Sensitivity of Resistant Human Tumor Cell Lines to Tumor Necrosis Factor and Adriamycin Used in Combination: Correlation between Down-Regulation of Tumor Necrosis Factor-Messenger RNA Induction and Overcoming Resistance

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ABSTRACT

A number of human tumor cell lines of various histological origin were examined for their sensitivity and resistance to tumor necrosis factor-a (TNF) and Adriamycin (ADR). Six ovarian lines, and one each of a renal, lung, and B-cell line, were tested for putative mechanisms of resistance to these agents. Cytotoxicity resulting from TNF or ADR showed no overall correlation in these lines. The combination of TNF and ADR produced enhanced cytotoxicity against these tumor lines and furthermore resulted in overcoming the resistance of TNF or ADR alone in combination. A proposed mechanism of TNF resistance in tumor cells is the endogenous production of TNF mRNA and protein. There was a positive correlation between resistance to TNF and the constitutive production of TNF mRNA and protein. The TNF-resistant lines that did not constitutively produce TNF mRNA and protein and the three TNF-sensitive tumor lines exhibited up-regulation of their TNF mRNA in the presence of TNF or phorbol myristate acetate/ionophore, but did not secrete any detectable protein. Due to the enhanced cytotoxicity seen with the combination of TNF and ADR, the effect of this combination on the level of TNF mRNA was examined. ADR alone reduced the constitutive level of TNF mRNA and in combination with TNF reduced the level of induction produced by TNF. This down-regulation of TNF mRNA by ADR may play a role in the enhanced cytotoxicity seen with the combination of these 2 agents.

INTRODUCTION

Conventional therapy for the eradication of tumor cells such as chemotherapy, radiation, and surgery has resulted in prolongation of survival and cure in some cases. However, relapse and metastases occur frequently and in general they are not responsive to conventional therapy. Therefore, other modalities primarily involving immunotherapy with activated cells such as lymphokine-activated killer cells (1), tumor infiltrating lymphocytes (2), or magic bullets such as antibody-toxin conjugates (3), have been advocated as adjunct therapy to reach metastatic cancers and reverse the development of resistance. However, it is not clear whether drug resistance can be overcome by immune modalities. Recent studies from our laboratory suggest the existence of a hierarchy of tumor cell resistance to various cytotoxic modalities (4). Among other things, the hierarchy suggests that common mechanisms of sensitivity or resistance may underlie the ability of tumor cells to resist killing by a wide variety of agents. A number of recent studies have been designed with the aim of overcoming this resistance by combining effective modalities such as biological response modifiers, including tumor necrosis factor, and chemotherapeutic drugs such as Adriamycin or cisplatin (5–10).

TNF was originally found to be the serum mediator of tumor necrosis in animals given injections of endotoxin (11). Recently the use of TNF in clinical trials in the treatment of cancer has been possible because of the cloning and expression of TNF in Escherichia coli (12, 13). Although TNF is highly cytotoxic for some tumor lines, it has little or no effect on others (14, 15). The mechanism of this resistance, although not completely understood, has been associated with numerous findings such as the production of free radical scavengers like MnSOD (16), overexpression of the HER2 oncogene (17), differential degradation of TNF in sensitive versus resistant cells (18), and the induction of expression of TNF mRNA itself (19–21).

The development of resistance to a tumor cytotoxic agent results from many mechanisms. For instance, tumor lines that overexpress a membrane glycoprotein known as GP-170 (22, 23) have been shown to be cross-resistant to a number of anticancer agents. This MDR protein is thought to reduce drug accumulation by actively pumping toxic drugs out of the tumor cell (24, 25). Because of this, methods have been sought that will overcome these forms of resistance resulting in tumor cell cytotoxicity. Recent experiments by our laboratory and others have shown that combinations of certain chemotherapeutic drugs and TNF can synergistically kill resistant tumor lines, in vitro (5–8) and in vivo (9, 10). This ability to overcome drug and TNF resistance should be of tantamount importance in the treatment of resistant tumors. Unfortunately, little is known about the mechanisms involved in the reversal of resistance by the combination of TNF and drugs.

The present study was designed to examine the effect of the combination of TNF and ADR in several human tumor cell lines of various histological types. Specifically, we examined: (a) whether there was a correlation between their sensitivity and resistance to TNF and the TNF mRNA and TNF protein levels in these lines; (b) whether a similar correlation existed between their sensitivity and resistance to ADR; (c) whether drug resistance correlates with the MDR phenotype by examining mRNA of MDR-1; (d) whether the combination of TNF and ADR overcome resistance; and (e) as a possible mechanism of overcoming resistance, whether ADR down-regulates the TNF mRNA expressed and up-regulates the TNF mRNA in TNF-resistant lines.

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2 To whom requests for reprints should be addressed, at University of California, Department of Microbiology and Immunology, 10833 Le Conte Avenue, Los Angeles, CA 90024-1747.

3 The abbreviations used are: TNF, tumor necrosis factor; ADR, Adriamycin; MnSOD, manganese superoxide dismutase; MDR, multidrug resistance; rTNF, recombinant tumor necrosis factor; SSC, standard saline-citrate; SDS, sodium dodecyl sulfate; MTT, monocomazolium; ELISA, enzyme-linked immunosorbent assay.
MATERIALS AND METHODS

Cell Lines and Media. The human ovarian tumor lines A2780, AD10, C30, and OVC-8 were obtained from Dr. Robert Ozols, Philadelphia, PA. The human renal carcinoma cell line R4 was generously supplied by Dr. Arie Beldegrun at this Institute. The human lung line 322 was a gift from Dr. E. Grimm of the M. D. Anderson Cancer Center. The above lines including the human ovarian lines OVC-3 and SKOV-3, as well as the human B-cell line Raji, were maintained in RPMI 1640 supplemented with nonessential amino acids, glutamine, antibiotics, and 10% fetal bovine serum (Gibco/Bethesda Research Laboratories). All lines were grown in a humidified atmosphere at 37°C in 5% CO₂.

Reagents. The recombinant TNF was generously supplied by Smith-Kline-Beecham, Philadelphia, PA. Antiserum directed against rTNF was raised in rabbits by i.m. injection of 50 μg rTNF in complete Freund's adjuvant. The rabbits were boosted 3 weeks later, and 7 days hence were bled by venous puncture to test the serum for neutralizing activity. Rabbit care was in accordance with University of California at Los Angeles guidelines. Alkaline phosphatase-linked goat-anti-rabbit IgG was purchased from Caltag. Adriamycin was purchased from Sigma Chemical Co., St. Louis, MO. The TNF cDNA used in making probes for Northern blot analysis was a gift from Smith-Kline-Beecham. The MDR-1 cDNA was a gift from Carsten Lincke at The Netherlands Cancer Institute.

Northern Blot Analysis. Cytoplasmic RNA from the tumor lines was prepared following the method of Chomczynski and Sacchi (26). Forty μg/lane of tumor cell RNA were electrophoresed in 1.2% agarose-2.2 M HCHO gels in 1x MOPS buffer (27). The RNA was transferred to Zeta Probe nylon membranes (Bio-Rad) in 20x SSC (1 x SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Fifty to 100 ng of cDNA probe were labeled with [α-32p]dCTP (New England Nuclear) by random oligo-primer extension as described by Feinberg and Vogelstein (28). The nylon filters were UV cross-linked and then prehybridized at 42°C from 1 h overnight in 50% formamide (Bethesda Research Laboratories), 5x SSC/0.1% SDS, 100 μg/ml salmon sperm DNA, and 5x SSC. Radiolabeled probe was added at 1 x 10⁶ cpm/ml of hybridization fluid (6x SSC, 0.5% SDS, 5x Denhardt’s, 100 μg/ml salmon sperm DNA), and the blot was incubated overnight at 65°C. Hybridized filters were then washed twice for 15 min each in 2x SSC/0.1% SDS at room temperature and twice for 5 min each in 0.1x SSC/0.1% SDS at 65°C and exposed to Kodak XAR-5 X-ray film.

Cytotoxic Assays. The 51Cr-release assay was used to determine the extent of tumor cell lysis perpetrated by rTNF or Adriamycin. Briefly, tumor cells at 1-3 x 10⁶ cells/ml were incubated with 0.1 ml Na₂[51Cr]O₄ (Amershams, 1 mCi/ml) in a total volume of 1 ml of RPMI with 20% FCS at 37°C for 1-3 h. Cells were then washed 3 times and resuspended at 1 x 10⁶ cells/ml in culture media; 0.1 ml of 51Cr-labeled tumor cells at 1 x 10⁶ cells/ml plus 0.1 ml of effector cells, cytokines, or drugs was added to 96-well microtiter plates and incubated at 37°C for 18-22 h. The assays were harvested by centrifuging the microtiter plates at 200 x g for 5 min, after which 100 μl of cell-free supernatant were removed and counted for ⁵¹Cr content using a Beckman 5500 gamma counter. Cytotoxicity was estimated using the following formula:

\[
\text{% specific release} = \frac{\text{cpm experimental release} - \text{cpm spontaneous release}}{\text{cpm total} - \text{cpm spontaneous release}} \times 100
\]

Spontaneous release by the tumor cells lines varied between 15 and 30%. Spontaneous release >30% was not included in data analysis. In a few experiments, the MTT assay was used (29). Briefly, 100 μl of target cell suspension (2 x 10⁵ cells) were added to each well of a 96-well flat-bottomed microtiter plate (Corning Glass Works, Corning, NY), and each plate was incubated for 24 h at 37°C in a humidified 5% CO₂ atmosphere. After incubation, 100 μl of drug solution or complete medium for control were plates and each plate was incubated for an additional 24-72 h at 37°C. Following incubation, 20 μl of MTT working solution (5 mg/ml; Sigma) were added to each culture well, and the cultures were incubated for 4 h at 37°C in a humidified 5% CO₂ atmosphere. The culture medium was removed from the wells and replaced with 100 μl of isopropanol (Sigma) supplemented with 0.05 N HCl. The absorbance of each well was measured with a microculture plate reader (Titertek Multiskan MCC/340; Flow Laboratories, Finland) at 540 nm. Percentage cytotoxicity was calculated as follows:

\[
\text{% cytotoxicity} = \frac{1 - \frac{\text{absorbance of experimental wells}}{\text{absorbance of control wells}}}{100}
\]

Estimation of Synergistic Cytotoxicity. Calculations of synergistic cytotoxicity were estimated using isobologram analysis as described by Berenbaum (30).

TNF ELISA. TNF protein in the supernatant of the tumor cells was quantitated by ELISA. Monoclonal antibodies against TNF used in this assay were the generous gift of Dr. G. Trinchieri (Wistar Institute) (31). The murine IgG1 monoclonal antibody, B154.7.1 and B154.9.1, were each specific for a different epitope of the TNF molecule. B154.7.1 derived from ascites fluid was purified by affinity chromatography using protein A. B154.9.1 from ascites was partially purified by 50% ammonium sulfate precipitation. Wells of 96-well ELISA plates were coated with 50 μl of both antibodies for at least 1 day and then stored for up to 4 weeks at 4°C. To set up the assay, coated plates were washed 3 times and blocked with ELISA phosphate-buffered saline containing 1% bovine serum albumin for 1 h. Plates were washed twice, and 50 μl of both tumor cell supernatants and TNF standards were added to the wells. After overnight incubation, plates were washed 3 times, and 50 μl of polyclonal anti-TNF antibody were added to each well. After a 2-h incubation, alkaline phosphatase-conjugated goat anti-rabbit IgG (Caltag) was added to each well and incubated for an additional 2 h. Finally, plates were washed and incubated with the substrate (Sigma 104). Plates were read 2 h later at 405 nm using a Titertek Multiscan MCC/240 ELISA reader.

TNF Binding Assay. ¹²⁵I-labeled rTNF (specific activity, ~1 x 10⁸ cpm/μg) was prepared using Iodobeads (Pierce), following the procedure recommended by the manufacturer.

Tumor cells (1 x 10⁶/ml) were incubated in microtube tubes in 0.5 ml complete medium containing 1 nM ¹²⁵I-labeled rTNF with or without a 100-fold excess of unlabeled rTNF at 4°C for 2 h. The cells were then washed 3 times with ice-cold medium and the radioactivity remaining in the pellet was determined. To determine the number of receptors per cell, we calculated the amount of radioactivity displaced by cold TNF and determined the number of receptors for each bound TNF trimer.

RESULTS

Lack of Correlation between Sensitivity of Human Tumor Cell Lines to TNF and Adriamycin. Recent studies of tumor line sensitivity to TNF suggested that there is a cross-sensitivity or resistance between TNF and certain chemotherapeutic drugs (18, 32), whereas other reports do not find this correlation (33). Because this discrepancy may be due to the restricted number of tumor cell lines used, the relationship between TNF and ADR sensitivity was investigated using several human tumor cell lines of different histological origin. The lines were tested for sensitivity to TNF and ADR using the ¹⁵¹Cr-release assay (Table 1). A titration of each agent was used to determine the concentration required for 30% lysis (LC-30) versus each tumor cell line. Clearly, the lines were either sensitive to both agents (A2780, C30), sensitive to TNF but drug resistant (AD10), resistant to TNF but sensitive to the drug (OVC-3, OVC-8, R4, 322), or resistant to both agents (SKOV3, Raji). AD10 and C30 are subclones of the parental A2780 that were selected for drug resistance to either Adriamycin (AD10) or cisplatinum (C30),
respecitively. Although C30 is resistant to cisplatinum, it is sensitive to ADR. Based on these findings, we assigned a sensitivity and/or resistance phenotype to each cell line (Table 1). These results demonstrate that no overall correlation exists between the sensitivity or resistance to TNF and ADR in the various tumor cell lines tested.

Previous data have suggested that expression of the TNF receptor on the cell membrane does not correlate with TNF sensitivity or resistance in tumor cells (34, 35). Similarly, the observed resistance to TNF in all the lines tested except Raji could not be attributed to a lack of TNF receptor expression in these lines, inasmuch as both TNF-sensitive and -resistant lines expressed the TNF receptor on the cell surface (Table 2). These findings point out that resistance to TNF is most likely due to an event that follows TNF binding to the receptor and may involve the trigger for initiation of lysis.

Augmented Cytotoxicity of the Combination of TNF and ADR against Tumor Cell Lines. Recent findings from our laboratory have suggested that there exists a hierarchy of sensitivity and resistance of tumor cells to immune and nonimmune cytotoxic modalities (4). Based on these findings, we proposed a common mechanism of cytotoxicity and hypothesized that under certain circumstances, resistance to any one or more agents can be overcome by combinatorial treatment. Accordingly, we examined here whether TNF and ADR can synergize in their cytotoxicity against sensitive and resistant tumor cell lines.

Synergy is documented here by isobologram analysis (29) in a representative experiment using the TNF-resistant, ADR-sensitive human ovarian lines OVC-3 and OVC-8 and the double-resistant SKOV3 (Fig. 1, a–e). In other instances, the combination of these agents produced additive cytotoxicity (data not shown). Comparing the 51Cr release assay with the MTT assay revealed similarities as depicted in Fig. 1, f–g. The ability to achieve additive or synergistic cytotoxicity with the combination of TNF and ADR supports the hierarchy hypothesis and suggests that the resistance of tumor cells to the cytotoxic agents can be overcome by the mechanisms of these agents working in concert.

We examined the effect of sequential treatment on cytotoxic activity of 2 ovarian carcinoma lines, 222 and the TNF-resistant subline 222TR. The cells were first treated for 3 h with one of the agents, washed, and then incubated for 24 h with the other agent and cytotoxicity assessed. The results in Table 3 indicate that pretreatment with TNF followed by ADR results in an additive effect, whereas pretreatment with ADR followed by TNF results in a synergistic effect. These results indicate that sequential treatment sensitizes the cell to the effect of the other agent, a condition that mimics clinical administration of cytotoxic agents in humans.

### Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>LC-30 (µM)</th>
<th>Susceptibility to lysis (µM × 10^{-3})</th>
<th>LC-30 (µM)</th>
<th>Susceptibility to lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>Ovarian</td>
<td>0.11</td>
<td>+ b</td>
<td>0.86</td>
<td>+ b</td>
</tr>
<tr>
<td>C30</td>
<td>Ovarian</td>
<td>0.58</td>
<td>+</td>
<td>3.45</td>
<td>+</td>
</tr>
<tr>
<td>AD10</td>
<td>Ovarian</td>
<td>0.029</td>
<td>+</td>
<td>&gt;172</td>
<td>-</td>
</tr>
<tr>
<td>OVC-3</td>
<td>Ovarian</td>
<td>3.52</td>
<td>±</td>
<td>&gt;172</td>
<td>-</td>
</tr>
<tr>
<td>OVC-8</td>
<td>Ovarian</td>
<td>&gt;12</td>
<td>-</td>
<td>17.2</td>
<td>+</td>
</tr>
<tr>
<td>R4</td>
<td>Renal</td>
<td>&gt;12</td>
<td>-</td>
<td>17.2</td>
<td>+</td>
</tr>
<tr>
<td>322</td>
<td>Lung</td>
<td>5.8</td>
<td>+</td>
<td>&gt;172</td>
<td>-</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>Ovarian</td>
<td>&gt;12</td>
<td>-</td>
<td>&gt;172</td>
<td>-</td>
</tr>
<tr>
<td>Raji</td>
<td>B-cell</td>
<td>&gt;12</td>
<td>-</td>
<td>&gt;172</td>
<td>-</td>
</tr>
</tbody>
</table>

*Concentration needed for 30% lysis as measured by the 51Cr-release assay described in "Materials and Methods." Final value is an average of multiple experiments.

* +, sensitivity; –, resistance.

### Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TNF sensitivity</th>
<th>No. of TNF receptors/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>+</td>
<td>4.971 ± 1.091</td>
</tr>
<tr>
<td>C30</td>
<td>+</td>
<td>8.731 ± 242</td>
</tr>
<tr>
<td>AD10</td>
<td>+</td>
<td>4.002 ± 363</td>
</tr>
<tr>
<td>OVC-3</td>
<td>±</td>
<td>3.572 ± 909</td>
</tr>
<tr>
<td>OVC-8</td>
<td>–</td>
<td>4.972 ± 1.576</td>
</tr>
<tr>
<td>R4</td>
<td>–</td>
<td>7.397 ± 1.600</td>
</tr>
<tr>
<td>322</td>
<td>–</td>
<td>4.850 ± 1.250</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>–</td>
<td>11.399 ± 2.050</td>
</tr>
<tr>
<td>Raji</td>
<td>–</td>
<td>72 ± 60</td>
</tr>
</tbody>
</table>

* a, sensitivity; –, resistance.

* Measured by 125I-labeled TNF binding and competed by 100-fold excess cold TNF as described in "Materials and Methods."

* Mean ± SD.

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Fig. 1. Cytotoxicity of the combination of TNF and ADR versus OVC-3 (a and b), OVC-8 (c and d), and SKOV3 (e) measured by 51Cr-release as described in "Materials and Methods." The cytotoxicity is expressed as percent survival (a, c, and e), and isobologram analysis (29) was then generated (b and d) g and f, cytotoxicity by the MTT assay on AD10. Isobologram analysis could not be generated from SKOV3 data because of the lack of sufficient TNF cytotoxicity.
Table 3  Effect of sequential treatment on cytotoxicity
TNF (100 pm) or ADM (0.5 μg/ml) at 3 h were used for pretreatment followed by wash and incubation for 24 h in medium, and cytotoxicity was assessed by the MTT assay.

<table>
<thead>
<tr>
<th>Pretreatment (3 h)</th>
<th>Second treatment (21 h)</th>
<th>% cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>TNF</td>
<td>15.3 ± 2.1*</td>
</tr>
<tr>
<td>ADM</td>
<td>ADM</td>
<td>18.7 ± 1.6</td>
</tr>
<tr>
<td>TNF</td>
<td>Medium</td>
<td>13.8 ± 1.5</td>
</tr>
<tr>
<td>Medium</td>
<td>ADM</td>
<td>15.7 ± 1.8</td>
</tr>
<tr>
<td>TNF</td>
<td>Medium</td>
<td>32.0 ± 2.7</td>
</tr>
<tr>
<td>Medium</td>
<td>TNF</td>
<td>14.4 ± 2.0</td>
</tr>
<tr>
<td>ADM</td>
<td>Medium</td>
<td>14.6 ± 1.1</td>
</tr>
<tr>
<td>ADM</td>
<td>TNF</td>
<td>47.5 ± 3.1</td>
</tr>
</tbody>
</table>

\* Mean ± SD.

Fig. 2. Induction of TNF mRNA by TNF. Northern blot analysis of TNF mRNA was performed as described in "Materials and Methods." Each cell line was incubated in the presence or absence of TNF in complete medium for 1 h at 37°C, and the total RNA was isolated as described. Blots were stripped and reprobed with β-actin cDNA as a control for equal loading of the lanes (data not shown).

Expression and Induction of TNF mRNA and Secretion of TNF Protein. Previous studies have suggested that a possible mechanism of resistance to TNF is the expression of TNF mRNA and/or protein by the tumor cell line (19–21). However, the correlation between sensitivity and resistance and constitutive versus TNF-induced TNF mRNA or TNF protein secretion was not established. Therefore, several tumor lines of different histological origin were examined for the presence of TNF mRNA in several lines (Table 1), only OVC-3, OVC-8, Raji, and R4 constitutively expressed detectable levels of the mRNA (Fig. 2). The remaining lines, both TNF-sensitive and TNF-resistant, were negative for TNF mRNA. These findings demonstrate that resistance to TNF does not necessarily correlate with constitutive expression of the TNF gene. However, treatment of the tumor lines with rTNF for 1 h resulted in the induction of varying levels of TNF mRNA expression, whether the tumor line was resistant or sensitive to TNF, as shown in Fig. 2. Treatment of the TNF-sensitive lines with TNF resulted in less TNF mRNA induction than the resistant lines, suggesting that resistance positively correlates with TNF mRNA expression.

To correlate the induction of TNF mRNA expression and translation, we examined the secretion of TNF protein in sensitive and resistant lines by ELISA. As shown in Table 4, only TNF-resistant lines secreted detectable levels of TNF protein either constitutively (OVC-8 and R4) or by induction with TNF or phorbol myristate acetate/ionophore (Raji, OVC-3, and SKOV3). These results demonstrate that some TNF-resistant lines are easily inducible and suggest that translation of the message and secretion correlates with resistance. However, the 322 lung line does not secrete detectable levels of TNF protein, although it may express membrane-bound TNF. This suggests that TNF resistance in this line may be mediated by a mechanism that is related to TNF mRNA expression but does not require TNF protein secretion.

Table 4  Secretion of TNF by human tumor lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TNF sensitivity</th>
<th>Control</th>
<th>PMA/ionophore</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>+</td>
<td>0</td>
<td>15.5 ± 21.9*</td>
</tr>
<tr>
<td>AD10</td>
<td>+</td>
<td>0</td>
<td>11.4 ± 16.3</td>
</tr>
<tr>
<td>C30</td>
<td>+</td>
<td>0</td>
<td>25.5 ± 15.6</td>
</tr>
<tr>
<td>OVC-3</td>
<td>±</td>
<td>11.8 ± 14.2</td>
<td>131.8 ± 36.2</td>
</tr>
<tr>
<td>OVC-8</td>
<td>–</td>
<td>57.5 ± 14.1</td>
<td>1818 ± 65.7</td>
</tr>
<tr>
<td>R4</td>
<td>+</td>
<td>103.8 ± 20.8</td>
<td>1050 ± 50.2</td>
</tr>
<tr>
<td>322</td>
<td>–</td>
<td>0</td>
<td>4.0 ± 8.9</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>–</td>
<td>0</td>
<td>217.1 ± 66.5</td>
</tr>
<tr>
<td>Raji</td>
<td>–</td>
<td>5.7 ± 8.7</td>
<td>666.5 ± 25.5</td>
</tr>
</tbody>
</table>

\* +, sensitivity; –, resistance.
\* Measured by ELISA as described in "Materials and Methods."  
\* PMA, phorbol myristate acetate.
\* Phorbol myristate acetate (1 ng/ml) + A23187 (200 nm). Treatment of cells with 10 ng/ml TNF produced similar results (data not shown).
\* Mean ± SD.
ADR by the cell may be important for TNF mRNA down-regulation and increased sensitivity to TNF.

The kinetics of TNF mRNA down-regulation by ADR was examined in three of the tumor lines. In the ovarian line SKOV3 and the B cell line Raji, TNF message was induced at 1 and 2 h after TNF treatment and was subsequently down-regulated in the presence of TNF and ADR (Fig. 4c). The kinetics were further examined in OVC-8, where down-regulation by ADR was most effective when the cells were pretreated with ADR for 2 h prior to TNF addition (Fig. 4b). These results correlate with synergy experiments, whereby pretreatment with drug followed by TNF results in high enhancement of killing compared to the simultaneous combination of TNF and drug (data not shown). The addition of ADR for 2 h after 1 or 2 h pretreatment with TNF also markedly reduced the level of TNF mRNA induced by TNF alone (Fig. 4b). These results suggest that the down-regulation of TNF mRNA by ADR occurs whether ADR is present before or after induction of the mRNA by TNF. Thus, ADR may operate by inhibiting transcription of the mRNA and also by decreasing its stability.

The specificity of the down-regulation of TNF mRNA by ADR was examined in preliminary experiments with the R4 cell line. Because increased levels of MnSOD have also been linked to TNF resistance in tumor cells, we examined the effect of ADR on MnSOD mRNA. If the same R4 blot (Fig. 4a, upper blot) is probed with a MnSOD oligomer, there was no effect of the ADR on this mRNA (Fig. 4a, middle blot). There was also no effect on the β-actin mRNA levels in this or other combination treatment experiments (data not shown). Therefore, the effect of ADR on TNF mRNA may be specific.

The enhanced cytotoxicity of the combination of TNF and ADR in the TNF-resistant, ADR-sensitive, OVC-8 (Fig. 1, c and d) and TNF- and ADR-resistant SKOV3 (Fig. 1c) may, therefore, be a result of overcoming the TNF resistance by the reduction in the TNF mRNA level. Overall, these results suggest that the reduction in TNF mRNA seen in TNF-resistant cell lines may be related to the synergy seen with the combination of these 2 agents.

DISCUSSION

The present study examines several human tumor lines of various histological types for putative mechanisms of sensitivity and resistance to TNF and ADR. The tumor lines were either sensitive to both agents, sensitive to one but resistant to the other, or resistant to both. This heterologous response to TNF and ADR was even seen within one histological type, inasmuch as there were ovarian tumor lines in each category. Thus, there appears to be no correlation between the sensitivity and resistance to TNF and ADR in these tumor lines. Treatment of the tumor lines with TNF induced TNF message in all the lines regardless of their sensitivity to the cytokine. Although TNF message could be up-regulated in all the lines, only TNF-resistant lines were capable of secreting detectable levels of TNF protein. Because the combination of TNF and ADR resulted in enhanced cytotoxicity against the tumor lines, certain features of the lines were examined for clues to the possible mechanisms involved. The production of TNF message and/or TNF protein by tumor lines has been suggested as a possible mechanism of resistance to this cytokine (19–21). In the Adriamycin-sensitive and some resistant lines examined here, Adriamycin treatment resulted in down-regulation of constitutive TNF mRNA, and the combination of TNF and ADR reduced the level of TNF mRNA induced by TNF alone.

To determine the sensitivity and resistance phenotype of the various tumor lines, the correlation of the lysis caused by TNF and ADR was examined. Earlier reports have suggested both the presence (18, 32) and the absence (33) of a correlation between sensitivity and resistance to TNF and drugs. Our results suggest that a correlation is possible in the lines that are either sensitive (A2780, C30) or resistant (SKOV-3, Raji) to both agents, but the remaining lines appear to exhibit no correlation whatsoever. The lack of a correlation is strengthened when A2780 is compared to its ADR-resistant subline, AD10. Creating ADR resistance in AD10 results not in resistance to TNF, but, in fact, in enhanced killing by TNF, as also reported by Salmon et al. (37) in MDR-positive cell lines.

Although the use of TNF alone in clinical trials has produced disappointing results (38–40), the potential of TNF in combination with other biological response modifiers or chemotherapeutic drugs is now being examined (41–43). Previous reports attest to the ability of TNF and ADR to result in enhanced cytotoxicity in vitro and in vivo (5–7, 9, 10). For these reasons, the effect of the combination of TNF and ADR on these human tumor lines was examined. Enhanced lysis was seen with the combination of TNF and ADR in all the lines tested. Thus, the resistance to either agent used alone can be overcome when they are combined, suggesting possible clinical applications.

To better understand the possible mechanisms of enhanced cytotoxicity seen with TNF and ADR, resistance mechanisms...
DOWN-REGULATION OF TNF-mRNA INDUCTION BY TNF AND ADR

Fig. 4. Effect of ADR or TNF plus ADR on the TNF mRNA level of these tumor lines. The cell lines were incubated for the indicated times at 37°C with TNF or ADR alone or in combination. Total RNA was then isolated, Northern blotted, and probed for TNF as described in “Materials and Methods.” a, ADR-sensitive R4 TNF mRNA. Blot was stripped and reprobed with MnSOD. Plot of densitometry scan is adjacent to blot. b, ADR-sensitive OVC-8 TNF mRNA. Blot was stripped and reprobed with B-actin. c, ADR-resistant SKOV-3. d, Raji TNF mRNA.

attributed to each agent alone were examined in these tumor lines. One possible mechanism of the resistance to TNF is the induction of TNF message and/or protein in the tumor cell (19–21). In fact, transfection of a murine TNF-sensitive line with a human TNF gene resulted in stable transfectants that expressed endogenous TNF and acquired resistance to TNF (44). Of the tumor lines examined here, only the TNF-resistant cells constitutively produced TNF mRNA and protein. The remaining lines both sensitive and resistant to TNF could be induced to up-regulate TNF mRNA, however, only TNF-resistant lines secreted detectable levels of TNF protein. These results corroborate those of Beutler et al. (45) and Kronke et al. (46), in that the presence of TNF mRNA does not determine whether or not TNF protein will be translated and secreted. This ability to secrete TNF seems to be important for resistance to TNF in TNF mRNA-positive lines, although not a necessity. For example, the human lung line 322 is resistant to TNF and can be induced to up-regulate TNF mRNA, but does not secrete any protein. Membrane expression of TNF, however, has not been ruled out. Therefore, the resistance mechanism operating in this line may be related to TNF mRNA induction in the absence of TNF protein secretion. Furthermore, shedding of TNF receptors by the resistant lines may interfere with the sensitivity of the line to TNF.

If the regulation of the TNF gene is important to the resistance mechanisms of a tumor cell, agents that possibly affect this regulation may result in changes in the resistance pattern. Because TNF plus ADR has resulted in enhanced tumor cell cytotoxicity in many instances (5–7, 9, 10), the effect of ADR on the expression and induction of TNF mRNA was examined. In the ADR-sensitive tumor and some resistant lines examined here, ADR down-regulated the constitutive expression of TNF, and the combination of TNF and ADR reduced the induced level of the TNF mRNA. Even though these lines are resistant to its cytotoxic effects, ADR is still able to reduce the TNF mRNA level in SKOV3 and Raji. Thus, the enhanced cytotoxicity seen with the combination of TNF and ADR may be the result of a reduction in any protective effect of the TNF mRNA. Further studies are needed to ascertain whether this down-regulation also includes a reduction in the level of secreted TNF protein. The effect of ADR on TNF mRNA appears to be specific, inasmuch as ADR had no effect on MnSOD mRNA in the R4 cell line.

Because any therapeutic combination of cytotoxic agents will eventually need to overcome some form of multidrug resistance, the tumor lines were examined for the MDR phenotype. Only the ADR-resistant subline of A2780 (AD10) expressed detectable levels of MDR-1 mRNA. Thus, the ADR
resistance mechanisms seen in the non-MDR-expressing lines do not appear to be related to the MDR phenotype. In summary, we have examined several human tumor lines of various histological types for their in vitro response to TNF and ADR, and for their resistance mechanisms that may be related to these cytotoxic agents. In a result that may be related to the mechanism of enhanced cytotoxicity seen with TNF plus ADR, ADR reduced both the constitutive and the TNF-induced level of TNF mRNA in tumor lines resistant to TNF. Obviously, the relationship between a tumor cell and its sensitivity or resistance to TNF and/or ADR is a complex one. It is likely a combination of events that leads to resistance to these and other cytotoxic agents. Because the resistance mechanisms examined here are by no means the sum total, future directions include the screening of the lines for other possible mechanisms pertaining to TNF and ADR resistance. For example, to further delineate the specificity of the ADR down-regulation of the TNF mRNA, the effect of ADR on other inducible protective mechanisms in addition to MnsSOD are currently being examined. These and other studies of resistance mechanisms of tumor lines are necessary for the development of effective combination therapies.

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Sensitivity of Resistant Human Tumor Cell Lines to Tumor Necrosis Factor and Adriamycin Used in Combination: Correlation between Down-Regulation of Tumor Necrosis Factor-Messenger RNA Induction and Overcoming Resistance

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