Cytotoxic Activity of Tumor Necrosis Factor Is Mediated by Early Damage of Mitochondrial Functions

EVIDENCE FOR THE INVOLVEMENT OF MITOCHONDRIAL RADICAL GENERATION*

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Structural mitochondrial damage accompanies the cytotoxic effects of several drugs including tumor necrosis factor (TNF). Using various inhibitors of mitochondrial electron transport we have investigated the mechanism of TNF-mediated cytotoxicity in L929 and WEHI 164 clone 13 mouse fibrosarcoma cells. Inhibitors with different sites of action modulated TNF cytotoxicity, however, with contrasting effects on final cell viability. Inhibition of mitochondrial electron transport at complex III (cytochrome c reductase) by antimycin A resulted in a marked potentiation of TNF-mediated injury. In contrast, when the electron flow to ubiquinone was blocked, either at complex I (NADH-ubiquinone oxidoreductase) with amytal or at complex II (succinate-ubiquinone reductase) with thenoyltrifluoroacetone, cells were markedly protected against TNF cytotoxicity. Neither uncouplers nor inhibitors of oxidative phosphorylation nor complex IV (cytochrome c oxidase) inhibitors significantly interfered with TNF-mediated effects, ruling out the involvement of energy-coupled phenomena. In addition, the toxic effects of TNF were counteracted by the addition of antioxidants and iron chelators.

Furthermore, we analyzed the direct effect of TNF on mitochondrial morphology and functions. Treatment of L929 cells with TNF led to an early degeneration of the mitochondrial ultrastructure without any pronounced damage of other cellular organelles. Analysis of the mitochondrial electron flow revealed that TNF treatment led to a rapid inhibition of the mitochondria to oxidize succinate and NADH-linked substrates. The inhibition of electron transport was dose-dependent and became readily detectable 60 min after the start of TNF treatment, thus preceding the onset of cell death by at least 3–6 h. In contrast, only minor effects were observed on complex IV activity.

The different effects observed with the mitochondrial respiratory chain inhibitors provide suggestive evidence that mitochondrial production of oxygen radicals mainly generated at the ubisemiquinone site is a causal mechanism of TNF cytotoxicity. This conclusion is further supported by the protective effect of antioxidants as well as the selective pattern of damage of mitochondrial chain components and characteristic alterations of the mitochondrial ultrastructure.

Tumor necrosis factor (TNF), 1 primarily produced by activated macrophages, was originally described based on its ability to cause necrosis of Meth A sarcomas in vivo and selectively kill transformed and neoplastic cell lines in vitro (1). During the last few years, TNF has been shown to exert a wide range of biological activities mostly related to inflammatory and immunomodulatory functions. Beside its anti-tumor and anti-malignant cell effects, TNF has been reported to influence mitogenesis, differentiation, and immunoregulation of various cell types, which suggests an important role of this cytokine under several physiological and pathological conditions (reviewed in Refs. 2 and 3).

Although recently some mechanisms of TNF-mediated cell regulatory activities have become better understood, the biochemical basis of the cytotoxic action against tumor cell lines is still largely unknown. TNF binds to its target cell via high affinity surface receptors. Two different receptor types have been cloned recently, but their primary structure does not reveal any significant consensus sequences pointing to the involvement of one of the classical signaling pathways (4–6). However, studies mainly based on inhibitors have indicated that, probably depending on the cell type, multiple intracellular pathways may be involved in TNF-mediated killing. Among the reported effects, major steps seem to include G-protein-coupled activation of phospholipases (7, 8), generation of reactive oxygen radicals (9, 10), and DNA damage (11, 12). Furthermore, it has been shown that inhibitors of proteases (13, 14) and lysosomal enzymes (15), glucocorticoids (8, 16), and antioxidants (9, 10, 17) may confer some protection against TNF cytotoxicity. Certainly, the cytotoxic action of TNF does not depend on RNA or protein synthesis, and its activity is even considerably enhanced by transcription or translation inhibitors (18). In agreement with this observation, evidence has been obtained that cells may counteract...
TNF action by synthesis of rescue factors (19). Wong et al. (20) reported that, at least in some cell types, one of the proteins that can provide protection against TNF is manganese superoxide dismutase. Overexpression of this mitochondrial enzyme confers increased resistance against TNF. These findings further imply that superoxide radicals or other reactive oxygen species might participate in the TNF-initiated cytotoxic pathway.

In the present study we show that specific mitochondrial electron transport inhibitors can either potentiate or block TNF cytotoxic activity. The effects observed with the inhibitors implicate oxygen radicals which are produced by the respiratory chain as a key cause of TNF-induced cytotoxicity. This conclusion is confirmed by further experiments showing that early events of TNF cytotoxicity are a functional damage of selective components of the mitochondrial electron transport chain and structural alterations in mitochondrial morphology.

MATERIALS AND METHODS

Cell Cultures

L929, a murine fibrocartilage cell line, was grown in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated newborn calf serum, 100 units of penicillin/ml and 0.1 mg of streptomycin/ml. WEHI 164 clone 13, a murine fibrocartilage cell line which is highly TNF-sensitive (obtained from Dr. T. Espevik, University of Trondheim, Norway), was cultured in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. The cell lines were repeatedly found to be mycoplasma-free as judged by a DNA-fluorochrome assay.

TNF and Reagents

TNF Preparation—Recombinant murine TNF was produced in Escherichia coli and purified to at least 95% homogeneity (21). The preparation had a specific activity of 3.7 x 10^7 IU/mg protein and contained less than 4 ng of endotoxin/mg of protein. TNF activity was determined as described by Ostrove and Gifford (22) using an international standard TNF preparation (code No. 88/532, obtained from the United Kingdom) as a reference.

Reagents—The following agents, used without further purification, were purchased from Sigma Chemical Co. (Deisenhofen, Federal Republic of Germany): actinomycin D, adenine diphosphate, amytal (amobarbital), butylated hydroxyanisole, butylated hydroxytoluene, carbonyl cyanide-p-(trifluoromethoxy)phenyldrazon (FCCP), diethyldithiocarbamate (DDC), 2,4-dinitrophenol (DNP), 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), l-glutathione, , oligomycin, p-phenanthroline, rotenone, sodium nitroprusside, tetramethyl-p-phenylene diamine (TMPD), and thenoyltrifluoroacetone (TTFA). Antimycin A, succinate, and malate were from Serva (Heidelberg, Federal Republic of Germany) and desferrioxamine (Desferal) from Ciba Geigy (Basel, Switzerland). Stock solutions of the reagents were routinely prepared in medium, dimethyl sulfoxide, or ethanol as appropriate.

For use in cytotoxicity assays, stock solutions were diluted in culture medium such that the final concentration of the organic solvent never exceeded 0.05%. Control experiments demonstrated that this concentration did not affect TNF cytotoxicity.

TNF Cytotoxicity Essay

Cells were seeded in 96-microwell plates at 3 x 10^4 cells in 100 /ul of medium. Twelve to 16 h later 50 /ul of a drug solution was given. TNF with or without actinomycin D (1 ug/ml) was added 3 h later in a 50- /ul volume and a concentration range of 2-5000 IU/ml. After 18-24 h of further incubation, cell viability was routinely determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining (23). Similar results were obtained with crystal violet staining of the attached cells (24).

Measurement of Oxygen Consumption

L929 cells were harvested by trypsinization at the indicated times after TNF incubation and combined with the decanted medium. After centrifugation and two following washing steps in calcium-free phosphate-buffered saline, cells were resuspended at 1 x 10^6 cells/ml in respiration medium consisting of 0.25 M sucrose, 0.1% bovine serum albumin, 10 mM MgCl2, 10 mM K+-Hepes, 5 mM KH2PO4, pH 7.2, with or without 1 mM ADP, and kept on ice. Cell viability was determined by trypan blue exclusion.

Oxygen consumption was measured with a Clark electrode fitted in a 3.3-ml thermostated sample chamber (37 °C) under constant stirring (25). Briefly, cells were injected in a 200-μl volume and permeabilized by the addition of digitonin (final concentration 0.005%) in order to permit free entry of mitochondrial inhibitors and substrates. The latter were added in the following final concentrations: malate, 5 mM; rotenone, 100 nM; succinate, 5 mM; antimycin A, 50 mM; ascorbate, 1 mM; TMPD, 0.4 mM; KCN, 1 mM; oligomycin, 1 μM; DNP, 1 μM. Oxygen concentration was calibrated with air-saturated buffer, assuming 390 ng atoms/ml of O2 (25). Rates of oxygen consumption are expressed as nanogram atoms of oxygen/min/10^7 cells.

Electron Microscopy

L929 cells were seeded on thermanox coverslips (diameter 1 cm; GIBCO, Bio-Cult, Paisley, United Kingdom) at a density of 100,000 cells. 24 h later 10,000 IU/ml TNF were added. After various incubation times ranging from 1 to 17 h, cells were processed for electron microscopy. The growth medium was removed by two changes with serum-free medium and replaced by 100 mM cacaodylate buffer, pH 7.4, containing 2% glutaraldehyde and 4% formaldehyde. After fixation for 15 min at room temperature, coverslips were rinsed three times in cacodylate buffer for 5 min. Postfixation was carried out for 1 h with potassium ferricyanide in 1% OsO4. After a washing step in cacodylate buffer, specimens were stained en bloc in 2% aqueous uranyl acetate for 2 h and then washed with water. Dehydrations were performed with increasing concentrations (70–90–100%) of acetone. Cells were embedded overnight in a mixture of epoxy resin (LX112, Ladd) and acetone and polymerized at 60 °C for 60 h. After removal of the coverslips, sections (60–80 nm) were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined in an Jeol 1200 EX microscope at 60 KV.

RESULTS

Mitochondrial Respiratory Chain Inhibitors Strongly Modulate TNF Cytotoxicity—After analysis of the effects of various respiratory chain inhibitors, TNF cytotoxicity was found to be either increased or decreased, depending on the action site of the inhibitors. For reference, a simplified scheme of the mitochondrial respiratory chain is shown in Fig. 1. Inhibitors of complex III, which inhibit the electron flow from ubiquinone to cytochrome c1, strongly potentiated TNF-mediated cytolsis of L929 cells. Indeed, both antimycin A and HQNO gave a dose-dependent enhancement of TNF cytotoxicity (Table I; Fig. 2a). At the concentrations used, neither inhibitor significantly influenced cell viability on its own. However, myxothiazol, which inhibits electron flow via the Rieske iron sulfur center to cytochrome c1 (26), had no potentiating effect and was even marginally protective.

Most striking was the observation that inhibitors of complex I, which interfere with the electron flow from NADH-linked substrates and NADH dehydrogenase to the ubiquinone pool, were protective against TNF, in contrast to the effect of complex III inhibitors. Myxothiazol strongly decreased TNF susceptibility (Table I; Fig. 2b), and also rotenone, which inhibits the same step, was protective, albeit to a lesser extent (Table I). Furthermore, when the electron transport from succinate dehydrogenase was blocked by the addition of TPTA, TNF cytotoxicity was also reduced (Table I). Finally, the combined inhibition of both potential electron entry sites to ubiquinone with amytal plus TPTA resulted in an almost complete inhibition of TNF-induced cell lysis (Table I), which was still observed at a high TNF concentration of 10,000 IU/ml (not shown). On the other hand, inhibition of complex IV (cytochrome c oxidase) with azide or cyanide gave ambiguous results. Azide was found to be slightly protective, whereas cyanide had marginal synergistic effects on TNF cytotoxicity.
TABLE I

Influence of mitochondrial and other inhibitors on TNF cytotoxicity in L929 cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug concentration</th>
<th>-Act D, % rel. survival</th>
<th>+Act D, % rel. survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IU/ml</td>
<td>IU/ml</td>
<td>IU/ml</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>555</td>
<td>61</td>
</tr>
<tr>
<td>I. Mitochondrial chain inhibitors</td>
<td></td>
<td>6</td>
<td>0.7</td>
</tr>
<tr>
<td>1. Complex I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amytal</td>
<td>400 µM</td>
<td>87.6</td>
<td>99.3</td>
</tr>
<tr>
<td>Rotenone</td>
<td>25 µM</td>
<td>46.8</td>
<td>87.6</td>
</tr>
<tr>
<td>2. Complex II</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TTPA</td>
<td>250 µM</td>
<td>68.4</td>
<td>93.6</td>
</tr>
<tr>
<td>TTPA + amytal</td>
<td>99.8</td>
<td>99.7</td>
<td>94.2</td>
</tr>
<tr>
<td>3. Complex III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antimycin A</td>
<td>50 µM</td>
<td>1.8</td>
<td>16.2</td>
</tr>
<tr>
<td>HQNO</td>
<td>50 µM</td>
<td>1.4</td>
<td>28.6</td>
</tr>
<tr>
<td>Myxothiazol</td>
<td>1 µM</td>
<td>22.1</td>
<td>78.3</td>
</tr>
<tr>
<td>4. Complex IV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azide</td>
<td>500 µM</td>
<td>15.2</td>
<td>85.3</td>
</tr>
<tr>
<td>KCN</td>
<td>500 µM</td>
<td>8.5</td>
<td>68.3</td>
</tr>
<tr>
<td>II. Uncouplers</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Dinitrophenol</td>
<td>1 mM</td>
<td>10.4</td>
<td>64.9</td>
</tr>
<tr>
<td>FCCP</td>
<td>10 µM</td>
<td>13.8</td>
<td>72.3</td>
</tr>
<tr>
<td>III. F1-ATPase</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Oligomycin</td>
<td>25 µM</td>
<td>12.2</td>
<td>70.3</td>
</tr>
<tr>
<td>IV. Iron chelators</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Desferroxamine</td>
<td>100 µM</td>
<td>41.2</td>
<td>92.8</td>
</tr>
<tr>
<td>D-Phenanthroline</td>
<td>1 mM</td>
<td>27.8</td>
<td>96.3</td>
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<tr>
<td>V. SOD inhibitors</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nitroprusside</td>
<td>1 mM</td>
<td>4.6</td>
<td>46.3</td>
</tr>
<tr>
<td>DDC</td>
<td>2 mM</td>
<td>6.8</td>
<td>65.1</td>
</tr>
<tr>
<td>VI. Antioxidants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHA</td>
<td>200 µM</td>
<td>78.6</td>
<td>98.6</td>
</tr>
<tr>
<td>BHT</td>
<td>200 µM</td>
<td>70.3</td>
<td>92.3</td>
</tr>
</tbody>
</table>

All the inhibitors were tested in the presence and absence of actinomycin D. Inhibition of transcription, however, did not lead to qualitative differences in drug effects on TNF-mediated killing, although a much lower dose of TNF was necessary for a cytotoxic effect (Table I). Furthermore, in WEHI 164 clone 13 cells, which were studied as an additional cell line, similar effects of the mitochondrial inhibitors were observed (not shown).

Time Kinetics of the Influence of Mitochondrial Inhibitors on TNF Cytotoxicity—Antimycin A or amytal were added at various time points before and after TNF addition, in order to determine whether their effects occurred at an early step of TNF action (Fig. 3). Addition of antimycin A or amytal from 2 h before up to 4 h after start of the TNF treatment did not affect the inhibition or protection, respectively. But after 4 h, the potentiating effect of antimycin A and the protection by amytal gradually declined, although effects were still detectable 10 h after TNF addition.

TNF-mediated Cytotoxicity Is Not Dependent on ATP Synthesis—Protonophorous uncouplers of the mitochondrial electron flow, such as dinitrophenol or FCCP, were found to exert no effect on TNF cytotoxicity (Table I). This result indicates that the phenomena observed with mitochondrial chain inhibitors were not due to a blockade of ATP synthesis. Moreover, oligomycin, which directly blocks ATP synthesis by inhibiting the F1-ATPase subunit, did not influence the activity of TNF on L929 or WEHI 164 clone 13 cells.

Antioxidants and Iron Chelators Protect against TNF Cytotoxicity—The modulation of TNF cytotoxicity by respiratory chain inhibitors strongly points to an involvement of oxidative events in the cytotoxic action of TNF. We therefore analyzed the effects of antioxidants and iron chelators on
TNF activity. Iron ions have been shown to exert a strong influence on the toxicity of reactive oxygen species, as Fe(II) can catalytically dismute superoxide radicals (O_2^-) and hydrogen peroxide to the extremely toxic hydroxyl radical (reviewed in Refs. 27 and 28). We found indeed that the antioxidants butylated hydroxytoluene and butylated hydroxyanisole strongly protected against the cytotoxic effects of TNF (Table I). The importance of an involvement of reactive oxygen species was further supported by the finding that both desferrioxamine, a specific iron chelator, and o-phenanthroline, a chelator of heavy metal ions, inhibited TNF cytotoxicity (Table I).

On the other hand, inhibitors of superoxide dismutases, which counteract oxidative damage of superoxide radicals, rendered the cells more susceptible to TNF. Nitroprusside, which inhibits all classes of superoxide dismutases, including the mitochondria-located manganese superoxide dismutase (29), had a marked potentiating effect, whereas DDC, which blocks activity of Cu/Zn superoxide dismutase (30), was less synergistic (Table I).

**TNF Causes an Early Damage of Mitochondrial Electron Transport**—We next analyzed by polarographic measurements of oxygen consumption whether TNF affects mitochondrial electron flow. L929 cells were treated with digitonin, which selectively permeabilizes the plasma and outer mitochondrial membranes, while leaving the inner mitochondrial membrane intact. Three different substrates were used to study the characteristics of oxygen consumption: malate, which generates intramitochondrial NADH; succinate, which provides electrons at the ubiquinone level; and ascorbate plus TMPD, which reduces the respiratory chain at the cytochrome c level. Traces from typical measurements are shown in Fig. 4a. We found that L929 cells, treated with 250 IU/ml TNF, became markedly inhibited in their ability to oxidize a NADH-linked substrate. Already after 3 h, oxidation of malate had decreased to 61% of its original activity; after 6 h of TNF incubation, less than 31% of activity remained detectable (Fig. 4b). This rapid loss of electron transport activity preceded cell lysis by at least 3–6 h.

By blocking the electron flow through complex I with rotenone and by adding succinate as reducing substrate, the electron flow through complexes II, III, and IV (succinoxidase) was analyzed. Inhibition of the activity of succinoxidase was similarly observed, although it was somewhat delayed as compared with the rapidly decreased NADH-linked oxidation. After injection of antimycin A followed by TMPD plus ascorbate as electron donor, the activity of cytochrome c oxidase (complex IV) was measured. The activity of this enzyme complex was found to be relatively resistant to TNF, as more than 50% of its original activity was still detectable 12 h after TNF addition.

The observed decrease in the different mitochondrial enzyme activities could be due either to a direct effect on mitochondrial electron flow, to a block in ADP/Pi translocation, or to an inhibition of the F_1-ATPase complex. Addition of oligomycin, inhibition of the mitochondrial electron consumption was observed (data not shown). This indicates that TNF-treated mitochondria were still energy-coupled, since oligomycin would not have exerted any effects on uncoupled mitochondria.

**Dose Dependence and Time Course of Electron Transport Inhibition in Uncoupled Mitochondria**—In order to establish a dose dependence of TNF-induced inhibition of mitochondrial electron transport, oxygen consumption was measured in the presence of the protonophorous uncoupler dinitrophenol, which induces maximum electron transfer activity. Also under these conditions, TNF-induced inhibition of mitochondrial electron transfer was clearly dose-dependent (Fig. 5a).
trypan blue exclusion. Dose dependence of TNF on electron flow was determined 6 h after addition of TNF in a titration series from 50 to 10,000 IU/ml TNF (a). The time course of TNF effect on oxygen consumption was determined using a TNF concentration of 10,000 IU/ml TNF (b).

6 h after addition of 500 IU/ml TNF, the oxygen consumption was inhibited for more than 60%; at this time, cell death, as determined by trypan blue exclusion, was detectable in less than 20% of the cells.

The amplified system of TNF-induced decrease in oxygen consumption in the presence of an uncoupler was further used to investigate the time course of mitochondrial electron transport inhibition (Fig. 5b). Applying a high concentration of 10,000 IU/ml TNF, a marked decrease in electron transport could already be observed 1 h after TNF addition, reaching a plateau in about 5–6 h. Consistent with the results shown in Fig. 4b, the TNF-induced inhibition largely preceded the onset of cell death as defined by trypan blue exclusion.

Ultrastructural Changes Caused by TNF—L929 cells were grown in the presence of TNF for varying times ranging from 1 up to 17 h. After each hour, cells were fixed and analyzed by electron microscopy. Cells grown in the presence of TNF revealed an early degeneration of their mitochondrial ultrastructure, which became evident about 2 h after TNF addition. The most prominent and initial change was the appearance of onion-like structures inside the mitochondrial matrix, which could generally not be recognized in untreated cells (Fig. 6, A and B). The cristae became more electron-dense and rounded up, and appeared to protrude into the mitochondrial matrix. With prolonged TNF incubation, the stacking of cristae became increasingly pronounced, resulting in the gradual formation of multilamellar bodies inside the mitochondrion (Fig. 6, C and D). However, extensive swelling of mitochondria was not observed before 9 h after TNF addition. In the first 5 h, other cellular compartments still retained a generally normal ultrastructure. In some TNF-treated cells, a slight margination of nuclear chromatin could be observed in the nucleus. Sometimes, the endoplasmic reticulum also displayed alterations similar to those observed in the mitochondria (Fig. 6C).

The exposure of L929 cells to a combination of TNF and antimycin A seemed to potentiate the degenerating process of the mitochondria (Fig. 7A). The cells exhibited more swollen mitochondria with an increased number of disoriented cristae. Many cristae were apparently detached from the inner membrane. In comparison with TNF-treated cells, onion-like structures apparently were not increased by the combined treatment with antimycin A and TNF. On the other hand, the exposure of L929 cells to a combination of amytal and TNF seemed to diminish the deleterious TNF effects on the mitochondrial ultrastructure (Fig. 7B).
mitochondria seemed to be affected. The cristae of most mitochondria exhibited the normal transverse orientation.

**DISCUSSION**

Several cellular pathways have been suggested leading to cell death, including perturbation of ion homeostasis, activation of proteases and phospholipases, generation of reactive oxygen species, or degradation of DNA (31). Despite a growing number of investigations dealing mainly with immunomodulatory activities of TNF, its mechanism of cytotoxicity is still largely unknown. TNF has been described to initiate two forms of cell killing, although both forms presumably cannot be strictly separated for many cell types (32, 33). The necrotic form of cell death is mainly characterized by swelling of the cytoplasm and organelles followed by lysis of the plasma membrane. In apoptotic or so-called programmed cell death, which is exemplified by growth factor-deprived thymocytes and embryonic cells, the cytoplasm becomes condensed and the nucleus compact. Characteristic patterns of 183-base pair DNA fragments can be observed resulting from the activation of a nuclear endonuclease during the apoptotic process (34). We have also observed some typical DNA degradation patterns in L929 cells, but these arose as a rather late event in the cytotoxic action of TNF. L929 and other fibroblast cell lines seem to be more likely killed by necrotic processes.

One of the first consequences inflicted by TNF in L929 cells includes morphological alterations of the mitochondria. We observed that TNF-treated cells reveal a degenerative clumping of mitochondrial cristae, which resulted in the formation of multilamellar vesicles inside the mitochondrial matrix. In addition, Matthews et al. (35, 36) have shown that besides mitochondrial degeneration, TNF-treated cells respond with an increased rate of glycolysis and decreased rate of ATP synthesis. Conceivably, the increase in glycolysis compensates for the damage of mitochondrial electron transport as observed in this study. Functional inhibition of the respiratory chain has been demonstrated previously as a consequence of the cytotoxic action of macrophages on tumor cells (25, 37, 38). Moreover, Lancaster et al. (39) recently reported that TNF induces mitochondrial electron transfer inhibition in a number of transformed cell lines which may be a causative event in the cytotoxicity of TNF. We have detailed these studies in highly TNF-susceptible L929 cells, and we show that functional damage of the electron flow occurs as an early consequence of TNF action. The ability of mitochondria to oxidize succinate and NADH-linked substrates was seriously affected by TNF treatment, preceding cell death by at least 3-6 h. The activity of complex IV, however, was fairly resistant to TNF, with over 50% activity remaining even 12 h after incubation with 250 IU/ml TNF. Analysis of the electron flow in permeabilized cells with uncoupled mitochondria and high TNF concentrations allowed the detection of a TNF effect on the respiratory chain already after 60 min, without any evidence for compromised cell viability at this time point.

In the TNF cytotoxicity assay, we observed that inhibition of mitochondrial electron transport per se or depletion of ATP synthesis were not detrimental to the viability of L929 and WEHI 164 clone 13 cells. Moreover, we show that inhibition of mitochondrial electron transport at specific sites can differentially interfere with TNF-mediated cytotoxicity. Inhibition of the electron transport at complex I with amytal or rotenone or at complex II with TTFA markedly protected the cells against TNF. On the other hand, we found that electron transport inhibition behind the ubiquinone region with antimycin A potentiated TNF cytotoxicity. These results suggest that oxidative events generated in the mitochondrion, not inhibition of energy-coupled processes, are crucial in TNF-induced cytotoxicity.

It has long been recognized that mitochondria are an important source of reactive oxygen species under certain conditions. According to Boveris et al. (40) about 1-2% of the oxygen consumed in state 4 (resting respiration) is utilized by the formation of reactive oxygen species. In the presence of various drugs (i.e. mitochondrial inhibitors, quinoid compounds), mitochondrial generation of reactive oxygen radicals can increase severalfold.

Two sites of the mitochondrial respiratory chain have been identified as sources responsible for the formation of reactive oxygen species. One is dependent on the autooxidation of the flavin mononucleotide from the NADH-dehydrogenase (complex I), whereas the other, probably the most relevant one, depends on autooxidation of the unstable ubisemiquinone (complex III), which is an intermediate of the Q-cycle reaction (41, 42). Mitochondrial substrates and inhibitors have been described as effective modulators of reactive oxygen species. The production of oxygen radicals at the ubiquinone site supported by NADH-linked substrates is diminished by complex I and complex II inhibitors (43-45). On the other hand, formation of reactive oxygen species increases several times in the presence of antimycin A, proposing ubisemiquinone as the main reductant site of oxygen (46). The opposite effects observed with specific mitochondrial inhibitors in this study, viz. enhancement of TNF cytotoxicity with antimycin A and inhibition with amytal and TTFA, strongly indicate that TNF activates radical production in mitochondria mainly at the ubiquinone site.

The first oxygen reduction product generated in mitochondria under both physiological and pathological conditions appears to be the superoxide radical, which can subsequently be converted to hydrogen peroxide (47). Dismutation of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) can result in the production of the more deleterious hydroxyl radical and singlet oxygen. Conversion of superoxide and hydrogen peroxide to these harmful oxygen intermediates is catalyzed by transition metals such as iron ions in the Fenton reaction and the (metal-catalyzed) Haber-Weiss reaction (27, 28). Therefore, chelation of iron by desferrioxamine or \( \text{o-phenanthroline} \) reduces susceptibility of L929 and WEHI 164 clone 13 cells toward TNF.

That generation of radicals is probably a crucial event in TNF action is further supported by our finding that antioxidants and free radical scavengers protect against TNF-induced cytolsis (Table I; Refs. 8 and 13). As already noticed by Neale et al. (7), we confirmed that L929 cells kept under anaerobic conditions, or treated with succinylacetone in order to block heme synthesis and deplete cells from cytochromes, became less sensitive to TNF. Further support for the importance of radicals arises from our observation of the characteristic degeneration pattern of mitochondrial ultrastructure. Similar typical clumping and vesiculation of mitochondrial cristae has been reported to appear after perfusion of rat hearts with a free radical-generating system (48).

Normally, cells counteract the toxicity of reactive oxygen species by means of superoxide dismutases and other detoxifying enzymes. Indeed, TNF has been shown to induce synthesis of manganous superoxide dismutase, but not of other known antioxidant enzymes such as Cu/Zn superoxide dismutase, glutathione peroxidase, or glutathione transferase (49). On the other hand, inhibition of superoxide dismutase or depletion of reduced glutathione (50) leads to an enhance-

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ment of TNF cytotoxicity. Additionally, the rather selective specificity of TNF action on tumor cells may be related to the long-standing observation that tumor cells generally contain low levels of enzymes dealing with oxygen radicals and hydrogen peroxide (51).

Oxygen radicals escaping detoxifying enzymes are capable of initiating various damage (27, 28). Attack by radicals can result in lipid peroxidation, protein insults, and DNA degradation. Although some O2 and H2O2 will diffuse from the mitochondrion to damage more distant cellular components, the reactions of the highly toxic short-lived hydroxyl radical are largely diffusion controlled. It is conceivable that the toxicity of all three oxygen species should be greatest at the inner mitochondrial membrane. It has been shown that mitochondria treated with peroxidants have an impaired ability to retain calcium (52). Elevation of cytoplasmic calcium from intracellular stores has been further reported to cause activation of proteases and phospholipases, both of which seem to be involved in TNF cytotoxicity (7, 8, 12, 13). Furthermore, oxygen radicals on their own can directly impair the mitochondrial electron transport (53), which would suggest an autocatalytic inhibition of mitochondrial chain components.

The TNF-induced inhibition of the mitochondrial electron transport, together with the effects observed for different mitochondrial inhibitors, favor the explanation that TNF damages the mitochondrial chain at complex III, which consequently results in the increased production of oxygen radicals inside the mitochondrion. However, it remains unclear from our results in which way TNF or activated second messengers affect the mitochondrial electron transport. Further studies are under way in order to unravel primary effects of TNF on components of the electron transport chain.

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