Journal of Cellular Biochemistry 39:95–105 (1989)
Growth Inhibitory and Cytotoxic Polypeptides 67–77

Cytotoxicity Mediated by Tumor Necrosis Factor in Variant Subclones of the ME-180 Cervical Carcinoma Line: Modulation by Specific Inhibitors of DNA Topoisomerase II

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The mechanism of tumor necrosis factor (TNF)-induced cytotoxicity has been investigated using two clonal variants of the ME-180 human cervical carcinoma cell line. The clonal lines were characterized with respect to their expression of TNF receptors, kinetics of cell death, and their ability to communicate intercellularly through gap junctions. The ME-180.4 and ME-180.8 clones were identified by their relative sensitivity to TNF induced lysis in a 24-h assay. The dose of TNF required to kill 50% of the target cells was 60 pM for the sensitive ME-180.4 and 2.5 nM for the ME-180.8. However, when assay times were extended, the dose response for both clones was the same, indicating that a difference in the kinetics of cell death and not absolute TNF sensitivity existed between the ME-180.4 and ME-180.8 clones. Both clones were gap junction deficient as judged by their inability to transfer Lucifer yellow or 6-carboxyfluorescein, a characteristic phenotype of cells sensitive to cytotoxicity by TNF. The level of surface receptor expressed on these clones was nearly identical with a Kd = 0.3 nM and 5,000 binding sites per cell. Measurement of the kinetics of cell death revealed that the time between the addition of TNF and the onset of observed cell death (induction phase) was much shorter for the ME-180.4 (32–55 h) than for the resistant ME-180.8 (55–80 h). Mitomycin C, a DNA alkylating agent, significantly reduced the length of the induction phase for both clones, although the kinetic difference between the clones remained unchanged. Two epipodophyllotoxins, VP-16 and VM-26, which specifically inhibit the rejoining activity of DNA topoisomerase II, showed a 10–100-fold synergistic effect when combined with TNF as shown by isobologram analysis. VM-26 when added to the resistant ME-180.8 clones de-

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Received February 1, 1988; accepted August 18, 1988.

creased the length of induction phase and abolished the kinetic difference observed with the ME-180.4 clone. These results indicate that the variance in the TNF response of these two clones was closely associated with DNA topoisomerase II, and suggest that this enzyme may play an important role in TNF mediated cytotoxicity.

Key words: cell death mutants, kinetics of cell death, GAP junctions, receptor occupancy

T-lymphocytes, natural killer cells, and macrophages secrete several types of protein toxins in response to viral, bacterial, and other inflammatory stimuli [1]. Two of the cytotoxic lymphokines produced by activated lymphoid cells, lymphotoxin (LT) and its structural homologue tumor necrosis factor (TNF), are selectively cytotoxic for virus-infected cells and certain types of malignant cells while generally sparing normal tissue [2]. In addition to cytotoxic activity, LT and TNF also induce a myriad of biological responses (primarily growth and differentiation) in other tissues involved in promoting inflammatory responses [3]. The mechanism(s) of action of LT and TNF appear to be analogous to classical polypeptide hormones in that they bind to a common, specific high-affinity cell-surface receptor and the particular response elicited by these proteins depends on the differentiated state of the target tissue [2,4–6].

The cellular and molecular mechanisms controlling the selective cytotoxic function are not well understood but involve both receptor and postrecognitive events [7]. The LT/TNF receptor is expressed on a wide variety of tissue, indicating that expression of the receptor, although necessary for a biological response, is not sufficient to induce the cytolytic process [2]. The relationship between the sensitivity (or resistance) to cytolysis and the growth or differentiation status of the target cell is especially intriguing because these parameters are altered in malignant transformation and viral infection. We have recently identified a differentiated state common to several target cells that correlates strongly with their sensitivity/resistance to cytolysis induced by LT/TNF [7]. This differentiated state is measured by the cells' capacity to communicate intercellularly through gap junctions. Cells that are communication competent can resist cytolysis, whereas cells that are communication deficient, such as certain tumor or virus-infected cells, undergo cell death.

The postreceptor processes leading to cell death induced by LT/TNF remain obscure. However, the nucleus appears to be a major target since DNA fragmentation has been documented as a prominent even in LT/TNF induced cell death [8,9]. Thus, enzymes involved in DNA cleavage reactions, such as DNA topoisomerases or endonucleases, may be specific target proteins for the signal(s) generated by LT/TNF receptor interaction.

Here we present evidence that two subclones of the human tumor cell line ME-180 are kinetic variants in the cytotoxic response to TNF. The difference between these variants was not due to receptor expression or affinity, nor to differences in the ability to communicate with gap junctions. Further, two specific inhibitors of DNA topoisomerase II, teniposide (VM-26) and etoposide (VP-16), act synergistically with TNF to enhance TNF cytotoxicity and to abolish the kinetic difference between the variant clones. These results suggest that DNA topoisomerase II may play a key role in cell death induced by LT/TNF.

MATERIALS AND METHODS

Cell Lines

The human cervical carcinoma cell line, ME-180, was originally obtained from the American Type Culture Collection (ATCC #HTB33). The subclones ME-180.4

and ME-180.8 were derived by limiting dilution at 0.9 cell per well in RPMI-1640 medium containing 10% fetal bovine serum, antibiotics, Na pyruvate, and glutamine, and buffered with 5% CO_2/air. Cell monolayers were subcultured by removal from flasks with 25 mM EDTA in phosphate buffered saline every 3 days.

Reagents

Teniposide (VM-26) and etoposide (VP-16) were gifts from Bristol Meyers Company and concentrated stocks were made in dimethylsulfoxide. TNF was purified from *Escherichia coli* cells containing human recombinant TNF gene-encoded plasmids and was a gift from Dr. J. Browning, Biogen Research Corp. (Cambridge, MA) [10]. Cytotoxic activity of LT and TNF, both in 24-h and kinetic assay, was measured by the dye reduction assay using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) as previously described [11]. Purified recombinant TNF was radioiodinated with $Na^{125}I$ by the Iodogen method [12] to a specific activity of 5–8 $\times$ 10^7 cpm/μg of protein with retention of >50% of the initial cytotoxic activity as detected on the sensitive L929 cell line.

TNF Receptor Assay

TNF receptors were measured by a radioligand binding assay using ^{125}I-TNF. ME-180 clones were removed from tissue culture flasks by addition of 25 mM EDTA in PBS, washed and resuspended at 2 $\times$ 10^6 cells/ml in Hanks' balanced salt solution containing 10% FBS. Cells were incubated with graded amounts ^{125}I-TNF for 16 h at 4°C wtih continual mixing. Cells were then centrifuged and unbound ligand was removed by aspiration of the medium. Nonspecific binding was measured in the presence of 100-fold excess cold TNF and was less than 4% to 10% of the total bound. Scatchard analysis was used to determine receptor number and binding affinity of the iodinated ligand [13]. ^{125}TNF binding was saturable and the binding curves consistent with a single class of binding sites.

Cytotoxicity Assay

The cytotoxic assays measured at 24 h were performed by seeding 96-well plates with 20,000 cells/well in 0.1 ml and allowing them to adhere overnight at 37°C. Serial dilutions of TNF or other effectors were added in 0.1-ml aliquots, and the plates were incubated 20 h at 37°C. Four hours before termination, 20 μl of 5 mg/ml MTT was added to each well. MTT is a yellow tetrazolium salt which is reduced to a purple formazaen by mitochondrial dehydrogenase of live cells; the loss of MTT reductive ability parallels cell death as measured by other methods [11]. At the end of the incubation, the medium was aspirated from each well and replaced with 200 μl of 70% isopropanol—0.02 M HCl. Following a 1-h incubation at 37°C and rapid repipetting with a multiwell pipetter to solubilize the purple dye, the absorbance at 570 nM was read using an automatic multiwell spectrophotometer (BioRad Model 2550 EIA Reader). The percentage of cytotoxicity was determined by comparing the background-corrected absorbance of the TNF treated cells to that of cells which received medium only according to the following equation: percentage of cytotoxicity = [1 − (ODTNF-treated)/(ODcontrol)] $\times$ 100.

The MTT dye reduction assay was used to measure cell death during kinetic time courses. Kinetic assays were performed by seeding 96-well plates with 20,000 cells/well in 0.18 ml and allowing them to adhere overnight at 37°C. At specified intervals, 20-μl aliquots of medium or medium containing TNF were added to

replicate wells (n=6). Four hours before termination, 20 μl of 5 mg/ml MTT was added to each well, and the percentage of cytotoxicity was determined as described above. The data are presented as the means of six replicates, and the SD in all cases never exceeded 50% cytotoxicity. When analyzing kinetic time courses, the onset of cell death was determined as the time when greater than 20% cytotoxicity had been achieved, a value four times the standard deviation, yielding a confidence limit of ≥95%.

Isobole analysis to determine interactions between TNF and VP-16 or VM-26 was conducted as detailed by Berenbaum [14]. Serial dilutions were made containing different concentrations of TNF and VP-16 or TNF and VM-26 in medium and applied to the ME-180.4 or ME-180.8 clones. Cells were incubated overnight and the level of cytotoxicity was measured by MTT assay described above. For each agent alone or in combination, the concentration of TNF and either drug yielding an ID_{50} was determined by linear regression analysis. An isobologram was constructed by plotting these concentrations as a function of the ID_{50} value for the other agent.

The hyphenated diagonal line connecting the ID_{50} values for each agent alone defines additive effects and those points falling below this line are indicative synergistic interactions between two agents. Where appropriate, the interaction index was calculated according to $Ac/Ae \leqslant Bc/Be = 1$, where A and B are TNF or drug; Ae (or Be) represents the concentration of TNF (or drug) by itself; and Ac (or Bc) is the concentration of TNF (or drug) in the mixture. Accordingly, a synergistic interaction index will be < 1.

RESULTS

We have examined TNF-mediated cytotoxicity in several subclones of the human cervical carcinoma cell line ME-180, which was modestly sensitive to the cytotoxic effects of LT/TNF [2]. Subclones were derived by limiting dilution culture without additional selective pressure and showed a surprising range of sensitivities towards TNF when cell death was measured in a 24-h assay in the presence of the DNA synthesis inhibitor, mitomycin C. The sensitivity of each subclone remained stable through months of continuous culture and at least 1 year storage in liquid nitrogen. The most sensitive subclone ME-180.4 required 60 pM TNF to kill 50% of the cells in 24 h; the least sensitive, ME-180.8, required 2,500 pM (50-fold increase) (Fig. 1). Other subclones showed sensitivities to TNF ranging between these two. However, when the dose response to TNF was measured over longer periods of time (5 days) without mitomycin C there was very little difference in the amount of TNF required to kill 50% of the cells (60 pM for ME-180.4 compared to 110 pM for ME-180.8) (Fig. 2).

The expression of specific TNF receptors on the sensitive and resistant ME-180 subclones as measured by ^{125}I-TNF binding and Scatchard analysis revealed no significant differences (Table I). The values for both Kd and receptor number were within the ranges reported by others for a number of human cell lines [2,4,5].

Dye transfer experiments to assess the gap junctional competence of the ME-180 clones revealed that both clones were unable to transfer (transit time >30 min) either Lucifer yellow (457 MW) or 6-carboxyfluorescein (376 MW) (data not shown). Multiple ME-180 cells were injected with the dye, and neither clone exhibited any significant dye transfer as judged by equal levels of fluorescence intensity of different

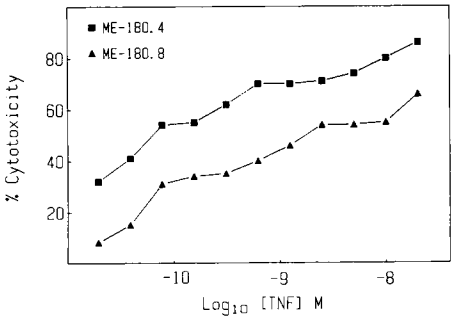

Fig. 1. Dose response of TNF-mediated cytotoxicity of ME-180.4 and ME-180.8 clones in a 24-h assay with mitomycin C. Cells were seeded in 96-well microtiter plates at 20,000 per well with 0.5 μg/ml mitomycin C and allowed to incubate overnight at 37°C. Serial dilutions of TNF were then added, and the plates incubated 24 h at 37°C. Cytotoxicity was determined by the MTT assay, in which dead cells lose the ability to reduce the yellow tetrazolium dye MTT to a purple formazan product. Each data point represents a mean of six replicates and the SD did not exceed ±5% cytotoxicity.

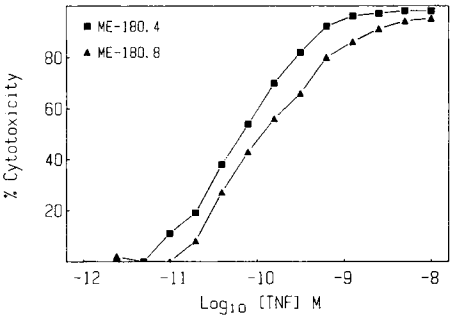

Fig. 2. Dose response of TNF-mediated cytotoxicity of ME-180.4 and ME-180.8 clones in a 5-day assay. Cells were seeded in 96-well microtiter plates at 1,500 per well and allowed to adhere to 37°C for 6 h. Serial dilutions of TNF were added and the plates incubated five days at 37°C. Cytotoxicity was determined by the MTT assay.

TABLE I. TNF Receptor Analysis of Sensitive and Resistant ME-180 Clones*

			ID_{50}[b] (pM)	
Clone	Kd[a] (nM)	Sites/cell[a]	24 h	5 days
ME-180.4	0.3	4,300	60	60
ME-180.8	0.25	5,350	2,500	110

*TNF receptor was measured by a radioligand binding assay using ^{125}I-TNF as described in Materials and Methods.

[a]Scatchard analysis was used to determine the equilibrium dissociation constant (Kd) and the number of binding sites per cell.

[b]The amount of TNF required to kill 50% of the cells was determined in a 24-h cytotoxic assay in the presence of mitomycin C or at 5 days without mitomycin C. These values were obtained from data presented in Figures 1 and 2.

cells over the observation period. Primary porcine ovarian granulosa cells were used as a positive control for dye transfer and showed transfer to secondary, tertiary, and quarternary neighbor cells within 1–2 min [15].

The results of the 5-day TNF dose-response experiments suggested that the difference between these two clones in their sensitivity to TNF was related to the time course of cell death. Measurements of the kinetics of cell death in the ME-180.4 and ME-180.8 clones at saturating and subsaturating levels of TNF revealed striking differences in the time required to initiate cell lysis. Significant cell death ($\geqslant$20% cytotoxicity) in the TNF sensitive ME-180.4 clone was detected between 32 and 55 h, whereas the onset of lysis in the ME-180.8 clone was evident at 55–80 h (Fig. 3).The time from the addition of TNF to the appearance of $\geqslant$20% cell death as determined by the MTT assay was defined as the induction phase. The length of the induction phase was dependent upon the dose of TNF for both clones. For example, the ME-180.4 the length of the induction phase was 48 h at 0.3 nM and 32 h at 30 nM (Fig. 3A). When the cells were growth-arrested by overnight preincubation in 0.5 μg/ml mitomycin C, significant cell death in the sensitive ME-180.4 clone was detected by 8 h. In contrast, the ME-180.8 clone did not exceed 20% lysis until approximately 20 h after the addition of TNF (Fig. 4). Mitomycin C also reduced the TNF dose dependency of the length of the induction phase; e.g., the induction phase was approximately 10–12 h at both 0.3 and 30 nM TNF for the ME-180.4 clone. Collectively, these data indicated that these two clones represent variants in the lethal event induced by TNF and that with the LT/TNF-sensitive, gap-, junction-deficient phenotype additional control mechanism(s) exist that modulate the pace of the lethal event(s). The results with mitomycin C, a DNA cross-linking agent, indicate that nuclear processes exert a strong influence on the events associated with cell death.

Previous studies have indicated that fragmentation of DNA occurs in target cells killed by TNF and LT [8,9]. We have also observed DNA degradation in the ME-180 clones as a consequence of TNF treatment using alkaline sucrose gradients (unpublished observations). These results indicate that DNA is a major target of TNF-induced damage and suggested to us that enzymes involved in DNA cleavage may be

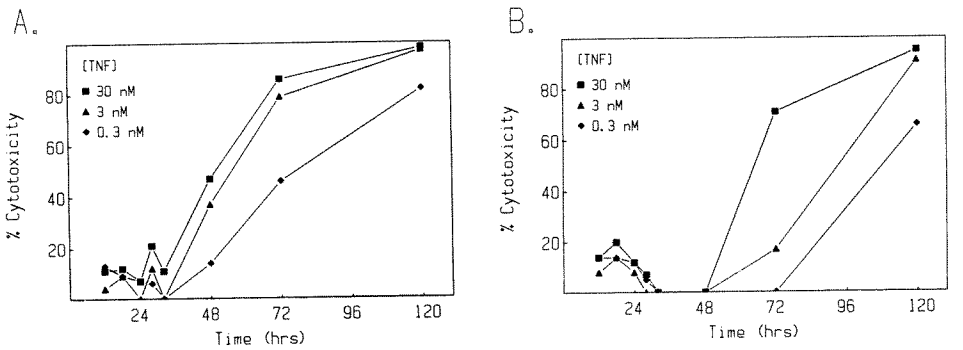

Fig. 3. Kinetics of TNF-mediated cytotoxicity without mitomycin C. ME-180.4 **(A)** and ME-180.8 **(B)** cells were seeded in microtiter plates at 10,000 per well (for the 12–32-h time points), 7,500 per well (48 h) 5,000 per well (72 h), and 1,500 per well (120 h) and allowed to incubate 6 h at 37°C to adhere. Aliquots of TNF were added to give final concentrations of 0.3, 3, and 30 nM, and the plates were incubated for the indicated times at 37°C. Cytotoxicity at each end point was determined by the MTT assay.

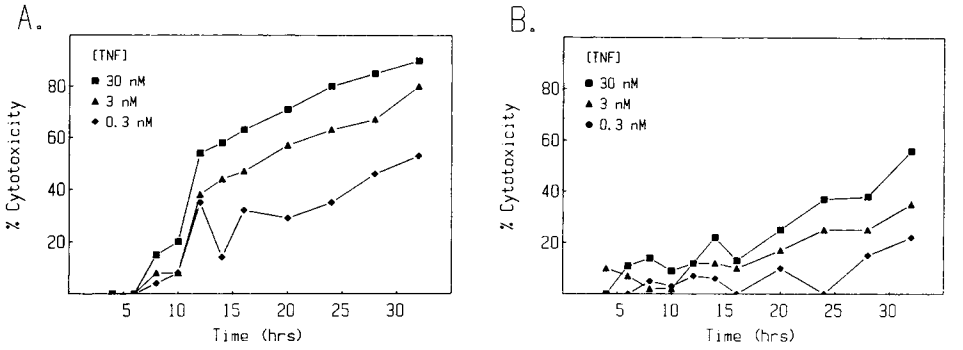

Fig. 4. Kinetics of TNF-mediated cytotoxicity in the presence of mitomycin C. ME-180.4 **(A)** and ME-180.8 **(B)** cells were seeded in microtiter plates at 20,000 per well in the presence of 0.5 μg/ml mitomycin C and allowed to incubate overnight at 37°C. Aliquots of TNF were added to give final concentrations of 0.3, 3, and 30 nM, and the plates were incubated at 37°C for the indicated times. Cytotoxicity at each end point was determined by the MTT assay.

potential sites for TNF action. DNA topoisomerase II is a major nuclear enzyme involved in DNA cleavage and reunion reactions essential for chromatin structure and gene expression and cell division [16,17]. This enzyme has phosphorylation sites, and thus could be regulated by signals generated by hormone-receptor interactions. TNF has also been reported to augment the cytotoxicity of several antitumor drugs which are thought to act on DNA topoisomerases [18]. To investigate the hypothesis that DNA topoinsomerase II may play a role in TNF-induced cytotoxicity, two specific inhibitors of mammalian DNA topoisomerase II, the epipodophyllotoxins VM-26 and VP-16, were tested for their effects on TNF induced cell killing. VM-26 and VP-16 are specific nonintercalating compounds which block enzyme activity by binding to and stabilizing the covalent intermediate complex between DNA and the enzyme, thus effectively inhibiting DNA strand rejoining [19,20]. This leads to extensive DNA fragmentation in VM-26- and VP-16-treated cells [20]. If DNA fragmentation mediated by DNA topoisomerase II is a critical element in the events leading to cell death in TNF-treated cells, then VM-26 or VP-16 should potentiate the cytolytic action of TNF.

The toxicity of these compounds was tested on the ME-180 clones under varying conditions (Table II). At concentrations of < 500 μM VP-16 was not directly cytotoxic for these cells in a 24-h assay; however, VM-26 exhibited toxicity for both the ME-180 clones. Cocentrations of VM-26 below 20–40 μM exhibited less than 10% cytotoxicity for the ME-180 clones. Inhibition of cell division by mitomycin C reduced the observed toxicity of VM-26 in the ME-180.8 clone by fourfold. A marked potentiation of the cytotoxic effects of TNF was observed in the presence of 40 μM of either VM-26 or VP-16 for both clones. This concentration of VP-16 and VM-26 were selected because it is very close to the K_I for inhibition of DNA topoisomerase II activity [20]. The sensitive ME-180.4 clone did not manifest a cytotoxic response to TNF in 24 h in the absence of mitomycin C (see Fig. 3A); however, in the presence of VP-16 or VM-26 an ID_{50} for TNF was measurable within 24 h. In the presence of mitomycin C the dose response to TNF was reduced by nearly ten fold in both clones

TABLE II. Toxicity and Enhancement of TNF Cytotoxicity by DNA Topo-Isomerase II Inhibitors VM-26 and VP-16*

		ID_{50} (μM)	
	Mitomycin C	VP-16	VM-26
A. Toxicity of VP-16 and VM-26			
ME-180.4	−	>500	80
	+	>500	100
ME-180.8	−	>500	60
	+	>500	250

		TNF ID_{50} (pM)		
	Mitomycin C	TNF	TNF + VP-16	TNF + VM-26
B. Enhancement of TNF cytotoxicity				
ME-180.4	−	—[a]	300	20
	+	60	6	≤1
ME-180.8	−	—[a]	nd[b]	nd[b]
	+	2,500	210	20

*ME-180.4 and ME-180.8 cells were added to 96-well (2×10^4 per well) mitotiter plates in the presence or absence of 0.5 μg/ml mitomycin C and allowed to incubate overnight at 37°C. **A:** Serial dilutions of VP-16 or VM-26 were prepared in medium in separate microtiter plates and 100-μl aliquots were transferred to cells and cultured for 24 h at 37°C (total volume was 200 μl). Following the incubation the percentage of cytotoxicity was determined by the MTT dye reduction assay as described in Materials and Methods. ID_{50} values were determined by linear regression analysis with at least 6 points in each titration curve. **B:** Serial dilutions of TNF or TNF mixed with either VP-16 or VM-26 (40 μM final concentration) in medium were added to cells and incubated for 24 h, and the percentage of cytotoxicity was determined as described above.

[a]ID_{50} values were not achieved in 24-h assay at 30 nM TNF.

[b]Not determined.

by VP-16 and by nearly 100-fold for VM-26. These results suggested that a combination of TNF with either VP-16 or VM-26 acted in a synergistic fashion.

To extend the quantitative aspects of the effects of these compounds on cell killing simultaneous dose titrations of combinations of TNF at different molar ratios with either VP-16 or VM-26 were performed and analyzed by an isobologram (Fig. 5). The treatments were performed in the presence of mitomycin C in order to have a detectable cytotoxic effect by both agents in a 24-h assay (a prerequisite for defining the term synergy) [see reference 14]. The data for both inhibitors on either cell line fall below the diagonal line (which defines the point of additive effects between two agents), indicating a synergistic effect between TNF and VM-26 or VP-16 at all doses tested. VM-26 showed a 5–10-fold greater effect than the VP-16 on both clones as measured by their interaction index. The effects of both drugs on TNF cytotoxicity was significantly more pronounced on the resistant ME-180.8 clone.

The basis for the synergistic effects of the DNA topoisomerase II inhibitors on TNF-induced killing was investigated by examining the effect of VM-26 on the kinetics of cell death. VM-26 (40 μM) when added to the TNF resistant ME-180.8 clone abolished the kinetic difference with the ME-180.4 cells and enhanced the level of killing such that the resulting time course of cell death was indistinguishable from that of the sensitive subclone (Fig. 6). VM-26 did not significantly alter the kinetics of lysis of the ME-180.4 clone (data not shown). Mitomycin C was included in this experiment to decrease the overall time course of cell death while maintaining the

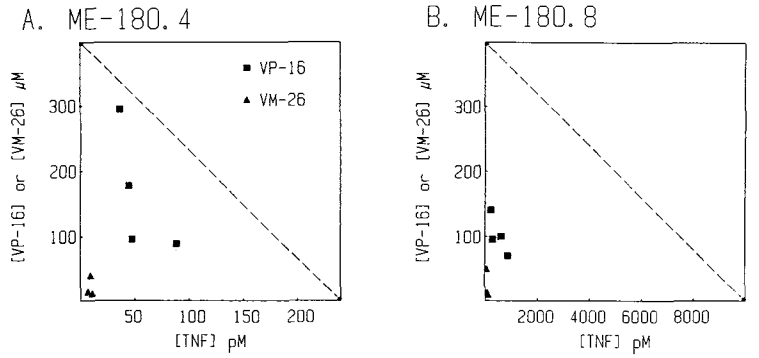

Fig. 5. Isobologram depicting synergistic interactions between TNF and VP-16 or VM-26. Isobolograms were constructed from dose-response curves of TNF in combination with different concentrations of VP-16 or VM-26 as described in Materials and Methods. Cytotoxicity as measured by MTT assay was assessed after an 18-h incubation on **(A)** the ME-180.4 or **(B)** the ME-180.8 clone.

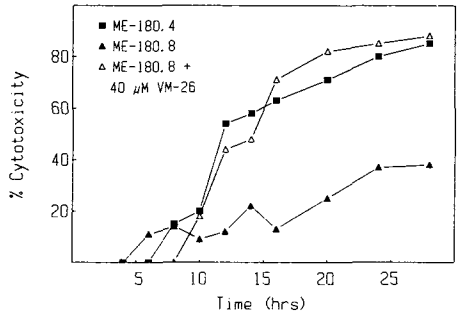

Fig. 6. Effect of VM-26 (teniposide), a DNA toposisomerase II inhibitor, on the time course of TNF-medicated killing of ME-180.8 cells. The kinetic assay was performed as described in Figure 4, except that the aliquots added to the ME-180.8 cells contained both TNF and VM-26, and gave final concentrations of 30 nM TNF and 40 μM VM-26. This concentration of VM-26 showed negligible (< 15%) cytotoxicity at 24 h when assayed under the same conditions without TNF. For comparison, the time courses of ME-180.4 and ME-180.8 killing by 30 nM TNF alone are also presented.

relative kinetic difference between the clones (see Fig. 4) and because the toxicity of VM-26 was less than 15% at 40 μM.

DISCUSSION

We have analyzed two clones of the ME-180 cell line which appear to represent kinetic variants in the lethal event induced by TNF. The major difference distinguishing the TNF-sensitive ME-180.4 clone from its resistant counterpart, ME-180.8, was the length of the induction phase (defined as the length of time following the addition of toxin to the observed onset of cell death). This contention is supported by the kinetics of cell death (Figs. 3, 4) and the similarity of the ID_{50} when measured over longer periods (compare Fig. 1 with Fig. 2). The Kd and the number of TNF receptors expressed on these two clones were nearly identical, indicating that neither differences in receptor expression nor receptor affinity could account for the differ-

ence between these two clones. Both clones were gap junction deficient as measured by fluorescent dye transfer, ruling out differences in intercellular communicative ability as the site of their difference in response to TNF.

The effect of mitomycin C on the kinetics of cell lysis was twofold: it decreased the length of the induction phase for both clones without changing the overall kinetic differences between them and diminished the dose-dependent differences in the length of the induction phase. For proliferating cells increasing concentrations of TNF induced a higher level of cytotoxicity, and the length of time to the onset of cell death (induction phase) was decreased at higher levels of TNF (Fig. 3). Mitomycin C treatment decreased the length of the induction phase for both clones: the ME-180.4 induction phase went from 32 h (30 mM TNF) to 10 h, and the ME-180.8 induction phase decreased from 55 h to 20 h. This effect has been seen in other cell lines treated with antiproliferative agents; for example, L929 fibrosarcoma cells showed a similar decrease in the induction phase when treated with mitomycin^3 [21] or actinomycin D [22]. These results are intriguing as they directly link agents which modulate the structure of DNA, such as the covalent cross-linking of DNA by mitomycin C, to the mechanism of TNF cytotoxicity. However, the retention of kinetic differences between the ME-180.4 and ME-180.8 clones after mitomycin C treatment indicates that DNA cross-linking alone was insufficient to overcome the kinetic difference(s) in the TNF cytotoxic responses between these two cell lines.

The link between nuclear processes and TNF cytotoxicity was further strengthened by the enhancing effects of the specific DNA topoisomerase II inhibitors VP-16 and VM-26. For both cell lines, concentrations of these inhibitors which had little or no cytotoxicity by themselves reduced the TNF ID_{50} values from 10- to 100-fold in 24 h assays (Table II; Fig. 5). VM-26 showed greater synergy with TNF and reduced the ID_{50} values by 5–10-fold more than an equivalent concentration of VP16. The synergistic effect was more pronounced on the ME-180.8 cells than the ME-180.4 cells for both VP-16 and VM-26.

The crucial role of DNA topoisomerase II activity in the kinetic variance between the ME-180.4 and ME-180.8 cell lines was demonstrated by the abolishment of this difference in the presence of VM-26 (Fig. 6). In separate experiments, VM-26 did not alter the length of the induction phase of the ME-180.4 cells (data not shown), suggesting a minimum duration for the induction phase which was demonstrated by the time course of cell death of the ME-180.4 cells. It should be emphasized that the sensitivity towards TNF was enhanced for both ME-180.4 and ME-180.8 by the topoisomerase II inhibitors, while only the ME-180.8 cells show a change in the kinetics of cell death. The enhanced synergy of both inhibitors on the ME-180.8 cell line (as compared to the ME-180.4 cell line) see in 24-h cytotoxicity assays can be explained by this kinetic shift. These results strongly suggest a major role for DNA topoisomerase II in the process of TNF-induced cytoxicity.

DNA strand breakage is a common observation among several forms of receptor-mediated cell death, including corticosteroid induced lysis of thymocytes, cell killing mediated by cytotoxic T lymphocytes and LT/TNF [8,9,23–25]. The epipodophyllotoxins, VP-16 and VM-26, appear to induce cytotoxicity by potentiating DNA strand breaks through inhibition of the DNA topoisomerase II strand rejoining activity [9]. Insufficient information currently exists on the DNA cleavage induced by TNF to compare it with those changes seen with these topoisomerase inhibitors. The results presented here showing the synergistic interaction between TNF and VP-

16 or VM-26 indicate the mechanism of synergy between the epipodophyllotoxins and TNF may be through their ability to induce DNA strand breakage. The extent of DNA fragmentation would be expected to be enhanced by a combination of TNF with this class of DNA topoisomerase II inhibitors which act by preventing the DNA rejoining reaction. In contrast, an inhibitor of the DNA cleavage reaction mediated by topoisomerase II, such as novobiocin, might be expected to decrease the cytotoxic activity of TNF. Novobiocin has been shown to block DNA strand breakage induced by VP-16 and VM-26 in vitro and in vivo [26] by inhibiting the ATPase activity on topoisomerase II, thereby blocking the initial DNA strand breaks caused by the enzyme. Our preliminary studies with novobiocin on murine L929 target cells showed a dramatic ability to decrease TNF cytotoxicity, suggesting that the DNA strand breaks induced by TNF are mediated at least in part by topoisomerase II. Experiments currently in progress will determine whether protection against TNF cytotoxicity by novobiocin correlates with a decrease in TNF-induced DNA damage.

ACKNOWLEDGMENTS

The authors would like to thank Dr. W. Fletcher and Ms. T. Ishida (Molecular Cytology Laboratory, J.L. Pettis Memorial Veterans Hospital, Loma Linda, CA) for their technical assistance in measuring dye transfer and Dr. Dale Stringfellow, Bristol Myers Company, for providing the inhibitors of DNA topoisomerase II. Mr. P. Breton is gratefully acknowledged for word processing. This work was supported by PHS grant CA35638 (C.F.W.), PHS Biomedical Research Support grant RR05816, and NIH postdoctoral training grant AM07310 (F.D.C.).

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