Anti-Transferrin Receptor Antibody Linked to Pseudomonas Exotoxin as a Model Immunotoxin in Human Ovarian Carcinoma Cell Lines

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ABSTRACT

The present in vitro study was performed to evaluate the potential usefulness of immunotoxins in treating human ovarian carcinomas. A monoclonal antibody against the human transferrin receptor was covalently linked to Pseudomonas exotoxin. The activity of this immunotoxin (anti-TFR-PE) was studied in five ovarian carcinoma cell lines, a breast carcinoma cell line (MCF-7), and in KB cells. The ovarian carcinoma cell lines included one previously established cell line (A1847) and four recent isolates obtained from the malignant ascites of patients with metastatic ovarian carcinoma (OVCAR cell lines). While all cell lines showed inhibition of protein synthesis by anti-TFR-PE, there were quantitative differences when the level of protein synthesis was assayed after a 12-hr incubation with the immunotoxin. These differences resulted from different kinetics of anti-TFR-PE activity in the various cell lines. Higher levels of cellular binding and internalization of anti-TFR were shown to contribute to increased toxicity of anti-TFR-PE. Verapamil increased the rate of protein synthesis inhibition and thus enhanced the toxicity of anti-TFR-PE in the OVCAR cell lines.

INTRODUCTION

Monoclonal antibodies have opened new perspectives in cancer chemotherapy because they allow for targeting toxins (11, 27) or drugs (25) to cancer cells. Some encouraging results targeting toxins to specific cells have been published previously (3, 11, 15), and ex vivo treatment of donor bone marrow with anti-T-cell immunotoxins was recently reported to prevent graft-versus-host disease after allogeneic bone marrow transplantation in humans (6).

Immunotoxins presumably enter cells via receptor-mediated endocytosis (17), and the toxin moiety, either free or still linked to the antibody, must escape from endocytic vesicles into the cytoplasm to be cytotoxic. Antibody-toxin conjugates are often less toxic than the native toxins and, therefore, it is important to devise ways of increasing the toxicity of immunotoxins. Adenovirus enhances the cytotoxicity of anti-TFR-PE3 in KB cells by facilitating the penetration of the immunotoxin into the cytoplasm (7), where PE inhibits protein synthesis by inactivating elongation factor 2. Other investigators showed increased activity of immunotoxins in the presence of ammonium chloride (5) or chloroquine (18). The toxicity of ricin A chain-antibody hybrids can be enhanced by adding free ricin B chain (28).

We have started to investigate the effect of immunotoxins on human ovarian carcinoma cells, the eventual goal of this being the in vivo treatment of human ovarian cancer with immunotoxins. Ovarian cancers are difficult to treat adequately with current therapies and, thus, new approaches are highly desirable. This need, coupled with the propensity of ovarian cancer to remain localized within the peritoneal cavity even late in the course of the disease (29), makes this tumor a good prototype cancer to explore such a novel therapeutic approach as antibody-toxin conjugates. To study in detail the factors that guide the activity of immunotoxins in ovarian carcinoma, the transferrin receptor, which is mainly expressed on rapidly proliferating cells (10, 22), was used as a target.

In the present paper, we report the effect of anti-TFR-PE on 5 human ovarian carcinoma cell lines, 4 of which (OVCAR-2, -3, -4, -5) were recently isolated from the malignant ascites of patients with metastatic ovarian cancer (13, 19). Three of the OVCAR cell lines were derived from patients resistant to combination chemotherapy and showed in vitro resistance to Adriamycin (19). Because in a clinical setting immunotoxins will probably be first used in patients resistant to conventional chemotherapy, these cell lines additionally offer the possibility to evaluate whether drug-resistant cancer cells are still susceptible to immunotoxin treatment. We show that differences in cellular binding and uptake of the antibody correlate with the activity of anti-TFR-PE in the various cell lines and that the activity of the antibody-toxin conjugate in the OVCAR cells can be enhanced by verapamil which was recently reported to modulate the toxicity of anti-TFR-PE in KB cells (1).

MATERIALS AND METHODS

Cell Culture. NIH:OVCAR-2, -3, -4, and -5 are human ovarian carcinoma cell lines recently isolated from the malignant ascites of 4 patients with ovarian carcinoma. OVCAR-5 was from an untreated patient, whereas all the other cell lines were from patients who had received cytotoxic chemotherapy. All of these patients received cisplatin and cyclophosphamide; Patients 3 (OVCAR-3) and 4 (OVCAR-4), in addition, received Adriamycin prior to the isolation of the cell lines (13, 19). The ovarian cancer cell line A1847 from a previously untreated patient was obtained from S. Aaronson, National Cancer Institute, Bethesda, MD.

The ovarian cells were grown in RPMI Medium 1640 (Grand Island Biological Co., Grand Island, NY) containing 10% fetal bovine serum (Grand Island Biological Co.), insulin (10 μg/ml; Elanco Products Co., Indianapolis, IN), penicillin (100 units/ml), and streptomycin (100 μg/ml). MCF-7 cells were kept as monolayers in improved minimum essential
medium zinc option medium (Grand Island Biological Co.) with gentamicin sulfate (40 µg/ml), 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). KB cells (American Type Culture Collection) were maintained as monolayers in DMEM (Grand Island Biological Co.) supplemented with 10% calf serum, 2 µM glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml).

If not otherwise stated, cells were plated out at a density of 2 x 10^6 cells (OVCAR, A1847), 4 x 10^5 cells (MCF-7), and 5 x 10^5 cells (KB)/35-mm dish 24 hr prior to use. Before each experiment, cells were washed twice with DMEM containing BSA (2 mg/ml) (DMEM:BSA) and incubated in the new medium for 30 min.

Monoclonal Antibody against the Human Transferrin Receptor (HB21). HB21 was obtained from the American Type Culture Collection (14), propagated as ascites in BALB/c mice, and purified by precipitation at ammonium sulfate 50% saturation and affinity chromatography using Staphylococcus aureus Protein A. HB21 is of the IgG1 subclass. In the present paper, HB21 is referred to as "anti-TFR." The antibody was radiolabeled using the Bolton-Hunter Reagent (New England Nuclear, Boston, MA). Briefly, 50 to 100 µg of HB21 in 10 to 20 µl of PBS (Grand Island Biological Co.) were incubated with 0.25 to 1 mCi of radiiodinated ester for 1 to 2 hr at 0-4°C. Then, additional HB21 (475 µg) was added as carrier, and the unbound Bolton-Hunter Reagent was separated from the antibody by gel filtration on a PD-10 column (Pharmacia Fine Chemicals, Uppsal, Sweden).

Toxin. Purified PE (M, 66,000) was a generous gift of Dr. S. Leppa, USAMRIID, Fort Detrick, Frederick, MD.

Conjugates of PE and Anti-TFR. PE was linked to anti-TFR via a disulfide bond using a disulfide exchange reaction (4, 23); 2 mg of PE were incubated with 0.9 mw NAD* and 86 mw MMB (Pierce Chemical Co., Rockford, IL) in a final volume of 1.1 ml of 0.1 m phosphate buffer (pH 8.0) for 2 hr at 37°C. Derivatized PE was separated from free MMB using Sephadex G-25M (Column PD-10; Pharmacia) eluted with 0.1 m phosphate buffer (pH 7.5), 0.1 m NaCl, and 1 m EDTA. Fractions containing PE were pooled and concentrated by ultrafiltration (Centricon 30 microconcentrator, Amicon Corp., Danvers, MA).

For activation of antibody, 0.04 mw anti-TFR (3 mg) and 0.05 mw N-succinimidyl-3-(2-pyridyldithio)propionate (dissolved in dimethylformamide; Pierce Chemical Co.) were incubated in 0.5 ml of 0.1 m borate buffer (pH 9.0) containing 0.1 m NaCl at room temperature for 15 min. Then derivatized antibody was separated from free N-succinimidyl-3-(2-pyridyldithio)propionate by gel filtration on a PD-10 column equilibrated and eluted with 0.1 m acetic acid buffer (pH 4.5), 0.1 m NaCl, and 1 m EDTA. The fractions containing antibody were pooled and concentrated (Centricon 30 microconcentrator). To change the buffer, the antibody was now passed over a column PD-10 equilibrated and eluted with 0.1 m phosphate buffer (pH 7.5), 0.1 m NaCl, and 1 m EDTA. After concentration, the antibody was incubated with the derivatized toxin in a final volume of about 0.3 ml for 12 hr at room temperature.

The immunotoxin was purified by high-performance liquid chromatography using a 300- x 7.5-mm BioSil TSK-250 column (Bio-Rad Laboratories, Richmond, CA) equilibrated and eluted with 0.1 m phosphate buffer (pH 7.5). The elution profile is shown in Chart 1. At a flow rate of 0.5 ml/min, the 1:1 conjugate (Peak C) could be separated from both unrelated antibody (Peak D) or toxin (Peak E) and larger aggregates (Peaks A and B). The purity of the immunotoxin was determined by sodium dodecyl sulfate:5% polyacrylamide gel electrophoresis under nonreducing conditions. Molecular weight standards were run with each gel. The proteins in the gel were stained with Coomassie Brilliant Blue. The immunotoxin (anti-TFR-PE), containing 1 mol of toxin/mol of antibody, was only slightly contaminated with free antibody, and no free toxin could be detected (see gel in Chart 1).

Cytotoxicity Assay for Anti-TFR-PE Conjugate or PE. Inhibition of protein synthesis was used to measure the cytotoxic effect of anti-TFR-PE or PE in OVCAR, A1847, MCF-7, and KB cells. After a 30-min preincubation with DMEM:BSA, cells were incubated with 1 ml DMEM:BSA containing different concentrations of either anti-TFR-PE (0.01 to 10 µg/ml) or PE. Both native and derivatized PE were studied. Control dishes received medium without conjugates. Incubation was carried out at 37°C for various time periods, usually for 12 hr. Then, the medium was replaced by 1 ml of DMEM:BSA containing [3H]leucine (specific activity, 146.5 Ci/mmol; 3 to 7 µCi/dish; New England Nuclear), and the cells were incubated for 60 to 75 min. Next, they were washed twice with PBS and dissolved in 0.1 N NaOH, and the proteins were precipitated with trichloroacetic acid. The precipitated proteins were washed twice and dissolved in 2 ml of 0.1 N NaOH. An aliquot was then counted in a Packard scintillation spectrometer. Protein synthesis was expressed as a percentage of controls. Experiments were done in duplicates which varied by <10%.

Kinetics of Protein Synthesis Inhibition by Anti-TFR-PE. The time dependence of protein synthesis inhibition by anti-TFR-PE was evaluated by incubating the cells with anti-TFR-PE (1 µg/ml in OVCAR-2, OVCAR-3, OVCAR-5, and KB; 0.1 µg/ml in OVCAR-2, OVCAR-3, and KB) at 37°C for various time periods (1 to 19 hr). After the end of each incubation period, protein synthesis was measured as described above and expressed as a percentage of the controls incubated for the same time period in the absence of anti-TFR-PE.

Effect of Verapamil on the Toxicity of Anti-TFR-PE. Verapamil-HCl was a gift of Knoll Pharmaceutical Co., Whipping, NJ. OVCAR cells were incubated with anti-TFR-PE in the presence of verapamil (20 µg/ml) for 4 hr at 37°C. Then, protein synthesis was measured as described above. In addition, the time dependence of protein synthesis inhibition by anti-TFR-PE (1 µg/ml) in the presence of increasing concentrations of verapamil (1 to 20 µg/ml) was studied in OVCAR-3.

Cellular Binding of Anti-TFR Antibody. Binding studies were performed in PBS containing BSA at 2 mg/ml (PBS:BSA). After the usual 30-min preincubation with DMEM:BSA, cells (OVCAR-2, OVCAR-3, and KB) were incubated with 0.85 ml of cold PBS:BSA containing various amounts (0.01 to 20 µg/ml) of [125I]-anti-TFR (specific activity, 50,000 cpm/µg), and the cells were incubated at 4°C for 3 hr. This period of time was chosen because previous experiments showed that, within 3 hr, binding reaches a plateau under these conditions in all cell lines tested (data not shown). The monolayers were washed with PBS and solubilized in 0.1 N NaOH, and the radioactivity was counted. Nonspecific binding, obtained by parallel incubations with excess unlabelled anti-TFR (300 µg/ml) in addition to the appropriate concentrations of radiolabeled anti-
ACTIVITY OF AN IMMUNOTOXIN IN OVARIAN CARCINOMA

TFR, was subtracted. Analysis of binding was done according to the method of Scatchard (20).

Cell-associated Anti-TFR Antibody after incubation at 37°. Cells (OVCAR-2, OVCAR-3, A1847, and KB) were seeded as usual. After preincubation with DMEM:BSA and replacement by 1 ml of DMEM:BSA containing 10 μg of anti-TFR and 0.17 μCi of 125I-anti-TFR (specific activity, 1.3 μCi/μg/dish), cells were incubated at 37° for various time intervals (20 min to 16 hr). Then, the monolayers were washed with PBS and trypsinized with 0.05% trypsin:0.02% EDTA (Grand Island Biological Co.) at 37° for 10 min, and the cell-associated radioactivity was counted. Nonspecific uptake, measured by incubating cells with radiolabeled antibody in the presence of excess unlabeled anti-TFR (210 μg/dish), was subtracted. Results are expressed as cpm/10^5 cells. To determine the number of cells per dish, parallel incubations were done for appropriate time intervals in medium without antibody, and cells were trypsinized and counted in a Coulter Counter.

RESULTS

Cytotoxicity of Anti-TFR-PE Conjugates. Anti-TFR-PE was tested for its ability to inhibit protein synthesis in 5 ovarian carcinoma cell lines (OVCAR-2, -3, -4, -5, and A1847) and in a breast carcinoma cell line (MCF-7). These results were compared with those in KB cells which were shown previously to be very sensitive to anti-TFR-PE (7). Cells were incubated with various concentrations of anti-TFR-PE for 12 hr at 37°, and then assayed for protein synthesis. In all cell lines tested, there was a dose-dependent inhibition of protein synthesis by anti-TFR-PE with 50% inhibition of protein synthesis at anti-TFR-PE concentrations varying from approximately 0.03 μg/ml in KB cells to about 2 μg/ml in OVCAR-3 (Chart 2). KB cells were more sensitive than the ovarian cells, with OVCAR-3 being the least sensitive cell line. OVCAR-5 was generally slightly more sensitive than OVCAR-3 (Chart 3), but its response was somewhat variable (Chart 4). OVCAR-2, OVCAR-4, A1847, and MCF-7 showed about the same sensitivity to anti-TFR-PE (Chart 2).

The specificity of anti-TFR-PE was shown in 2 ways. (a) The toxicity of anti-TFR-PE could be competed by adding excess anti-TFR antibody as shown for OVCAR-2 and A1847 cells in Table 1. In A1847 cells, which were of particular interest because of their high sensitivity to native PE, anti-TFR-PE at 0.1 μg/ml reduced protein synthesis to 46% of the control, and this effect was prevented by excess anti-TFR (50 μg/ml). Anti-TFR antibody did not affect protein synthesis by itself or compete for the toxicity of native PE. (b) PE linked to an irrelevant antibody such as the antibody against the human T-cell growth factor receptor (8) was used as a further control. At 1 μg/ml, no inhibition of protein synthesis was seen in OVCAR-2 (Table 1). These controls suggested that anti-TFR-PE bound to the transferrin receptors
ACTIVITY OF AN IMMUNOTOXIN IN OVARIAN CARCINOMA

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Immunotoxin or PE</th>
<th>Anti-TFR antibody (µg/ml)</th>
<th>Protein synthesis (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVCAR-2</td>
<td>Anti-TFR-PE</td>
<td>(0.1)</td>
<td>22</td>
</tr>
<tr>
<td>Anti-TFR-PE</td>
<td>(0.1)</td>
<td>200</td>
<td>97</td>
</tr>
<tr>
<td>(0)</td>
<td>200</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>(0.1)</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>PE</td>
<td>(0.1)</td>
<td>200</td>
<td>14</td>
</tr>
<tr>
<td>Irrelevant</td>
<td>(1)</td>
<td>0</td>
<td>103</td>
</tr>
<tr>
<td>A1847</td>
<td>Anti-TFR-PE</td>
<td>(0.1)</td>
<td>46</td>
</tr>
<tr>
<td>Anti-TFR-PE</td>
<td>(0.1)</td>
<td>50</td>
<td>93</td>
</tr>
</tbody>
</table>

* This conjugate was from another batch and was slightly more active than the one used for the experiments shown in Chart 2.

b Numbers in parentheses, µg/ml.

A complete dose-response curve (in the absence of anti-TFR) is shown in Chart 2.

Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Native PE (nM)</th>
<th>Derivatized PE (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVCAR-2</td>
<td>1.2</td>
<td>&gt;75</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>7.8</td>
<td>&gt;75</td>
</tr>
<tr>
<td>OVCAR-5</td>
<td>7.9</td>
<td>ND</td>
</tr>
<tr>
<td>A1847</td>
<td>0.1</td>
<td>&gt;75</td>
</tr>
<tr>
<td>KB</td>
<td>0.3</td>
<td>32</td>
</tr>
</tbody>
</table>

* Concentration (nM) of PE resulting in 50% inhibition of protein synthesis.

* Extrapolated value.

nd, not done.

and also that there was no contamination of anti-TFR-PE with unconjugated PE.

Cytotoxicity of PE. To find possible reasons for the different toxicity of anti-TFR-PE in the various cell lines, we evaluated the effect of both native and derivatized PE on protein synthesis in these cell lines. Cells were incubated with different concentrations of PE for 12 hr at 37°C and then assayed for protein synthesis. Controls were incubated without toxin. Mean values of duplicates are expressed as percentage of the controls.

Kinetics of Protein Synthesis Inhibition by Anti-TFR-PE. Cells were incubated with anti-TFR-PE for various time intervals and also that there was no contamination of anti-TFR-PE with unconjugated PE.

Cytotoxicity of PE. To find possible reasons for the different toxicity of anti-TFR-PE in the various cell lines, we evaluated the effect of both native and derivatized PE on protein synthesis in these cell lines. Cells were incubated with different concentrations of PE for 12 hr at 37°C and then assayed for protein synthesis. The values of the concentration resulting in 50% inhibition of protein synthesis for native PE were lowest in A1847 and KB, intermediate in OVCAR-2, and highest in OVCAR-3 and OVCAR-5 (Table 2). Derivatization of the toxin significantly reduced its toxicity.

Kinetics of Protein Synthesis Inhibition by Anti-TFR-PE. Cells were incubated with anti-TFR-PE for various time intervals at 37°C. Then, protein synthesis was measured as usual. Results for 2 different immunotoxin concentrations (0.1 and 1 µg/ml) are shown in Chart 3. After a certain lag period (its duration was dependent on both the dose and the cell line), inhibition of protein synthesis followed first-order kinetics and was more rapid at the higher concentration of anti-TFR-PE. At 1 µg/ml, a 50% inhibition was seen in OVCAR-2 after about 7 hr, in OVCAR-3 after 16 hr, in OVCAR-5 after about 13 hr, and in KB cells after about 2 hr.

Influence of Verapamil on the Toxicity of Anti-TFR-PE. Cells were incubated with anti-TFR-PE in the presence of verapamil (20 µg/ml) for 4 hr. Then, protein synthesis was determined as usual. Verapamil by itself had little (<10% inhibition) or no effect on protein synthesis.

In all OVCAR cell lines, verapamil enhanced the cytotoxicity of anti-TFR-PE (Chart 4). Comparing the values of the concentration resulting in 50% inhibition of protein synthesis, the enhancement was about 6-fold in OVCAR-2, 7-fold in OVCAR-3, and about 10-fold in OVCAR-4. Enhancement was dependent not only on the cell line, but also on the verapamil concentration. In the range tested (1 to 20 µg/ml), enhancement correlated with the concentration of verapamil (Chart 5). Also in the presence of verapamil, excess anti-TFR antibody competed for the toxicity of anti-TFR-PE (data not shown).

A kinetic analysis of protein synthesis inhibition by anti-TFR-PE (1 µg/ml) in the presence of increasing concentrations of verapamil (0 to 20 µg/ml) is shown for OVCAR-3 in Chart 5. This cell line was chosen because it was the least sensitive cell line to anti-TFR-PE. Verapamil increased the rate at which protein synthesis was inhibited. In the experiment shown, 50% inhibition of protein synthesis was seen after 13 hr in the absence of verapamil, and after about 6 hr in the presence of verapamil (20 µg/ml). First-order kinetics of protein synthesis inhibition was observed both in the presence and absence of verapamil.

Binding of Anti-TFR Antibody to the Cells. Binding of anti-TFR antibody to OVCAR-2, OVCAR-3, and KB cells was studied at 4°C. Within 3 hr, binding of the antibody reached equilibrium in all cell lines (data not shown). Thus, cells were incubated with increasing concentrations of 125I-anti-TFR for 3 hr, washed, and then counted for bound radioactivity.

Scatchard analysis revealed approximately 470,000 binding sites/cell for OVCAR-2; 250,000 for OVCAR-3; and 590,000 for KB cells assuming equivalent binding in all cells (Table 3). The dissociation constants were in the range of 10⁻⁸ mol/liter, and were slightly different for the 3 cell lines (Table 3).

Cell-associated Anti-TFR Antibody at 37°C. To measure cellular uptake of the monoclonal antibody, cells (OVCAR-2, OVCAR-3, A1847, and KB) were incubated with radiolabeled
anti-TFR for various time intervals at 37°C. After washing and trypsinization, cell-associated radioactivity was measured. Most of the radioactivity was not released by trypsin treatment, suggesting it was inside the cell. To be certain that trypsin was capable of removing surface-bound antibody, the amount of anti-TFR antibody present on the cell surface before and after trypsin treatment was assessed by indirect immunofluorescence. At least 80% of the antibody was removed by this treatment (data not shown). Therefore, under the conditions used, most of the cell-associated radioactivity was due to internalized anti-TFR.

The initial rate of uptake (antibody molecules internalized per cell during the first hr) was highest in KB and lowest in A1847 cells (Chart 6; Table 3). Cell-associated anti-TFR reached a plateau after about 4 hr in A1847, after 6 hr in KB, and after 12 hr in the OVCAR cells. Maximum uptake was highest in OVCAR-2 cells, which are larger than the other cells (data not shown). Therefore, under the conditions used, most of the cell-associated radioactivity was due to internalized anti-TFR.

and may, therefore, finally accumulate more antibody molecules per cell.

**DISCUSSION**

In the present study, PE was linked to anti-TFR to make an immunotoxin (anti-TFR-PE) active against ovarian carcinoma cell lines (OVCAR-2, -3, -4, -5, and A1847) and a breast carcinoma cell line (MCF-7). The ovarian carcinoma cell lines used in the present study include recent isolates (OVCAR) from patients with ovarian carcinoma, with 3 of the cell lines derived from patients resistant to conventional chemotherapy, a situation commonly seen in clinical oncology. Because the therapeutic options for such patients are at present limited and because cross-resistance to various treatment regimens is often seen (29), we chose to examine the sensitivity of these cells to anti-TFR-PE in an in vitro system. Anti-TFR-PE has been recently shown to be cytotoxic for established KB cell lines (7), and these cells were used for comparison.

Our results show that in a 12-hr incubation with anti-TFR-PE, the concentrations of anti-TFR-PE needed for 50% inhibition of protein synthesis ranged from 0.025 μg/ml for KB to 2 μg/ml for OVCAR-3 cells. These differences may reflect the different kinetics of anti-TFR-PE activity for the various cell lines because the rate of protein synthesis inhibition was greatest in KB cells, followed by OVCAR-2 and then OVCAR-3 cells. In all cell lines studied, the inhibition of protein synthesis by anti-TFR-PE followed first-order kinetics, in agreement with previous reports on other antibody-toxin conjugates (18, 28).

The binding and uptake of antibody-toxin conjugates presumably occurs via receptor-mediated endocytosis (17). Differences in binding and internalization can account for the kinetic differences seen in our studies. To investigate this, the binding and uptake of radiolabeled anti-TFR antibody was measured in the OVCAR-2 and OVCAR-3 cells, and compared with KB cells. A Scatchard analysis of cellular binding showed similar dissociation constants but differences in the number of antibody binding sites per cell. The number of binding sites per cell correlated with the
ACTIVITY OF AN IMMUNOTOXIN IN OVARIAN CARCINOMA

sensitivity of the 3 cell lines to anti-TFR-PE (Table 3). In addition, in these 3 cell types, the initial (first hr) rate of anti-TFR uptake at 37° also correlated with the activity of anti-TFR-PE (Table 3). However, A1847 cells, which were as sensitive to anti-TFR-PE as OVCAR-2, had the lowest rate of anti-TFR internalization, suggesting that additional factors play a role in sensitivity to immunotoxins. A1847 cells may have a greater inherent sensitivity to PE (Table 2). Possible reasons for this might include a greater efficiency of exotoxin delivery from the endocytic vesicle to the cell cytoplasm. That the toxic activity of anti-TFR-PE was mediated via the PE cell surface receptor seems to be unlikely because excess anti-TFR antibody significantly reduced the toxicity of anti-TFR-PE. Further, derivatizing PE with MMB reduces its cytotoxicity (Table 2), presumably by diminishing cellular binding, but not its ADP-ribosylating activity (7).

After internalization, immunotoxins must escape from the endocytic vesicle into the cytoplasm, where the toxins interfere with protein synthesis. Verapamil, a calcium antagonist which is used clinically in cardiovascular diseases, enhances the activity of anti-TFR-PE in the OVCAR cells. This is in agreement with a recent report in which verapamil was shown to modulate the activity of anti-TFR-PE in KB cells, probably by allowing the internalized conjugates greater access to the cytoplasm (1). Although in the OVCAR cells the enhancement by verapamil was especially pronounced in the cell line that was least sensitive to anti-TFR-PE (OVCAR-3), there is no clear evidence that the enhancement of immunotoxin activity by verapamil is more pronounced in cells less sensitive to immunotoxins. In previous reports, verapamil enhanced the daunorubicin effect in vivo only in daunorubicin-resistant tumors (21), and enhanced the cellular content of vincristine in resistant P388 leukemia cells more than in sensitive P388 cells (26). Similar studies have recently been reported using ovarian cancer cell lines (including OVCAR-2, OVCAR-3, and OVCAR-4) with varying sensitivity/resistance to Adriamycin (19).

Verapamil increases the rate of inhibition of protein synthesis, shortening the incubation time needed for activity of the immunotoxin. Although, in a 4-hr incubation, enhancement of immunotoxin activity required verapamil concentrations which are too high to be clinically tolerable, it is probable that, in vivo, some enhancement could be obtained at therapeutic levels when present over a longer time period. A more promising application for a drug such as verapamil would be in the ex vivo use with any immunotoxin to eliminate specific cells from bone marrow during autologous (9) or allogeneic bone marrow transplantation (6). Lengthy incubation times which might be harmful to stem cells (18) could thus be shortened.

Some comments should be made about the usefulness of the anti-TFR antibody for constructing immunotoxins. Because TFR are also expressed on rapidly growing nonmalignant cells, but only in a restricted pattern (10), there might be limitations for the clinical use of anti-TFR antibodies for targeting toxins to cancer cells. The ideal antibody would recognize an antigen expressed only on tumor cells. Whether such tumor-specific antigens exist still has to be critically evaluated (24). Nevertheless, some properties of the TFR could be useful in making it a preferential target for immunotoxins. (a) It is essential for cell growth. Thus, the problem of antigen heterogeneity and antigen modulation on tumor cells, both of which could limit the usefulness of other antibody-toxin conjugates (27), might not arise with immunotoxins constructed by using an anti-TFR antibody. (b) The TFR is not rapidly shed from the cell surface (24), whereas some other tumor-associated antigens, including the recently reported ovarian cancer antigen CA 125 (2), are found in the circulation and might have to be removed before immunotoxin treatment in vivo (27). (c) Murine bone marrow stem cells and early progenitor cells were shown to be relatively insensitive to an anti-TFR-ricin A conjugate (16).

Our in vitro studies were done in ovarian carcinoma cell lines recently established from the malignant ascites of patients with ovarian cancer resistant to combination chemotherapy. These cells are resistant to cytotoxic drugs both in vivo and in vitro (13, 19). Immunotoxins such as anti-TFR-PE offer the possibility of salvage therapy by acting through a different mechanism, thus avoiding the problems of cross-resistance so characteristic of ovarian cancer after primary chemotherapy (29). Enhancement of the immunotoxicity by verapamil could make successful immunotoxin treatment of human cancer more likely. Animal studies using a xenograft model of human ovarian carcinoma in nude mice (12) should provide more information about the clinical relevance both of toxins linked to the anti-TFR antibody or to other monoclonal antibodies and of verapamil in enhancing the toxicity of immunotoxins. This will clarify whether there is sufficient differential cytotoxicity between tumor cells and normal tissues to make this novel approach clinically feasible.

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ACTIVITY OF AN IMMUNOTOXIN IN OVARIAN CARCINOMA


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