# Expression Cloning of cDNAs That Render Cancer Cells Resistant to *Pseudomonas* and Diphtheria Toxin and Immunotoxins

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## ABSTRACT

**Background:** Several immunotoxins in which antibodies are coupled to plant or bacterial toxins are now in clinical trials for the treatment of cancer. One of these is B3-LysPE38 in which MAb B3 which reacts with many human cancers, is coupled with a genetically modified form of *Pseudomonas* exotoxin (PE).
**Materials and Methods:** To investigate how cells can become resistant to PE-derived immunotoxins, we constructed an immunotoxin-sensitive MCF-7 breast cancer cell line that contains SV40 T antigen and allows episomal replication of SV40 origin containing plasmids. We transfected a pCDM8/HeLa cDNA expression library into these cells, thereby causing overexpression of the plasmid-encoded genes. The transfected cells were treated with immunotoxin to select for resistance-mediating plasmids, which were reisolated from these cells and amplified in *Escherichia coli*. The resulting plasmid pool was transfected into cells for two further rounds of selection and plasmid reisolation.
**Results:** Several plasmids that caused immunotoxin resistance were enriched by this selection procedure. Four plasmids were stably transfected into MCF-7 cells and found to increase their resistance to PE-derived immunotoxins by 5- to 20-fold. These plasmids also confer resistance to native PE and to diphtheria toxin but not to ricin or cycloheximide. Thus, they appear to specifically interfere with the action of ADP-ribosylating toxins.
**Conclusion:** Cancer cells can become resistant to immunotoxins by deregulated expression of normal genes. The clinical significance of this type of resistance will be evaluated in clinical trials.

## INTRODUCTION

Immunotoxins are conjugates of antibodies and toxins that selectively kill cancer cells by binding to target antigens on their surface. Many different types of immunotoxins have been constructed that have been shown to kill cancer cells in vitro and to cause the regression of human tumor xenografts grown in immunodeficient mice. Currently, there are several clinical trials in progress using immunotoxins directed at lymphomas, leukemias, and solid tumors (1–5). We have been developing immunotoxins in which antibodies are attached to genetically modified forms of Pseudomonas exotoxin A (6). One of these utilizes MAb B3, an antibody which recognizes $Le^y$ and related carbohydrate antigens which are present in large amounts on the surface of many carcinomas (7). Initially MAb B3 was coupled with LysPE38, a truncated mutant form of *Pseudomonas* exotoxin (PE) to make B3-LysPE38 (8). This immunotoxin is currently in clinical trials. B3(Fv)-PE38KDEL is an improved form of B3-LysPE38 which is a wholly recombinant molecule in which the Fv portion of MAb

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B3 in a single chain form is fused to PE38KDEL (9).

Because it is well known that cells can become resistant to anticancer drugs, we have begun to investigate the mechanisms by which cancer cells can become resistant to PE-based immunotoxins. Resistance to immunotoxins could occur by reduced expression of the target antigen of the immunotoxin, or the cell could become resistant to the toxin moiety by developing mutations that interfere with toxin internalization, intracellular transport and processing (10–11), or by alteration of elongation factor 2, the intracellular target of PE (12–16). Resistance to conventional chemotherapeutic agents has been widely studied, and many mechanisms have been elucidated. In some cases, resistance is due to structural gene mutations. In others, it is due to enhanced expression of a normal cellular gene. For example, overexpression of the human MDR1 gene or of the DHFR gene leads to drug resistance (17–21).

Here we describe the isolation of plasmids that render cells resistant to an immunotoxin composed of MAb B3 and PE. To do this, a human cDNA expression library with an SV40 origin was introduced into T antigen containing MCF-7 breast cancer cells which were then treated with the immunotoxin B3(Fv)-PE38KDEL to select for resistance-mediating cDNA plasmids. Plasmids mediating resistance are enriched by two additional cycles of transfection and selection and then used to make immunotoxin-resistant, stable MCF-7 cell lines. These plasmids also confer resistance to PE itself and to diphtheria toxin (DT), but not to ricin or cycloheximide. The approach described should also be useful in identifying cDNAs responsible for other types of toxin as well as for drug resistance.

## MATERIALS AND METHODS

### Materials

Monoclonal antibody B3, B3-immunotoxins, and PE were produced and purified in our lab (4,7,22). DT, ricin, and cycloheximide are from Sigma. A HeLa cDNA library in pCDM8 was from Clontech, pMC1neo/polyA from Stratagene and pCMV-TAg a gift from V. Ogryzko (23).

### Molecular Biology Techniques

Plasmids were transformed in *E. coli* (MC1061/P3, Invitrogen) by electroporation using a Biorad gene pulser/pulse controller, 1650 V, 200 ohm, 25 $\mu$FD, in 0.1 cm cuvettes. Isolation of plasmid DNA and from E. coli of DNA fragments was done by standard molecular biology techniques (24), plasmids for electroporation into eukaryotic cells were produced by Qiagen. Episomal plasmids were isolated from cells by the method of Hirt (25). For hybridizations, 1 $\mu$l (~20 ng) of DNA from *E. coli* plasmid minipreparations of 96 clones were denatured with NaOH, spotted on nitrocellulose, and neutralized. Hybridization probes were prepared from inserts isolated from the pCDM8 cDNA library clones as XbaI fragments, gel-purified, and labeled with digoxigenin using the Genius nonradioactive labeling kit (26) (Boehringer Mannheim). Hybridization (68°C, no formamide), washing and detection of hybridized labeled fragments with NBT/X-Phosphate was done according to the instructions of the Genius kit.

### Recombinant Cell Lines

MCF-7/T cells are derived from MCF-7 cells by cotransfection (three pools each) of 15 $\mu$g pCMV-TAg (23) and 3 $\mu$g pMC1neo/polyA into $5 \times 10^6$ MCF-7 cells using a Biorad gene pulser with 400 V, 960 $\mu$FD, and 0.4 cm cuvettes. Two days after transfection, transfectants were selected with 0.8 mg/ml G418. MCF-7/T is a pool of G418 resistant clones obtained from this cotransfection. MCF-7/T allows episomal propagation of plasmids carrying the SV40 replication origin (e.g., pCDM8 library clones). MCF-7/C, MCF-7/17, MCF-7/30, MCF-7/50, MCF-7/63 and MCF-7/90 are cell pools which were made by cotransfection of 3 $\mu$g pMC1 neo/polyA and 15 $\mu$g of either pCDM8, p17, p30, p50, p63, or p90, respectively, using the electroporation conditions and selection as for MCF-7/T. The MCF-7/N pool was made by transfecting only pMC1neo/polyA.

### Selection Cloning

Initially, 30 pools of MCF-7/T cells ($5 \times 10^6$ cells each pool) were electroporated with each 15 $\mu$g of pCDM8-HeLa cDNA library plasmids as described above and incubated for 15 hr without selection. Evaluation with a $\beta$ Gal expression plasmid revealed that our electroporation conditions result in >25% plasmid transfectants at ~20% cell survival. This results in $\sim 7.5 \times 10^6$ transfectants ($30 \times 5 \cdot 10^6$ cells $\times$ 20% surviv-

al × 25% positive transfectants), which is close to fully representing a cDNA library consisting of $10^6$ independent clones (cloned HeLa pCDM8 library). After immunotoxin treatment (see Fig. 1 and Results), plasmids were isolated from surviving cells from Hirt supernatant and transformed and amplified in *E. coli* (see above). The second and third round of transfection was done under identical conditions with the *E. coli*-derived plasmid pool from the preceding round of selection into 10 pools of $5\times 10^6$ cells in the second round and four pools of $5 \times 10^6$ cells in the third (last) round of electroporation.

### ELISA

Sixteen thousand cells of the MCF-7 controls and of the resistant transfectants were grown in 96 well plates, fixed with formaldehyde, blocked for 1 hr with 0.2% BSA in PBS, and incubated for 1 hr with various concentration of MAb B3. After being washed twice with TPBS (0.05% Tween 20 in PBS), they were incubated with goat anti-mouse IgG peroxidase conjugate (Jackson) and washed twice with TPBS and twice with PBS. Antibody binding was detected with ABTS, the reaction was stopped with 10% SDS and measured at 405 nm.

### Cytotoxicity Assays

Sensitivity of cells to toxins was assessed by MTS assays (Promega) (27) which measure dehydrogenases present in living cells. The signal at 490 nm in this assay is proportional to the number of live cells in the sample. Three thousand cells/well in 200 $\mu$l medium in 96-well plates were grown overnight and incubated with toxin for 72 hr. Then MTS solution was added, and after 1 hr, incubation at 37°C the A490 was determined. The background in wells with medium but without cells was subtracted.

## RESULTS

### Immunotoxin-Selection Cloning

Our goal was to identify whether cloned human cDNAs or cDNA fragments can confer resistance towards immunotoxins when overexpressed in immunotoxin sensitive MCF-7 cells. This resistance could be due to overexpression of resistance genes or inhibition of the expression or function of genes conveying toxin sensitivity. To accomplish our goal, we transfected a HeLa cDNA expression library (in pCDM8) into MCF-7/T cells which are MCF-7 cells containing the gene for the SV40 T antigen (see Materials and Methods for construction of MCF-7/T and the cDNA library and for details of the electroporation). HeLa cells are resistant to B3(Fv)-PE38KDEL, whereas MCF-7 cells and MCF-7/T-cells which permit episomal replication of plasmids with a SV40 origin of replication are sensitive to the immunotoxin. Two days after electroporation, cell pools were treated with 10 ng/ml of B3(Fv)-PE38KDEL, a concentration that has previously been found to kill >99% of MCF-7 cells (9). Three days later, microscopic inspection revealed that most of the cells were dead and had detached from the culture dish. The dishes were then washed, the remaining cells harvested by trypsinization, and the episomal plasmids recovered in Hirt supernatants. The reisolated plasmid pools were then transformed and amplified in *E. coli* and retransfected into MCF-7/T for two further rounds of immunotoxin selection and plasmid reisolation. This procedure of plasmid transfection, immunotoxin selection, and plasmid reisolation is summarized in Fig. 1. Because treatment of MCF-7 cells with immunotoxin results in the detachment of dying and dead cells (i.e., cells that do not contain resistance-mediating plasmids), these were removed in the washing step prior to plasmid reisolation. Therefore, this procedure enriches for plasmids that cause cells to remain alive and attached to the plate after immunotoxin exposure. In the three cycles of transfection and selection, we observed a 100-fold increase of plasmid clones recovered. After selection, we obtained $1.5 \times 10^5$ cfu (plasmid DNA in *E. coli*) from $1.5 \times 10^8$ transfected MCF-7 cells in the first round (cfu/cell = 0.1%), $3 \times 10^5$ cfu from $5 \times 10^7$ cells in the second round (cfu/cell = 0.6%) and $2 \times 10^6$ cfu from $2 \times 10^7$ transfected cells (cfu/cell = 10%) in the third round of selection (see legend to Fig. 1). This result indicates that this selection method enriches for plasmids which presumably mediate immunotoxin resistance.

### Identification of Selectively Amplified Plasmids

The plasmids that were recovered from MCF-7/T cells after three rounds of selection can be either resistance plasmids or other cDNA plasmids,

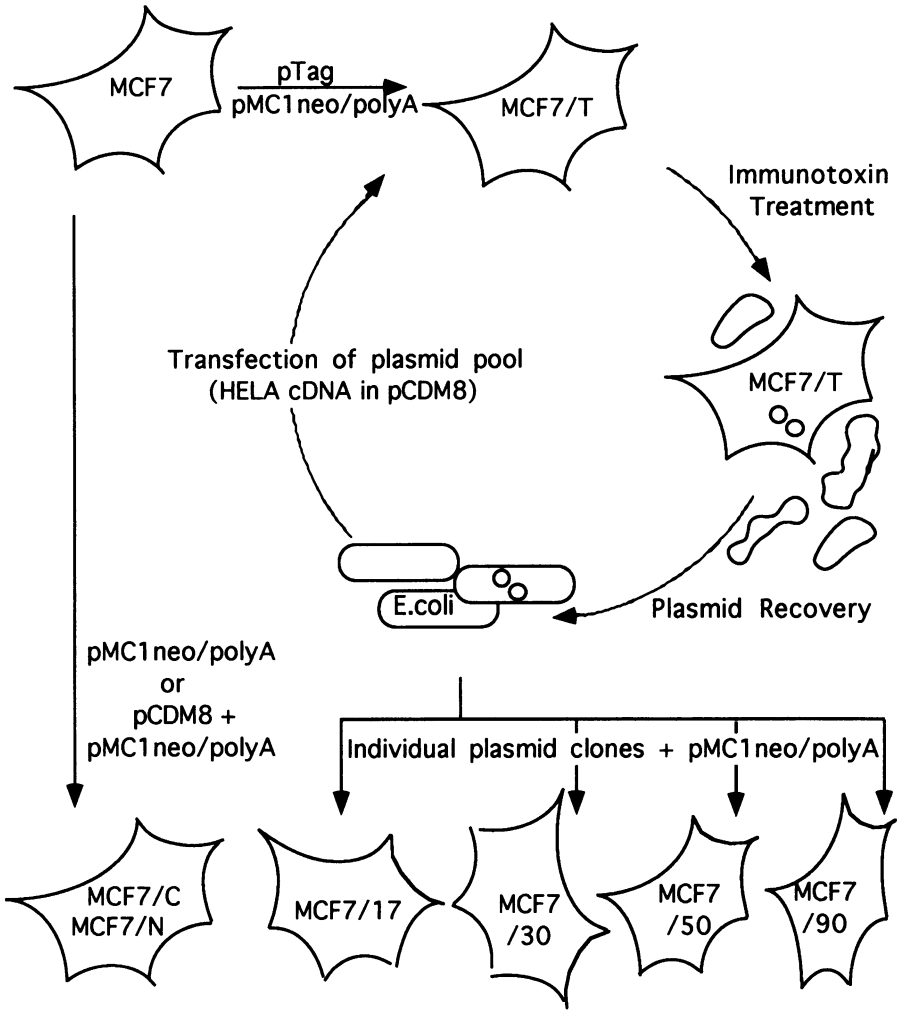


**FIG. 1. Cloning of cDNAs that mediate toxin resistance**

MCF-7/T cells expressing SV40 T antigen were obtained from MCF-7 cells by cotransfection of pCMV-TAg (23) and pMC1neo polyA (Stratagene) and subsequent G418 selection at 0.8 mg/ml as described in Materials and Methods. MCF-7 and MCF-7/T cells have the same morphology and sensitivity to PE and B3-derived immunotoxins but MCF-7/T appears to grow slightly slower. MCF-7/T were transfected with a pCDM8/HeLa cDNA expression library, treated with 10 ng/ml immunotoxin (B3(Fv)-PE38KDEL) (9); and three days later the flasks were washed with PBS, and the remaining cells were trypsinized, plasmids reisolated from them (25) and transformed into *E. coli* MC1061/P3. Aliquots of the transformations were used to determine the number of recovered plasmids (cfu) in *E. coli*, the remainder was grown for preparation of plasmid pools to be again transfected into MCF-7/T for more rounds of selection. After the third round of transfection-selection, stable MCF-7 transfectants were made by cotransfection of single isolated plasmids and pMC1neo/polyA.

which do not confer resistance but are rescued in MCF-7/T cells by the simultaneous presence of a resistance plasmid. Selectively amplified plasmids that confer resistance are likely to occur more frequently than contaminating random plasmids and thus should be easily detectable by hybridization analysis. We therefore randomly picked 96 plasmid clones from the final (*E. coli*) plasmid pool obtained after the third round of selection for further analysis and hybridized them with labeled cDNA inserts of 10 clones randomly chosen from these 96. The inserts of six of these plasmids hybridized to other plasmids (Table 1). These clones have apparently been selectively enriched and amplified in consecutive rounds of selection, and thus are candidates for resistance mediating plasmids. Each of the other clones only hybridized to itself.

**TABLE 1. Plasmid amplification by selection-cloning results in siblings with the same or similar cDNA insert**

| | Hybridization Probe | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Plasmid** | **90** | **25** | **30** | **17** | **50** | **63** | **31** | **33** | **68** | **99** |
| p90 | + | + | + | − | − | − | − | − | − | − |
| p25 | + | + | + | − | − | − | − | − | − | − |
| p30 | + | + | + | − | − | − | − | − | − | − |
| p35 | + | + | − | − | − | − | − | − | − | − |
| p96 | + | + | − | − | − | − | − | − | − | − |
| p55 | + | − | − | + | − | − | − | − | − | − |
| p17 | − | − | − | + | − | − | − | − | − | − |
| p50 | − | − | − | − | + | − | − | − | − | − |
| p28 | − | − | − | − | + | − | − | − | − | − |
| p40 | − | − | − | − | + | − | − | − | − | − |
| p63 | − | − | − | − | − | + | − | − | − | − |
| p60 | − | − | − | − | − | + | − | − | − | − |
| p81 | − | − | − | − | − | + | − | − | − | − |
| p31 | − | − | − | − | − | − | + | − | − | − |
| p33 | − | − | − | − | − | − | − | + | − | − |
| p68 | − | − | − | − | − | − | − | − | + | − |
| p99 | − | − | − | − | − | − | − | − | − | + |

Ninety-six plasmid clones from the third round of selection were hybridized with inserts of 10 randomly chosen clones that were labeled with digoxigenin as described in Materials and Methods. Six of these inserts hybridize not only with themselves but also with other plasmids ([+] hybridization signal; [−] no signal). Preliminary sequencing data (not shown) indicate that some hybridizing clones are identical siblings and others nonidentical clones with related sequences.

### Stable Transfectants Containing the Selected Plasmids Are Less Sensitive to B3-Immunotoxin

The rationale of plasmid enrichment under immunotoxin selection is that cells harboring "resistance plasmids" remain alive and attached to the dishes despite immunotoxin exposure, so that plasmids are preferentially reisolated from them and subsequently amplified. To determine which of the isolated plasmids confer immunotoxin resistance to cells, we chose five of the plasmids known to have one or more siblings (p17, 30, 50, 63, and 90, see Table 1) to make stable MCF-7 transfectants. Plasmid p17 is part of the "hybridization-group" containing p17 and p55; p50 and p63 each have two siblings (p28, 40, 50 and p60, 63, 81) and p30 and p90 are members of the largest group (p25, 30, 35, 55, 90, 96). Stable transfectants were obtained by cotransfection of MCF-7 cells not containing the T antigen with one of these plasmids together with pMC1neo/polyA followed by G418 selection as described in Materials and Methods. The resulting clones containing the plasmids pMC1neo/polyA and p17, p30, p50, p63, or p90 were called MCF-7/17, MCF-7/30, MCF-7/50, MCF-7/63, and MCF-7/90, respectively. Control transfections contained the library vector pCDM8 and pMC1neo/polyA (MCF-7/C) or only pMC1neo/polyA (MCF-7/N). To compensate for clonal variability of expression of transfected plasmids which might enhance or obscure the phenotype of the transfectants, we chose not to isolate single clones from transfections but instead pooled transfectants; greater than 10 colonies were pooled for our plasmid transfectant and >20 colonies for controls. The only selection those cell pools have undergone and with which the cells are stably maintained before examination of their toxin sensitivity was with G418.

The MCF-7 transfectants were then exposed to the immunotoxin B3(Fv)-PE38KDEL, which

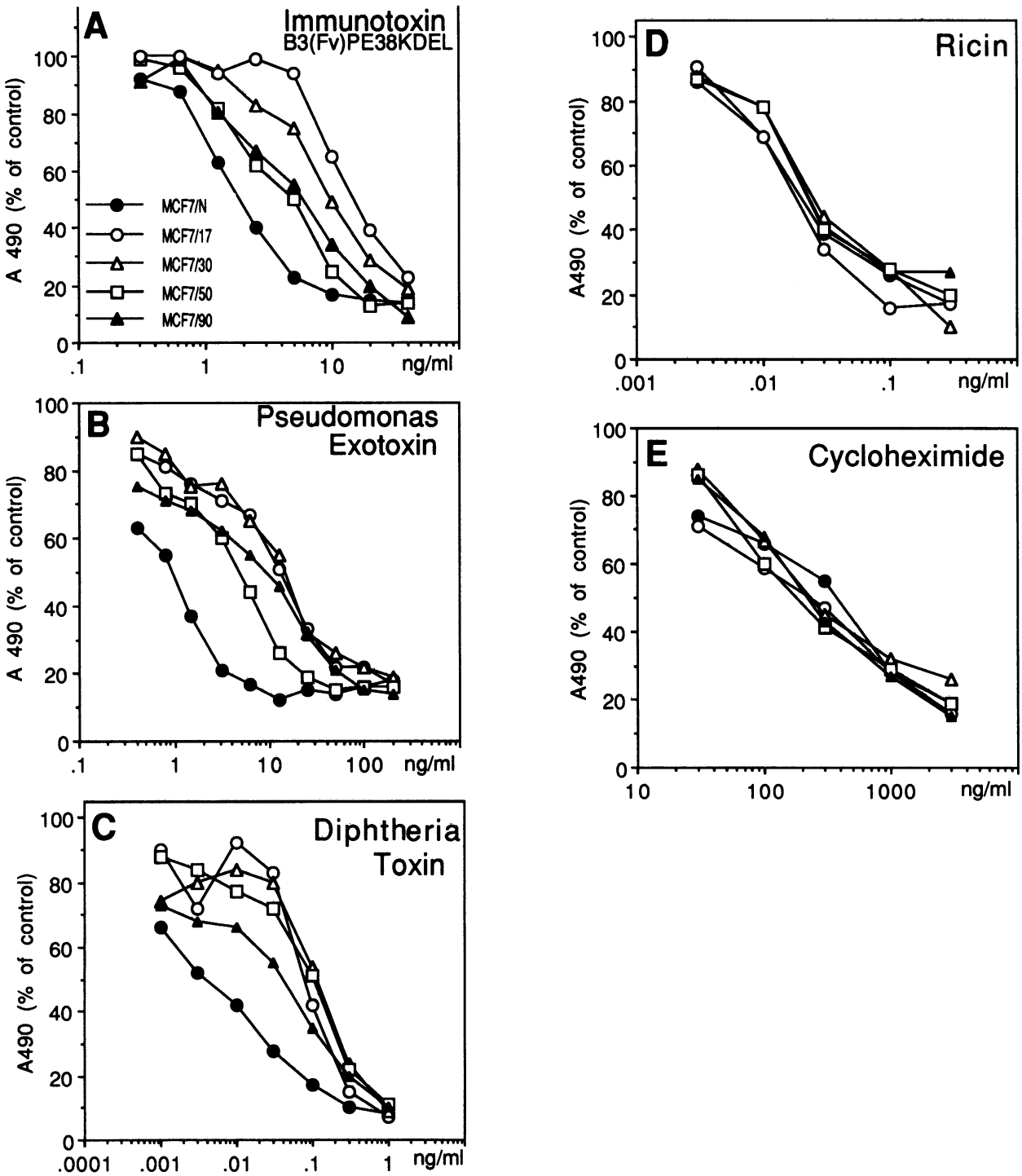


**FIG. 2. Sensitivity of MCF-7 and MCF-7 transfectants towards toxins**

MCF-7 control cells and the MCF-7-transfectants were exposed to various concentrations of toxins and their viability assessed 72 hr after toxin addition by MTS cell proliferation assays. The transfectants are less sensitive than controls to the immunotoxin B3(Fv)-PE38KDEL (A), PE (B), and DT (C), but equally sensitive to ricin (D) and cycloheximide (E).

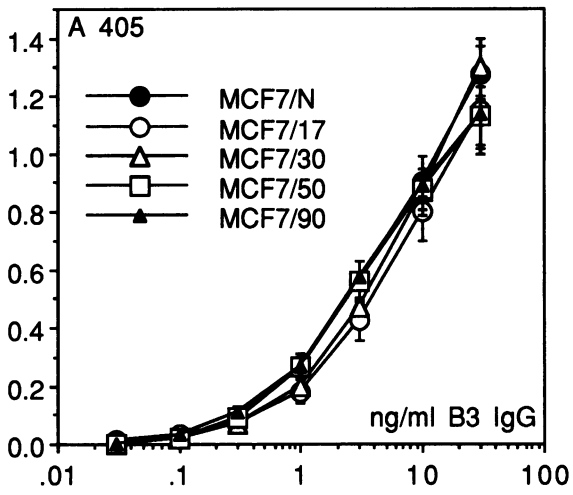


**FIG. 3. Determination of B3-antigen expression by ELISA**

The expression of the B3 antigen on the different MCF-7 transfectants was assayed by ELISA. Equal numbers of MCF-7 transfectant cells and control cells grown in 96-well plates were formaldehyde-fixed (fixation does not affect ELISA with B3 antigen; U. Brinkmann, unpublished) and incubated with various concentrations of (murine) MAb B3. Goat anti-mouse IgG⟨peroxidase⟩ was used as secondary antibody and antibody binding was colorimetrically detected with ABTS as described in Materials and Methods. The control cell lines KB and OVCAR3 do not express B3 antigen. In agreement with B3-immunofluorescence analyses of the MCF-7 transfectants (not shown), no differences in ELISA signals were observed, indicating the B3-antigen expression of the MCF-7 transfectants remains unchanged.

was used for the initial plasmid selection. With one exception (MCF-7/63, which was not further analyzed), the transfected cells were less sensitive to immunotoxin-mediated cell death than the controls. Figure 2A shows the results of cell viability assays with the various MCF-7 cell lines exposed to different concentrations of B3(Fv)-PE38KDEL. The immunotoxin concentration that resulted in a 50% reduction in cell number ($IC_{50}$) in MTS-assay was 0.5 ng/ml for control cells. The MCF-7 clone pools were 4- to 10-fold resistant with $IC_{50}$s between 2 ng/ml (MCF-7/30) and 5 ng/ml (MCF-7/17). Similar results were obtained with another B3-immunotoxin B3(dsFv)-PE38KDEL (28), which contains as targeting moiety a disulfide-stabilized Fv fragment instead of a single chain Fv.

## The Plasmids Cause Resistance to PE and DT but Not to Cycloheximide or Ricin

To determine the basis of immunotoxin resistance, we examined the levels of B3 antigen on the transfected cells since the cells could have become resistant due to reduced B3-antigen expression. As shown in Fig. 3, using an ELISA, the amount of B3 antibody bound was the same in the immunotoxin resistant and sensitive clones. This result was confirmed using an immunofluorescent assay which showed that all the cells reacted strongly with MAb B3 (data not shown). Thus, resistance is not due to loss of the antigen.

We next examined resistance to other cytotoxic agents. As shown in Fig. 2B, the transfectants also are resistant to PE. The $IC_{50}$s were 1 ng/ml PE for the controls and between 5 ng/ml and 15 ng/ml for the plasmid containing cells. Decreased sensitivity of the transfectants was also demonstrated by colony formation assays in which cells were treated with various amounts of PE for two days, and then after trypsinization their ability to form colonies were assessed (Table 2). Compared with the control cell line, the numbers of colonies obtained in these assays was 4- to 11-fold higher at 3 ng/ml PE. This increase in resistance is in accord with the decreased toxin sensitivity observed in the MTS proliferation assays. At 10 ng/ml PE, the number of colonies of the MCF-7 transfectants was between 36- and 80-fold higher than the controls and at 30 ng/ml PE no colonies were observed with control cells, while the transfectants still yielded between 100 and 250 colonies each.

Finally, we analyzed whether resistance was

**TABLE 2. Viability of MCF-7 transfectants after toxin exposure**

| PE (ng/ml) | cfu After a 48-hr Exposure to PE | | | | |
|---|---|---|---|---|---|
| | MCF-7/N | MCF-7/17 | MCF-7/30 | MCF-7/50 | MCF-7/90* |
| 0 | 200,000 | 200,000 | 200,000 | 200,000 | 200,000 |
| 3 | 4,500 | 50,000 | 20,000 | 35,000 | 30,000 |
| 10 | 250 | 9,000 | 10,000 | 20,000 | 15,000 |
| 30 | 0 | 200 | 150 | 250 | 100 |

A hundred thousand cells in 24-well plates were incubated 48 hr with toxin, washed twice with PBS, trypsinized, and plated at serial dilutions in medium without toxin.

Colonies were visualized 6 days after plating by methylene-blue staining, and the colony numbers (cfu) calculated by the colonies/well × dilution factor. *MCF-7/90 was treated with identical conditions but in a separate experiment.

limited to PE and PE containing immunotoxins or if the plasmids also mediated resistance to other toxins that inhibit protein synthesis. Figure 2 C–E shows experiments (MTS assays) in which the cells were treated with diphtheria toxin (DT), ricin, or cycloheximide. We found that the transfectants were also less sensitive to DT, a toxin that, like PE, inhibits protein synthesis by ADP-ribosylation of EF2 (2,5,12–14). In contrast, the transfectants exhibited no resistance to ricin or cycloheximide, which both inhibit protein synthesis by interacting with ribosomes. Table 3 shows a summary and comparison of the sensitivity of the control cells and transfectants towards B3(Fv)-PE38KDEL, PE, DT, ricin, and cycloheximide.

## DISCUSSION

We have isolated by immunotoxin selection cDNAs that render MCF-7 breast carcinoma cells less sensitive to an immunotoxin composed of the Fv fragment of MAb B3 fused to a truncated mutant form of PE. These cDNAs do not reduce the expression of the B3-antigen. Instead, they reduce the lethal effect of the toxin moiety of the immunotoxin. The cDNAs also confer resistance to native *Pseudomonas* exotoxin and diphtheria toxin, but not to ricin or cycloheximide. Thus, they appear to specifically interfere with toxins that inhibit protein synthesis by ADP-ribosylating EF2.

The cloning strategy used to isolate resis-

**TABLE 3. Sensitivity of MCF-7 and transfectants towards toxins that inhibit protein synthesis**

| Cell Line | $IC_{50}$ (ng/ml) | | | | |
|---|---|---|---|---|---|
| | IT | PE | DT | RC | CY |
| MCF-7/N | 1.5 | 1.0 | 0.006 | 0.027 | 300 |
| MCF-7/17 | 15 | 15 | 0.07 | 0.02 | 220 |
| MCF-7/30 | 10 | 15 | 0.1 | 0.027 | 220 |
| MCF-7/50 | 6 | 5 | 0.1 | 0.024 | 200 |
| MCF-7/90 | 7 | 10 | 0.04 | 0.024 | 220 |

The toxicity of the immunotoxin B3(Fv)-PE38KDEL (IT), *Pseudomonas* exotoxin (PE), Diphtheria toxin (DT), ricin (RC), and cycloheximide (CY) was determined by MTS assays (24) (see Materials and Methods). $IC_{50}$ is the concentration that reduces the signal to 50% of cells that were not incubated with toxin.

tance-mediating cDNAs was designed to directly isolate cDNAs derived from normal cellular genes that cause resistance, rather than to isolate resistant mutants. Such cDNA plasmids might contain expressed cDNAs of resistance genes or produce antisense transcripts of cDNAs or cDNA fragments that interfere with the expression of "sensitivity genes." The isolation of resistance plasmids was accomplished by transfecting a HeLa cDNA expression library (i.e., a collection of expressed human genes), into MCF-7 breast cancer cells that contain the SV40 T antigen. The T antigen allows the transfected library plasmids to replicate as episomes in these cells. This leads to transient overexpression of the plasmid encoded cDNA, and also allows rescue of the plasmids from cells (29). Plasmids that render cells resistant were selected in three rounds of immunotoxin exposure, plasmid reisolation, and amplification in *E. coli*. In contrast to other approaches that obtain PE-resistant cells with mutations in their genome (30–33), we isolated plasmids with cDNA inserts that are responsible for the resistance. This is advantageous because cDNAs can be directly analyzed to elucidate the cause of the resistance. Another advantage is that by using several consecutive rounds of transfection and selection of plasmids, instead of directly isolating plasmid containing resistant colonies (36,37), resistance conferring mutations in the host cells are eliminated in our approach, because the resistance phenotype is directly linked to the plasmid and only plasmids are carried on to the next round of selection. Also the number of colonies obtained in *E. coli* after each round of selection is a measure of plasmid enrichment in consecutive rounds and thus an indicator of selective amplification. Godkuv et al. (34,35) have described a method of cloning genes mediating drug-resistance by inserting cDNAs into a retroviral vector and selecting for etoposide (VP16) resistance. Using this approach, they have isolated antisense fragments called genetic suppressor elements. Our method obviates the need for producing retroviruses containing a cDNA library. Also, with the episomal plasmid library, growth of plasmid containing cells is not required to obtain resistance conferring plasmids, since the rescue of plasmids occurs shortly after transfection. Thus, it should be possible to obtain plasmids which prevent toxin or drug mediated cell death or simply detachment from dishes, but simultaneously lead to growth inhibition or growth arrest. Such plasmids cannot be obtained from a retrovirus library, since cell growth is required to produce retroviruses.

The plasmids that we isolated from the HeLa cDNA library contain cDNAs that mediate resistance not only to the selecting immunotoxin but also to *Pseudomonas* and diphtheria toxin (DT). However, resistance to ricin or cycloheximide, which both inhibit protein synthesis but by different mechanisms, was not affected. We also did not observe significant differences in sensitivity towards various low molecular weight anticancer drugs (VP16, actinomycin D, vincristine, colchicine, taxol, daunorubicin, or mitomycin C, data not shown). This suggests that the mechanism(s) by which these resistances arise is related to the common site of action of PE and DT which both inhibit protein synthesis by ADP-ribosylation of EF2.

Although their amino acid sequences are dissimilar, PE and DT have many similarities in the manner in which they kill target cells. Both must be internalized by receptor-mediated endocytosis and undergo proteolytic processing and reduction in order to generate an active fragment that can translocate to the cytosol where EF2 is located (2,5,10–16). Furthermore, both can only ADP-ribosylate diphthamide modified EF2. Cellular mutations that mediate toxin resistances have been described which include resistances to PE caused by a processing defect (10) or alterations in vesicle acidification and transport or by mutations in EF2 which do not allow toxin mediated ADP-ribosylation (11–15). Furthermore, it is possible that mutations that affect other, so far uncharacterized cellular factors (possibly unfolding enzymes) (16) can cause toxin resistance. We have investigated and eliminated some of these possibilities. It is unlikely that proteolytic processing is affected by the resistance plasmids, because the cells are resistant to both proteolytically "nicked" as well as "unnicked" DT (data not shown). We can also exclude expression of a mutated, toxin-resistant EF2 gene, since preliminary analysis of the sequences of the isolated clones shows they do not contain sequences resembling the EF2 gene. In fact, all the sequences analyzed so far encode unknown cDNAs (data not shown). We think that another more likely explanation for resistance could be that the plasmids contain cDNAs or cDNA fragments derived from normal cellular genes, whose "deregulated" overexpression cause the resistance phenotype. It is well known, for example that overexpression of the multidrug-resistance gene (*MDR*1) in human cancers can cause resistance of

cells towards various chemotherapeutic agents (17). Alternatively, the plasmids might contain and express cDNA or short cDNA fragments in an inverse orientation; such antisense transcripts have been found to reduce expression of a corresponding cellular gene and produce resistance to VP16 (34). We are presently analyzing some of the resistant MCF-7 transfectants and plasmids in detail to elucidate their resistance mechanisms.

The results presented here suggest that cancer cells can become toxin resistant by deregulated expression of normal cellular genes. It is possible that resistances based on these mechanisms could develop in vivo and limit the success of immunotoxin therapy. Clinical trials that have already been initiated and will be expanded in the next few years will show if the mechanism of toxin resistance identified by selection cloning will be an impediment to the immunotoxin therapy of cancer.

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