The Cytotoxicity of Tumor Necrosis Factor Depends on Induction of the Mitochondrial Permeability Transition*

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Complete prevention of the killing of L929 fibroblasts by tumor necrosis factor α (TNF) in the presence of 0.5 μg/ml actinomycin D (ActD) was obtained with cyclosporin A (CyA), an inhibitor of the mitochondrial permeability transition (MPT), and aristolochic acid (ArA), a phospholipase A₂ inhibitor. Peripheral benzodiazepine receptor (PBzR) agonists (PK11195, FGIN 1–27, or chlorodiazepam), agents known to potentiate induction of the MPT, potentiated the cytotoxicity of TNF in the absence of ActD, an effect prevented by CyA plus ArA. The MPT was demonstrated independently of its effect on viability as the CyA-sensitive loss of rhodamine 123 fluorescence from cells preloaded with the dye. Treatment with TNF and ActD resulted in the loss of 80% of rhodamine fluorescence within 6 h, a time prior to any loss of viability. CyA plus ArA completely prevented this effect of TNF. Potentiation of the cytotoxicity of TNF by PBzR agonists was associated with induction of the MPT, as assessed by the loss of rhodamine fluorescence. CyA plus ArA completely prevented the loss of rhodamine 123. Ceramide replaced TNF in killing L929 fibroblasts, an effect also prevented by CyA plus ArA. Ceramide in the presence of ActD resulted in the loss of rhodamine fluorescence, an effect that was again prevented by CyA plus ArA. In addition, CyA plus ArA prevented the ability of PBzR agonists to potentiate the cytotoxicity of ceramide. In the presence of each PBzR agonist, ceramide caused the loss of rhodamine fluorescence, an effect completely prevented by CyA plus ArA. D609, an inhibitor of phosphatidylcholine-specific phospholipase C, completely prevented the killing by TNF, but not by ceramide, in the presence of ActD. D609 prevented induction of the MPT occurring with TNF, but not with ceramide. Inhibitors of endocytosis, as well as lysosomotropic amines, prevented the cytotoxicity of TNF, but not that of ceramide. It is concluded that the MPT is causally linked to the genesis of irreversible cell injury with TNF. In the face of an inhibition of protein synthesis, the MPT occurs as a consequence of the formation of ceramide.

Tumor necrosis factor α (TNF)¹ is a pleiotropic cytokine, which is produced primarily by activated macrophages and lymphocytes (1). Known to induce physiologic effects in a variety of cells and tissues, TNF is also implicated in the pathogenesis of certain diseases, most notably septic shock. In addition, TNF kills cancer cells in intact animals and a variety of cell lines in vitro. Although attributed to both apoptosis and necrosis, the biochemical basis of the cytotoxic action of TNF is still largely unknown. The difficulty in unraveling the mechanism of cell killing lies in the fact that TNF activates many signaling molecules and second messengers, including phospholipases, kinases, phosphatases, oxygen radicals, and transcription factors (2).

The L929 line of mouse fibroblasts has been widely used to explore the mechanism of the cytotoxicity of TNF. In these cells, the signaling pathways initiated by TNF lead to a death that is better characterized as necrosis rather than apoptosis (3, 4). An alteration in mitochondrial structure and function with the resultant formation of reactive oxygen species seems to be an important step in the cytotoxic mechanism of TNF (5–9). The reported ability of antioxidants to protect against TNF cytotoxicity supports this hypothesis (10–13). Nevertheless, the specific nature of the mitochondrial alteration induced by TNF and how it relates, in turn, to the initial signal transduction events remain to be defined.

The mitochondrial permeability transition (MPT) is the regulatable opening of a large, nonspecific pore in the inner mitochondrial membrane (reviewed in Refs. 14–17). Although the molecular elements that form this pore have not been definitively established, they are presumed to derive from well known membrane constituents, including the adenine nucleotide translocator, porin molecules, and the complex forming the peripheral benzodiazepine receptor (14–17). The MPT is a critical event in the killing of cultured hepatocytes that follows the inhibition of electron transport by anoxia, rotenone, cyanide, or N-methyl-4-phenylpyridinium (18, 19). Cyclosporin A (CyA) prevents induction of the MPT in isolated mitochondria (20, 21). Similarly, CyA prevented the MPT observed in intact cells made anoxic or treated with rotenone (22). In turn, CyA prevented the killing of hepatocytes by anoxia, rotenone, or cyanide (18, 22).

In the present study, we have utilized the L929 line of mouse fibroblasts to document that the MPT is an essential event in the pathogenesis of the lethal cell injury induced by TNF. In addition, we provide an account that ceramide is an important

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¹ The abbreviations used are: TNF, tumor necrosis factor α; MPT, mitochondrial permeability transition; CyA, cyclosporin A; DMEM, Dulbecco’s modified Eagle’s medium; PBS, calcium-free/magnesium-free phosphate-buffered saline; CCCP, carbonyl cyanide m-chlorophenylhydrazine, ActD, actinomycin D; ArA, aristolochic acid; PBzR, peripheral benzodiazepine receptor; PhAsO, phenylarsine oxide; PC-PLC, phosphatidylcholine-specific phospholipase C; DAG, diacylglycerol.
part of the signal transduction pathway by which TNF leads to
induction of the MPT.

MATERIALS AND METHODS

The L929 line of mouse fibroblasts (ATCC-CCL-1, American Type
Culture Collections) was maintained in 25-cm² polystyrene flasks
(Corning Costar Corp., Oneonta, NY) with 5 ml of Dulbecco’s modified
Eagle’s medium (DMEM) (high glucose; without pyruvate) (Life
Technologies, Inc.), containing 100 units/ml penicillin, 0.1 mg/ml streptomycin,
and 10% heat-inactivated fetal bovine serum and incubated under
an atmosphere of 95% air, 5% CO₂. All experiments were performed 2
days after plating 1.0 × 10⁵ cells in 500 μl of the above medium into
1.88 cm² wells of a 24-well microtiter plate (Corning Costar). By
the second day, the cells were growing exponentially and had achieved a
density of 2.5–3.0 × 10⁶ cells/well. Prior to treatment, fibroblasts were
washed twice with Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS),
after which 500 μl of DMEM without serum was added to the wells.

Cells were pretreated for 30 min with one or more of the following
chemicals. D609 (Biomol Inc.) and cyclosporin A (Sandoz, 50 mg/ml in
cremophore) were dissolved in deionized, pyrogen-free water and added
to wells in a 0.2% volume for a final concentration of 50 μg/ml and 5 μM,
respectively; aristolochic acid (Biomol) was dissolved in PBS and added in
and added in a 0.2% volume for a final concentration 50 μM; rotenone, antimycin A,
and colchicine (Sigma) were dissolved in dimethyl sulfoxide and added
to wells in a 0.5% volume for a final concentration 25 μM, 50 μM, and 1 mM,
respectively. Oligomycin and monensin (Sigma) were dissolved in
Me₂SO and added in a 0.2% volume for a final concentration of 0.1
μg/ml and 10 μM, respectively. Ammonium chloride (Sigma) was
dissolved in deionized pyrogen-free water and added in a 0.2% volume
for a final concentration of 10 mM.

Thirty minutes following treatment with the above chemicals,
TNF + ActD or ceramide + ActD was added. TNF (Sigma; 22 units/ng)
dissolved in PBS and added to the wells in 0.2% volume at the final
concentration indicated in the text. C₂-ceramide or C₂-dihydroceramide
(Biomol) was dissolved in Me₂SO and added in a 0.2% volume to give a
final concentration of 6 μM. ActD (Sigma) was dissolved in Me₂SO,
further diluted in PBS, and added to wells in 0.2% volume to a final
concentration of 0.5 μM. Where indicated in the text, the cells were
treated at the same time they were given either TNF or ceramide with
one of the following peripheral benzodiazepine receptor (PBzR) ago-
nists. PK11195, FG13 1–27, and chlorodiazepam (Research Biochemi-

tical) were dissolved in ethanol and added to the wells in a
0.2% volume for a final concentration of 10, 25, and 50 μM, respectively.
Clonazepam (Sigma) was dissolved in Me₂SO and added in a 1% volume
for a final concentration of 100 μM.

Cell viability was determined at the times indicated in the text by the
release of lactate dehydrogenase into the culture medium as described
previously (23). Cell viability measured in this manner correlated
closely with that determined by the uptake of propidium iodide or
trypan blue (data not shown). The data from triplicate wells for each
experimental point were averaged to obtain a single value for each point
in each experiment. All experiments were repeated three times. Protein
synthesis was determined by the incorporation of [³H]leucine into an
acid-insoluble precipitate as described previously (24).

The MPT was demonstrated in intact cells as a CyA-sensitive loss of
rhodamine 123 fluorescence. Cells in a 24-well microtiter plate were
washed twice with PBS and returned to DMEM without serum. Rho-
damine 123 from a 500 μM stock solution in water was added to give a
final concentration of 5 μM. The cells were incubated at 37 °C for 1 h,
then washed twice with PBS and placed in fresh DMEM without serum.
The cells were treated as described in the text below, after which
the medium was removed by aspiration and the cells washed again twice
with PBS. Digitonin (Sigma) was dissolved in H₂O and added in a 0.2% volume
to give a final concentration of 7.5 μM. CCCP (Sigma) was
dissolved in Me₂SO and added in a 0.2% volume to give a final concen-
tration of 10 μM. Phenylarsene oxide (PhAsO) (Sigma) was dissolved in
Me₂SO and added in a 0.2% volume to give a final concentration of 100
μM. The cells were covered with a solution of 0.05% trypsin in PBS.
Following aspiration of the trypsin, the cells were collected in 500 μl of
PBS, transferred to a microcentrifuge tube, and centrifuged at 700 × g
for 5 min. Following aspiration of the supernatant, the cell pellet was
resuspended in 600 μl of PBS. The cell suspension was transferred to a
quartz cuvette, and the fluorescence of cell-associated rhodamine 123
was read in a Perkin-Elmer spectrofluorimeter at 505 nm (excitation)
and 534 nm (emission).

RESULTS

The Killing of L929 Fibroblasts by TNF—Conditions for killing
L929 fibroblasts by TNF are illustrated in Fig. 1. With
these cells, the cytotoxicity of TNF does not depend on RNA or
protein synthesis; rather, it is enhanced by inhibitors of tran-
scription or translation (25). Accordingly, doses of TNF from 0.1
to 1.5 ng/ml did not lethally injure the fibroblasts for at least
14 h (Fig. 1A). However, in the presence of 0.5 μg/ml ActD, the
same doses of TNF killed an increasing proportion of the
fibroblasts. With 1.5 ng/ml TNF, almost 80% of the cells died within
14 h. The time course of the killing of L929 fibroblasts by 1
ng/ml TNF in the presence of ActD is shown in Fig. 1. Dead
cells were first detected between 6 and 8 h, and their number
increased steadily between 8 and 14 h. Cycloheximide (1 μM)
similarly sensitized L929 fibroblasts to the cytotoxicity of TNF
(data not shown).

Prevention by Cyclosporin A of the Cytotoxicity of TNF—The cell killing by TNF (in the presence of ActD or cycloheximide)
depends on induction of the MPT. Three different criteria were
used to document the participation of the MPT in the cytotoxic-
ity of TNF: 1) prevention of the cell killing by CyA, an inhib-
itor of the MPT; 2) potentiation of the cytotoxicity of TNF by...
Cytotoxicity of TNF

**Fig. 2. Prevention by cyclosporin A of the cytotoxicity of TNF and ceramide.** Where indicated, the cells were pretreated for 30 min with CyA plus ArA. The cells were treated with 2.0 ng/ml TNF or with 6 μM ceramide in the presence of 0.5 μg/ml ActD. Cell killing was determined 18 h after addition of TNF or ceramide. The data are the mean ± S.D. from three independent experiments.

Peripheral benzodiazepine receptor agonists, agents known to induce the MPT, and 3) measurement of the MPT in L929 cells treated with TNF.

Inhibition by CyA of the MPT induced in isolated mitochondria de-energized by cyanide required the additional presence of a phospholipase A₂ inhibitor (18). Likewise, preservation by CyA of the viability of cultured hepatocytes treated with cyanide also required the presence of a phospholipase A₂ inhibitor (18). Similarly, complete protection against the cell killing by TNF was obtained in the presence of CyA plus aristolochic acid, a phospholipase A₂ inhibitor (26) (Fig. 2). CyA alone decreased the cytotoxicity of TNF by 45% and ArA alone by 35%. Other phospholipase inhibitors in combination with CyA also effectively prevented the cell killing by TNF (in the presence of ActD), including CDP-choline (27), ON-RS-082 (28), 3-(4-octadecyl)benzoylauric acid (29), and trifluoperazine (30) (data not shown).

The ability of CyA to prevent the cytotoxicity of TNF is not a consequence of the its binding to and, thus, inhibition of calcineurin, a calcium-dependent phosphatase. The immunosuppressive drug FK506 similarly inhibits calcineurin, but is inactive against the MPT (16). FK506 alone or in combination with a phospholipase A₂ inhibitor was without effect on the cytotoxicity of TNF (Table I). In addition, cypermethrin, another potent inhibitor of calcineurin, was unable to prevent the cytotoxicity of TNF (Table I).

**Table I**

| Treatment | Dead cells |
|------------|------------|
| TNF and ActD | 97 ± 14 |
| TNF and ActD + 1 μM FK506 and ArA | 98 ± 17 |
| TNF and ActD + 1 μM cypermethrin and ArA | 94 ± 8 |
| TNF and ActD + CyA and ArA | 8 ± 3 |

FK506 and cypermethrin, inhibitors of calcineurin, to prevent the cytotoxicity of TNF

The cells were pretreated with FK506, cypermethrin, or CyA (all in the presence of ArA). Thirty min later, the cells were treated with 2 ng/ml TNF and 0.5 μg/ml ActD. Cell killing was determined 18 h after addition of TNF. The data are the mean ± S.D. from three independent experiments.

(34, 35) potentiates induction of the MPT in isolated mitochondria as well as in intact hepatocytes (36). Table II details the killing of L929 fibroblasts treated with TNF in the presence of PK11195, FGIN 1–27 (37), or chlorodiazepam (38). In this experiment, the cells were not treated with ActD. Whereas TNF alone was again not toxic, almost 90% of the cells died in the combined presence of PK11195 and TNF. CyA plus ArA prevented the cell killing in the presence of TNF and PK11195. Similarly, FGIN 1–27 and chlorodiazepam potentiated the cytotoxicity of TNF (Table II), effects prevented by CyA plus ArA. By contrast, the central benzodiazepine receptor agonist clonazepam was without effect (Table II).

Cycloheximide potentiates the cytotoxicity of TNF as a consequence of an inhibition of protein synthesis. However, the potentiation of the cytotoxicity of TNF by PBzR agonists is not a consequence of a similar inhibition of protein synthesis. Table III shows that neither PK11195, FGIN 1–27, nor chlorodiazepam had an effect on the rate of the incorporation of [³H]leucine into protein at either 6 or 18 h after treatment with each agent. By contrast, treatment of the cells with 0.5 μg/ml ActD resulted in a 90% inhibition of protein synthesis after 6 or 18 h.

**Demonstration of the MPT in L929 Fibroblasts Treated with TNF.**—An assay was developed to demonstrate the MPT in intact L929 fibroblasts independently of the effect of the transition on cell viability. The assay is based on the ability of the fluorescent dye rhodamine 123 to accumulate in the mitochondria as a consequence of the mitochondrial membrane potential. The MPT causes the loss of the mitochondrial membrane potential, resulting in the release of the accumulated rhodamine. PhAsO is a potent inducer of the permeability transition in isolated mitochondria (39). Intact cells, however, are impermeable to PhAsO, but respond to it following their permeabilization with digitonin. Fig. 3 shows that 7.5 μM digitonin had no effect on the rhodamine 123 fluorescence accumulated by cells preloaded with the dye. However, following permeabilization with digitonin, treatment with PhAsO resulted in the loss of 80% of the rhodamine fluorescence, an effect that was completely prevented by CyA (with or without ArA). Digitonin and CyA in the absence of PhAsO increased the fluorescence yield 25% compared with cells treated with digitonin alone. The basis for this increase is not known, but it may reflect inhibition by CyA of transient, physiological pore openings that reduce rhodamine retention. In any case, these data show that the CyA-inhibitable marked loss of rhodamine fluorescence can document the MPT under conditions where the loss of the mitochondrial membrane potential is a consequence rather than a cause of the transition.

Fig. 4 details the effect of TNF (in the presence of 0.5 μg/ml ActD) on the fluorescence of rhodamine-labeled cells. Treatment with TNF resulted in the loss of 40% of the rhodamine fluorescence within 2 h and 80% within 6 h. CyA plus ArA
Potentiation of the cytotoxicity of TNF by peripheral benzodiazepine receptor agonists

Where indicated the cells were pretreated with 5 \(\mu\)M CyA and 50 \(\mu\)M ArA for 30 min. All cells were treated with 2 ng/ml TNF and a peripheral benzodiazepine receptor agonist as indicated. The extent of cell killing was determined 18 h later. The data are the mean \(\pm\) S.D. from three independent experiments.

| Treatment | Dead cells |
|-----------|------------|
| TNF alone | \(4 \pm 1\) |
| TNF + 10 \(\mu\)M PK11195 | \(88 \pm 15\) |
| TNF + PK11195 + CyA and ArA | \(5 \pm 2\) |
| TNF + 25 \(\mu\)M FGIN 1–27 | \(79 \pm 5\) |
| TNF + FGIN 1–27 + CyA and ArA | \(9 \pm 8\) |
| TNF + 50 \(\mu\)M chlorodiazepam | \(69 \pm 5\) |
| TNF + chlorodiazepam + CyA and ArA | \(13 \pm 7\) |
| TNF + 100 \(\mu\)M clonazepam | \(7 \pm 1\) |

Inability of peripheral benzodiazepine receptor agonists to inhibit protein synthesis in L929 fibroblasts

L929 fibroblasts were treated with PK11195, chlorodiazepam, FGIN 1–27, or ActD. Protein synthesis was measured 6 and 18 h after the respective treatments. Untreated control cells incorporated 50,119 \(\pm\) 2590 dpm \[^{3}\text{H}]\text{leucine/mg of protein. The data represent the mean \(\pm\) S.D. from three separate experiments.}

| Treatment | % of control |
|-----------|-------------|
| 10 \(\mu\)M PK11195 | 94 \(\pm\) 6 |
| 25 \(\mu\)M FGIN 1–27 | 97 \(\pm\) 8 |
| 50 \(\mu\)M chlorodiazepam | 100 \(\pm\) 9 |
| 0.5 \(\mu\)g/ml ActD | 8 \(\pm\) 7 |

Cytotoxicity of TNF

A lipid ceramide has been implicated as a second messenger in various pathways of TNF signal transduction (40, 41). In the presence of ActD, 6 \(\mu\)M ceramide killed L929 fibroblasts (see Fig. 2). In the absence of ActD, this dose of ceramide was not toxic. An inactive analogue of ceramide, dihydroceramide, did not kill fibroblasts in the presence of ActD (9 \(\pm\) 2% cells died over 18 h). The cell killing by ceramide depended on induction of the MPT, as assessed by the same criteria used in the case of TNF.

Ceramide Substitutes for TNF in Killing L929 Fibroblasts—The lipid ceramide has been implicated as a second messenger in various pathways of TNF signal transduction (40, 41). In the presence of ActD, 6 \(\mu\)M ceramide killed L929 fibroblasts (see Fig. 2). In the absence of ActD, this dose of ceramide was not toxic. An inactive analogue of ceramide, dihydroceramide, did not kill fibroblasts in the presence of ActD (9 \(\pm\) 2% cells died over 18 h). The cell killing by ceramide depended on induction of the MPT, as assessed by the same criteria used in the case of TNF.

CyA plus ArA completely prevented the cell killing by ceramide (see Fig. 2). Treatment with ceramide and ActD resulted in a time-dependent loss of rhodamine fluorescence from the fibroblasts (Fig. 7). More than 80% of the rhodamine was lost from the cells within 6 h. Again, CyA plus ArA completely prevented the loss of rhodamine fluorescence caused by ceramide and ActD (Fig. 7).

\[ \text{FIG. 3. Induction by PhAsO of the MPT in L929 fibroblasts permeabilized with digitonin.} \]
PBzR agonists potentiated the cytotoxicity of ceramide. PK11195, FGIN 1–27, and chlorodiazepam potentiated the cytotoxicity of ceramide in the absence of ActD (Table IV). In each case, CyA plus ArA prevented the loss of viability with ceramide in the presence of a PBzR agonist (Table IV). Finally, with each PBzR agonist, ceramide caused the loss of rhodamine 123 fluorescence from the fibroblasts, an effect that was completely prevented by CyA plus ArA (Table IV).

Ceramide is generated as a consequence of the hydrolysis of sphingomyelin by either a neutral or an acidic sphingomyelinase (42). In the case of the acidic sphingomyelinase, binding of TNF to its 55-kDa cell surface receptor activates a phosphatidylinositol-specific phospholipase C, a plasma membrane enzyme that hydrolyzes phosphatidylinositol to yield phosphorylcholine and 1,2-diacylglycerol (40, 42–44). Following binding of TNF to the cell surface receptor and the activation of PC-PLC, the receptor complex is internalized within an endosomal vesicle (45) by an energy-dependent mechanism, which also acidifies the vesicle (46). Once activated by DAG and in the acidic milieu required for optimal activity, acidic sphingomyelinase liberates ceramide (42, 43).

The xanthate D609 specifically inhibits PC-PLC (42, 47). Fig. 8 shows that D609 completely prevents the killing of L929 fibroblasts by TNF in the presence of ActD. Whereas over 90% of the cells died within 18 h of exposure to TNF and ActD, only 15% of the cells died over the same time course in the presence of 50 μg/ml D609. By contrast, D609 did not prevent the cell killing by ceramide in the presence of ActD (Fig. 8). In the absence of ActD, ceramide and D609 were not toxic.

D609 prevented the induction of the MPT in fibroblasts treated with TNF and actinomycin D (ActD), as shown by the ability of D609 to prevent the loss of rhodamine fluorescence (Fig. 8). By contrast, D609 did not prevent the loss of rhodamine fluorescence that occurred in fibroblasts treated with ceramide and ActD. Finally, Fig. 8 indicates that ceramide alone (in the absence of ActD) did not induce the MPT, a result indicating that with ceramide, as with TNF, the effect of ActD is to promote the permeability transition. Importantly, CyA plus ArA still prevented both the loss of viability and the MPT in cells treated with ceramide and D609 (data not shown). In other words,
D609 did not change the mechanism of cell killing by ceramide. Metabolic inhibitors that deplete the fibroblasts of ATP can interfere with receptor-mediated endocytosis, as well as prevent the acidification of endosomal vesicles. Accordingly, rotenone, oligomycin, or antimycin A substantially reduced the cytotoxicity of TNF (Table IV). As receptor-mediated endocytosis also depends upon intact microtubules (46), the depolymerization of microtubules by colchicine similarly prevented TNF-induced cell killing (Table V). Lysosomotropic agents such as monensin and monensin protect against the cytotoxicity of TNF (Table V). Importantly, the cell killing by ceramide was not affected by rotenone, oligomycin, or antimycin A, colchicine, monensin, or ammonium chloride (Table V).

FIG. 7. Induction of the MPT in fibroblasts treated with ceramide in the presence of ActD. The cells were preloaded with rhodamine 123, washed, and treated as shown. Where indicated, 5 μM CyA and 50 μM ArA were added 30 min prior to 6 μM ceramide and 0.5 μg/ml ActD. At the times indicated, the content of rhodamine in the cells was determined as described under “Materials and Methods.” The data are the mean ± S.D. from three independent experiments as described.

**TABLE IV**

Potentiation of ceramide cytotoxicity and induction of the mitochondrial permeability transition by benzodiazepine receptor agonists

Where indicated the cells were pretreated with 5 μM CyA and 50 μM ArA for 30 min. All cells were treated with 6 μM ceramide with or without the PBzR agonist indicated. The extent of cell killing was determined 18 h later. For determination of the MPT, the cells were preloaded with rhodamine 123, washed, and treated as shown. After 6 h, the content of rhodamine in the cells was determined as described under “Materials and Methods.” The data are the mean ± S.D. from three independent experiments.

| Treatment | Dead cells (18 h) % | Rhodamine retention (6 h) % | % control |
|-----------|---------------------|----------------------------|-----------|
| Ceramide  | 5 ± 1               | 108 ± 15                   | 15 ± 8    |
| + 10 μM PK11195 | 82 ± 2             | 15 ± 8                     | 8 ± 3     |
| + PK11195 + CyA and ArA | 8 ± 3 | 119 ± 19                 | 18 ± 8    |
| + 25 μM FGIN 1–27 | 77 ± 8            | 18 ± 8                     | 7 ± 2     |
| + FGIN + CyA and ArA | 75 ± 7            | 92 ± 7                     | 9 ± 2     |
| + 50 μM chlorodiazepam | 71 ± 10           | 16 ± 9                     | 8 ± 6     |
| + Chlorodiazepam + CyA and ArA | 8 ± 6 | 121 ± 16                | ND*       |
| + 100 μM clonazepam | 12 ± 5            | ND*                        | ND*       |

*Not determined.

**TABLE V**

Prevention of the cytotoxicity of TNF but not that of ceramide by inhibitors of endocytosis and lysosomotropic amines

The cells were pretreated with the either rotenone, oligomycin, antimycin A, colchicine, monensin, or ammonium chloride as described under “Materials and Methods.” Thirty min later, the cells were treated with 2 ng/ml TNF and 0.5 μg/ml ActD or with 6 μM ceramide and ActD. Cell killing was determined 18 h after addition of TNF or ceramide. The data are the mean ± S.D. from the average of duplicate determinations for each point from three separate experiments.

| Treatments | Dead cells |
|------------|------------|
| TNF + ActD | Ceramide + ActD |
| %          | %          |
| No additions | 88 ± 4 | 84 ± 4 |
| 25 μM rotenone | 24 ± 5 | 84 ± 1 |
| 0.1 μg/ml oligomycin | 28 ± 3 | 86 ± 4 |
| 50 μM antimycin A | 25 ± 6 | 85 ± 8 |
| 1 μM colchicine | 13 ± 5 | 85 ± 8 |
| 10 μM monensin | 21 ± 2 | 80 ± 7 |
| 10 mM ammonium chloride | 26 ± 6 | 82 ± 9 |

**DISCUSSION**

The data presented above document that the MPT is an essential feature of the mechanism of the cytotoxicity of TNF. Evidence from three different studies supports this conclusion. CyA plus ArA, which inhibits the MPT, prevented the cytotoxicity of TNF (Fig. 2). Peripheral benzodiazepine receptor agonists, agents that potentiate induction of the MPT, potentiated the cytotoxicity of TNF, an effect that was prevented by CyA plus ArA (Table II). Finally, the MPT in response to TNF was assessed in intact fibroblasts as the CyA-sensitive loss of rhodamine 123 fluorescence (Fig. 4). In all cases, TNF-induced cell death was correlated with MPT occurrence.

Alternative explanations, other than an effect on the induction of the MPT for the protection afforded by CyA and the potentiation occurring with PBzR agonists, were ruled out. An interpretation of the protective effect of CyA as a consequence of an inhibition of calcineurin was excluded by the inability of two other inhibitors of this enzyme, FK506 and cypermethrin, to prevent the cell killing by TNF. Similarly, an interpretation...
of the potentiation by PBzR agonists as a consequence of the inhibition of protein synthesis was excluded by the inability of PBzR agonists to prevent the incorporation of $[^3]H$leucine into protein. Thus, the data in this report provide compelling evidence that the MPT is causally linked to the genesis of irreversible injury with TNF in L929 fibroblasts.

Whereas the specific mechanism whereby the MPT develops in response to TNF is not known, the data presented here suggest that the generation of ceramide plays a role. Ceramide replaced TNF in both inducing the MPT (Fig. 7) and in killing L929 fibroblasts (Fig. 2). As with TNF, the cytotoxicity of ceramide was potentiated by PBzR agonists (Table II) and prevented by CyA plus ArA (Fig. 2). Finally, the data presented here are consistent with the mechanism whereby ceramide is generated by the activation of an acidic sphingomyelinase by DAG, which was formed, in turn, as a result of activation of PC-PLC. An inhibitor of PC-PLC prevented the cytotoxicity of TNF, but not that of ceramide (Fig. 8). Inhibitors of either receptor-mediated endocytosis or endosomal acidification prevented the cytotoxicity of TNF, but again not that of ceramide (Table V). These data are consistent with the scenario whereby the binding of TNF to its 55-kDa surface receptor activates PC-PLC, with the resultant formation of DAG. After the TNF-receptor complexes are actively internalized within acidic endosomal vesicles, DAG activates acidic sphingomyelinase. As a consequence, sphingomyelin is hydrolyzed, releasing ceramide, an event that leads, in turn, to induction of the MPT.

The mechanism of the cytotoxicity of TNF proposed here is consistent with previous observations utilizing L929 mouse fibroblasts. In particular, we confirm that mitochondrial inhibitors modulate the cytotoxicity of TNF (7). However, our data imply that the mechanism whereby such agents act is different from that proposed previously (7). Rotenone, oligomycin, and antimycin A prevent the cell killing by TNF (Table V). By depleting the cells of ATP, these agents prevent receptor-mediated endocytosis and, thus, prevent the formation of ceramide, the metabolic responsible for promoting induction of the MPT. Importantly, the inability of these same inhibitors to prevent the cell killing by ceramide (Table I) argues that a perturbation of mitochondrial function that leads to the formation of reactive oxygen intermediates (7) is not the primary mechanism of their protective effect. The alternative conclusion that ATP depletion is the relevant consequence of the action of these inhibitors also accounts for the recent observation that glutamine starvation protects against the cytotoxicity of TNF (48). In L929 fibroblasts, glutamine is the major energy source that drives ATP formation. Nevertheless, it is noteworthy that oxidants are potent inducers of the MPT (25), and antioxidants protect against the cytotoxicity of TNF (10–13). Thus, it remains to be determined whether ceramide promotes the MPT by increasing the flux of activated oxygen species, or alternatively, whether such species form as a consequence of the MPT induced by ceramide by a mechanism unrelated to these reactive oxygen intermediates.

Finally, it deserves emphasis that the cytotoxicity of ceramide, like that of TNF, depends on the presence of either ActD or cycloheximide. According to our hypothesis, ceramide acts to promote induction of the MPT, an event linked to the loss of cell viability by a mechanism that remains to be defined. The MPT does not occur with TNF or ceramide alone. Accordingly, the action of ActD or cycloheximide must be to modulate the induction of the MPT by ceramide. ActD or cycloheximide most likely prevents the synthesis of a protective protein constitutively present in L929 fibroblasts that turns over rapidly or of a protein that is induced by TNF. Clearly, the induction of such a protein by TNF would allow cells to react to this cytokine without loss of viability, and the loss of such a response could readily account for the sensitivity of cancer cells to the cytotoxicity of TNF.