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Mechanisms of the cytotoxic actions of tumor necrosis factor (TNF) in cultured cancer cells

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The University of Arizona, 1987

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MECHANISMS OF THE CYTOTOXIC ACTIONS OF TUMOR NECROSIS FACTOR (TNF) IN CULTURED CANCER CELLS

by

James Duncan Liddil

A Thesis Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN TOXICOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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STATEMENT BY AUTHOR

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APPROVAL BY THESIS DIRECTORS

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19 Nov 1987
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11/18/87
Date

Dedicated to my wife, Nancy,
to my parents and to my brother, Bruce,
who have provided unending support
of my quest to further my education.

ACKNOWLEDGMENTS

I would like to thank the members of my committee for their assistance and guidance in completing this project. Thanks to Dr. Robert T. Dorr for his continual financial and moral support and enthusiasm for which I will always be grateful. I would also like to thank Dr. William Dalton for his thoughtful insight and Dr. I. Glenn Sipes for supporting my educational goals.

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ABSTRACT

Tumor necrosis factor's (TNF) cytotoxic mechanism of action was examined using cultured cancer cell lines. TNF demonstrated cytolytic and cytostatic effects on L929 fibrosarcoma and MCF-7 adenocarcinoma cells. TNF failed to show any specific effects on RNA, DNA or protein synthesis or ATP content in tumor cells in vitro. It did not cause DNA single strand breaks. Decreased cellular levels of reduced thiols did not predict sensitivity to the cytotoxic effects of TNF. Depletion of cellular glutathione failed to increase the sensitivity of TNF-sensitive or resistant cells. However, various non-specific and specific lysosomotropic agents lead to an inhibition of TNF's cytotoxic action. Differences in enzyme activity, primarily lysosomal, were noted between TNF-sensitive and resistant cells. These changes involved a general halving of lysosomal proteins and enzymes in the TNF-resistant cells. The antitumor activity of TNF does not involve specific inhibition of macromolecular synthesis but may involve alterations in lysosomes.

CHAPTER I

INTRODUCTION

Historical Background

Reports of endotoxins, a major component of the cell walls of gram negative bacteria, having antitumor activities were first published in the late 1800's (Bruns, 1888; Coley, 1891). These authors described the regression of certain tumors in patients either recovering from bacterial infection or in patients intentionally injected with mixtures of bacterial toxins from streptococci and serratia. Similar observations were made in the 1940's when it was shown that endotoxins could induce hemorrhagic necrosis of certain murine tumors (Shear et al., 1943).

Over the last 100 years, knowledge of the mechanisms involved in endotoxin-induced antitumor effects has increased significantly. In 1975 it was reported that the sera of mice infected with Bacillus Calmette-Guerin (BCG) and subsequently treated with endotoxin contained a substance which caused tumor necrosis independent of endotoxin (Carswell et al., 1975). The substance was called tumor necrosis factor (TNF) and besides causing necrosis of transplanted murine tumors it also inhibited the growth of human and murine neoplastic cell lines in culture (Fiore et al., 1975). Further research indicated that monocytes and macrophages were the principal cellular source of TNF (Mannel et al., 1980; Matthews et al., 1981; Kelker et al., 1985). The human promyelocytic leukemia cell line HL-60, can be induced with

phorbol myristate acetate to synthesize large amounts of TNF and this method has been utilized for the purification and molecular cloning of the human TNF gene using recombinant DNA technology (Wang et al., 1985).

Chemistry

Purified human TNF is a nonglycosylated protein consisting of 157 amino acids with a molecular weight of approximately 17 kilodaltons and isoelectric point of 5.3. It contains two cysteine residues which form a single intramolecular disulfide bond (Aggarwal et al., 1985) and occurs as a trimer in its active form (Smith et al., 1987) with a molecular weight of approximately 52,000 (Arakawa and Yphantis, 1987).

Pharmacokinetics

At the present time no information is available concerning the metabolism of TNF in man. In mice, the plasma half-life of radioiodinated TNF is reported to be in the range of 6 to 7 minutes with most of it being taken up by the liver, skin, gastrointestinal tract and kidney and then quickly metabolized (Beutler et al., 1985).

Cellular Uptake

Specific high-affinity receptors for TNF have been identified on cells both susceptible and resistant to the cytotoxic action of TNF. Binding of TNF is followed by receptor-mediated endocytosis and intracellular degradation in lysosomes. This latter process is inhibited by the lysosomotropic agent chloroquine producing a reduction in TNF cytotoxicity (Tsujimoto et al., 1985).

Antineoplastic Action

In Vivo

TNF now appears to be the major mediator of hemorrhagic necrosis of tumors, an effect once thought to be due to the action of endotoxin (Parr et al., 1973). Intravenous injection of TNF in mice causes the necrosis of transplanted solid tumors such as Meth A sarcoma, EL-4 leukemia and P815 mastocytoma (Carswell et al., 1975). It also causes necrosis and regression of a number of human tumor cells heterotransplanted into nude mice including malignant melanoma, gastric carcinoma and nasopharyngeal carcinoma (Haranaka et al., 1984).

In Vitro

Numerous investigators have reported the ability of TNF to have cytotoxic and/or cytostatic effects on tumor cells in culture (Carswell et al., 1975; Wang et al., 1985; Sugarman et al., 1985). It has also been shown that a large number of tumor cell lines and nearly all normal cell lines are resistant to TNF's effects (Sugarman et al., 1985) This lack of sensitivity is not due to a decreased number of TNF receptors or low binding affinity (Sugarman et al., 1985; Tsujimoto et al., 1986). Thus resistance is distal to receptor binding and internalization and is possibly related to lack of activation intracellularly.

Augmentation

The sensitivity of tumor cells to TNF cytotoxicity can be enhanced using RNA synthesis inhibitors like actinomycin D or DNA

alkylating agents such as mitomycin C (Ruff and Gifford, 1981; Matthews and Watkins, 1978; Kull and Cuatrecasas, 1981). Also, incubating cells at 39-40°C increases their sensitivity to TNF over that of cells incubated at 37°C (Tsujimoto et al., 1985). Inhibitors of protein synthesis such as cycloheximide also cause an increase in cellular sensitivity to TNF's cytotoxic actions (Kull and Cuatrecasas, 1981). In addition, normal cells such as human fibroblasts can be made susceptible to the cytolytic effects of TNF by preexposing them to cycloheximide (Kirsten and Baglioni, 1986).

Mechanism of Action

Currently, the molecular mechanism of TNF cytotoxicity has not been explained. Since the cytotoxic action of TNF is not decreased by the inhibition of cellular RNA or protein synthesis it appears that activation of cellular gene products is not involved in the cytolytic process. Also because there is an actual increase in cytotoxicity in the presence of RNA or protein synthesis inhibitors it may be that most cells are able to repair TNF-induced damage when cellular processes are allowed to function normally. A number of hypotheses have been put forward regarding the mechanism by which TNF might be acting. These include free radical generation followed by lipid peroxidation, mitochondrial injury and concomitant interference with energy production (Jones, 1986). Others have proposed that TNF may lead to degradation of nuclear DNA followed by cell death (Dealtry et al., 1987; Maury, 1986). Recently, it has been proposed that glutathione levels may be related to TNF sensitivity (Sherwin, 1987).

It has been reported that the cytotoxic effects of TNF on L929 cells can be inhibited by lysosomotropic agents such as chloroquine and cytoskelatal disrupting agents such as colchicine and cytochalasin B (Kull and Cuatrecasas, 1981; Niitsu et al., 1985). These authors have suggested that the cellular uptake of TNF involves internalization by receptor mediated endocytosis followed by the fusion of pinosomes with lysosomes (Figure 1). Further support for this idea comes from studies which demonstrate that TNF is rapidly degraded into small fragments after internalization and that chloroquine inhibits this process (Tsujimoto et al., 1985).

Hypothesis

The foregoing information leads to the hypothesis that the antitumor activity of TNF does not involve specific inhibition of macromolecular synthesis but may involve alterations to or in lysosomes. It does not seem likely that a polypeptide such as TNF would directly inhibit macromolecular synthesis since they usually act as growth factors to stimulate cellular processes as in the case of epidermal growth factor (Goldstein et al., 1979; Dickson et al., 1983). Support for the role of receptor-mediated endocytosis and lysosomal involvement in the cytotoxic action of TNF comes from studies demonstrating that direct microinjection of TNF into the cytoplasm or nucleus fails to cause cell death (Niitsu et al., 1985). Figure 1 depicts how TNF may be taken up into the cell and then processed (Goldstein et al., 1979). The TNF molecule is believed to bind to a specific plasma membrane receptor which is located in clathrin coated

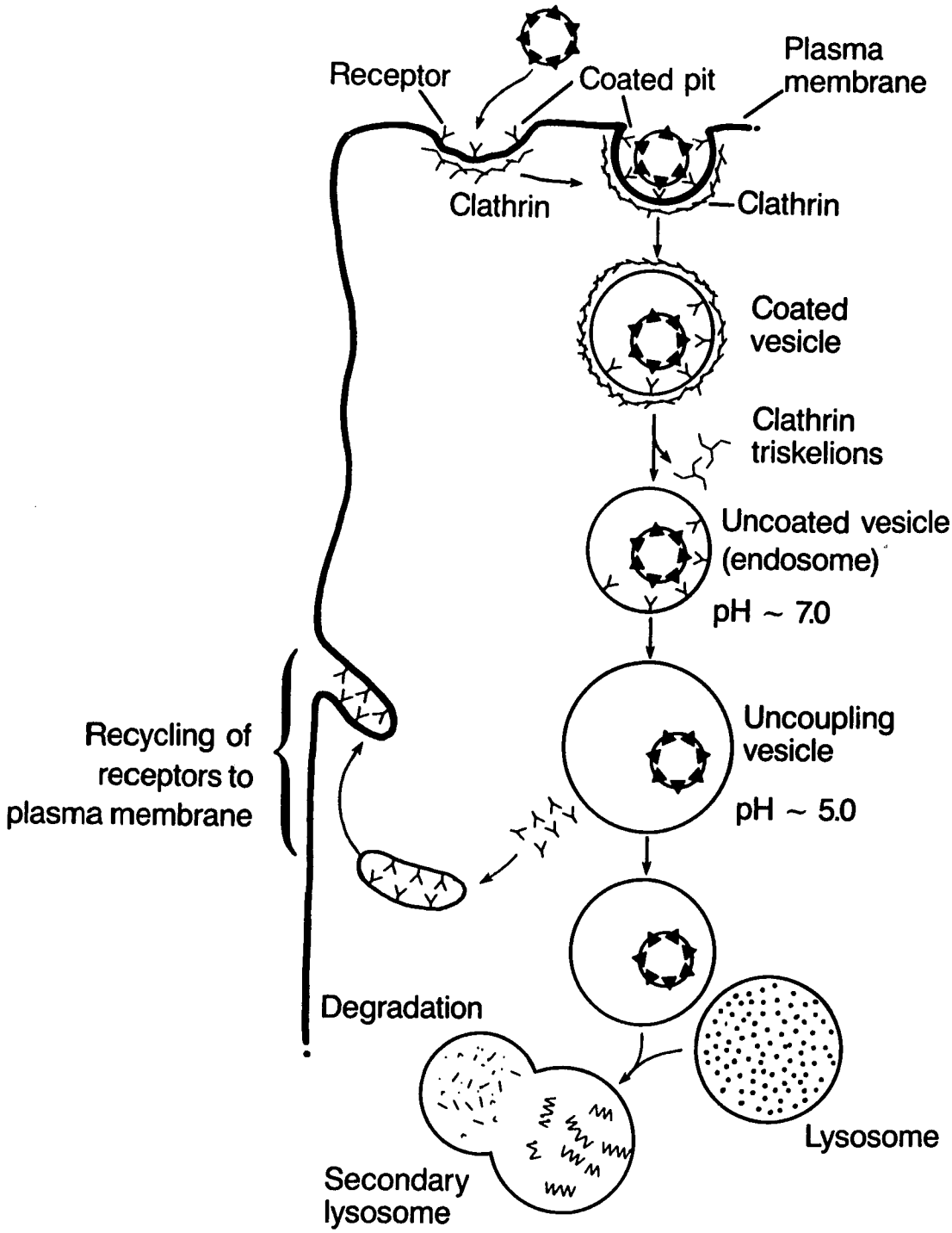

Figure 1. TNF cellular uptake.

Proposed fate of TNF showing internalization by receptor-mediated endocytosis and degradation by lysosomes. Adapted from Lodish et al., 1986.

pits. After TNF binds to the receptor the pits invaginate to from a coated vesicle. The clathrin depolymerizes resulting in the formation of an uncoated vesicle or endosome. The endosome fuses with an uncoupling vesicle and the receptors are recycled back to the cell surface. The vesicle containing the TNF fuses with a primary lysosome and a secondary lysosome results. It is here that TNF undergoes degradation into fragments which may lead to lysosomal alterations and/or cell death.

Experimental Approach

Due to the lack of an understanding of the cytotoxic mechanisms of tumor necrosis factor in vitro, a number of studies were performed to examine the correlation between death and other effects on the cell. Initial experiments were performed to demonstrate the cytotoxicity of TNF in various tumor cell lines including the L929 fibrosarcoma and the MCF-7 adenocarcinoma. The L929 and MCF-7 cells were chosen because of their known sensitivity to the cytotoxic effects of TNF (Ostrove and Gifford, 1979; Sugarman et al., 1985). Also, a sensitive human and animal cell line were chosen to determine if TNF was species specific. Next, a series of experiments were carried out to determine the effect of TNF on various biochemical processes including DNA, RNA and protein synthesis. Its effect on the ATP content of tumor cells was also determined. The potential for TNF to cause DNA damage was examined through the use of the alkaline elution technique. Other mechanistic studies involved determining the role of non-protein sulfhydryls in ameliorating the cytotoxic actions of TNF. Because the lysosomes

appear to be important in mediating cytotoxic effects of TNF the level of lysosomal activity was compared in TNF-sensitive and resistant cells using both histochemical and spectrophotometric techniques. In addition, a group of experiments were performed to look at the effect of various lysosomal inhibitors on TNF cytotoxicity in vitro.

To determine the possible role of lysosomal enzymes in TNF toxicity, a wide range of non-specific and specific lysosomal enzyme inhibitors as well as a membrane stabilizer were investigated (Table 1). The pan-lysosomal enzyme inhibitors included chloroquine (Gonzalez-Noriega et al., 1980), ammonium chloride (de Duve et al., 1974) and verapamil (Akiyama et al., 1984). These agents are taken up by the cell and are selectively sequestered in the lysosomes. They cause the pH of the lysosomes to rise above 5 and thereby inactivate the acid-dependent enzymes present. In contrast, leupeptin is a rather specific inhibitor of the lysosomal enzyme cathepsin B while antipain inhibits cathepsins A and B. Bestatin is a highly specific inhibitor of aminopeptidase B and leucine aminopeptidase and pepstatin is an inhibitor of the lysosomal enzyme cathepsin D (Aoyagi et al., 1977). D-sacchric-1,4 acid lactone (DSAL) is a specific inhibitor of the enzyme β-glucuronidase (Aronson et al., 1968). L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) is another specific lysosomal enzyme inhibitor which inactivates cathepsins B, H and L (Barrett et al., 1982). Monensin is a monovalent cationophore that alters cellular monovalent cationic trans-membrane flow and has been shown to disturb the Golgi apparatus. This reduces the intracellular transport of newly synthesized secretory proteins and plasma membrane glycoproteins. In

Table 1

Lysosomotropic Agents

Inhibitor	Enzyme(s) Inhibited
Ammonium Chloride	Pan-lysosomal
Chloroquine	Pan-lysosomal
Monensin	Pan-lysosomal
Verapamil	Pan-lysosomal
Antipain	Cathepsin A and B
Bestatin	Aminopeptidase B
	Leucine Aminopeptidase
Leupeptin	Cathepsin B
Pepstatin	Cathepsin D
DSAL	β-glucuronidase
E-64	Cathepsin B, H and L
Dexamethasone	membrane stabilizer

addition monensin can inhibit some of the post-translational modifications of proteins which are believed to occur in the Golgi apparatus (Tartakoff, 1983). Monensin has also been shown to disrupt lysosomal function and to reduce pinocytotic activity (Wilcox et al., 1983). The glucocorticoids such as dexamethasone act as stabilizers of cellular membranes thereby preventing their fusion and the release of lysosomal enzymes (Weissmann, 1969).

Purpose of Study

The purpose of this thesis was to carry out studies to determine the possible mechanism(s) involved in the killing of cancer cells in vitro by tumor necrosis factor. Specific objectives set to fulfill this purpose included the following:

Evaluation of the time course and dose-response for cytotoxicity of recombinant human tumor necrosis factor in cultured tumor cell lines using the following parameters:

1. Inhibition of colony formation
2. Cytolysis indicated by decreased protein dye binding
3. Intracellular ATP content

The mechanism of TNF-induced cell killing was studied by:

1. Evaluation of the effects of TNF on tumor cells in vitro involving macromolecular synthesis of DNA, RNA and protein.
2. Evaluating the role of reduced thiols in TNF cytotoxicity.

3. Determining whether DNA strand breakage is produced by TNF.
4. Studying TNF's effect on cellular ATP content.
5. Elucidating the role of lysosomes in the mechanism of TNF cytotoxicity in sensitive and resistant cells by using:
    a. Lysosomal enzyme inhibitors
    b. Lysosome isolation and enzyme fractionation studies

CHAPTER II

MATERIALS AND METHODS

Cell Lines and Culture

The L929 cells (Certified Cell Line 1) a murine tumorogenic fibroblast and the MCF-7 (Human Tumor Cell Bank 22) a human breast adenocarcinoma, were obtained from the American Type Culture Collection, Rockville, MD. L929 cells were grown as adherent monolayers on plasticware (Costar, Cambridge, MA) in RPMI 1640 (Irvine Sci. Santa Ana, CA) supplemented with 7.5% fetal bovine serum (M.A. Bioproducts Inc., Walkersville, MD). MCF-7 cells were also grown as adherent monolayers in McCoy's 5A (Irvine Sci, Santa Ana, CA) supplemented with 10% fetal bovine serum. All cells were maintained in a humidified incubator at 37°C in an atmosphere of 95% air-5% carbon dioxide. L929 cells resistant to tumor necrosis factor at a concentration of 1 X 10^5 units/ml, were the gift of Dr. Phillip Scuderi at the Arizona Cancer Center. TNF receptor content was found to be equivalent in the sensitive and resistant lines with approximately 1000 receptors/cell as determined by Scatchard analysis of radioiodinated TNF binding (Scuderi, 1987). Lehmann and Droge (1986) have reported similar findings in TNF-resistant HeLa cells.

Reagents

Recombinant human tumor necrosis factor-alpha (rHuTNF-α) was the gift of Genentech Inc. (San Francisco, CA). It had an activity of

5 X 10^7 units/mg, a protein concentration of 0.49 mg/ml and an endotoxin level of <.123 Endotoxin units (EU)/ml by Limulus amebocyte lysate (LAL) assay as specified by the manufacturer. Culture media containing fetal bovine serum was used as a diluent. All other reagents and inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO) except where noted.

Cytotoxicity Assay

Tumor Necrosis Factor

Test materials serially diluted in media were added to microtiter wells containing 2 X 10^4 L929 cells. After 48 hours of incubation the number of living cells was assessed by the Coomassie G250 method (Bearden, 1978). Briefly, wells were washed 3 times with Hank's balanced salt solution (HBSS) to remove lysed and nonadherent cells and then 50 μl H_2O added to each well. Then the plate was frozen and thawed 3 times and 200 μl of a 1:4 dilution of Coomassie blue G250 solution (Bio-Rad Laboratories, Richmond, CA) in H_2O was added to each well. Finally, the absorbance of protein in each well was measured at 590 nm using a microtiter plate reader (Dynatech MR600).

Enzyme Inhibitors

For the studies of the various enzyme inhibitors, 2 X 10^4 cells were seeded in microtiter wells along with the appropriate concentration of inhibitor and incubated for 24 hours. The wells were then washed and fresh media along with the inhibitor and tumor necrosis factor were added. The cells were incubated at 37°C for another 48

hours. At the end of this time the plates were processed for protein determination as already described. The inhibitors were used at noncytotoxic concentrations (>95% survival) as determined in protein dye cytotoxicity assays.

A significant change in the cytotoxicity of TNF alone vs the cytotoxicity of TNF combined with the various inhibitors was defined as a one $\log_{10}$ or greater difference in the respective dose response curves. This criteria was used to assure that the differences in cytotoxicity were not due only to variability in the assays.

Macromolecular Synthesis Inhibition

The inhibition of DNA, RNA and protein synthesis was investigated using a modification of the nuclide incorporation method of Li et al. (1984). Sterilized glass scintillation vials (Research Products International Corp., Mount Prospect, IL) were seeded with 1.5×10^5 cells in 1.0 ml media. Vials were incubated at 37°C overnight. Following incubation, spent media was discarded and 1.0 ml warmed media containing the desired concentration of rHuTNF was pipetted into each vial. The vials were then placed back into the incubator. During the last hour of exposure 1.0 μCi ^{3}H-thymidine, ^{3}H-uridine or ^{14}C-valine (ICN Radiochemicals, Irvine, CA) for the measurement of DNA, RNA and protein synthesis, respectively, was added to the appropriate vials and the incubation continued. After one hour the supernatants were removed. Vials were then rinsed 2 times with cold Hank's balanced salt solution (HBSS) and 2 times with cold 5% trichloroacetic acid (TCA). Following aspiration of the TCA a final

0.5 ml 5% TCA was added and the vials were incubated in an oven at 80°C for 30 minutes. Upon cooling 15 ml of scintillation fluid (Westchem, San Diego, CA) were added to each vial. Vials were counted in a scintillation counter and % of control of ^{3}H-thymidine, ^{14}C-valine or ^{3}H-uridine counts per minute (cpm) were calculated. As positive controls actinomycin D was used for RNA synthesis (1 μg/ml), cycloheximide for protein synthesis (5 μg/ml) and doxorubicin for DNA synthesis (1 μg/ml).

ATP Determination

The cellular content of ATP is a reliable indicator of general cell viability and of cellular energy metabolism (Strehler et al., 1952). ATP was measured by a modification of the firefly-luciferase reaction (Lundin et al., 1975). Specifically, 6-well tissue culture plates were seeded with 1 X 10^5 cells/well in 2.0 ml media. The plates were incubated overnight at 37°C. Following incubation, spent media was discarded and 2.0 ml of warmed media containing the desired concentration of rHuTNF was pipetted into each well. Plates were incubated for the appropriate time period and supernatants removed. Wells were rinsed one time with HBSS and then 1.0 ml of 90% DMSO:10% .025M HEPES buffer pH 7.75 was added to each well and the plate placed in an ultrasonicator bath for 3 minutes to extract cellular ATP. The supernatant was removed and diluted 1:100 in .025M HEPES buffer pH 7.75. ATP content was then determined using the ATP luciferin-luciferase assay with a luminometer (Turner Designs, Mountain View, CA).

Cytochemical Staining

TNF-sensitive and resistant L929 cells were prepared on slides by cytocentrifugation (Shandon Cytospin 2, Pittsburgh, PA) at 600 rpm for 10 minutes. The slides were then stained for acid phosphatase and β-glucuronidase as described previously (Culling et al., 1985). The substrate for acid phosphatase was naphthol AS-BI phosphoric acid and for β-glucuronidase the substrate was naphthol AS-BI glucuronide. These substrates, when acted on by their respective enzymes, form hydrolysis products which can then be reacted with a fast red diazonium dye for visualization. Cells (100) were visualized at random using light microscopy and cellular enzyme content determined based on staining intensity. Those cells staining the darkest were given a score of 3 while cells staining less intensely were given lesser scores of 2,1 or 0. The staining scores were totaled for each cell type and those cells with a score of 300 were said to have the highest enzyme levels and cells with lower scores contained lesser amounts.

Alkaline Elution Experiments

The potential for TNF-induced DNA damage was assessed by quantitation of DNA single strand breaks (SSBs) as described (Kohn et al., 1976). Cellular DNA was labeled by logarithmic cell growth in complete media containing [2-^{14}C]thymidine (0.1 μCi/ml), followed by growth for 4 hours in nonradioactive media. Following 24 hours of TNF treatment cells were diluted in PBS at 4°C and layered on 25 mm 2.0 μm pore size polycarbonate filters (Nucleopore Corp., Pleasanton, CA). Control cells were x-irradiated with 150-650 cGy prior to loading to

calibrate the number of SSBs per unit dose. Cells were irradiated on ice at a rate of 300 cGy/min using a 4 MeV linear accelerator (Varian Assoc., Palo Alto, CA). For SSB assays 1 X 10^6 cells were lysed on filters with 2% sodium dodecyl sulfate (SDS):20 mM disodium EDTA (pH 10.0). Proteinase K (0.5 mg/ml, E. Merck Co., Darmstadt, Germany) was added to the lysis solution to remove any DNA-protein crosslinks which retard the elution of DNA in a SSB assay. The DNA was eluted from the filter with a solution of 1% SDS adjusted to pH 12.1 with tetrapropyl ammonium hydroxide which was pumped through the filters at a rate of 2.5 ml/hr for 15 hours. Fractions were collected hourly for ^{14}C-DNA quantitation using scintillation counting.

Lysosomal Purification

Cellular material was kept at 4°C throughout the lysosome isolation procedures and during subsequent enzyme analysis. The lysosomal isolation was carried out as previously reported with some modifications (Jonas et al., 1983). Adherent cells were initially scraped from the flask with a rubber cell scraper, and then washed twice with HBSS. Next the cells were washed once with 0.25 M sucrose and then disrupted by repetitive pipetting in 10 ml of 0.25 M sucrose. The resulting crude cell homogenate was centrifuged for 10 min at 750 X g followed by removal of the supernatant. The pellet was resuspended and the entire procedure was repeated two more times. The supernatants containing subcellular organelles and plasma membrane fragments were combined and centrifuged in a Beckman SW28 rotor for 10 min at 20,000 X g_{av}. The pellet was resuspended in 40 ml of 0.25 M sucrose, 20 mM

HEPES, pH 7.0 and centrifuged again at 20,000 X g_{av}. The pellet was resuspended in 4 ml HEPES/sucrose buffer and further purified using Percoll gradients. The resuspended pellet in buffer was added to 6 ml buffered Percoll (90% Percoll:10% 2.5 M sucrose with 200 mM HEPES, pH 7.0) and mixed by inversion. The suspension was centrifuged for 90 min at 40,000 X g_{av} in a Beckman T70.1 rotor. Density of the fractions was determined using calibrated density marker beads (Pharmacia Fine Chemicals, Uppsala, Sweden).

Enzyme Assays

A variety of enzymes were assayed in the gradient fractions to determine the distribution of organelles and membranes in the preparations. Sample amounts were chosen to yield enzyme activities within a linear range for each assay. All enzyme activities were determined after solubilization of samples in 0.1% Triton X-100 which did not interfere with any of the assays. Protein levels were determined spectrophotometrically by the method of Smith et al. (1985) using a commercially available kit (Pierce Chemical Co., Rockford, IL).

For plasma membranes, alkaline phosphodiesterase I was determined using p-nitrophenyl-5'-thymidylate as a substrate (Aronson and Touster, 1974). Hexosaminidase (Li and Li, 1972), acid phosphatase (DiPietro et al., 1967) and β-glucuronidase (Fishman et al., 1967) were used as lysosomal markers. Succinate-p-iodonitrotetrazolium violet reductase was used as a marker for mitochondrial enzyme activity (Morre et al., 1971). For golgi apparatus, the enzyme α-D-mannosidase II was

measured with p-nitrophenyl-α-D-mannopyranoside as the substrate (Tulsiani et al., 1977). All assays were performed in triplicate and enzyme activities expressed as milliunits/mg cellular protein.

Colony Forming Assay in Soft Agar

Inhibition of tumor cell colony formation was determined using the soft-agar cloning method of Hamburger et al. (1977). Briefly underlayers were prepared in 35 mm petri dishes using 0.3% molten agar (Difco Bacto-Agar) in culture media. After these had gelled a single cell suspension of tumor cells in 0.3% molten agar and culture media was pipetted on to the underlayer. All tumor necrosis factor exposures were continuous with the appropriate concentration of cytokine present in the final plating medium. All drug exposures were performed in triplicate with the plates being incubated at 37°C in a humidified incubator with an atmosphere of 5% CO_2-air for 7-9 days. At the end of this time the tumor cell colonies (>60 μm in size) were enumerated using an automated image analysis instrument (FAS II, Omnicon^R, Bausch and Lomb, Rochester, NY) (Salmon et al., 1984).

Non-protein Bound Sulfhydryl Determination

Non-protein sulfhydryl group (NPSH) concentrations were determined as previously described (Sedlak and Lindsay, 1968) but with certain modifications. TNF-sensitive and resistant L929 cells were prepared by scraping them from the culture flask and then washing them 2 times with ice-cold phosphate-buffered saline pH 7.4 (PBS). An aliquot of 5×10^6 cells was then resuspended in 1.0 ml distilled deionized water. The cells were lysed using an ultrasonic probe and

100 μl 5% sulfosalicylic acid added. The tube was cooled on ice for 10 minutes to precipitate cellular proteins and spun at 12,000 X g for 5 minutes in a Fisher microcentrifuge. A 1.0 ml aliquot of the supernatant was removed and used for the NPSH determination as previously reported (Sedlak and Lindsay, 1968). Results were expressed as nmole/10^6 cells.

Statistics

Experiments for determination of enzyme activity in both whole cells and lysosomal fractions were carried with triplicate samples in all instances. Data are expressed as mean ± standard deviation. Differences in enzyme activity between the fractions for TNF-sensitive and resistant cells were analyzed using a Student's t-test for paired samples with significance being designated for $p < 0.01$.

Dose response curves for TNF cytotoxicity were analyzed for variation in the per cent survival using the formula for variance of a ratio (Slyman, 1987).

CHAPTER III

RESULTS

TNF Cytotoxicity Studies

Sensitive L929 Cells

Previous studies have demonstrated the need for continuous exposure to tumor necrosis factor for >15 hours to achieve maximal cytotoxic responses in vitro (Kull and Cuatrecasas, 1981). Therefore, all cytotoxicity assays were carried out with cells continually exposed to TNF throughout the 5-7 day colony-forming assays or the 48 hour dye cytotoxicity assays. Figure 2 compares the dose response curves for TNF against sensitive L929 cells using both assays. With each procedure, the dose response curve was sigmoidal but relatively shallow. This is unlike the steeper survival curves produced by most cytotoxic antitumor agents but not unlike those seen for steroids (Alberts et al., 1981). The flat secondary phase of sensitivity may be due to the existence of various subpopulations of cells resistant to the effects of TNF (Nakano et al., 1986).

Using either the soft-agar cloning or Biorad assay, a 50% cytotoxic concentration (IC_{50}) of TNF was found to be in the range of 2-6 ng/ml by continuous exposure. However, the IC_{50} was consistently lower with the colony forming assays. From this analysis, the colony-forming assay appears to be a more sensitive method of measuring the response of cells to TNF. This is probably due to the ability of this assay to measure both cytolytic as well as cytostatic effects of TNF on

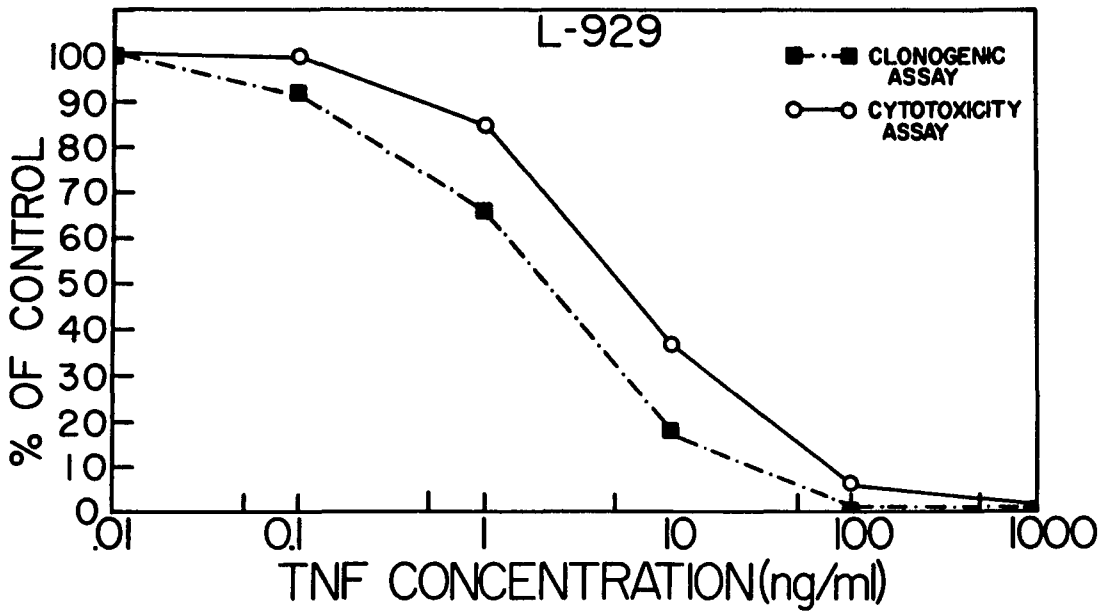

Figure 2. Dose response curves for TNF in TNF-sensitive L929 cells.

Dose response curves for TNF in TNF-sensitive L929 cells generated using colony-forming and cytotoxicity assays. TNF was present throughout the course of the experiment. Each data point represents the mean of two experiments performed in triplicate. Where no error bars are present they are contained within the symbol.

the cells (Leonessa et al., 1986). In contrast, the cytotoxicity assay for protein content measures only the cytolytic activity of TNF. Nonetheless, both assays demonstrated similar cell killing activity for TNF in L929 cells. Because of the simplicity and time savings involved in its use, the Coomassie blue dye cytotoxicity assay was chosen for measuring cell killing by TNF in the various mechanistic studies.

Resistant L929 Cells

A TNF-resistant L929 subclone was produced by serial passage in increasing concentrations of recombinant human tumor necrosis factor over several months. This resistant line showed no response to the cytolytic effects of TNF at doses up to 10,000 ng/ml as determined in the dye cytotoxicity assay. Differences between the resistant subclone and the sensitive parent were then investigated to help determine reasons for the loss of sensitivity to the actions of TNF.

MCF-7 Cells

The human breast adenocarcinoma cell line MCF-7 was also tested for sensitivity to the lethal effects of TNF. Using the dye cytotoxicity assay, little response was noted even after 72 hours of continuous TNF exposure. This differs from results published by others which showed good sensitivity of MCF-7 cells to TNF using mitochondrial function assays rather than the cytolytic method (Sugarman et al., 1985). Using the colony-forming assay, MCF-7 cells did show sensitivity to TNF with a concentration of 5 ng/ml producing 50% inhibition of colony formation (IC_{50}) by continuous exposure (Figure 3). Again, the slightly greater sensitivity of the colony

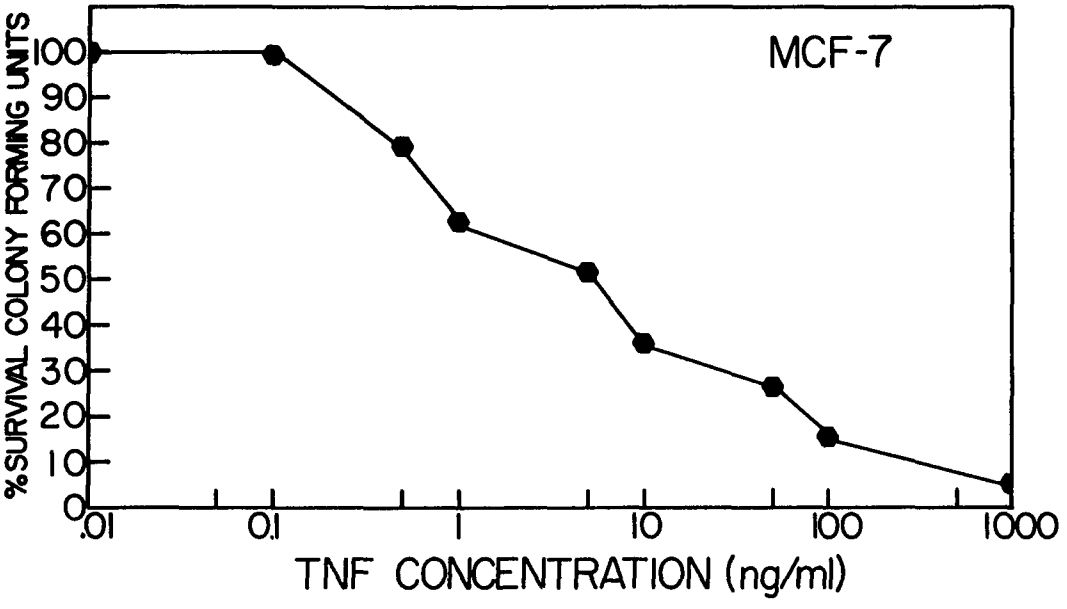

Figure 3. Dose response curve for TNF in MCF-7 cells.

Dose response curve for TNF in MCF-7 cells using the colony-forming assay. TNF was present throughout the course of the experiment. Each data point represents one experiment performed in triplicate. Where no error bars are shown they are contained within the symbol.

forming assay can be attributed to its ability to measure both the cytostatic and cytolytic effects of TNF (Leonessa et al., 1986). Because greater inhibition was noted in the colony-forming assays, it appeared that MCF-7 cells are more sensitive to the cytostatic properties of TNF than to its cytolytic effects. The phenomenon of cells undergoing mainly a cytostatic response to TNF has been previously reported in melanoma, lung and ovarian tumor cell lines (Williamson et al., 1983).

Mechanism of Action Studies

DNA, RNA and Protein Synthesis and ATP Content

Macromolecular synthesis experiments were done by exposing L929 and MCF-7 tumor cells continually to a cytotoxic concentration of TNF (IC_{100}). The incorporation of radiolabeled nuclides into acid insoluble DNA, RNA and protein was then quantitated. Cellular energy stores were approximated using the firefly luciferin-luciferase assay for ATP. Using these methods it was possible to monitor the effect of TNF on biochemical cellular processes including those leading to high energy phosphate generation.

L929 Cells

Figure 4 illustrates the results of these experiments carried out on TNF-sensitive L929 cells. Initially, there is a lag time of approximately 5-10 hours before any perturbation is seen in the synthesis of DNA, RNA or protein as well as in ATP content. This is compatible with previous reports indicating a requirement for binding,

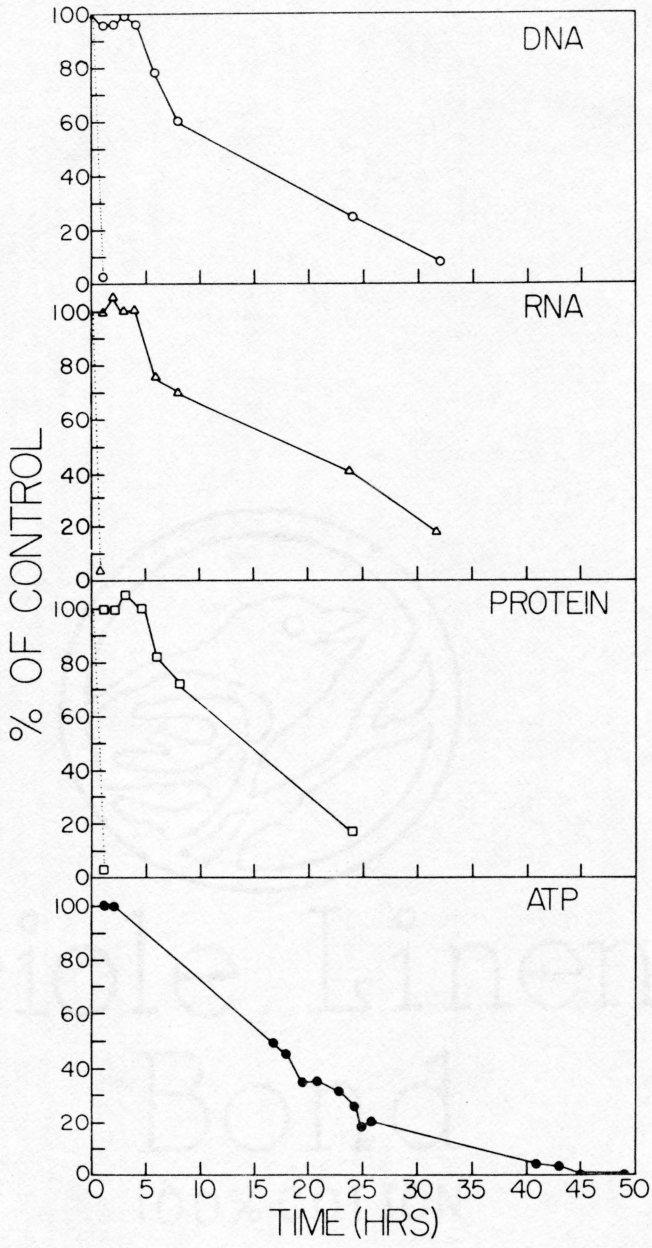

Figure 4. Effect of TNF on macromolecular synthesis and ATP content of TNF-sensitive L929 cells.

Effect of TNF and specific macromolecular synthesis inhibitors on DNA, RNA and protein synthesis and ATP content of TNF-sensitive L929 cells. TNF and inhibitors were present throughout the course of the experiment. Solid lines represent data for TNF exposure and dotted lines represent data for inhibitor exposure. See Table 2 for details.

internalization and degradation of the TNF molecule before a cytotoxic response is seen (Tsujimoto et al., 1985). After the initial lag time, there is a proportional decrease in all four parameters (Figure 4). Thus, TNF simultaneously suppressed the synthesis of each macromolecule. There was no evidence for an early "selective" inhibition of DNA, RNA or protein synthesis or cellular ATP content in the L929 cells (Table 2). This suggests that none of these parameters are specifically involved in the mechanism of cytotoxicity of tumor necrosis factor in L929 cells.

MCF-7 Cells

The same experiment was carried out using the MCF-7 tumor cell line (Figure 5). As with the L929 cells there was an initial lag time during which no effect on macromolecular synthesis was seen. Following this period, there was a steady and parallel decrease in all four parameters. The transient increase in DNA, RNA and protein synthesis 30 hours following exposure may in fact represent cells that are undergoing mitosis which increases the DNA, RNA and protein pools. This phenomenon has been reported previously in the case of RNA synthesis following treatment of L929 cells with tumor necrosis serum (Ostrove et al., 1979). After the initial increase, the parallel non-specific decrease in all four parameters continues. Thus, in the MCF-7 cells TNF again does not specifically inhibit macromolecular synthesis or cellular ATP content (Table 2). The slower decrease in ATP content as compared to the other parameters is intriguing and may be due to the cells being more sensitive to macromolecular synthesis inhibition than

Table 2

Time Points for 50% Decrease in DNA, RNA and Protein Synthesis and ATP Content in L929 and MCF-7 Cells Produced by TNF and Specific Macromolecular Synthesis Inhibitors

Parameter	Time at which 50% decrease occurs (hrs)*			
	L929**	L929***	MCF-7**	MCF-7***
DNA Synthesis	12.5	≤0.5	50	≤0.5
RNA Synthesis	19	≤0.5	50	≤0.5
Protein Synthesis	14	≤0.5	60	≤0.5
ATP Content	16.5	ND	95	ND

* Time at which a reduction of 50% occurs in the parameter for the treated cells vs the control cells.

** Cells were exposed continuously to 100 ng/ml of TNF during the entire experiment.

*** Cells were continuously exposed to various inhibitors: Actinomycin D for RNA (1μg/ml), Doxorubicin for DNA (1 μg/ml) and Cycloheximide for protein (5 μg/ml)

ND Not Determined

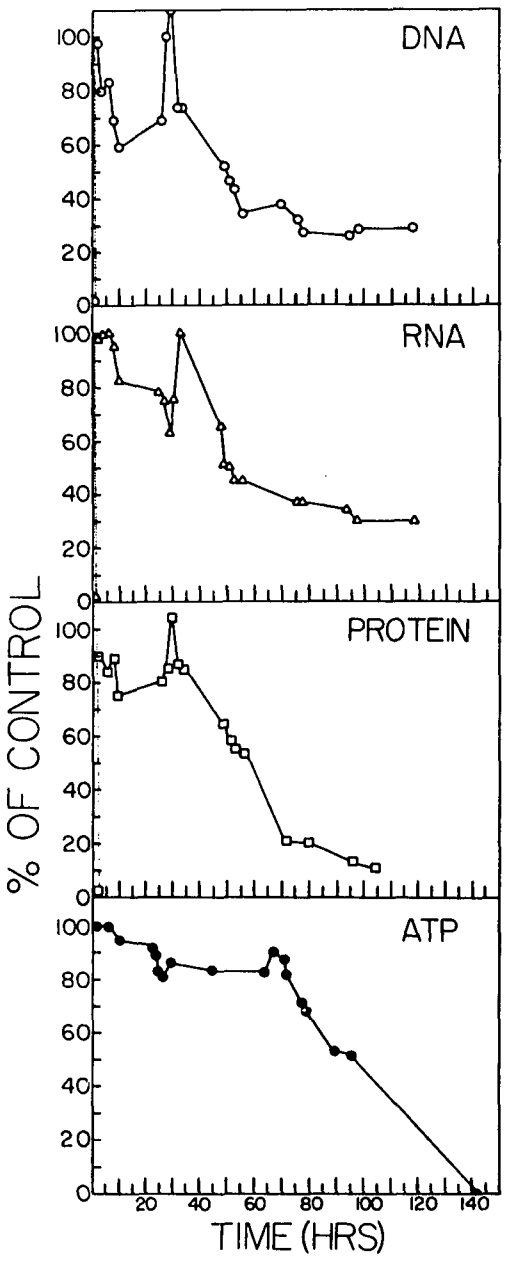

Figure 5. Effect of TNF on macromolecular synthesis and ATP content of MCF-7 cells.

Effect of TNF and specific macromolecular synthesis inhibitors on DNA, RNA and protein synthesis and ATP content of MCF-7 cells. TNF and inhibitors were present throughout the course of the experiment. Solid lines represent data for TNF exposure and dotted lines represent data for inhibitor exposure. See Table 2 for details.

to energy depletion. These observations lead to the conclusion that TNF must be causing cytostasis and/or cytolysis through mechanisms other than inhibition of macromolecular synthesis.

Specific Inhibitors

Use of specific metabolic inhibitors such as actinomycin D, which inhibits RNA synthesis, doxorubicin which inhibits DNA synthesis and cycloheximide, which inhibits protein synthesis, at appropriate concentrations caused a greater than 90% inhibition of each parameter with in one hour of exposure for both L929 and MCF-7 cells (Figures 4 and 5). TNF did not produce the sudden marked decrease in any one of the parameters as was seen with the specific inhibitors. The use of these positive controls supports the interpretation of the results that suggests that TNF does not kill cells by specifically inhibiting macromolecular synthesis. TNF should cause a decrease in one of the parameters over the others if it was specifically inhibiting one of the processes.

The use of an oxidative phosphorylation inhibitor such as 2,4-dinitrophenol (2,4-DNP) would cause a rapid decrease in ATP content of the cells, just as the other inhibitors specifically cause decreases in their respective processes. The problem in the use of 2,4-DNP is that it also causes a rapid decrease in macromolecular synthesis as do other oxidative phosphorylation uncouplers and this precludes its use as a positive control in whole cell experiments (Snoeij et al., 1986).

Cytochemical Staining

TNF-sensitive and resistant L929 cells were prepared by cytocentrifugation and stained for the lysosomal enzymes acid phosphatase and β-glucuronidase. Traditionally, the level of acid phosphatase is used as a marker of lysosomal activity. Thus, I felt this would be a quick method to determine if the two subclones differed in the numbers of lysosomes. As the data in Table 3 show, both the sensitive and resistant L929 cells have equal staining scores for acid phosphatase. They differed substantially however, in their staining scores for β-glucuronidase. The resistant cells contained no stainable enzyme at all. The results of this experiment led us to pursue determinations of the role that β-glucuronidase might play in TNF resistance. It also provided us with evidence that lysosomes may play an important role in TNF's mechanism of action.

Lysosomal Inhibitor Studies

Because earlier studies had indicated a possible role for lysosomes in the cytotoxic actions of a crude tumor necrosis serum (Kull and Cuatrecasas, 1981), experiments were conducted to determine the effect of various specific and non-specific lysosomal enzyme inhibitors on the cytolytic action of TNF in L929 cells. These were performed to determine whether lysosomes are directly involved in TNF's mechanism of action and whether specific lysosomal enzymes are more crucial than others.

The pan-lysosomotropic agents verapamil, ammonium chloride and chloroquine each inhibited the cytolytic activity of TNF to varying

Table 3

Lysosomal Enzyme Cytochemical Staining Scores for TNF-Sensitive and Resistant L929 Cells

L929 Cell Type	Enzyme Score	
	Acid phosphatase*	β-glucuronidase*
Sensitive L929	300	124
Resistant L929	300	0

* Substrate for acid phosphatase was naphthol AS-BI phosphoric acid.

** Substrate for β-glucuronidase was naphthol AS-BI glucuronide. Both reaction products were visualized using fast red diazonium dye. Scores represent the total from 100 cells which were assessed for enzyme content by light microscopy.

degrees (Table 4). Ammonium chloride was the most effective and chloroquine the least at maximal non-cytotoxic concentrations.

The various specific cathepsin inhibitors did not decrease the cytolytic action of TNF to the extent that the pan-lysosomotropic agents did (Table 4). Only DSAL which inhibits β-glucuronidase and leupeptin which blocks cathepsin B produced any appreciable (>0.5 $\log_{10}$) change in TNF sensitivity. This finding tends to support the view that the lysosomal enzymes which are important in the action of TNF probably include β-glucuronidase and few, if any cathepsins.

Dexamethasone was also moderately effective in reducing the toxicity of TNF. This is presumably due to its membrane stabilization properties which would block the degradation of lysosomal membranes and prevent the exposure of cells to toxic lysosomal products. However, dexamethasone induces the synthesis of a number of other proteins and these may also effect TNF activity.

Overall, none of the lysosomotropic agents produced a total inhibition of TNF cytolytic action other than ammonium chloride. These findings seem to indicate that a number of enzymes may be involved in TNF's action. It is not clear however whether it is through lysosomal degradation of the TNF polypeptide into active (toxic) fragments, and/or their release into the cytoplasm that causes cell death.

Glutathione Studies

The chemical L-buthionine-S,R-sulfoximine (BSO) is a specific inhibitor of γ-glutamylcysteine synthetase, the enzyme which catalyses the rate-limiting step in glutathione (GSH) synthesis. The

Table 4

Effect of Enzyme and Metabolic Inhibitors on TNF Cytotoxicity in L929 Cells

Treatment	Concentration	Sensitivity (Log Change)*
BSO	10 μM	-0.6
Dexamethasone	5 μM	+0.6
NH_4Cl	20 μM	+6.0
Chloroquine	10 μM	+1.3
Verapamil	22 μM	+3.0
Leupeptin	50 μM	+0.6
Antipain	50 μM	0.0
Bestatin	50 μM	-0.3
Pepstatin	5 μM	+0.1
DSAL	5 mM	+1.3
E-64	100 μM	0.0
Monensin	1 nM	+0.35

* (-) indicates increase in sensitivity, (+) indicates decrease in sensitivity, both determined at IC_{50} concentration. A significant change is defined as a one log or greater difference in sensitivity.

administration of BSO thereby leads to glutathione depletion (Meister et al., 1981). Sherwin (1987) had suggested an association between cellular GSH levels and TNF sensitivity in cultured cell lines. Thus, a series of experiments were performed to test this hypothesis. The results showed that BSO reduced the IC_{50} for TNF in sensitive L929 cells by just over 0.5 $\log_{10}$. The TNF-sensitive and resistant L929 cells were also analyzed for non-protein sulfhydryl (NPSH) content to see if this might account for the differences in sensitivity to TNF since it had been suggested that resistant cells had higher NPSH levels. The results (Table 5) however, show that there is no clear association between GSH content and TNF sensitivity in the L929 cells, since both cell lines contained similar levels of NPSHs. Thus, at least in the L929 cells, the content of cellular GSH does not appear to correlate to the sensitivity to TNF in vitro.

DNA Damage Experiments

DNA Single Strand Breaks

The ability of TNF to induce single strand breaks (SSBs) in the DNA of L929 cells was investigated using alkaline elution techniques. L929 cells were exposed for 24 hrs to 1, 10, 100 and 1000 ng/ml TNF and then the incidence of SSBs was determined using the method of Kohn et al. (1976). A radiation dose of 250 cGy was used as a positive control. Figure 6 illustrates a typical DNA elution pattern for these experiments. It is apparent that, there is no significant increase in the rate of DNA elution for the TNF-treated cells versus that of the controls. Even at a TNF concentration of 1000 ng/ml (which is

Table 5

Non-Protein Sulfhydryl Group (NPSH) Determination of TNF-Sensitive and Resistant L929 Cells

Cell Line	NPSH (nmole/10^6cells)*
TNF-sensitive L929	5.7±2.7
TNF-resistant L929	5.3±0.7

* mean of 3 determinations (SD) on 5 X 10^6 cells.

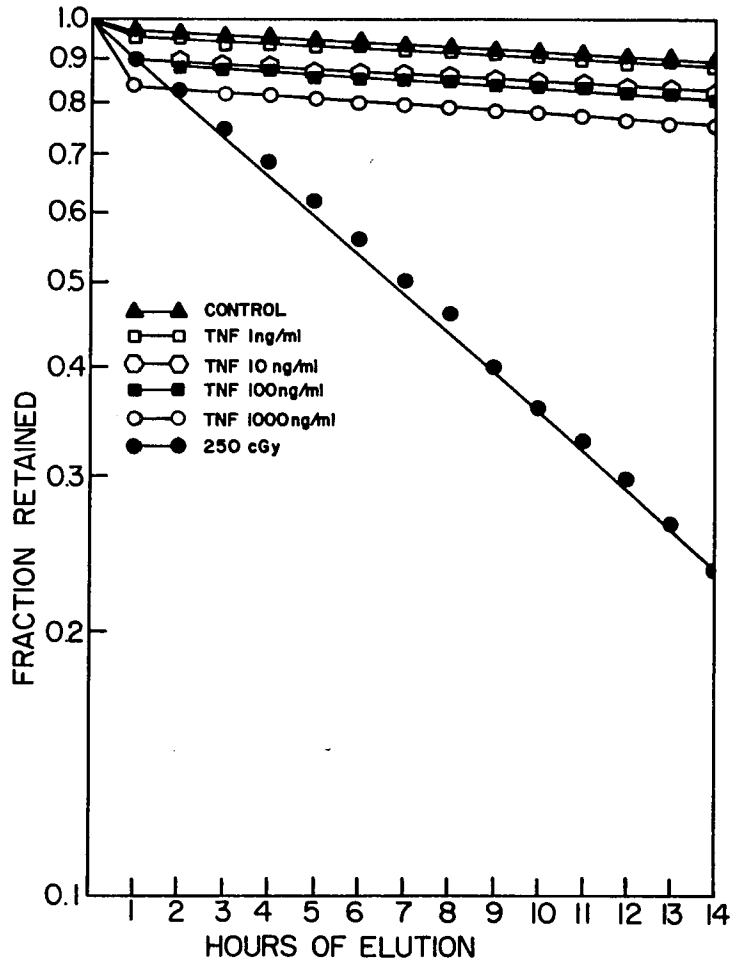

Figure 6. Effect of TNF on incidence of DNA single-strand breaks in TNF-sensitive L929 cells.

Effect of TNF on the incidence of DNA single strand breaks in TNF-sensitive L929 cells as assessed by alkaline elution. Cells were exposed to TNF for 24 hrs prior to start of experiment. The fraction of ^{14}C-labeled DNA retained on the filter is plotted against the time of elution.

cytotoxic to over 90% of the cells), there is no increase in elution indicating no significant production of SSBs. This leads to the conclusion that TNF does not directly cause SSBs in the DNA of lethally-treated cells. These observations provide further evidence for the hypothesis that TNF acts at a cytoplasmic target rather than one in the nucleus. It is possible that any DNA strand breaks, whether single or double, which become apparent after TNF treatments, may be due to the action of lysosomal enzymes on DNA released from lethally damaged cells.

Lysosomal Isolation

Cellular Enzyme Determinations

TNF-sensitive and resistant L929 cells were prepared and lysosomes isolated as described in the methods section. A portion of the whole cell lysate was set aside for protein and cellular enzyme determinations. Table 6 lists these enzymes and their relative activities in both the TNF-sensitive and TNF-resistant L929 cells. In the whole cells there was a slightly greater amount of hexosaminidase activity per milligram of protein in the TNF-resistant cells as compared to the sensitive ones ($p < 0.01$). There was also a level of β-glucuronidase activity in the sensitive cells which was twice that of the resistant clones ($p < 0.01$). The other lysosomal enzyme marker, acid phosphatase, was slightly elevated in the resistant cell line.

The enzymes used as markers for mitochondria (succinic-INT-reductase), golgi apparatus (α-mannosidase II), and plasma membrane

Table 6

Cellular Enzyme Activities of TNF-Sensitive and Resistant L929 Cells

	ENZYME LEVELS Whole Cells	
	TNF-sensitive L929 (mU/mg protein)*	TNF-resistant L929 (mU/mg protein)*
Hexosaminidase	5.93±0.07	7.29±0.50**
β-Glucuronidase	27.90±0.71	11.71±1.52**
Acid Phosphatase	2.34±0.04	2.53±0.05**
Succinic-INT-Reductase	1.00±0.03	1.09±0.06
α-Mannosidase	2.51±0.02	2.70±0.04**
Alkaline Phosphodiesterase	0.24±0.01	0.21±0.03
Cellular Protein ($mg/10^8$ cells)	27.5	25.8

* mean of 3 determinations (SD) on 10^8 cells.

** $p < 0.01$ Student's t-test for difference between sensitive and resistant cells.

(alkaline phosphodiesterase I) did not show any biologically significant differences between the sensitive and resistant cells.

Lysosomal Fraction Enzyme Determinations

The lysosomal fractions obtained by differential density centrifugation (density 1.050 g/l) showed a greater difference in activity between the sensitive and resistant subclones using the various marker enzymes (Table 7). The fraction obtained from the TNF-resistant cells had twice the level of hexosaminidase activity as compared to the sensitive cell's lysosomal fraction. Importantly, the resistant cells had only half the β-glucuronidase activity when quantitated on a basis of total protein. The lysosomal fraction from the sensitive cells contained approximately two-thirds the acid phosphatase activity and over three times the succinic-INT-reductase activity indicative of mitochondria. The level of α-mannosidase was approximately equal for both fractions and the level of alkaline phosphodiesterase, though low, was twice as high in the sensitive (Table 7).

The enzyme activities determined spectrophotometrically for both β-glucuronidase and acid phosphatase are consistent with the results described earlier using cytochemical staining. It should be recalled that the resistant cells had less β-glucuronidase staining than in the sensitive cell line. Indeed the staining technique failed to detect any β-glucuronidase in the resistant cells. This is most likely due to the fact that the cytochemical staining requires that enzymes be localized (i.e., in an organelle) rather than diffused

Table 7

Cellular Enzyme Activities of Lysosomal Fractions from TNF-Sensitive and Resistant L929 Cells

	ENZYME LEVELS Lysosomal Fraction	
	TNF-sensitive L929 (mU/mg protein)*	TNF-resistant L929 (mU/mg protein)*
Hexosaminidase	13.03±0.06	27.90±1.46**
β-Glucuronidase	52.61±2.70	22.52±6.20**
Acid Phosphatase	2.07±0.01	3.09±0.05**
Succinic-INT-Reductase	2.45±0.77	0.74±0.39**
α-Mannosidase	2.08±0.00	2.01±0.02
Alkaline Phosphodiesterase	0.12±0.01	0.06±0.01**
Lysosomal Protein (mg/10^8 cells)	1.94	0.97

* mean of 3 determinations (SD) on 10^8 cells.

** $p < 0.01$ Student's t-test for difference between sensitive and resistant cells.

throughout the cytoplasm. It should be further stressed that staining techniques can be subjective and and are clearly dependent on consistent cytocentrifugation techniques.

The more pronounced differences in the various marker enzymes for the lysosomal fractions vs the whole cells can be attributed to a number of factors. First, whole cells usually contain the enzymes in both an organelle distribution as well as in a diffuse cytoplasmic distribution. Depending on the state of the cell and the enzyme being assayed, a significant amount of total enzyme content can be cytoplasmic in distribution. Also in the isolation procedure, some organelles can be ruptured with a loss of their enzymes prior to analysis. Table 7 shows that the sedimentation isolation procedure for the lysosomes does not produce a completely pure lysosomal fraction free of all other organelle contamination since there were detectable concentrations of plasma membrane and especially mitochondrial enzymes. This may account for some of the differences in mitochondrial activity between fractions from the two cell lines. Nonetheless, the procedure concentrated the lysosomal fraction efficiently provided clear distinctions between the TNF-sensitive and resistant L929 cells.

CHAPTER IV

DISCUSSION

Tumor necrosis factor (TNF) is a natural human polypeptide with a molecular weight of approximately 17 kilodaltons which exhibits broad cytotoxic activity against various tumor cells both in vitro and in vivo. It was first described by Carswell et al. (1975) and since that time a large body of research has been directed toward the characterization of TNF and its effects on various normal and neoplastic cells. Much work has also been dedicated to elucidation of the mechanism by which it kills tumor cells. Currently, little is known concerning this process. It may have both cytostatic and cytolytic effects on some tumor cells, while in others, including normal cells, it may produce no toxic effects. Various researchers have tried to elucidate why this is and have put forward a number of hypotheses in an effort to arrive at an answer. As yet there is no definitive explanation for these effects.

TNF Cytotoxicity

In these studies, the first task was to develop a system for testing the cytotoxicity of TNF in tumor cells. It was also necessary to obtain cell lines which were sensitive and resistant to the cytotoxic effects of the cytokine. Previous reports indicated that the L929 and MCF-7 tumor cell lines were sensitive to TNF (Carswell et al., 1975; Yamamoto et al., 1984) and that both colony forming

(Watanabe et al., 1985) and dye-uptake (Carswell et al., 1975) assays were useful for quantifying its cytotoxic effects. Using these methods it was possible to demonstrate the cytotoxicity of TNF in both cell lines. The dye binding assay was used primarily due to its simplicity and time savings although some sensitivity to the cytostatic actions of TNF may have been sacrificed.

Effects on DNA, RNA and Protein Synthesis and ATP Content

Many of the drugs used in the treatment of neoplastic diseases act by specifically inhibiting various biochemical processes such as macromolecular synthesis (Chabner, 1982). When any new antineoplastic agent is introduced it is important that the mechanism by which it acts be understood in order to aid in its being used most effectively. The initial approach in the current study involved looking at the effect of TNF on primary cell processes such as RNA, DNA and protein synthesis. It was also important to assess the effect of TNF on cellular energy content using measurements of intracellular ATP levels.

Early data suggested that TNF could cause a transient increase in RNA synthesis even though it ultimately killed the cells (Ostrove et al., 1979). The current findings are unique since there are no other reports which comprehensively evaluate the effect of TNF on cellular DNA, RNA and protein synthesis as well as ATP content using highly purified recombinant human TNF rather than the crude serums used in earlier mechanistic studies.

In these experiments TNF failed to fit the classical pattern of a specific metabolic inhibitor. A given concentration of TNF did not

produce an immediate decrease in synthesis of a single class of macromolecules and even failed to produce a broad spectrum of effects in a short time period (<1 hr). At concentrations which were lethal to all cells specific inhibition of macromolecular synthesis also failed to occur. DNA, RNA and protein synthesis rates all decrease at about the same rate. Thus in comparison to classical macromolecular synthesis inhibitors, TNF does not fit the pattern.

Specific metabolic inhibitors can effect a broad range of biochemical processes other than the one they are specific for when used at high concentrations. For example, actinomycin D selectively inhibits RNA synthesis at concentrations below 1 μg/ml but as the dose increases inhibition of DNA and protein synthesis also occurs. Also inhibition of specific biochemical processes usually occurs in less than an hour after cells are exposed to these agents.

TNF also does not seem to specifically inhibit oxidative phosphorylation or other cellular energy production. Again, classical inhibitors such as 2,4-dinitrophenol produce a reduction in ATP content of cells which is greater than 90% of the control value in less than one hour. TNF does not cause a rapid depletion of ATP in this manner. Rather it causes a slow loss of energy which seems to follow cell death. So in the case of TNF exposure, ATP content determination appears to simply be an indicator of cell viability.

Overall, these data confirm previous reports that TNF has no specific effect on DNA or RNA synthesis (Darzynkiewics et al., 1984). The current results also refute the hypothesis that TNF's primary mechanism of action involves disturbance of cellular energy metabolism

(Jones, 1986). It is therefore concluded that TNF's cytotoxic action does not involve specific effects on DNA, RNA or protein synthesis or energy production of tumor cells.

Role of Reduced Thiols

One preliminary report stated that reduced thiols, such as glutathione, may be important in determining sensitivity to the cytotoxic effects of TNF (Sherwin, 1987). It was also believed that TNF could cause mobilization of polyunsaturated fatty acids and subsequent lipid peroxidation which would lead to free radical production and cell death (Fiers et al., 1986). These researchers argued that cells with lower levels of glutathione would have decreased abilities to scavenge free radicals and would therefore be more sensitive to the cytotoxic actions of TNF.

Conversely, cells selected for resistance to TNF should have higher levels of reduced thiols as compared to their sensitive counterparts. Therefore, glutathione depleting agents such as BSO should make TNF-sensitive cells even more susceptible to its cytotoxic effects and should reverse the resistance of cells which can withstand the cytotoxic effects of TNF.

However, the current studies of the non-protein sulfhydryl levels of TNF-sensitive and resistant L929 cells showed that these cell lines did not have significantly different amounts of GSH. This is contrary to what would be expected if thiols were important in mediating TNF cytotoxicity. Furthermore, BSO did not significantly augment TNF sensitivity in normal L929 cells and it did not reverse the

resistance of TNF-resistant L929 (Data not shown). These data seem to clearly refute the hypothesis that TNF's mechanism of action is dependent on the amount of reduced thiols present in cells.

DNA Effects

The possibility that TNF causes cell death by DNA strand breakage had been suggested by several groups (Maury, 1986; Ruddle, 1987). However, there was little convincing evidence brought forward to support this hypothesis. For example, previous reports suggested that lymphotoxin, a cytokine with 30% homology with TNF, caused fragmentation of target cell DNA (Schmid et al., 1986). This suggested that TNF's cytotoxic mechanism of action might similarly involve damage to nuclear DNA which ultimately caused cell death (Ruddle, 1987).

In an attempt to test this hypothesis a series of studies were performed to document any DNA damaging ability of TNF using the alkaline elution technique (Kohn et al., 1976). Looking at the incidence of single strand breaks (SSBs) caused by TNF in L929 cells, it was found that high concentrations of TNF over a sufficient time period for adequate internalization did not produce significant DNA SSBs. This suggests that TNF's primary mechanism of action does not involve damage to nuclear DNA through single strand breaks at least as indicated by alkaline elution experiments in L929 cells. These results do not exclude the possibility of other types of DNA lesions such as DNA-DNA or DNA-protein crosslinking.

TNF Cytotoxicity and Lysosomes

Lysosomes are primarily involved in the digestion of biosynthetic material. The numerous hydrolytic enzymes present in these organelles usually exert their actions inside the cell, in the vacuolar system of the cytoplasm, either on exogenous material engulfed by endocytosis or on endogenous cell constituents segregated by autophagy. Normally, intralysosomal digestion leads to products that are able to leave the lysosomes by passing unhindered through lysosomal membranes. Non-digestible materials that cannot pass through the membrane accumulate and can cause pathological overloading and swelling of these organelles. This is the situation found in genetic lysosomal storage diseases which involve the congenital absence of a single lysosomal enzyme. In a disease such as Tay-Sachs the missing enzyme is hexosaminidase A and due to its absence, the ganglioside GM_2 accumulates in such amounts as to cause cell death.

This suggests that the alteration of one lysosomal enzyme may be sufficient to confer resistance to a cell to the cytotoxic effects of TNF. Thus, TNF may be degraded to toxic polypeptide fragments by a single enzyme. The deletion of the one enzyme would allow the cell to avoid death by not forming these toxic species. Conversely, there may be the presence of an enzyme in TNF-resistant cells which metabolizes TNF to fragments which can then undergo exocytosis allowing the cell to avoid cell death. The absence of this enzyme in the sensitive cells does not allow them to degrade the TNF. The TNF may then accumulate in the lysosome leading to swelling, eventual rupture and cell lysis.

Both of these theories are viable considering the current data. The lysosomal enzyme inhibitors used in the various experiments may have either prevented enzymatic degradation of TNF to cytotoxic species or they may have inhibited the enzymes which could lead to cell lysis. Further investigations are needed to differentiate which of these is the case.

The various pan-lysosomotropic agents all produced at least a one log or greater loss of sensitivity to TNF's cytotoxic action in the L929 cells. Of these, ammonium chloride produced almost complete inhibition of TNF cytotoxicity as compared to the other agents. Surprisingly, verapamil also decreased the cytotoxicity of TNF by three logs. This was not entirely expected, though some of this effect may be due to verapamil's membrane altering properties. Chloroquine was least effective at blocking TNF cytotoxicity probably due to its toxicity at high concentrations.

In contrast, the more specific lysosomal enzyme inhibitors did not produce as large an inhibition of the cytotoxic activity of TNF in the L929 cells. Results similar to these for leupeptin have been published before (Kull and Cuatrecasas, 1981). Importantly, D-sacchric acid-1,4-lactone (DSAL) produced the greatest inhibition of TNF's cytotoxic action. This is intriguing considering that it is an inhibitor of β-glucuronidase and that the TNF-resistant L929 have a decreased level of the enzyme as compared to the sensitive parent.

The lysosomal enzyme β-glucuronidase is involved in cleaving non-reducing terminal β-glucuronosyl residues from glycosaminoglycans and conjugated steroids, drugs and other xenobiotics (Barrett and

Heath, 1977). TNF should not be a substrate for β-glucuronidase since it is a polypeptide. One possible explanation for the decrease in β-glucuronidase in the TNF-resistant cells is that the enzyme is involved in membrane digestion. (Barrett and Heath, 1977). By having a lower amount of enzyme activity the cells are protected to some degree from autodigestion of their own membranes as a result of TNF exposure. How and if β-glucuronidase is involved in TNF resistance will require further investigations to determine.

The results with the various lysosomotropic agents suggest that the lysosomes are involved in a functional way with the cytotoxic actions of TNF. Further research is needed to determine whether they simply process the TNF to a cytotoxic species or whether release of their enzymes following TNF exposure is what leads to cell death.

In order to further characterize the differences between the TNF-sensitive and resistant L929 cells experiments were performed to compare various enzymes in both the whole cells and their isolated lysosomes in the two subclones. Standard marker enzymes were examined in the two cell lines. It was noted that in the whole cells as well as the lysosomal fractions that there was again a decreased level of β-glucuronidase activity in the resistant cells as compared to the sensitive clone. Differences were also noted in the levels of the other marker enzymes between the sensitive and resistant cells. In particular, both hexosaminidase and acid phosphatase were significantly elevated in the TNF-resistant lysosomal fraction as compared to the sensitive cell fraction (Tables 6 and 7).

At the present time no other reports exist concerning levels of cellular enzymes, particularly lysosomal, and their relationship to TNF sensitivity or resistance. One can only speculate as to why the activities of the various enzymes are different between the two clones. It may be that these enzyme activity changes were brought about by the exposure of the cells to TNF and this led to the cells increasing or decreasing the amounts of the various enzymes in order to aid in detoxifying the TNF. Thus, enzyme changes may be a consequence and not a cause of TNF resistance. It is possible that the cells selected by the continuous exposure to TNF had different amounts of enzyme to start with. It has been reported by others that various subclones selected for TNF-resistance have different characteristics even though they demonstrate the same level of resistance (Lehmann and Droge, 1986). Thus, it is still possible that the differences in enzyme activities may have nothing to do with the cellular mechanisms for TNF resistance or that there is more than one mechanism involved.

TNF: Mechanism of Action

At the present time no researchers have made any definitive statements concerning the mechanism(s) by which TNF kills tumor cells. Some have postulated that DNA may be the primary target (Maury, 1986). Others have suggested that inhibition of cellular energy production may be the route by which TNF kills cells (Jones, 1986). Still others feel that cells are susceptible to TNF due to the production of free radicals and decreased levels of reduced thiols (Sherwin, 1987). To date few investigators have presented data to support their hypotheses.

Kull and Cuatrecasas (1981) and Niitsu et al. (1985) have been some of the only scientists to report results which support their views. These groups feel that the lysosomes are involved in the cytotoxic action of TNF either directly or indirectly. Their experiments as well as the current findings demonstrate the need for some type of lysosomal processing before TNF can kill a cell.

Previously published data suggest that TNF is internalized by cells using receptor mediated endocytosis. The receptor-TNF complex is incorporated into pinosomes which then undergo fusion with lysosomes. Upon entering the lysosome, the TNF-receptor complex is most likely cleaved so the receptor can be recycled to the cell surface (Dickson et al., 1983). The fate of the TNF molecule is not as yet known. The molecule may be degraded by proteolytic enzymes in the lysosome into various polypeptide fragments. These fragments may be cytotoxic in and of themselves and lead to cell death by unknown mechanisms. It is possible that TNF directly causes rupture of the lysosomes which leads to release of the acid hydrolases and leads to cell death by self digestion. If TNF is cleaved into smaller peptide fragments in the lysosome, these fragments may then be taken up by the nucleus where they induce the synthesis of new mRNA species which lead to the synthesis of proteins which are cytotoxic. This would account for the lag time between the exposure of the cell to TNF, the transient increase in DNA and RNA synthesis and the time of cell death. This hypothesis is unlikely due to the observation that protein synthesis inhibitors such as cycloheximide or RNA inhibitors such as actinomycin D actually enhance the cytotoxicity of TNF. This may be due to the

fact that the synthesis of repair proteins is inhibited. It may also be that the lysosomotropic agents that have been used are inhibiting other enzymes and organelle processes outside of the lysosome. These may actually be the areas or enzymes which are involved in TNF cytotoxicity. Only through more intense research with an emphasis on identification of subcellular distribution of the TNF molecule will this question be answered

Summary

Through my research I have shown that TNF is indeed cytotoxic to cancer cells and that its mechanism of action does not seem to involve specific effects on RNA, DNA or protein synthesis or ATP content of tumor cells. I have also demonstrated that reduced thiols do not play a role in inhibiting the cytotoxic actions of TNF. My studies of TNF's effect on DNA seem to indicate that damage to nuclear DNA through single strand breaks is not the primary mechanism by which TNF acts. Furthermore, the data show that there are consistent enzymatic differences between TNF-sensitive and resistant cells and that these differences occur primarily in the lysosomes. Lysosomotropic agents, both specific and non-specific, can inhibit the cytotoxic actions of TNF supporting the theory that lysosomes are involved in its cell killing mechanism.

Future Studies

Future studies should be directed toward a more thorough examination of the enzyme and subcellular differences between TNF-sensitive and resistant cells. These should include determinations of

the levels of a wider range of enzymes, specifically the proteases which might be involved in the degradation of TNF. Also experiments should be carried out to further refine the procedures for isolation of subcellular organelles and the assays for their enzymatic activities. Studies should be done to determine the fate of TNF once it has been internalized by a cell. These could be carried out using iodinated or tritiated TNF to allow for enhanced detection of the protein. ELISA and bioassays may also have to be employed to determine the biological activity of internalized TNF. It would be worthwhile to study further the effect of TNF on various cellular processes other than macromolecular synthesis and ATP production. Electron microscopy might also prove useful in determining the subcellular distribution of TNF and its effect on various organelles within the cell. The selection of other resistant cell lines from sensitive parental lines may also be useful in further elucidating the mechanism by which TNF kills cancer cells. The ultimate goal of all of this research would be an understanding of the cytotoxic mechanism of TNF which would greatly aid in its use as an antineoplastic agent.

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BSO	L-buthionine-S,R-sulfoximine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E-64	L-trans-epoxysuccinyl leucylamido (4-guanidino) butane
EDTA	ethylenediaminetetraacetic acid
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
LAL	Limulus amebocyte lysate
μg	microgram
μM	micromolar
mg	milligram
ml	milliliter
mM	millimolar
mU	milliunits
M	molar
ng	nanogram
nm	nanometer
nM	nanomolar
NPSH	non-protein sulfhydryl
PBS	phosphate-buffered saline

LIST OF ABBREVIATIONS--Continued

rpm	revolution per minute
RNA	ribonucleic acid
DSAL	D-sacchric acid 1,4-lactone
SSB	single strand break
SDS	sodium dodecyl sulfate
SD	standard deviation
TCA	trichloroacetic acid
TNF	tumor necrosis factor

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