Isolation of Human KB Cell Lines Resistant to Epidermal Growth Factor-
*Pseudomonas* Exotoxin Conjugates

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ABSTRACT

Mutants of the human KB carcinoma cell line resistant to a cytotoxic conjugate of epidermal growth factor (EGF) and *Pseudomonas* exotoxin (PE) were selected. EGF-PE and the drug verapamil, which enhanced EGF-PE cytotoxicity, were used in the selection process. These mutants also showed some cross-resistance to PE. All of the EGF-PE resistant variants displayed lower levels of 125I-EGF binding, 20–50% of parental KB levels, without altered affinity for EGF and grew at a slower rate than the parental cell line KB-3-1. These results indicate that EGF-PE resistant KB cells have a complex phenotype which includes a reduction in the number of EGF receptors and reduced sensitivity to unconjugated PE. Resistance to toxin-conjugates, although pleiotropic, is specific and does not lead to resistance to multiple other anticancer drugs, nor are independently selected multidrug resistant KB lines resistant to PE. These results argue that protocols for cancer treatment could effectively use specifically designed cytotoxic toxin conjugates as an adjunct to conventional chemotherapy.

INTRODUCTION

Cell growth is controlled by growth factors that interact with specific receptors on the cell surface. One of the best studied of these is the epidermal growth factor receptor (1). Recently this receptor was shown to be structurally related to the v-erbB oncogene (2). Human KB cells which contain about 150,000 EGF receptors per cell have been used to determine the pathway by which EGF and the EGF receptor are internalized and degraded (3, 4). It has also been shown that following EGF-induced down regulation of the EGF receptor, the level of EGF receptor mRNA and the rate of EGF receptor synthesis is increased (5). The EGF receptor in KB cells also appears to be a good target for conjugates of EGF with toxins. To learn more about the mechanism of action of EGF and EGF toxin conjugates, we have begun to isolate mutants of KB cells resistant to a cytotoxic conjugate composed of EGF and *Pseudomonas* exotoxin. Six EGF-PE resistant clones were isolated and characterized. This paper describes the isolation and preliminary biological characterization of these clones. Their biochemical characterization will be reported elsewhere.

Recently, toxins coupled to monoclonal antibodies or growth factors have been proposed as agents for the treatment of human cancer (6). It is believed this treatment method will supplement conventional chemotherapeutic agents to which cancer cells often become resistant. Therefore, one aspect of the EGF-PE resistant cells that has been given special attention in this work is whether or not these EGF-PE resistant cells are cross-resistant to conventional chemotherapeutic agents such as vinblastine, Adriamycin, and colchicine, and whether multidrug resistant cell lines which were specifically selected for resistance to these chemotherapeutic agents show any cross-resistance to EGF-PE.

MATERIALS AND METHODS

Materials. Mouse EGF was purchased from Bethesda Research Laboratories. A monoclonal antibody against the transferrin receptor, designated HB21, was obtained from American Type Culture Collection, propagated as ascites in BALB/c mice, and purified as described (7). *Pseudomonas* exotoxin A was a gift from Dr. S. Leppa, USA MR110, Fort Detrick, MD. Conjugates of EGF and HB21 with PE were prepared as previously described (7, 8). Verapamil was a gift from Knoll Pharmaceutical Co., Whippany, NJ. Ethyl methanesulfonate was purchased from Sigma Chemical Co. Colchicine, Adriamycin, and vinblastine were purchased from Sigma. EGF was radioiodinated by using a method described by Hunter and Greenwood to a specific activity of 2.7 x 10⁶ cpm/nmol (9).

Cells and Culture. KB cells were obtained from the American Type Culture Collection, subcloned twice, and a single recloned cell line, KB-3-1, was used as the parent cell line in this study. On the basis of karyotype analysis and cell surface antigens, these KB cells appear to be a HeLa variant. A multidrug resistant subline was derived from KB-3-1 by a series of step selections in colchicine (10) and grown in 1 μg/ml colchicine (KB-C1). Sublines of KB-3-1 resistant to 1 μg/ml of Adriamycin or vinblastine (KB-A1 and KB-V1, respectively) were selected as previously described (11). KB-3-1 and sublines were cultured in monolayer using DMEM (GIBCO) containing 10% FBS (GIBCO), penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively; GIBCO) and glutamine (GIBCO) at 1 mM in a controlled atmosphere of 5% CO₂ (v/v) and 95% air (v/v) at 37°C.

Toxin-conjugate resistant sublines were maintained in selective medium containing EGF-PE (1.7 nm) and verapamil (10 μg/ml). Drug resistant lines were maintained in medium containing the selecting drug added at 1 μg/ml. Stock drug solutions (2 mg/ml) of Adriamycin, vinblastine, and colchicine were prepared in dimethyl sulfoxide (Aldrich) and were added to the growth medium at the concentrations indicated. Cells were propagated as described (10–12).

Selection and Isolation of Mutant Sublines Resistant to EGF-PE. Mutant sublines of KB-3-1 were selected for resistance to EGF-PE and isolated as follows. Briefly, cells were treated for 24 h with 150 μg/ml of the mutagen ethyl methanesulfonate, and then transferred to fresh DMEM containing 10% FBS and incubated for 10 days at 37°C. EGF-PE (17 nm) and verapamil (20 μg/ml) were added, and after 10 h at 37°C the medium was replaced by fresh medium. Colonies were picked 10–14 days later and recloned in selected medium. Resistant colonies were designated ET (EGF-toxin resistant). Some clones were recloned several times in selective medium. Clone 28 was remutanogenized with ethyl methanesulfonate and reselected in medium containing 6.8 nM EGF-PE and 10 μg/ml verapamil.

Cell Killing Assay. KB-3-1 cells and sublines of KB-3-1 were grown for 1 day in medium without toxic conjugate or drugs before use. Relative plating efficiency was determined by plating 300 cells in 60-mm dishes overnight in complete medium and then adding the indicated amounts of EGF-PE, either with or without verapamil (10 μg/ml) or drug. After incubation at 37°C for 8 days, the dishes were stained with 0.5% methylene blue in 50% ethanol and the number of colonies was counted.
determined. The relative plating efficiency is the number of colonies formed in the presence of the agent being tested divided by the number of colonies formed in the absence of any additions.

**Measurement of Cell Killing Effects of Various Toxins.** KB-3-1 and EGF-PE resistant subline cells were plated in 24-well tissue culture dishes (Falcon) at a cell density of $2 \times 10^4$ cells/well in 2 ml DMEM containing 10% FBS. The following day, the medium was replaced by fresh DMEM and incubation was continued at 37°C for 1 h. Cells were then incubated on ice for 15 min prior to the addition of various amounts (from 0.5 nm to 8 nm) of a mixture of unlabeled EGF (150 nm) and $^{125}$I-labeled EGF (40 nm) (specific activity, $5.7 \times 10^8$ cpm/nmol) in a volume not greater than 50 µl. The radioactivity of $^{125}$I-EGF added in the binding assay varied from $0.2 \times 10^8$ cpm to $1.6 \times 10^8$ cpm. Binding was carried out on ice for 2h. Following this period, cells were washed three times with ice-cold medium and then solubilized in 1 ml NaOH for counting of $^{125}$I radioactivity in a mini-gamma counter (LKB). Nonspecific binding was estimated by adding unlabeled EGF in excess (350 nm) and this value was subtracted to give specific binding. Nonspecific binding linearly increased as a function of the ligand concentration and was less than 30% of the total binding at the highest ligand concentration. Cell number was estimated for duplicate wells using a Coulter Counter and protein concentrations were determined by the method of Bradford (13) using a dye reagent from Bio-Rad.

**EGF Uptake.** Measurement of uptake of $^{125}$I-EGF at 37°C was measured as previously described (14). Wild-type and mutant cells were plated in duplicate at $2 \times 10^4$ cells/well in 24-well tissue culture trays in 0.5 ml DMEM containing 10% FBS. The following day the medium was replaced with 0.5 ml DMEM. After 1 h at 37°C, the cells were chilled on ice for 15 min prior to the addition of $^{125}$I-EGF ($2.7 \times 10^9$ cpm/nmol). $^{125}$I-labeled EGF was added to a final concentration of 0.5 nm, cells were incubated on ice for 2h, and washed three times with ice-cold PBS to remove unbound EGF. The cells, prebound with $^{125}$I-

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**Table 1 Relative resistance of EGF-PE resistant cells to other toxins**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGF-PE</th>
<th>PE</th>
<th>HB21-PE</th>
<th>Ricin Diphtheria toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>300</td>
<td>3.3</td>
<td>10</td>
<td>0.3</td>
</tr>
<tr>
<td>12</td>
<td>300 (1,000)</td>
<td>33</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>19</td>
<td>30 (3,000)</td>
<td>10</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>28</td>
<td>30 (10,000)</td>
<td>3.3</td>
<td>10</td>
<td>0.3</td>
</tr>
<tr>
<td>30</td>
<td>300 (2,000)</td>
<td>&lt;10</td>
<td>25</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* The approximate 50% lethal dose of ET lines as determined from spot tests was divided by the 50% lethal dose of the parental KB-3-1 cell line.

* Numbers in parentheses, relative resistance calculated by using the colony-forming assay.

EGF, were transferred to a 37°C incubator, removed at various periods, acid washed with 0.2 M acetic acid, 0.5 M NaCl, pH 4.0, followed by two washes with ice-cold PBS, solubilized in 1 ml 1 N NaOH, and their content of $^{125}$I radioactivity was determined. Nonspecific uptake of radioactivity was estimated in cultures containing unlabeled EGF at 350 nm.

**Growth of Cells in Soft Agar.** Tissue culture dishes (60 mm in diameter) (Falcon) were layered with 2 ml of 0.5% agarose (Difco) at 45°C in DMEM containing 10% FBS. These plates were allowed to cool to room temperature. Cells were added to these dishes by mixing 3 ml of 0.5% agarose in DMEM plus 10% FBS at 45°C with 1.5 ml of
EGF-TOXIN CONJUGATE-RESISTANT HUMAN CELLS

Fig. 3. Binding of $^{125}$I-EGF to sublines of KB-3-1 as a function of $^{125}$I-EGF concentration (left) and Scatchard analysis of $^{125}$I-EGF binding to KB-3-1 sublines (right). KB-3-1 cells (O), 12 (A), 19 (Δ), 22 (x), 28 (□) top) and KB-3-1 (C), sublines 10 (○), and 30 (□) (bottom) were each plated in duplicate at a cell density of 2 x 10^6 cells/well in 24-well tissue culture dishes. The medium was replaced with 0.5 ml of fresh DMEM containing 10% FBS. After 1 h at 37°C, plates of cells were chilled on ice for 15 min prior to the addition of various amounts of a mixture of $^{125}$I-EGF and EGF from 0.5 nM to 8 nM and processed as described in "Materials and Methods."

Table 2 Binding of $^{125}$I-EGF to parental and mutant KB cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>$K_d$ (nM)</th>
<th>Binding capacity (receptors/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KB-3-1</td>
<td>1.01</td>
<td>148,000</td>
</tr>
<tr>
<td>ET-12</td>
<td>0.79</td>
<td>54,600</td>
</tr>
<tr>
<td>ET-19</td>
<td>0.62</td>
<td>81,000</td>
</tr>
<tr>
<td>ET-22</td>
<td>0.54</td>
<td>33,000</td>
</tr>
<tr>
<td>ET-28</td>
<td>0.65</td>
<td>33,600</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KB-3-1</td>
<td>1.46</td>
<td>141,000</td>
</tr>
<tr>
<td>ET-10</td>
<td>1.50</td>
<td>71,400</td>
</tr>
<tr>
<td>ET-30</td>
<td>1.33</td>
<td>84,000</td>
</tr>
</tbody>
</table>

RESULTS

Isolation of EGF-PE Resistant Cells. Six independent EGF-PE resistant clones of KB cells were isolated in a single step as described under "Materials and Methods." Verapamil, which increases the killing of KB cells by EGF-PE (12), was included in the selection medium; no resistant clones could be isolated in the absence of verapamil. The resistance to EGF-PE of clones ET-12, ET-19, ET-22, ET-28, and ET-30 was determined in the presence and absence of verapamil (Fig. 1). All clones were at least 100-fold resistant to EGF-PE; ET-22 and ET-28 were over 1000-fold resistant. Verapamil decreased the amount of EGF-PE required to kill both the parent KB-3-1 and the resistant clones. However, resistance to EGF-PE was apparent whether or not verapamil was present. Cell lines cultivated in the absence of EGF-PE for 3–6 months gradually lost their drug resistance, so for these studies, all lines were maintained in selective medium containing 1.7 nM EGF-PE and 10 μg/ml verapamil until 1–2 days prior to use. Lines growing in the presence of EGF-PE and verapamil maintained their phenotype as described below for more than 2 years, but minor variations in $^{125}$I-EGF binding have been observed (see below).

Cross-Resistance of EGF-PE Resistant Cells to Other Toxins. Because cells selected with one toxin are frequently cross-

Fig. 4. Uptake of $^{125}$I-EGF at 37°C in sublines of KB-3-1. Uptake of $^{125}$I-EGF by KB-3-1 (○), ET-28, subclone 4 (A), and ET-30, subclone 1 (□) was determined as described in "Materials and Methods."

Fig. 5. Growth curves of KB-3-1 and three PE-EGF resistant cell lines. Cells were plated in 60-mm dishes and at various times duplicate dishes were trypsinized and counted with a Coulter Counter. Points, mean. KB-3-1 (○); ET-10 (□); ET-19 (Δ); ET-28 (□).

Fig. 6. Cell number vs. days in culture. KB-3-1 (○); ET-10 (□); ET-19 (Δ); ET-28 (□).
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Fig. 6. Growth of wild-type and mutant cells in agar containing 20% serum. Cell lines were as follows: A, KB-3-1; B, ET-10; C, ET-12; D, ET-19; E, ET-22; F, ET-28; G, ET-30.

Table 1. Relative resistance or sensitivity of ET and multidrug resistant lines to colchicine, Adriamycin, and vinblastine

<table>
<thead>
<tr>
<th>Cloning efficiency (%)</th>
<th>Fold-resistance (50% lethal dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colchicine</td>
</tr>
<tr>
<td>KB-3-1</td>
<td>48</td>
</tr>
<tr>
<td>ET lines</td>
<td></td>
</tr>
<tr>
<td>ET-10</td>
<td>31</td>
</tr>
<tr>
<td>ET-12</td>
<td>4</td>
</tr>
<tr>
<td>ET-19</td>
<td>16</td>
</tr>
<tr>
<td>ET-22</td>
<td>26</td>
</tr>
<tr>
<td>ET-28</td>
<td>23</td>
</tr>
<tr>
<td>ET-30</td>
<td>4</td>
</tr>
<tr>
<td>Multidrug resistant lines</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>14</td>
</tr>
<tr>
<td>A1</td>
<td>19</td>
</tr>
<tr>
<td>V1</td>
<td>18</td>
</tr>
</tbody>
</table>

resistant to other toxins or toxic substances, we examined the cross-resistance of the EGF-PE resistant cells to *Pseudomonas* exotoxin, ricin, diphtheria toxin, and PE coupled to an antibody to the transferrin receptor (HB21-PE). These studies were done in a simplified spot assay for cell killing using multiwell dishes. Cells were incubated with increasing amounts of toxin for 3 days and the amount of toxin reducing cell growth by 50% (LD50) was determined visually. A typical result is shown in Fig. 2 and the data from several experiments are summarized in Table 1. As expected, the cells also showed a high degree of resistance to EGF-PE in this assay, although the numbers using this assay are semiquantitative and not as accurate as those obtained using the colony-forming assay. These results have been confirmed in some cases using a colony-forming assay (data shown in parentheses in Table 1). The ET cells also showed some cross-resistance to PE and to HB21-PE. There was minimal cross-resistance to diphtheria toxin. Three of the resistant clones (ET-10, ET-28, and ET-30) showed slightly increased sensitivity to ricin.

EGF Binding of Resistant Cells. Because the resistant cells were much more resistant to EGF-PE than to PE alone or to other toxins, it seemed likely that they would have a decreased number of EGF receptors. The number of EGF receptors and their affinity for EGF was measured as described under “Materials and Methods.” The data are shown in Fig. 3 and summarized in Table 2. When compared to the parental KB-3-1 cells, all the resistant lines had a decrease in the number of EGF receptors. Mutant cell lines contained from 22–54% as much receptor as the parental line. No significant changes in the KA of EGF binding was detected. Some variation in the number of receptors was observed in different experiments. Each cell line was studied at least twice and was always compared with the parental cell. The data shown in Table 2 are representative.

Uptake of 125I-EGF in EGF-PE Resistant Cells. One possible defect that could account for resistance to EGF would be the failure to endocytose EGF-containing conjugates. 125I-EGF uptake was measured in KB-3-1 cells and two resistant lines (ET-28 and ET-30). Cells were incubated at 4°C for 2 h in the presence of 0.5 nm EGF, then washed with ice-cold PBS, and
their ability to accumulate and degrade EGF was measured (Fig. 4). Both resistant lines took up less EGF than the parent line but the kinetics of uptake appeared to be similar to that of the parental line. Cell supernatants collected after 240 min at 37°C were analyzed on a PD-10 sizing column for I25I-EGF degradation (results not shown) and I25I-EGF from parent and mutant cell lines was completely degraded. These data indicate that the ability of 2 EGF-PE resistant cells to endocytose EGF is normal. The reduced amounts of EGF taken up appears to be related to a decrease in EGF receptor number on the cell surface.

Growth of EGF-PE Resistant Cells. During the characterization of these EGF-PE resistant cell lines, we noted that they appeared to grow more slowly than the parental KB-3-1 cell line, even in the absence of EGF-PE. To confirm this observation, the growth of the resistant cells was measured under standard culture conditions on plates and in agar.

Fig. 5 shows growth curves for KB-3-1 and three resistant cell lines on tissue culture dishes in medium containing 10% fetal bovine serum (ET-10, 19, and 28). It is evident that the three clones grow more slowly than the parent and ET-10 and ET-19 achieve a much lower saturation density. The growth of the cells was also evaluated in agar containing 10% serum (Fig. 6). After 7 days of growth, all the EGF-PE resistant cells were found to form fewer colonies and the colonies were small except for those formed by ET-22. These studies show that most of the ET resistant cells have a slower growth rate and grow less well in agar than KB-3-1 cells. There is no correlation, however, between the rate of growth and their degree of resistance to EGF-PE.

Resistance of EGF-PE Resistant Sublines to Colchicine, Adriamycin, and Vinblastine. Because growth factor-toxin conjugates or antibody-toxin conjugates may eventually be used to treat some types of human cancers, cross-resistance of EGF-PE resistant lines to conventional chemotherapeutic agents was examined. As shown in Table 3, the EGF-PE resistant lines were very slightly resistant to colchicine but not to Adriamycin and vinblastine. Also shown in Table 3 is the level of drug resistance of three multidrug resistant KB cell lines we have developed (10, 11). They are highly resistant to colchicine, Adriamycin, and vinblastine (11).

Resistance of Multidrug Resistant Cells to EGF-PE. We have isolated multidrug resistant KB cells by selection in either colchicine (C1), Adriamycin (A1), or vinblastine (V1). These mutant cells were examined for their resistance to EGF-PE (Fig. 7). Very little cross-resistance to EGF-PE was detected in A1 and V1. Cell line C1 appeared to be about 8-fold more resistant to EGF-PE than KB-3-1. In this experiment ET-10 and ET-22 were found to be 330-fold and >33,000-fold resistant to EGF-PE. We conclude that cell lines with high resistance to vinblastine, Adriamycin, and colchicine do not show a high level of resistance to EGF-PE.

DISCUSSION

Human KB carcinoma cells which express the EGF receptor were exposed to a toxic conjugate of EGF and PE (EGF-PE) and six independent resistant clones were isolated. These clones were at least 100 and in some cases more than 10,000-fold more resistant than the parental KB-3-1 cell line to PE-EGF. All the clones had reduced EGF binding, but the binding was reduced only 2- to 5-fold, not enough to account for the very high level of resistance to EGF-PE. The mutants were also moderately cross-resistant to unconjugated Pseudomonas exotoxin and another conjugate of Pseudomonas exotoxin with an antibody to the human transferrin receptor. This cross-resistance could reflect a defect in the ability of the cell to respond to Pseudomonas toxin, as could occur if the toxin were not entering the cytoplasm, or if the translation apparatus of the cell were altered.

The rate of EGF uptake and degradation was determined in two mutants. They displayed the same kinetics as the parental line, although the total amount of EGF internalized and degraded was diminished as expected from their somewhat decreased capacity to bind EGF. Thus, resistance to EGF-PE appears to result from a pleiotropic phenotype involving a change in sensitivity to PE, reduced EGF receptor number, and other unknown changes in the cell, not including a defect in endocytosis. Since most of the mutants were selected in a single step, and appeared independently, we assume that these changes are the consequence of a single mutation. In addition, most of the mutants have a decreased growth rate and grow poorly in agar. These changes are also probably due to the same mutation.

To date we have not been able to isolate complete revertants to see if all these properties are linked. We did not obtain any mutants which had very low or undetectable levels of EGF receptor as described in A431 cells (16) and 3T3 cells previously (17). This result may indicate that KB cells require the EGF receptor for survival and loss of the receptor is lethal.

Growth factor-toxin conjugates and antibody-toxin conjugates (immunotoxins) are currently being evaluated as alternative agents for therapy of cancer. Therefore, it was of interest to determine if the EGF-PE resistant mutants also became resistant to drugs used for conventional chemotherapy. Some of the lines were slightly resistant to colchicine but not to Adriamycin or vinblastine. Conversely, mutant cells with a high level of resistance to Adriamycin, vinblastine, and colchicine were effectively killed by EGF-PE, although the multidrug resistant cells initially selected in colchicine were slightly resistant to EGF-PE. These results indicate that conventional drugs and growth factor-toxin conjugates could be used together since resistance to one agent does not necessarily confer resistance to the other class of agents.

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