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R Pirker, ... , M C Willingham, I Pastan

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Research Article

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Characterization of Immunotoxins Active against Ovarian Cancer Cell Lines

Robert Pirker, David J. P. FitzGerald, Thomas C. Hamilton, Robert F. Ozols, Walter Laird, Arthur E. Frankel, Mark C. Willingham, and Ira Pastan

Laboratory of Molecular Biology and Medicine Branch, National Cancer Institute, Bethesda, Maryland 20205; and Cetus Corporation, Emeryville, California 94608

Abstract

The purpose of the present study was to develop immunotoxins directed against human ovarian carcinoma cells. Four monoclonal antibodies (260F9, 454C11, 280D11, and 245E7) were chosen because they were found to bind to various ovarian carcinoma cell lines. These antibodies were covalently linked to either *Pseudomonas* exotoxin (PE) or ricin A chain (RTA), and the conjugates were tested against five ovarian cancer cell lines (OVCAR-2, -3, -4, -5; A1847). The ability of the immunotoxins to inhibit both protein synthesis and colony formation was evaluated. Qualitatively similar results were obtained for both types of assays. Usually, PE conjugates were more toxic than their corresponding RTA conjugates. 454C11-PE was very toxic for all ovarian carcinoma lines, whereas 454C11-RTA had low activity. Both 260F9-PE and 260F9-RTA were active in all OVCAR cell lines but not in A1847 cells. 280D11-PE was toxic for OVCAR-4; otherwise, 280D11-PE and RTA conjugates of both 280D11 and 245E7 had little activity. Specificity of immunotoxin action was shown by competition by excess antibody, nontoxicity in nontarget cells, and inactivity of an irrelevant immunotoxin. To investigate the basis of antibody-dependent differences in activity of the various immunotoxins, antibody uptake was studied in OVCAR-2 cells, and the results indicate that antibody internalization is one important factor in the activity of immunotoxins.

Introduction

The availability of monoclonal antibodies (1) has opened new possibilities for cancer therapy because they can be used for targeting drugs (2) or toxins (3, 4) to cancer cells. We have begun to study the effects of antibody-toxin conjugates on human ovarian carcinoma cell lines in order to explore their potential usefulness as an alternative therapeutic approach to human ovarian cancer. Ovarian cancer is difficult to treat because these tumor cells often develop resistance to a broad spectrum of cytotoxic drugs (5). These cells should be sensitive to antibody-toxin conjugates because the mechanism by which toxins kill cells is different from the mechanism of action of conventional chemotherapeutic agents. Because ovarian cancer remains localized in the peritoneal cavity even late in the course of the disease, immunotoxins could be administered intraperitoneally

and, due to lymphatic obstruction (6), would only be cleared slowly from the peritoneal cavity. Thus, high local concentrations of the agent could be achieved and maintained, and systemic toxicity could be limited.

We recently described the effect of an antitransferrin receptor (anti-TFR)¹-*Pseudomonas* exotoxin (PE) conjugate on five human ovarian carcinoma cell lines (7). Although this immunotoxin inhibited protein synthesis in all cell lines tested, there were quantitative differences in the response of various cell lines which were explained by differences in the binding and internalization of the immunotoxin and by different sensitivities to the toxin. Currently, it is not known whether the expression of transferrin receptors on normal tissues (8) would exclude the clinical use of an antitransferrin receptor conjugate. Therefore, we are also investigating the activity of other immunotoxins. The monoclonal antibodies used in this study were originally obtained against human breast cancer cells (9) and, when conjugated to ricin A chain (RTA), gave immunotoxins active against human breast cancer cell lines (10). Because these antibodies were found to cross-react with some other carcinomas, and in particular with ovarian carcinomas (9), we used them to construct immunotoxins directed against human ovarian carcinoma cells by linking these antibodies to either PE or RTA. Both PE and RTA must be delivered to the cytoplasm, where they inhibit protein synthesis either by inactivating elongation factor 2 through ADP-ribosylation, as in the case of PE (11), or by blocking the ribosomal binding site for elongation factor 2, as in the case of RTA (12). In the present paper, we describe the cytotoxic effects of these antibody-toxin conjugates on human ovarian carcinoma cell lines.

Methods

Cell culture. National Institutes of Health/OVCAR-2, -3, -4, and -5 are isolates from the malignant ascites of patients with ovarian carcinoma (13, 14). The ovarian cancer cell line A1847 was obtained from Dr. S. Aaronson (National Cancer Institute, Bethesda, MD). The ovarian cells were grown in RPMI medium 1640, 10% fetal bovine serum, 10 µg/ml insulin, and penicillin-streptomycin. MCF-7 human breast cancer cells were cultivated in Improved MEM (modified Eagle's medium) Zinc Option Medium, 10% fetal bovine serum, and penicillin-streptomycin. KB cells were grown in Dulbecco's modified Eagle's medium (DME), 10% calf serum, glutamine, and penicillin-streptomycin. Tissue culture media, sera, glutamine, and antibiotics were purchased from Gibco Laboratories, Grand Island, NY, and insulin was obtained from Elanco Products Co., Indianapolis, IN. For protein synthesis inhibition assays, cells were plated at 2×10^5 cells/35-mm dish 1 d before use. Before adding immunotoxins, cells were washed twice with DME containing bovine serum albumin (2 mg/ml) (DME-BSA).

Address correspondence and reprint requests to Dr. Pastan, Laboratory of Molecular Biology, National Cancer Institute, Building 37, Room 4E 16, Bethesda, MD 20205.

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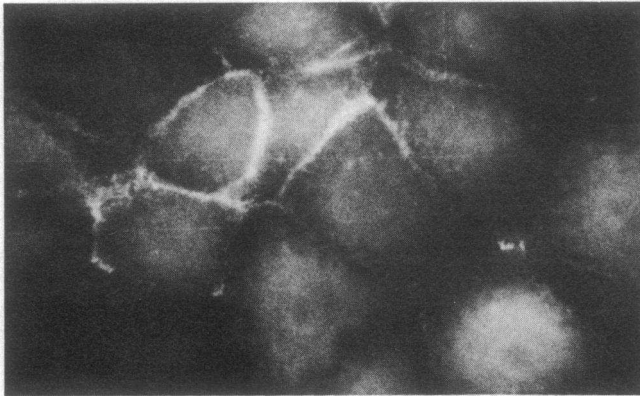
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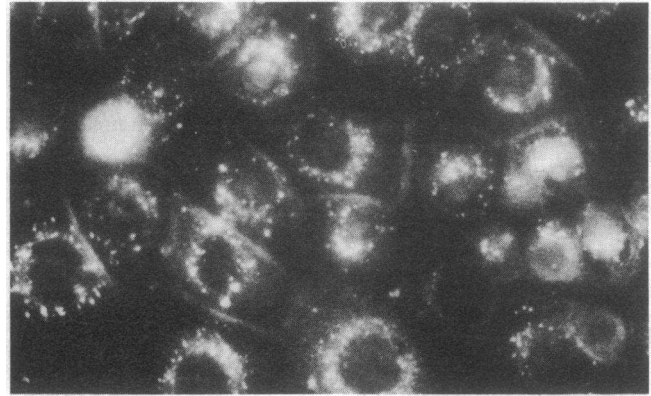
1. **Abbreviations used in this paper:** Anti-TFR, antibody against the transferrin receptor; DME, Dulbecco's modified Eagle's medium; DTNB; 5,5'-dithio-bis(2-nitrobenzoic acid); HPLC, high performance liquid chromatography; PE, *Pseudomonas* exotoxin; RTA, ricin A chain.

0 minutes

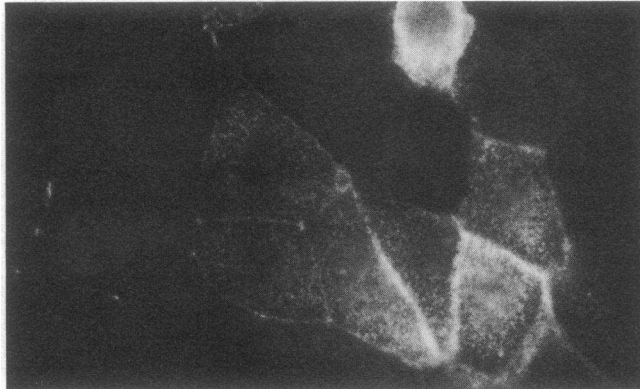


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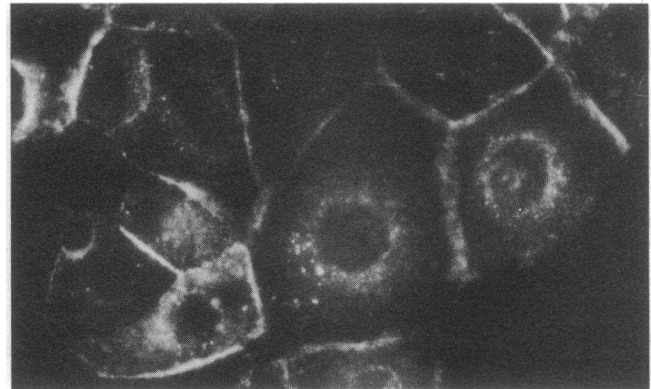
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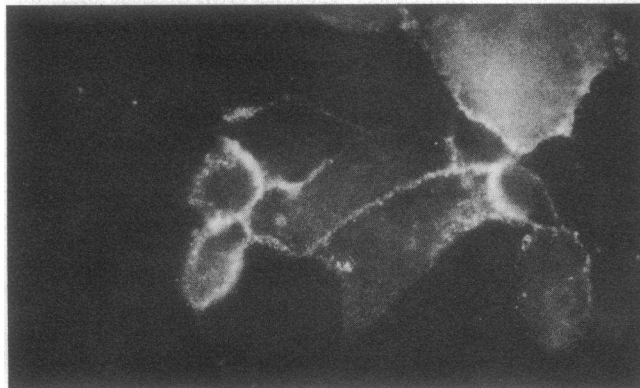
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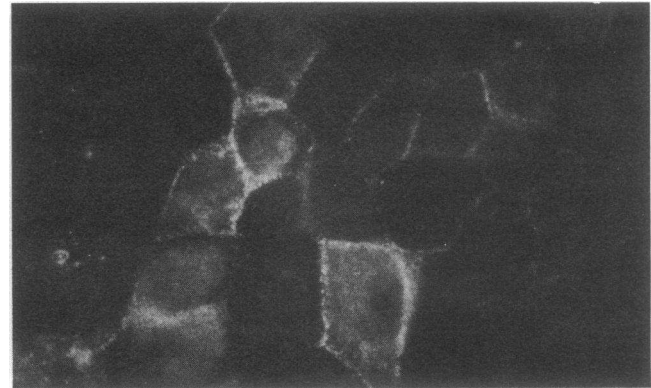
260F9



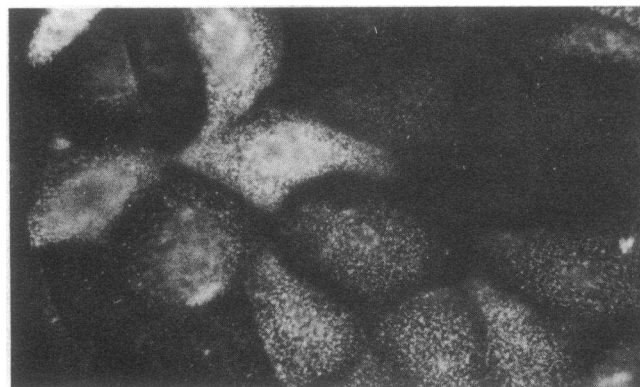
260F9



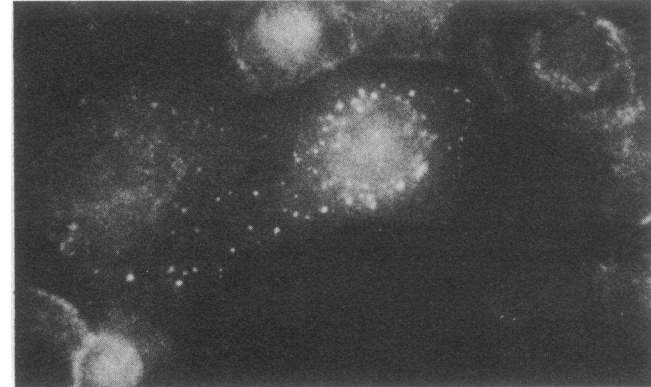
280D11



280D11



454C11



454C11

Figure 1. Binding and internalization of monoclonal antibodies by OVCAR-2 cells. Cells were incubated with antibodies (10 $\mu\text{g/ml}$) at 4°C for 1 h. Then cells were warmed up to 37°C for 0 or 60 min, fixed with 3.7% formaldehyde, permeabilized with Triton X-100, and then incubated with rhodamine-labeled goat anti-mouse IgG for 15

min at room temperature. Each of the antibodies bound to the OVCAR-2 cells (*left*). After the warm-up, anti-TFR, 260F9, and 454C11 were detected intracellularly, whereas no internalization of 280D11 could be observed (*right*).

Antibodies. Monoclonal antibodies were obtained as described (9). In addition to binding to human breast cancer cells, some of the antibodies were also found to bind to other carcinomas (9). Four of these antibodies (260F9, 454C11, 280D11, and 245E7) were chosen for the present study because they were found to react with ovarian carcinoma cell lines as assessed by indirect immunofluorescence. The antibody 454C11 is of the IgG-2a subclass, and the others are of the IgG-1 subclass. Anti-TFR was purified from the ascites of nude mice bearing HB21 (American Type Culture Collection, Rockville, MD) as previously described (7).

Antibody-toxin conjugates. PE was a gift of Dr. S. Leppla (Ft. Detrick, Frederick, MD). PE conjugates were constructed and purified by a modification of a method previously described (7). PE (30 nmol) was reacted with 5,000 nmol 2-iminothiolane-HCl (Pierce Chemical Co., Rockford, IL) and 500 nmol NAD⁺ in 1 ml 0.1 M phosphate buffer (pH 8.0) containing 1 mM EGTA at 37°C for 1 h. The derivatized PE was then separated from the reactants using high performance liquid chromatography (HPLC) and activated by the addition of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to a final concentration of 1 mM. Antibodies (40–50 nmol) were incubated with 100–200 nmol 2-iminothiolane-HCl in 0.75 ml 0.1 M phosphate buffer (pH 8.0) containing 1 mM EGTA at 37°C for 1 h. Then antibodies were reacted with the activated PE, and the conjugates were purified using HPLC as described (7). A peak containing a one-to-one conjugate of PE with the antibody was recovered and used for all studies described below. RTA conjugates were prepared by incubating RTA with antibody which had been activated by incubation with 2-iminothiolane and DTNB. They were purified by gel filtration chromatography to remove unreacted RTA and affinity chromatography to remove unreacted antibody. A detailed description of this procedure and the resulting product will be published (Bloch, W., R. Ferris, D. Birch, and J. Ellingson, manuscript in preparation).

Protein synthesis assay. Inhibition of protein synthesis was used to measure the activity of the immunotoxins. Cells were incubated with DME-BSA containing various concentrations of immunotoxins at 37°C for 24 h and then assayed for incorporation of [³H]leucine (New England Nuclear, Boston, MA; specific activity, 140.8 Ci/mmol) into TCA-insoluble material as described previously (7). Mean values of duplicates were expressed as a percentage of controls that did not receive immunotoxins.

Colony formation assay. About 500 cells were plated in a 60-mm dish containing 5 ml medium and various concentrations of immunotoxins. The cells were incubated at 37°C for 8–14 d. Then, the medium was removed and the colonies were stained with 0.5% methylene blue in ethanol/water (1:1). After washing and drying, the colonies were counted and the results were expressed as a percentage of the controls that were incubated without immunotoxin.

Binding and internalization of antibodies assessed by indirect im-

munofluorescence of OVCAR-2 cells. 1 d after plating, cells were washed with cold Dulbecco's phosphate-buffered saline (PBS) (Gibco Laboratories) containing bovine serum albumin (2 mg/ml) (PBS-BSA), and then incubated with 10 µg antibody (anti-TFR, 260F9, 280D11, and 454C11) in 1 ml PBS-BSA at 4°C for 1 h. Then the cells were washed, incubated in DME-BSA at 37°C for 0 or 60 min, fixed with 3.7% formaldehyde (15 min, room temperature), washed, permeabilized with 0.1% Triton X-100 (5 min, room temperature), washed, and incubated with rhodamine-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Avondale, PA) at room temperature for 15 min. After washing, the cells were examined using a Zeiss RA microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with rhodamine epifluorescence optics and a 63 times, numerical aperture 1.4, oil planapochromat objective. Photographs were prepared using Kodak Tri-X film (Eastman Kodak Co., Rochester, NY) and Diafine (Acufine, Inc., Chicago, IL) development.

Results

Effect of immunotoxins on protein synthesis. From a bank of monoclonal antibodies prepared against human breast cancer cells (9), four antibodies that reacted strongly with the ovarian cancer cell lines, as assessed by indirect immunofluorescence (Fig. 1 and data not shown), were selected for further study. Immunotoxins were constructed by linking each antibody to either PE or RTA. In the cases of 260F9, 454C11, and 280D11, both PE and RTA conjugates were studied; with 245E7, only RTA conjugates were investigated. The activity of each immunotoxin was initially assessed by measuring inhibition of protein synthesis on five ovarian and two nonovarian cell lines. Results from a representative experiment are shown in Figs. 2–7, and the average ID₅₀ values of all experiments are provided in Table I.

Fig. 2 shows experiments in which OVCAR-2 cells were incubated with various immunotoxins. 454C11-PE, 260F9-PE, and 260F9-RTA were very active, with ID₅₀ values (Table I) of 2 ng/ml for 454C11-PE, 3 ng/ml for 260F9-PE, and 38 ng/ml for 260F9-RTA. The unconjugated antibody competed for the toxicity of the corresponding immunotoxin. 280D11-PE inhibited protein synthesis only at concentrations >100 ng/ml. RTA conjugates of either 454C11, 280D11, or 245E7 showed little activity (Fig. 2 and Table I).

For OVCAR-3 cells (Fig. 3), 454C11-PE was very active;

Table I. ID₅₀ Values* for Protein Synthesis Inhibition

Cells	260F9-PE	260F9-RTA	454C11-PE	454C11-RTA	280D11-PE	280D11-RTA	245E7-RTA	RTA
OVCAR-2	3	38	2	>1,000	835	>5,000	>5,000	3,900
OVCAR-3	42	92	4‡	>1,000	805	800	>5,000	1,200
OVCAR-4	5	29	1	>1,000	54	2,700	>5,000	2,200
OVCAR-5	17	36	10	>1,000	3,450	>4,000	5,000	4,200
A1847	385	>1,000‡	3	>1,000	2,200	>5,000	>5,000	ND§
MCF-7	2	42	<10‡	1,000‡	9	70	230‡	ND§
KB	660	28,000‡	15‡	>1,000	1,200	19,000	>5,000	1,400

Cells were incubated with immunotoxins at 37°C for 24 h and then assayed for protein synthesis. Each experiment was done in duplicates which varied by <10%. Concentrations of the immunotoxins leading to 50% inhibition of protein synthesis (ID₅₀ values) were determined. If not otherwise mentioned, the values shown in the table represent mean values of at least two experiments. Results for RTA are from a 12-h incubation assay, and results for both native PE and derivatized PE have been published previously (7). * Values are given in nanograms per milliliter.

‡ Results from one experiment. § ND, not done. ^{||} A manuscript by Dr. M. J. Bjorn et al., Cetus Corp., describing in detail the activity of these immunotoxins on MCF-7 cells is in preparation.

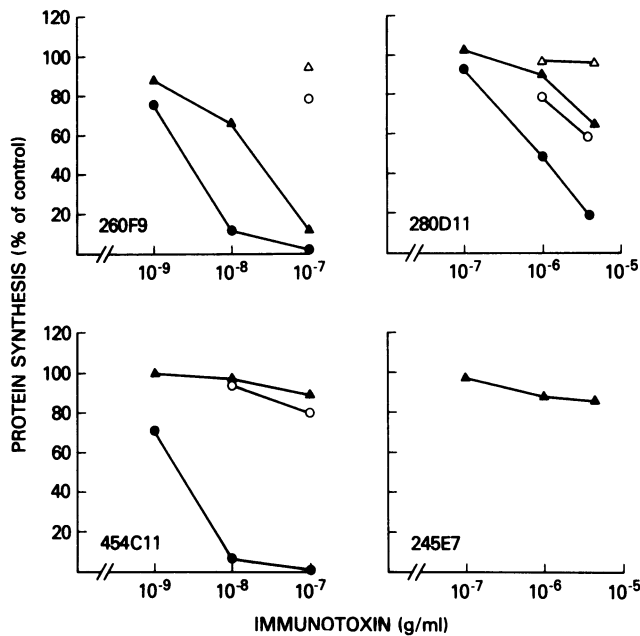


Figure 2. Inhibition of protein synthesis by immunotoxins in OVCAR-2 cells. Cells were incubated with immunotoxins in the absence (closed symbols) or presence (open symbols) of excess unconjugated antibody at 37°C for 24 h and then assayed for protein synthesis. Mean values of duplicates are expressed as percentage of controls that did not receive immunotoxins. The antibodies used for constructing immunotoxins are shown in the lower left corner of each part of the figure. Open symbols represent incubations with immunotoxins in the presence of excess corresponding antibody: 100-fold excess of 260F9 for 260F9 conjugates, 10-fold excess of 280D11 for 280D11 conjugates, and 50-fold excess of 454C11 for 454C11 conjugates. ●, ○, PE conjugates; ▲, △, RTA conjugates.

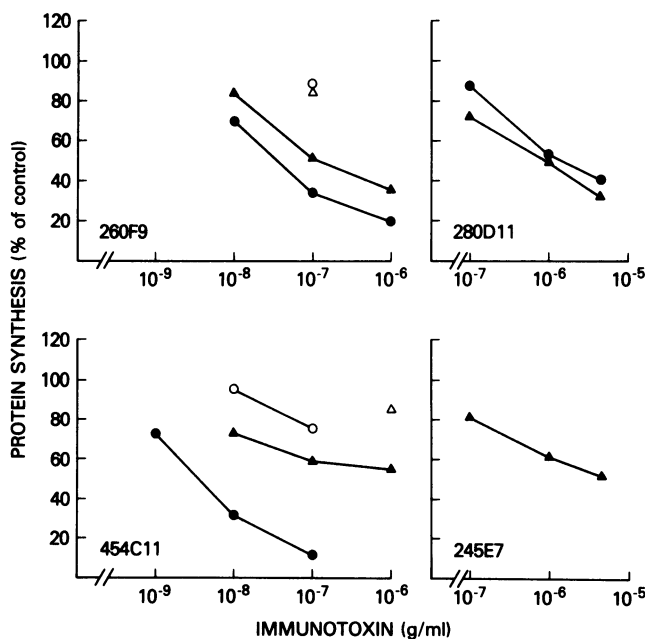


Figure 3. Inhibition of protein synthesis by immunotoxins in OVCAR-3 cells. The experiment was performed as described for Fig. 2. Open symbols represent experiments in which cells were incubated with immunotoxins in the presence of excess antibody as described for Fig. 2. ●, ○, PE conjugates; ▲, △, RTA conjugates.

conjugates of 260F9 showed intermediate activity, with 260F9-PE being slightly more active than 260F9-RTA. 280D11 conjugates and RTA conjugates of either 454C11 or 245E7 inhibited protein synthesis only at high concentrations (ID_{50} values ≥ 800 ng/ml). Fig. 4 shows the results for OVCAR-4 cells. These cells were very sensitive to 260F9-PE and to 454C11-PE. 260F9-RTA and 280D11-PE showed intermediate activity, whereas RTA conjugates of either 454C11, 280D11 or 245E7 antibodies had low activity. For OVCAR-5 cells (Fig. 5), 454C11-PE was very active. Conjugates of 260F9 showed intermediate activity, with ID_{50} values in the range of 10–40 ng/ml. The other immunotoxins affected protein synthesis only at high concentrations (ID_{50} values $> 1,000$ ng/ml).

The results for A1847 cells are shown in Fig. 6. Again, 454C11-PE was very active, and its toxicity was competable by excess antibody. It required high concentrations of 260F9-PE (or 280D11-PE) to inhibit protein synthesis. The effect of 260F9-PE appeared to be nonspecific because it could not be blocked by excess antibody. The ID_{50} values for RTA conjugates were $> 1,000$ ng/ml. In KB cells (Fig. 7), 260F9-PE, 260F9-RTA, 280D11-PE, and 280D11-RTA inhibited protein synthesis only at concentrations > 100 ng/ml. The toxicity of both 260F9-PE and 280D11-PE could not be blocked by excess antibody (Fig. 7 and data not shown). However, 454C11-PE inhibited protein synthesis in this cell line, and this effect was also competable by free antibody. KB cells express the antigen recognized by antibody 454C11 as assessed by indirect immunofluorescence (data not shown). 454C11-RTA showed little activity.

Colony formation assay. Immunotoxins were also studied for their cell-killing ability in a colony formation assay. The results of the colony formation assay reflected the toxicity of the conjugates seen in the protein synthesis inhibition assay. The results with OVCAR-2 cells are shown in Fig. 8. Conjugates of

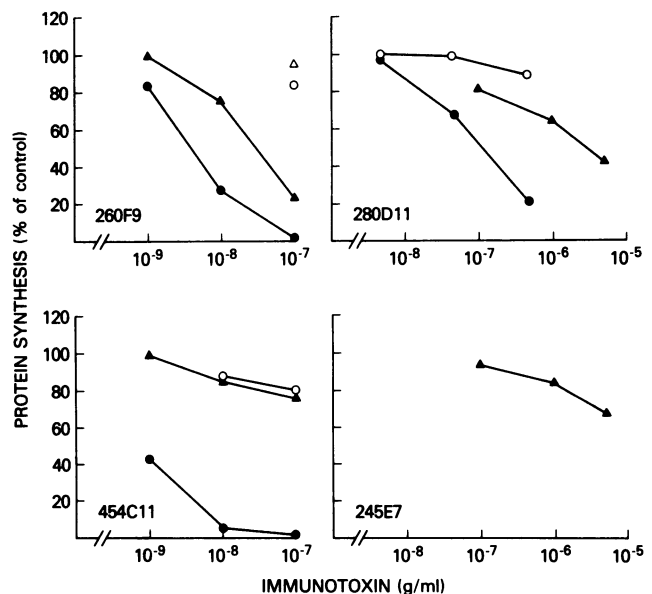


Figure 4. Inhibition of protein synthesis by immunotoxins in OVCAR-4 cells. For experimental details, see legend for Fig. 2. Competition with excess 260F9 or 454C11 antibody was performed as described for Fig. 2. For 280D11-PE, competition was done with 50 μ g/ml 280D11. Open symbols refer to immunotoxins plus excess antibody. ●, ○, PE conjugates; ▲, △, RTA conjugates.

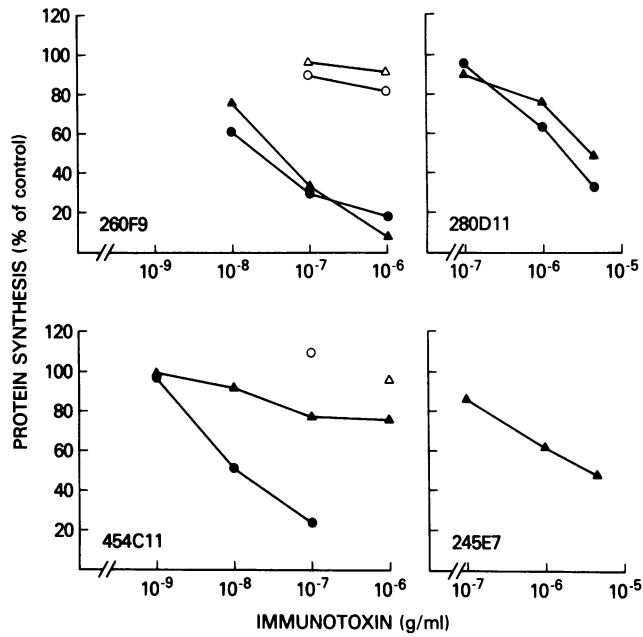


Figure 5. Inhibition of protein synthesis by immunotoxins in OV-CAR-5 cells. For experimental details, see legend for Fig. 2. Open symbols refer to inhibition of protein synthesis by immunotoxins in the presence of excess corresponding antibody as described for Fig. 2. ●, ○, PE conjugates; ▲, △, RTA conjugates.

antibody 260F9 were very efficient immunotoxins, with 50% inhibition of colony formation at 0.1 ng/ml 260F9-PE and 0.5 ng/ml 260F9-RTA, respectively. The toxicity was blocked by excess antibody 260F9. 454C11-PE was slightly more active than the 260F9 conjugates. 280D11-RTA and 245E7-RTA were toxic

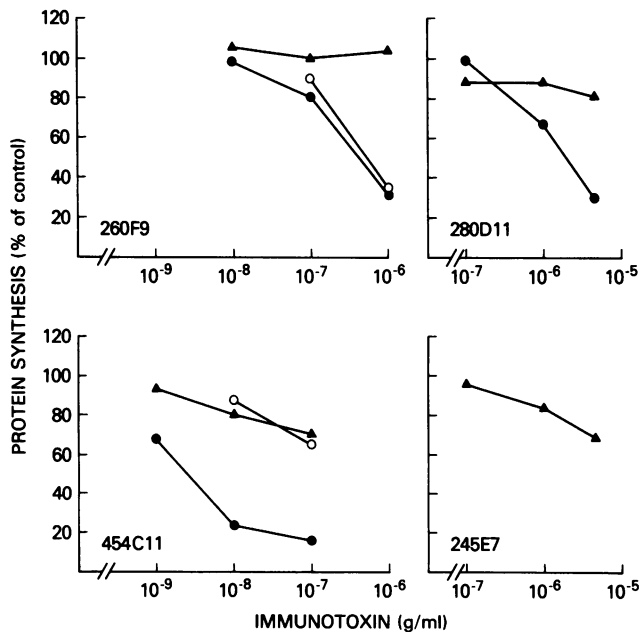


Figure 6. Inhibition of protein synthesis by immunotoxins in A1847 cells. For experimental details, see legend for Fig. 2. Open symbols refer to competition of the activity of immunotoxins by excess corresponding antibody. ●, ○, PE conjugates; ▲, RTA conjugates.

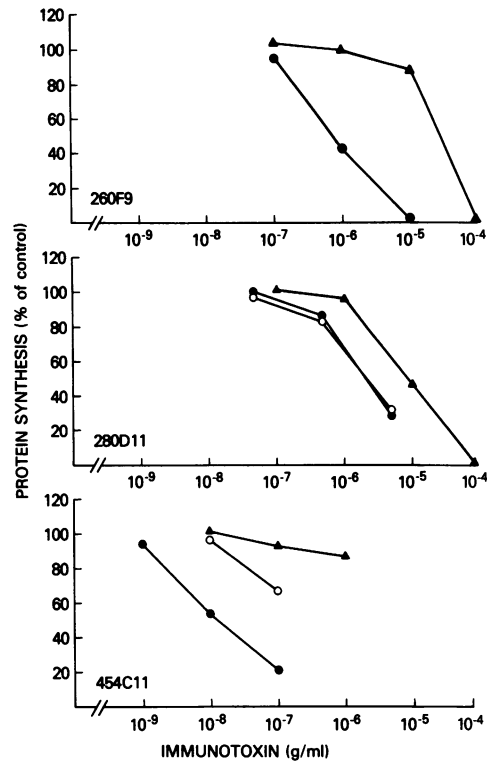


Figure 7. Inhibition of protein synthesis by immunotoxins in KB cells. For experimental details, see legend for Fig. 2. Open symbols refer to competition by excess free antibody as described for Fig. 4. ●, ○, PE conjugates; ▲, RTA conjugates.

only at very high immunotoxin concentrations, and were >100-fold less active than 260F9 conjugates or 454C11-PE.

Average ID₅₀ values of all of the colony formation experiments are summarized in Table II. In the OVCAR cell lines tested, 454C11-PE and conjugates of 260F9 had the highest activity. Also, in this assay, 260F9-PE was more active than 260F9-

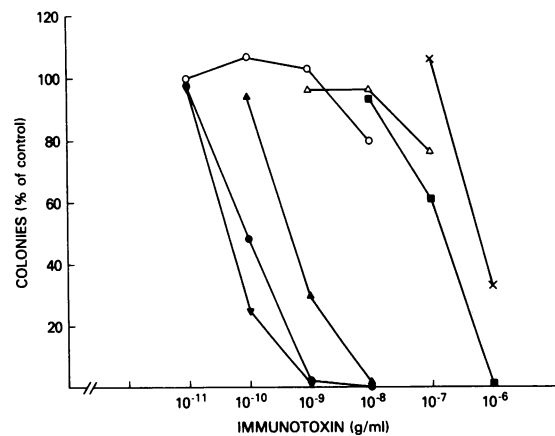


Figure 8. Inhibition of colony formation of OVCAR-2 cells by immunotoxins. Cells (500 cells/60-mm dish) were incubated with various immunotoxins plus or minus excess unconjugated antibody for 13 d, and then the colonies were counted and expressed as percentage of controls that did not receive immunotoxins. ▼, 454C11-PE; ●, 260F9-PE; ○, 260F9-PE plus 1,000-fold excess 260F9; ▲, 260F9-RTA; △, 260F9-RTA plus 100-fold 260F9; ■, 280D11-RTA; ×, 245E7-RTA.

Table II. ID₅₀ Values* for Inhibition of Colony Formation

Cells	260F9-PE	260F9-RTA	454C11-PE	280D11-RTA	245E7-RTA
OVCAR-2	0.1	0.7	0.05	91	390
OVCAR-3	0.8	0.6	0.7	12‡	ND§
OVCAR-4	0.02	0.2	0.1	24	100‡
OVCAR-5	0.2	0.5	0.2	210	107
A1847	>10	>80	0.4	>1,000	>1,000
MCF-7	0.05	0.8	0.03	4‡	7‡
KB	210	4,000‡	29	3,600‡	ND§

500 cells/60-mm dish were incubated with immunotoxins at 37°C for 8–14 d. Then, colonies were stained with 0.5% methylene blue in ethanol/water (1:1) and counted. Concentrations of the immunotoxins leading to 50% inhibition of colony formation (ID₅₀ values) were determined. If not otherwise mentioned, the values shown in the table represent mean values of at least two experiments. * Values are given in nanograms per milliliter. ‡ ID₅₀ value of one experiment. § ND, not done.

RTA (except in OVCAR-3). In A1847 cells, 454C11-PE at 0.4 ng/ml led to 50% inhibition of colony formation, whereas the ID₅₀ values for the other immunotoxins were >10 ng/ml.

Internalization of monoclonal antibodies. In OVCAR-2 cells, 454C11-PE was the most active immunotoxin, followed by 260F9-PE and 260F9-RTA, whereas 454C11-RTA and both conjugates of 280D11 showed little or no activity. To investigate possible reasons for these different activities, we studied the internalization of the following antibodies in OVCAR-2 cells: 260F9, 280D11, 454C11, and anti-TFR. When studied by indirect immunofluorescence, all these antibodies bound to the cell surface, and there were often quantitative differences in reactivity among cells from a single cell line. Only anti-TFR reacted in a uniform manner with all cells from the same cell line. As shown in Fig. 1, anti-TFR was efficiently internalized, and after a 1-h incubation at 37°C a punctate and presumably lysosomal distribution of the antibody was observed (15). Both 260F9 and 454C11 showed a much brighter surface pattern than anti-TFR at time zero (no warmup). After 1 h at 37°C, definite uptake of antibody into vesicles, although less than that observed with anti-TFR, was seen. No internalization of 280D11 antibodies could be detected under the same conditions.

Discussion

We have identified three immunotoxins that were very active against human ovarian carcinoma cell lines: 454C11-PE was toxic for all five ovarian cell lines studied, whereas 260F9-PE and 260F9-RTA were active in all OVCAR cell lines but not in A1847 cells. In contrast to these potent immunotoxins, 454C11-RTA as well as immunotoxins constructed from either 280D11 or 245E7 showed little or no activity towards the ovarian carcinoma cell lines except 280D11-PE in OVCAR-4 (Tables I and II). In a given cell type, the toxicity of the various antibody-toxin conjugates was dependent on both the antibody and the toxin components. Specificity of our active immunotoxins was shown in several ways: (a) Excess antibody competed for the toxicity of the corresponding immunotoxin, indicating that the conjugates bound to the cell surface via the antibody. (b) The immunotoxins had low activity on nontarget cells. In the colony formation assay, OVCAR cells were 260-fold to 20,000-fold more sensitive to 260F9-PE or 260F9-RTA than nontarget KB cells (Table II). In addition to our results with KB cells, RTA conjugates of the antibodies used in this study were found to be inactive in two human fibroblast cell lines (10). (c) An irrelevant

immunotoxin, such as PE linked to an antibody against the human T cell growth factor receptor (16), has previously been shown to be inactive in the OVCAR cells (7).

Although qualitatively similar results were obtained for both types of assays, ID₅₀ values were up to 250-fold lower in the colony formation assay. One explanation for this finding is that cells exposed longer die because the toxin is catalytic, and even a single molecule in the cytoplasm will eventually kill the cell (17). Because very high concentrations of immunotoxins showed nonspecific inhibition of protein synthesis in KB cells, the difference in ID₅₀ values between target ovarian cells and nontarget KB cells was less in the protein synthesis inhibition assay than in the colony formation assay where lower immunotoxin concentrations were used.

To be cytotoxic, PE or RTA must be delivered to the cytoplasm, where it can inactivate protein synthesis. In a given cell line, therefore, antibody-dependent differences in toxicity of immunotoxins containing one particular toxin could be due to the differences in rate and extent of antibody internalization. To investigate this, we looked at the cellular uptake of the antibodies by OVCAR-2 cells. Under the conditions used, anti-TFR, 260F9, and 454C11 were internalized, but 280D11 could not be detected intracellularly. These results could explain why in OVCAR-2 cells PE conjugates of anti-TFR (7), 260F9, or 454C11 were much more toxic than those of 280D11. However, despite internalization of 454C11, the RTA conjugate of this antibody had low activity, suggesting that additional factors influence the activity of immunotoxins. Nevertheless, poor internalization of an antibody indicates that immunotoxins constructed with this antibody will have low activity.

For a given antibody, PE conjugates were usually more active than their RTA counterparts. These differences may be due to the fact that PE conjugates contain the entire toxin molecule, whereas RTA conjugates lack the B subunit that facilitates membrane permeation (18). However, we cannot exclude the possibility that subtle differences in the disulfide linkage favored the retention of a higher binding affinity by the antibodies when linked to PE.

This study suggests that 454C11-PE, 260F9-PE, and 260F9-RTA are potential candidates for therapy of human ovarian carcinoma. Ongoing *in vivo* studies using a xenograft model of human ovarian carcinoma in nude mice (19) should answer whether sufficient *in vivo* targeting can be achieved and, additionally, will provide more data on the pharmacokinetics and side effects of these immunotoxins. Finally, it remains to be seen

whether the activity of these immunotoxins, in particular of the ones with low activity, could specifically be increased by one of the methods reported to enhance the toxicity of immunotoxins (18, 20–25).

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