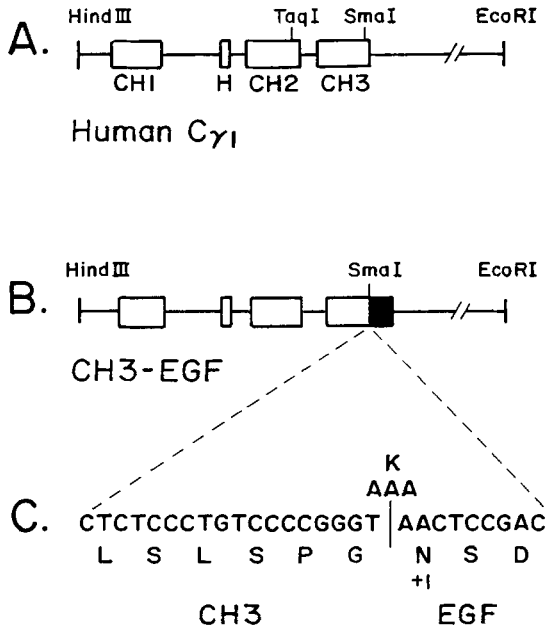

Figure 1. Construction of a fusion protein between the human Cγ1 Ig H chain and EGF. *A*, The restriction map of a Cγ1 gene fragment cloned in plasmid pBR322. *B*, The Cγ1 gene fused at the *SmaI* site to a synthetic EGF-encoding sequence. *C*, The sequence at the junction of the Ig CH3 domain and the amino terminus of EGF. Note that the carboxyl-terminal lysine codon was not included in the fusion protein.

for expression by ELISA as described (15). Human tumor cell lines A431 (epidermal carcinoma, ATCC no. CRL 1555), M24 (metastatic melanoma, originally obtained by D. C Morton, UCLA, Los Angelos, CA, and kindly provided by Ralph Reisfeld, Scripps Clinic, La Jolla, CA) and IMR-32 (neuroblastoma, ATCC no. CCL 127) were maintained in RPMI 1640 containing 10% FBS. The human TIL line 660, derived from a human melanoma patient, and the W-1 human PBL-derived CTL line were cultured in AIM V medium (GIBCO, Grand Island, NY) containing IL-2 (a gift of Hoffmann-LaRoche, Nutley, NJ) as described (16). More than 90% of the cells in both lines were $CD3^+$ and $CD8^+$ when examined by fluorescence microscopy.

Protein purification. Chimeric antibody and antibody/EGF conjugates were purified by affinity chromatography using protein A Sepharose (Repligen, Cambridge, MA).

EGF competitive binding assay. M24 melanoma cells (2×10^5 cells in a final volume of 0.1 ml) were mixed on ice in HBSS containing 0.1% BSA and 20 mM HEPES together with ^{125}I-EGF (Amersham Corp., Arlington Heights, IL, 10 ng/ml final concentration) and varying concentrations of unlabeled competitor (either EGF, anti-EGF antibody 225 (17), chimeric anti-CD3, or antibody conjugate). Mouse antibody 225 was generously provided by Dr. John Mendelsohn (Memorial Sloan-Kettering Cancer Center, New York, NY). After a 2-h incubation at 4°C, cells were washed three times by centrifugation and the cell-associated radioactivity was counted. A non-specific background, determined by incubation with a 200-fold excess of unlabeled EGF (< 10% of the experimental value), was subtracted from all data points. All assays were done in duplicate with SD between 2 to 8%. The results were expressed as the percent inhibition of binding relative to the no competitor control.

Cytotoxicity assays. Cytotoxicity assays were carried out using ^{51}Cr-labeled tumor targets and either TIL 660 or W-1 CTL as effectors. A fixed number of labeled targets (10^4/well) in 50 μl and varying numbers of effectors in 50 μl were mixed with 100 μl of diluted antibody or conjugate in the wells of a microtiter plate. The plates were centrifuged and assayed for released ^{51}Cr after a 4-h incubation at 37°C. Spontaneous release (generally <10% of the total) was subtracted from experimental values and the percent of specific lysis was determined by dividing the corrected value by the total (released with detergent lysis), and multiplying by 100. SD of duplicate determinations ranged from 1 to 7%, depending on the cell line.

RESULTS

Expression of Ig/EGF fusion proteins. Mouse hybridoma cells were transfected with plasmids encoding anti-CD3 chimeric antibody or anti-CD3/EGF fusion proteins. Transfectants secreting human antibody determinants were identified by ELISA and their culture supernatants were tested further for anti-CD3 reactivity by their ability to stain TIL 660 cells in the presence of a fluoresceinated anti-human Ig antiserum. Both the chimeric and conjugated antibodies were found to stain these cells as well as the original mouse antibody (OKT3-, Ortho Diagnostic Systems Inc., Raritan, NJ).

Cell culture media were used as a source of material for the purification of the chimeric and conjugated antibodies by protein A-Sepharose chromatography. Electrophoretic analyses of the purified proteins showed that they were both fully assembled into antibody molecules and that the conjugated H chain migrated as would be expected for the fusion of the Ig and EGF sequences (data not shown).

The ability of the conjugate to bind to EGFR was then examined in a competitive binding assay (Fig. 2). The anti-CD3 antibody alone showed little or no inhibition activity whereas the anti-CD3/EGF conjugate competed well with EGF for its receptor. A positive control anti-EGFR antibody, 225 (17), competed more effectively than either the conjugate or EGF itself for EGFR.

Killing of tumor cells expressing EGF receptors. The epidermal carcinoma cell line, A431, expresses a very high number (2×10^6/cell) of EGFR on its cell surface (18) and this overexpression has been correlated with its ability to form tumors in nude mice (8). We tested the ability of the anti-CD3/EGF conjugate to mediate the killing of labeled A431 cells by a human TIL cell line (660) in a 4-h chromium release assay (Fig. 3*A*). The parameters that were varied in the first studies were the E:T ratio and the concentration of the conjugate. No killing of the A431 targets was seen in the absence of the conjugate, demonstrating that the TIL 660 line has no specificity for these cells. Significant levels of lysis were seen with concentrations of conjugate as low as 0.1 ng/ml (6×10^{-13} M) and this killing increased as a function of conjugate concentration or E:T ratio. Very little additional killing was seen at concentrations above 25 ng/ml (1.5×10^{-10} M).

Exactly the same results were obtained when the constructs were made with the human Cγ1 or Cγ4 H-chain genes. The Cγ4 H chain was used for the conjugate

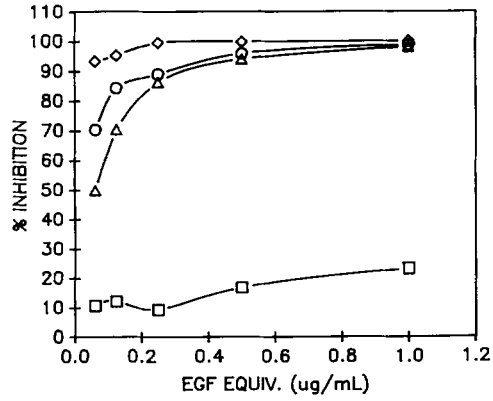

Figure 2. EGF receptor binding activity of the anti-CD3/EGF conjugate. The ability of the conjugate (Δ) to compete with labeled EGF for its receptor was measured using M24 melanoma cells and compared to unlabeled EGF (○), unconjugated anti-CD3 antibody (□) and anti-EGFR antibody 225 (◇). The results are normalized to the molar equivalents of EGF based on binding sites.

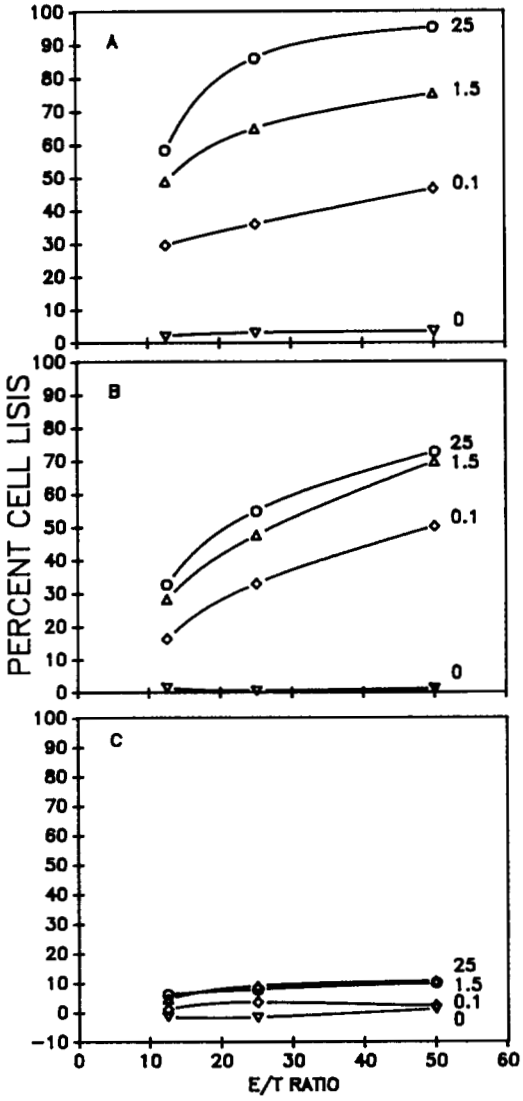

Figure 3. Anti-CD3/EGF conjugate-induced killing of tumor cell targets by TIL 660 cells. ^{51}Cr-labeled targets were incubated for 4 h with the indicated amounts of conjugate (ng/ml) and varying ratios of E:T. The amount of released radioactivity was used to calculate the percent of target cell lysis. The targets were (*A*) A431 epidermal carcinoma cells, (*B*) M24 metastatic melanoma cells, or (*C*) IMR-32 neuroblastoma cells.

TABLE I

^{125}I-EGF binding to tumor targets[a]

Cell Line	^{125}I-EGF Bound (pg/2 × 10^5 Cells)
A431 (epidermal carcinoma)	236.8
M24 (metastatic melanoma)	34.1
IMR-32 (neuroblastoma)	0.72

[a] Cells were incubated for 2.5 h in 100 μl of buffer (HBSS, 0.1% BSA, 20 mM HEPES, pH 7.4) at 4°C with 700 pg of ^{125}I-EGF, washed three times with buffer and the pellet counted in a gamma-counter. Nonspecific binding (that obtained in the presence of a 200-fold excess of unlabeled EGF) was subtracted.

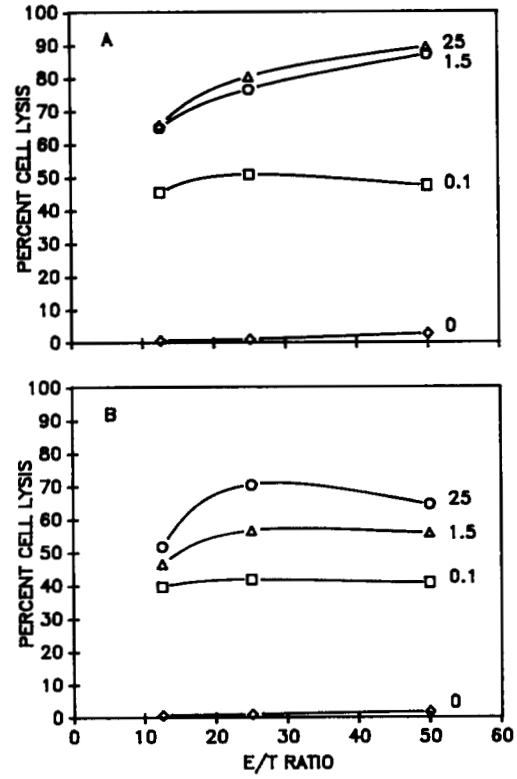

Figure 4. Anti-CD3/EGF conjugate-induced killing of A431 and M24 cells by peripheral blood-derived CTL. Killing assays were carried out as in Figure 3 using (*A*) A431 cells or (*B*) M24 melanoma cell targets.

because of its inability to fix human complement (19). Any subsequent studies in vivo might be complicated by the complement lysis of CD3$^+$ T cells, although we have found that the TIL 660 line is not lysed by the chimeric anti-CD3 antibody or by the conjugate and human complement (not shown).

Additional tumor cell lines were tested for their susceptibility to TIL lysis in the presence of the anti-CD3/EGF conjugates. These include a human metastatic melanoma line (M24) expressing a moderate level of EGFR, as well as a neuroblastoma line (IMR-32) that is very sensitive to lysis in an ADCC assay (lysis by FcR-bearing cells in the presence of an anti-tumor antibody; our unpublished observations) but expresses little or no detectable EGFR (Table I). The killing of these cell lines by the TIL 660 effectors was found to be directly related to the expression of EGFR (Fig. 3 *B* and *C*). The M24 line expresses EGFR, although 10-fold less than A431 cells, and is killed almost as well at low conjugate concentrations. The killing of A431 cells increased at higher concentrations of the conjugate (>1.5 ng/ml) whereas the killing of M24 cells did not. This difference may reflect the saturation of M24 cell receptors at the lower concentration. The neuroblastoma line, IMR-32, does not express EGFR (Table I) and was not killed by TIL 660 cells in the presence of the anti-CD3/EGF conjugate (Fig. 3*C*).

We extended our study to include a second CTL line, W-1, which was derived from peripheral blood and is both CD3$^+$ and CD8$^+$. It has specific cytotoxic activity for autologous EBV transformed lymphoblastoid cells (16). These cells also killed the EGFR-bearing A431 and M24 cells very efficiently in the presence but not in the absence of the conjugate (Fig. 4).

Cell killing specificity of anti-CD3/EGF conjugate. The specific lysis of the A431 and M24 tumor cell lines was measured in the presence or absence of the conjugate, as well as its component parts (Table II). EGF alone or anti-CD3 antibody alone or in combination were unable to mediate CTL killing of the tumor targets. Concentrations of antibody that were 100-fold higher also did not significantly increase the specific lysis above background levels (not shown). Clearly, the physical linkage of the antibody and EGF is required for killing activity

TABLE II

Specificity of anti-CD3/EGF conjugate

Additions	Percent Maximum Lysis of Cell Line	
	A431	M24
None	0	0
EGF (0.5 ng/ml)	0	0
Anti-CD3 (5 ng/ml)	0	0
EGF + anti-CD3	1	0
Anti-CD3/EGF (5 ng/ml)	100	100
Conjugate + 100× EGF (50 ng/ml)	123	114
Conjugate + anti-CD3 (0.5 μg/ml)	71	48
Conjugate + anti-CD3 (10 μg/ml)	10	15

The 4-h cytotoxicity assays were carried out using an E:T (TIL 660 cells) ratio of 50:1 with the indicated additions. Values represent the amount of lysis obtained in a particular reaction expressed as the percentage of that obtained with the anti-CD3/EGF conjugate.

since only the conjugate was able to mediate the lysis of the EGFR-bearing targets.

We have found that it is very difficult to block the killing activity of the conjugate with a 100-fold excess of anti-CD3 antibody. Inasmuch as this represents only 0.5 μg/ml, it is possible that there may still be CD3 molecules available for binding. When the concentration was increased to 10 μg/ml, significant inhibition was observed. This may indicate that only a small number of receptors is needed to cross-link the target and effector cells. The addition of unconjugated EGF did not block but actually enhanced the killing of both targets by the conjugate (Table II). Inasmuch as all EGFR would not be occupied at the concentrations used, this again suggests that only a small number of available receptors (in this case on the target cell) is required for lysis.

To demonstrate that the killing activity of the conjugate is mediated through binding of EGFR, we tested various concentrations of conjugate and increased the concentration of free EGF to very high levels. We also tested the ability of the anti-EGFR antibody 225 to block killing. The TIL 660 line was used as effectors and the M24 melanoma line was used as the target. This target was used because it expresses fewer receptors than the A431 line and also expresses the disialoganglioside GD2, recognized by the mouse antibody 14.18 (20) and the chimeric mouse/human antibody ch14.18 (14). The binding of ch14.18 to M24 cells serves as a specificity control that should not block EGFR-mediated killing. In these assays, all competitors were mixed with the target cells and incubated on ice for 30 min before the addition of effector cells in order to establish equilibrium of receptor/ligand binding.

Figure 5 shows the results obtained with a conjugate concentration of 100 ng/ml (20-fold higher than that used in Table II). Inhibition was observed at concentrations of EGF above 5 μg/ml (500-fold molar excess) and at the equivalent concentration (and higher) of the anti-EGFR antibody 225. The same concentrations of ch14.18 anti-GD2 antibody, which also binds to M24 cells, had no inhibitory effect on killing activity. Thus, inhibition is specific for those ligands that bind to EGFR.

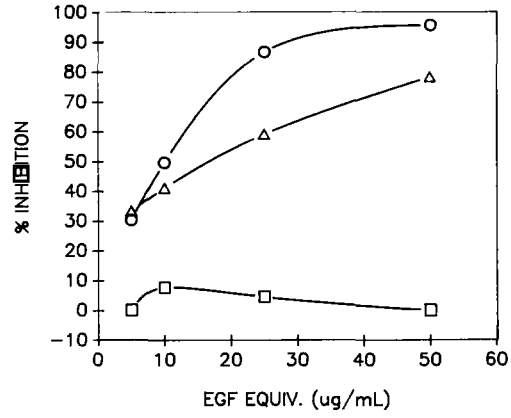

Figure 5. Inhibition of anti-CD3/EGF conjugate-mediated killing by free EGF or anti-EGF antibody. Killing assays were carried out as in Figure 3 except that M24 melanoma target cells were mixed with the conjugate (100 ng/ml) and varying concentrations of EGF (○), anti-EGF antibody 225 (△) or control anti-GD2 antibody ch14.18 (□) and incubated on ice for 30 min before the addition of effector cells (50:1 ratio). The specific lysis in the absence of inhibition was 70%.

DISCUSSION

Hormone receptors have been used as tumor-specific markers for the delivery of cytotoxic agents (21, 22). *Pseudomonas* exotoxin and diptheria toxin have been coupled chemically (and genetically as fusion proteins) to peptide hormones and the conjugates have been shown to be highly cytotoxic and specific for receptor-bearing cells (23, 24). An alternative targeting approach involves the use of a hetero-bifunctional antibody/hormone conjugate that both physically bridges a receptor-bearing tumor target and a cytotoxic T cell and activates the killing mechanism (7). In this way it is possible to confer upon a population of activated T cells an anti-tumor specificity that they do not normally have and would lose as soon as the reagent is withdrawn or metabolized in vivo. Thus, such a reagent might be useful in an adoptive immunotherapeutic approach either alone or in conjunction with the administration of a patient's lymphokine-activated killer cells or TIL.

We have used a population of TIL, derived from a patient with malignant melanoma, as a source of activated T cells for testing a genetically engineered anti-T cell/hormone (EGF) conjugate. These cells had little or no cytolytic activity against the tumor targets that we tested. In the presence of very low concentrations of the conjugate, those cells expressing EGFR were readily killed. This activity was seen at concentrations (10^{-12} to 10^{-11} M) that were significantly lower than the K_d for EGF binding to its receptor (2×10^{-10} M). A second CTL line, derived from peripheral blood and specific for autologous EBV-transformed cells, could also be induced to kill these tumor cells for which it has no specificity. These cells have been maintained in culture for an extended period of time in the presence of IL-2 and with bi-monthly stimulation with mitomycin C-treated autologous EBV-transformed B cells (16). The ability of these cells to kill

EGFR-bearing tumor cells over an extended period of time has not diminished, thus making this EBV-specific CTL system generally useful for testing hormone conjugates.

The specificity of the conjugate was examined by testing the activity of the anti-CD3 antibody alone or in combination with unconjugated EGF. These results clearly demonstrate that the two need to be physically linked for activity. It was more difficult to show inhibition of killing with free ligand. The killing was blocked by the addition of unconjugated anti-CD3 antibody, but only at concentrations that were in vast molar excess, relative to the conjugate. Initially we found that at low concentrations of conjugate (e.g., 5 ng/ml), inhibition of killing by a 100-fold molar excess of free EGF was not observed. In fact, we found that the addition of EGF significantly increases the killing of both cell lines. This enhanced killing suggests that these tumor cells, when treated with high levels of EGF, may become more susceptible to lysis by CTL.

At higher concentrations of either EGF or anti-EGFR antibody we were able to demonstrate inhibition of killing, even at much higher (saturating) concentrations of the conjugate (100 ng/ml). This inhibition was shown to be specific for EGFR by demonstrating that equivalent amounts of another antibody, binding to a different Ag on the same cell, has no inhibitory effect on conjugate-mediated killing. The fact that killing was not observed at the lower concentration range strongly suggests that the most important factor for inhibition is the blocking of receptors on the target cell, not simply the molar ratio of ligand and competitor. The fact that the conjugate is effective at concentrations that are well below the K_d for EGF receptor binding supports the idea that only a very small percentage of receptor occupancy is required for cytolysis. Thus, the blocking of as much as 95% of all receptors with competitor may not be sufficient to inhibit killing because the other 5% would be available to bind the conjugate.

The use of genetically engineered conjugates between anti-T cell antibodies and peptide hormones should prove useful in testing the feasibility of adoptive immunotherapy whereby a patient's TIL line or peripheral blood-derived CTL line is given an additional target specificity. Inasmuch as many different tumors overexpress the EGFR, the use of conjugates containing EGF may be particularly useful for many different cancers. The preclinical testing of such conjugates will require unique animal model systems that allow for growth of both the tumor xenograft and human CTL. Such a system is currently under development using severe combined immunodeficient mice into which are transplanted both a metastatic melanoma cell line and human T cells (B. Mueller and R. Reisfeld, personal communication). Such studies will also be important in determining whether the conjugate will also mediate the killing of normal cells expressing EGFR.

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