

Mechanism of TNF- α Cytotoxicity in a Leukemia Virus Transformation Model

by

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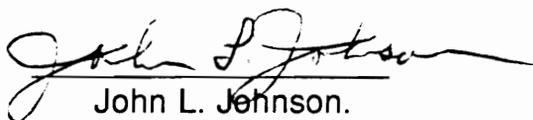
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ABSTRACT

Abelson murine leukemia virus (A-MuLV)-induced transformation was investigated to determine whether cells not sensitive to TNF- α could be made sensitive to the cytolytic action of TNF- α when infected with this retrovirus. Mouse embryonic fibroblast cell line CL.7 was found to be relatively insensitive to TNF- α . Upon transformation with A-MuLV, these cells gave rise to a clone (3R.1) which was found to be insensitive to TNF- α and another clone (6R.1) which had an increased sensitivity to TNF- α . The differential cytotoxicity was observed when cells were treated with TNF- α , for 18 hr, at 0 to 100 units/ml, at 37°C.

The mechanism of this differential cytotoxicity was further investigated. Thus, TNF-R levels on the cell surface were found to be not correlated with the differential TNF- α response. The A-MuLV transformation suppressed the epidermal growth factor-receptor (EGF-R) in 3R.1 clone and induced its levels significantly in the 6R.1 clone ($p < 0.05$). Cell surface EGF-receptor (EGF-R) levels in CL.7 and 3R.1 clones were lower than the 6R.1 clone ($p < 0.05$). Although the EGF-R levels in all the clones were induced with TNF- α , the expression of EGF-R correlated with the susceptibility to TNF- α .

The role of antioxidants, such as α -tocopherol and β -carotene, (known anti-cancer agents) in modulating TNF- α -induced EGF-R expression was investigated. In both the untransformed and the transformed clones, β -carotene suppressed the constitutive and the TNF- α induced EGF-R levels whereas α -tocopherol was found to have an enhancing effects. Studies with metabolic inhibitors on TNF-R and EGF-R expression indicate that inhibitors of the arachidonic acid cascade and modulators of protein kinase-C (PK-C), could influence the binding and internalization of TNF- α and thereby controlling the physiologic future of the cells.

The A-MuLV specific *V-abl* protein, p120, tyrosine phosphorylation was determined by a radio-labelled anti-phosphotyrosine antibody in an antigen capture assay. TNF- α had little effect on p120 phosphotyrosine levels of TNF- α insensitive CL.7 and 3R.1 clones. The, TNF- α sensitive, 6R.1 clone, however, was found to induce its p120 specific phosphotyrosine upon exposure to TNF- α

for 8 hr. Thus, TNF- α modulated the tyrosine phosphorylation of p120 only in the TNF- α -sensitive cell line.

The mitochondrial toxicity of TNF- α was determined by monitoring the rate of quenching of a cationic spin probe CAT 16. Mitochondrial preparation from CL.7 and 3R.1 clones had higher ability to quench CAT 16 signal with TNF- α incubation time than mitochondria from the 6R.1 cells. This indicates that the differential TNF- α cytotoxicity manifested in A-MuLV transformed clones may, in part, be due to the differential mitochondrial toxicity of this cytokine.

The hypothesis that TNF- α cytotoxicity was mediated via an oxidative process was tested on the TNF- α sensitive L929 cells. Using a flow cytometric detection system it was determined that TNF- α produced intracellular hydrogen peroxide in these cells which was sensitive to concentration and incubation time of TNF- α . Superoxide radicals were also generated during TNF- α action on L929 cells, as determined by the use of the spin trap PBN in conjunction with EPR spectroscopic techniques. The PBN-OOH spin adduct spectrum peaked at 9 hr of TNF- α incubation and was inhibitable upto 30 % with 10 μ M of desferal-Mn complex (a known SOD mimic). These data indicate that superoxide and hydrogen peroxide are common events in TNF- α dependent cell killing process.

The differential TNF- α cytotoxicity was found to depend on differences in the antioxidant status of the target clones. Thus, it was found that Cu/Zn-SOD, Mn-SOD, GSH-Peroxidase and GSH-Reductase enzymes were all induced significantly in the CL.7 clone ($p < 0.05$) upon incubation with 100 units/ml of TNF- α for 18 hrs. TNF- α had little effect on the antioxidant enzymes of both 3R.1 and 6R.1 cells. However, the constitutive levels of most antioxidant enzymes were found to be higher in 3R.1 cells than in the 6R.1 cells. Therefore, the susceptibility of 6R.1 to TNF- α may, in part, be due to a low level of antioxidant enzymes present in this clone.

In conclusion we found that the differential cytotoxicity of TNF- α may, in part, be due to: (1) differential EGF-R expression, (2) differential mitochondrial cytotoxicity, and (3) differential ability to modulate the tyrosine phosphorylation in untransformed and A-MuLV transformed cells and (4) differential antioxidant status of these cells to handle oxidative stress imposed by TNF- α .

DEDICATION

I dedicate this dissertation to all the cancer patients of the world who contribute to clinical research in search of anti-cancer therapies.

SIGNIFICANCE OF THE FINDINGS

The results of this investigation have potential clinical implications. Firstly, TNF- α might not be a good immunotherapeutic drug for those tumors that possess high levels of defense enzymes. Secondly, the tumors of oncogenic origin with an active tyrosine kinase activity could possibly be sensitized to the action of TNF- α . Thirdly, antioxidants like vitamin A could be effective in modulating the growth of cancers of epidermal origin.

Assay systems developed in this investigation would benefit investigators to quantitate the defense enzymes accurately by using a microtiter plate reader. The membrane lipid peroxidation as well as mitochondrial toxicity assays were developed by use of spin labels in conjunction with EPR spectroscopy. These assay systems would be of immense importance in immunotoxicological applications at the cellular level.

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TABLE OF ABBREVIATION

| | |
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| 6-PG | 6-Phosphogluconate. |
| A-MuLV | Abelson murine leukemia virus. |
| AEV | Avian erythro-blastosis virus. |
| ATCC | American Type Culture Collection. |
| BSA | Bovine serum albumin. |
| CAT 16 | 4-(N,N-dimethyl-N-hexadecyl) ammonium 2,2,6,6-tetramethyl piperidine-1-oxyl, iodide . |
| CCS | Cell culture supernatant. |
| CF | Center field. |
| CIF | Cytotoxin production inhibition factor. |
| CSF-1R | Colony-stimulating factor-1 receptor. |
| DAG | Diacyl glycerol. |
| DCF | 2',7' dichlorofluorescin. |
| DCF-DA | 2',7' dichlorofluoroscein-diacetate. |
| DMBA | Dimethyl benzo anthracine. |
| DMEM | Dulbecco's modified Eagle's medium. |
| DMSO | Dimethylsulfoxide. |
| EGF | Epidermal growth factor. |
| EGF-R | Epidermal growth factor-receptor. |
| EPR | Electron paramagnetic resonance. |
| ETI | 5,8,11-eicosatrynoic acid. |
| FALS | Forward angle light scatter. |
| FBS | Fetal bovine serum. |
| FGF | Fibroblast growth factor. |

| | |
|------------------|---|
| G-6-P | Glucose-6-phosphate. |
| G-6-PDH | Glucose-6-phosphate dehydrogenase. |
| GSH | total glutathione. |
| GSH-Px | Glutathione peroxidase. |
| GSH-Rx | Glutathione reductase. |
| HBSS | Hank's balanced salt solution. |
| I-R | Insulin-receptor. |
| IGF-1R | Insulin-like growth factor-1receptor. |
| IL-1 | Interleukin-1. |
| LI 90 | 90 ⁰ light scatter. |
| LPS | Lipopolysaccharide. |
| M-MuLV | Moloney-murine leukemia virus. |
| mAb | Monoclonal antibody. |
| MDA | Malonyldialdehyde. |
| MOPS | 3-(N-morpholino) propane sulfonic acid. |
| MTT | 3,4,5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide. |
| OAG | Oleylacylglycol. |
| ODC | Ornithine decarboxylase. |
| PBS | Phosphate buffer saline. |
| PDGF-R | Platelet-derived growth factor receptor. |
| PI | Propidium iodide. |
| PK-C | Protein kinase-C. |
| PMA | Phorbol myristate acetate. |
| rh-TNF- α | Recombinant human Tumor necrosis factor factor- α . |
| SDS-PAGE | Sodium dodecyl-sulphate-polyacrilamide gel electrophoresis. |
| SEM | Scanning electron microscope. |
| SOD | Superoxide dismutase. |
| SW | Sweep width. |
| TEMPO | 2,2,6,6-tetramethyl-1-piperidine-n-oxyl. |
| TGF- β | Transforming growth factor- β . |
| TNF- α | Tumor necrosis factor- α . |
| TNF-R | Tumor necrosis factor-receptor. |
| TX-100 | Triton X-100. |
| W-7 | N-(6-aminohexyl)-5-chloro-1 naphthalene-sulfonamide,HCl. |

INTRODUCTION

Tumor necrosis factor- α (TNF- α) is one of the most extensively studied polypeptide cytokines which has a wide spectrum of biological activity. This 17 Kd hormone is produced by activated macrophages and monocytes and has a half life of six minutes in the circulation. One of the major reasons why TNF- α has caught the attention of many investigators is its anti-tumorogenic property. Phase I and Phase II clinical trials with TNF- α for treatment of human cancers met with only partial success. TNF- α was found to be anti-tumorogenic in approximately one third of the tumors for which it was administered. At an optimal dose its anti-tumorogenicity was accompanied by severe endotoxic side effects. To date, the use of TNF- α as an immunotherapeutic drug is best at a suboptimal dose in conjunction with other anti-tumorogenic cytokines like γ -interferon.

The *in vivo* anti-tumorogenicity of TNF- α corresponds with its *in vitro* cytotoxicity to many tumor cell lines. TNF- α is not only cytotoxic to some tumor cell lines but it has also been found to act as a growth factor for normal fibroblasts. The mechanism of action of TNF- α in terms of its marked ability to act as a growth factor, a cytostatic agent, as well as a cytotoxic factor is not well understood. To understand this differential action of TNF- α , it is of vital importance to establish why TNF- α which is not toxic to a normal cell, such as a fibroblast, acts as a cytotoxic agent when the same cell is subjected to a transformation. The model in which the untransformed cells are TNF- α insensitive whereas its transformed daughter clones are sensitized to the cytokine will facilitate in the understanding of this switching action of TNF- α .

The **overall objective** of this investigation was to understand the mechanism of the differential cytotoxic action of TNF- α on normal and transformed cells. The transforming agent was a replication defective RNA retrovirus called the Abelson-murine leukemia virus (A-MuLV) which has the ability to transform murine fibroblasts in culture.

The specific aims of the dissertation were

1. To investigate whether transformation of a cell line, determined to be TNF- α insensitive, with A-MuLV could potentially alter its sensitivity to TNF- α .
2. To determine whether the differential sensitivity to TNF- α under A-MuLV transformation observed in specific aim (1) could be explained in terms of :
 - (a) the role of extracellular protein factors in rendering protection against TNF- α action,
 - (b) the membrane receptors and proteins including TNF-R, EGF-R, and the viral surface antigen p120 in modulating TNF- α cytotoxicity, and
 - (c) the intracellular events including mitochondrial toxicity, defense enzyme status, and production of reactive oxygen species in the cytotoxic pathway.

LITERATURE REVIEW

Tumor Necrosis Factor- α And Its Receptor:

Infectious diseases as well as tissue injury trigger the production of numerous cytokine mediators and endogenous hormones. Cytokine tumor necrosis factor- α (TNF- α) plays a major role not only in the host response to tissue injury but also in the inflammatory response to various infections. Immune effector cells of the monocyte/macrophage lineage play a central role in the production of cytokines aimed at the modulation of many aspects of inflammatory response. TNF- α is among the best studied of all cytokines and is one of the most abundant products of activated macrophages. TNF- α can not only elicit the production of a host of endogenous mediators but also is capable of triggering the diverse clinical syndromes of cachexia and shock. Detail reviews are available which have described in detail the biochemical basis of production and the pleotropic effect of TNF- α (Fong et al. 1989; Beutler et al., 1989; Rosenblum et al., 1989; Kunkel et al., 1989; Larrik et al., 1990).

Injury to host tissue by bacterial endotoxins has been implicated to be mediated by the macrophage derived polypeptide hormone TNF- α (Ha et al., 1983; Torti et al., 1985; Beutler et al., 1985). It is a multifunctional protein that is secreted by activated macrophages as well as monocytes in circulation and elicits hemorrhagic necrosis of certain kinds of tumors *in vivo*, as well as cytotoxicity to several murine and human cell lines *in vitro* (Carswell, 1975; Haranaka et al. 1981; Williamson et al. 1983). Apart from it's cytostatic, cytotoxic and null effects on several human tumor cell lines, TNF- α has also been shown to be growth promoting in normal human fibroblasts (Sugarman et al. 1985; Vilcek et al. 1986). The mechanism by which TNF- α elicits such a diverse response is unknown. To understand the diversity in the mechanism of TNF- α action it is important to establish that a normal cell line that is insensitive to TNF- α exhibits altered TNF- α cytotoxicity when it is malignantly transformed. The transforming agent could

be a chemical carcinogen, UV radiation or even a virus.

The structure of TNF- α from rabbit, mouse and human sources show a remarkable inter-species conservation of its amino acid residues (Fransen et al., 1985; Pennica et al., 1984). Human TNF- α is expressed as a 233 amino acid prohormone which is then cleared during biological processing to form the 157 amino acid active TNF- α protein (Buetler et al., 1987). The 76 additional amino acids in the prohormone sequence are attached to the N-terminus of the mature protein which has as yet an unknown biological activity. The mature protein has been known to exist as dimers, trimers or pentamers in solution (Beutler et al., 1986). The trimeric form is the active form of the hormone. TNF- α is a relatively hydrophobic protein containing one intra-chain disulfide linkage (Aggarawal, et al., 1985). Upon heating, it is irreversibly denatured and also it is known to lose its biological activity upon freezing and thawing.

Specific receptors for TNF- α have been found in a wide variety of cell types (Baglioni et al., 1985; Aggarawal et al., 1985, Tsujimoto, et al., 1985). Cultured adipocytes (3T3-L1 cells) have been shown to have approximately 10,000 TNF- α receptors per cell, with a dissociation constant (Kd) of 3×10^{-9} M (Beutler, B. et al., 1985). Murine fibrosarcoma L929 cells, which are sensitive to the cytotoxic effects of TNF- α , have 2,200 receptors per cell with a Kd of 6.1×10^{-10} M (Isujimoto et al., 1985), and the TNF- α -resistant FS-4 fibroblasts are known to have 7,500 receptors per cell with a Kd of approximately 3.2×10^{-10} M (Isujimoto et al., 1985). This has been argued to be evidence against the correlation of the biological responsiveness of a cell type to TNF- α . A maximal biological response is elicited by occupancy of as few as 5% of the receptors by TNF- α (Tsujimoto M, et al., 1986). Studies indicate that TNF- α - like other polypeptide cytokines elicit their cellular response after binding to specific cell surface receptors. Investigations on TNF- α receptors (TNF-R) have been made possible by use of highly purified recombinant human (rh)TNF- α (Baglioni et al., 1985). The binding of TNF- α to its receptor facilitates internalization of TNF-receptor complex via the classical receptor-mediated endocytic pathway (Mosselmans, et al., 1988). Inhibition of internalization and degradation of receptor bound TNF- α by agents such as colchicine

and chloroquine have been shown to effectively inhibit TNF- α cytotoxicity (Ruff et al., 1981). Expression of TNF- α membrane receptors is necessary, but not sufficient to determine the responsiveness of a given target cell (Kull et al., 1985; Scheurich et al., 1986; Sugarman et al., 1985). With increasing knowledge of the diversity of cellular responses to TNF- α (Le et al., 1987) there is an overall lack of understanding of the mechanisms by which diverse TNF- α responses are mediated. Regulating mechanisms could occur at levels of receptor expression, affinity of ligand to receptor, efficiency of ligand-receptor complex, internalization and heterogeneity of the receptor, to name a few, in contributing to the differential response pattern of distinctive cell types. It is known with other cytokines such as γ -interferon (Berkovic, et al. 1986) that the magnitude of a particular biological response is proportional to the quantity of ligand receptor interactions thereby making the number of expressed membrane receptors a critical criterion in the determination of cellular sensitivity to the cytokine in question. In the case of TNF-R the assumption of constitutive expression of the receptor in cancer cells (Sugarman, et al., 1985) has been modified to include the myriad positive and negative regulatory mechanisms controlling the membrane TNF-R levels. For example, TNF-R have been known to be reversibly induced in T-cells and expressed in a stimulus dependent manner (Scheurich et al., 1987). Similarly membrane expression of TNF-R in activated T-cells can be rapidly modulated by activators of protein kinase-C resulting in loss of TNF-binding capacity (Scheurich et al., 1986). Phorbol myristate acetate (PMA) and oleylacylglycerol (OAG) have both been known to down regulate TNF-binding capacity of both normal and malignant cells (Unglabe et al., 1987). Differences in the constitutive expression of TNF-R have been reported for cells undergoing differentiation. TNF-R found on immature mouse myeloid leukemic cells were enhanced 2 fold upon spontaneous differentiation into macrophages (M ϕ) and 5 fold when these M ϕ were treated with γ -interferon (Michishita et al., 1990).

Oxidant Damage by TNF- α

Macrophages are known to be the principal mediators of the effect of endotoxic challenge (Torti, et al 1985). They elicit this response by generating a low molecular weight 17 K protein factor

called TNF- α (Beutler, et al. 1985). TNF- α is responsible for orchestrating the hemorrhagic necrosis of transplantable tumors *in vivo* (Carswell et al. 1975). TNF- α shows diverse biological effects on different cell types. Binding of TNF- α to its cell surface specific receptor (TNF-R) is required to elicit its pleotropic effects. TNF- α has an overall growth enhancing effect, null effect and cytotoxic effect on various murine and human cell lines (Sugarman et al. 1985; Creasey et al. 1987; Ruggerio et al. 1987). These differences have been investigated at the level of TNF- α binding to TNF-R and it was found that different responsiveness to TNF- α cannot be attributed to differences in affinity of TNF- α to TNF-R. At present it is not clear as to how TNF- α can act as a growth stimulating agent in certain cell lines as in WI-38 or U-373 and be at the same time cytotoxic to some others like L-929, WEHI-164 and MCF-7. It has been suggested that a 188k polypeptide is involved at the level of TNF- α binding to its receptor on the cell surface in mediating its cytotoxicity. Post receptor binding membrane events include increase in membrane fluidity, permeability (Anghileri et al., 1987), and activation of the arachidonic acid cascade via an activated phospholipase A₂ (Godfrey et al., 1987) a concomitant increase in prostaglandin E₂ production (Dayer et al., 1985) and activation of protein kinase coupled pathways (Hensel et al., 1987). In rat mesangial cells TNF- α was found to stimulate the synthesis of PGE₂, PGF₂ α and 6-keto-PGF₁ α as well as adenosine 3'-5' cyclic adenosine monophosphate (cAMP) levels in a dose dependent manner (Baud et al., 1988). Recent investigations have determined that cytotoxicity of TNF- α could be mediated by production of free radical species. Incubation of TNF- α with TNF- α sensitive mouse tumorigenic fibroblasts L-M cells were shown to produce hydroxyl radicals as detected by evolution of methane gas upon addition of dimethyl sulfoxide (Yamauchi et al, 1989). The production of the hydroxyl radical under TNF- α cytotoxicity was dependent upon the dose of TNF- α and was shown by the above investigators to be inhibited by an iron chelator like 2,2-bipyridine. The addition of an iron chelator inhibits iron-catalyzed Fenton reaction thus suppressing radical production. The production of free radical species under TNF- α cytotoxicity has been indirectly inferred by many other studies. For example TNF- α induces mitochondria to swell with an accompanied reduced number

of cristae (Matthews et al. 1987) indicative of free radical involvement. Similarly it has been shown that TNF- α cytotoxicity is suppressed by mitochondrial electron transport chain inhibitors (Kull et al. 1981, Watanabe et al 1988) and inhibitors of arachidonic acid metabolism (Matthews et al 1987). Both the mitochondrial electron transport chain (Cadenas et al, 1980) and the arachidonic acid cascade (Kuehl, et al 1980) are known to generate hydroxyl radicals. Thus it is possible that TNF- α induced production of hydroxyl radicals could be via activation of the above mentioned metabolic pathways. Peroxidation of membrane lipids (Suffys et al, 1987), release of lysosomal enzymes (Watanabe et al, 1988) and fragmentation of DNA (Dealtoy, et al. 1987), observed under TNF- α toxicity could all be mediated by the production of hydroxyl radicals. Cytotoxicity of TNF- α has also been known to be partially mediated by other reactive species of oxygen like superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) in macrophage activated cell killing (Grace H. et al 1988). The role of glutathione in mediating oxidant injury in tumor cells susceptible to TNF- α was investigated by measuring the intracellular levels of glutathione in these cells (Zimmerman, et al 1989). Production of oxyradical species in cells susceptible to TNF- α , results in a high oxidative stress. If the cellular machinery cannot gear itself to handle this oxidant attack, cell death ensues. It was shown that there is an inverse correlation between glutathione levels and cellular susceptibility to TNF- α . Thus if intracellular metabolism of TNF- α leads to the production of free radical species, the susceptibility of a cell to killing by TNF- α could be influenced by its content of enzymatic antioxidants such as Cu/Zn-superoxide dismutase (Cu/Zn-SOD), Mn-superoxide dismutase (Mn-SOD), glutathione peroxidase (GSH-PX), glutathione reductase (GSH-Rx), glucose-6-phosphate dehydrogenase (G-6-PDH), cytochrome P-450 reductase and non enzymic antioxidants like total glutathione content, α -tocopherol (vitamin E) and β -carotene (vitamin-A). For example, incubation of cells with TNF- α leads to a specific induction in the levels of mRNA as well as protein levels for Mn-SOD (Grace et al, 1988) as determined from *in vitro* as well as *in vivo* studies. The induction of Mn-SOD mRNA was independent of cell type. It was proposed by the above authors that Mn-SOD, by dismutating O_2^- , could protect the cells from the cytotoxic effects

of TNF- α . This further substantiates the free radical pathway of cell killing.

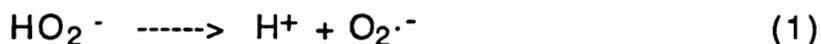
Reactive Species of Oxygen:

Aerobic life processes use oxygen for the controlled oxidation of carbon-containing molecules with the concomitant release of energy. Many of these oxidizable compounds are also the cellular "building blocks." It is not surprising, therefore, that oxygen will cause damage to most cell components. Such damage is normally limited by cellular antioxidant defence and by constant cell repair process. When these repair mechanisms fail, or are overwhelmed by oxidants, cell damage and death occur.

To understand why oxygen forms reactive species it is necessary to describe briefly the chemistry of the oxygen molecule (O_2). Molecular oxygen at ground state is a biradical that has two unpaired electrons. These two electrons have the same quantum spin number (parallel spin) and require incoming electrons to be also of parallel spin so as to fit into the vacant spaces in the π^* orbitals. In accordance with Pauli's exclusion principle, a pair of electrons from an atomic or molecular orbital would have antiparallel spins. Such chemistry imposes restrictions involving spin inversions on oxidations by O_2 that tend to make it accept its electrons one at a time. The advantage of this process for aerobic life is a considerable slowing down of reactions of oxygen with nonradicals. The disadvantage, however, is that one-electron reduction of O_2 leads to the formation of reactive oxygen species.

Superoxide Radical :

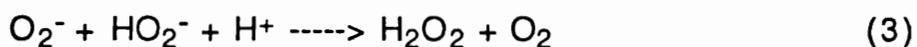
Hydroperoxyl radical ($HO_2\cdot$) is the protonated one-electron reduction product of $O_2\cdot$ since the pKa for its dissociation is approximately 4.8, there is likely to be little HO_2 present at physiological pH.



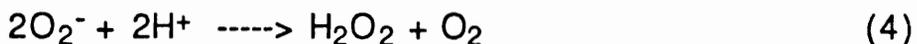
The superoxide anion ($O_2\cdot^-$), therefore, is the one-electron reduction product of dioxygen in aqueous biological media. Since

superoxide has only one unpaired electron (one less than O₂) the dot representing the unpaired electron in the superoxide anion is now usually omitted (ie, O₂⁻ and not O₂^{-·}). Generation of O₂⁻ in solution has been observed to kill or inactivate bacteria and other cells; stimulate lipid peroxidation; and damage DNA, carbohydrates, and proteins (Halliwell and Gutteridge, 1984a,b). Chemical studies, however, cast considerable doubt on the direct action of the superoxide radical, as in aqueous solution O₂⁻ is a weak oxidizing agent and moderately strong reducing agent. Most, if not all, damage associated with the generation of O₂⁻ must be due to other species whose formation depends on it. The hydroperoxyl radical has a greater oxidizing potential than O₂⁻ and may be important at sites with an acid pH or within the lipophilic membrane interior.

The major reaction of O₂⁻ is the dismutation reaction, which takes place in two stages (equations 2 and 3).



with the overall reaction reading



Since the concentration of H⁺ is low at physiological pH the dismutation reaction is slow, allowing O₂⁻ to diffuse from its site of formation.

Hydrogen Peroxide :

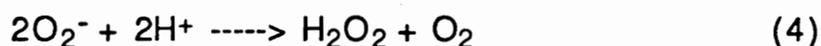
Hydrogen peroxide (H₂O₂) is the two-electron reduction product of O₂ and since it has no unpaired electrons it cannot be called a radical. Hydrogen peroxide is the most stable of the reactive intermediates formed in the pathway of reduction of O₂ to water. It thus can diffuse from its site of formation and can also cross cell membranes, unlike O₂⁻, which could cross the lipid bilayer only via specific membrane channels.

Hydroxyl Radical:

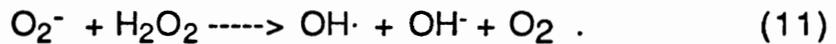
Addition of an electron to hydrogen peroxide breaks the O-O bond giving rise to hydroxyl radical (OH·) (equation 5). This is a highly reactive species, reacting at a near diffusion controlled rate with many biological molecules. Therefore, it cannot move far from its site of generation. Hydroxyl radicals react by hydrogen abstraction, addition, or electron transfer reactions (equations 6, 7, and 8).



Many early studies with O_2^- generating systems indicated the formation of a highly reactive species, $\text{OH}\cdot$, whose formation could be inhibited by the addition of superoxide dismutase or catalase and that could react with a variety of scavengers of the hydroxyl radical (Fridovich, 1975, 1978; McCord, 1974; Halliwell, 1978b). This led to the suggestion that O_2^- and H_2O_2 directly reacted to give $\text{OH}\cdot$ (Haber-Weiss reaction). However, it was soon clear that this reaction was not biologically feasible (Halliwell, 1976) but could occur through metal ion catalysis (Fong et al, 1976; McCord and Day, 1978; Halliwell, 1975, 1978a). Participation of catalytic amounts of iron salts in the reaction is essential and the reaction has since been known as the "iron-catalyzed Haber-Weiss" reaction or may be described as a superoxide-dependent Fenton reaction (equations 9, 4, and 10).



the overall reaction reads



Singlet Oxygen:

Singlet oxygen is a reactive form of O_2 in which one of the unpaired electrons has been moved from its ground state and as the direction of its spin is changed thus removing the spin restriction. There are two possible singlet states of O_2 , the $^1\Delta\text{O}_2$ and $^1\Sigma\text{O}_2$ (Foote, 1982). Singlet oxygen can arise in biological systems by photochemical reactions involving pigment sensitizers such as chlorophylls, retinal, flavins, porphyrins, and bilirubin or during the spontaneous dismutation of O_2^- radicals as shown (Stauff, et al. 1973)



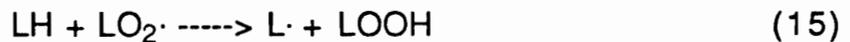
Cellular Interaction of Reactive Oxygen Species and Tissue Damage:

Many of the toxic effects associated with oxygen are due to the formation of oxygen radicals (Gerschman, 1981). This idea was developed by Fridovich into the "superoxide theory of oxygen toxicity" (Fridovich, 1974, 1975, 1978). The susceptible targets for the action of oxygen radicals are the polyunsaturated fatty acid (PUFA) side chains of cell and organelle membranes (specifically that of the mitochondria), cellular DNA, and cellular proteins. The superoxide dismutases (SOD) accelerate the dismutation reaction (equation 4) removing O_2^- at a tremendous rate ($2 \times 10^9 \text{ M}^{-1}\text{sec}^{-1}$) forming hydrogen peroxide and molecular oxygen. Hydrogen peroxide, which is also a very reactive species, is removed by glutathione peroxidase and the peroxisomal enzyme, catalase (equation 12).



Hydrogen peroxide produced by the dismutation of O_2^- by SOD can contribute to $\text{OH}\cdot$ formation via iron-catalyzed reactions (equation 10). The $\text{OH}\cdot$ radical so produced can initiate peroxidation of membrane lipids as well as cause damage to cellular DNA.

PUFA contains two or more carbon-carbon double bonds which makes them a good electron sink and therefore susceptible to oxidative damage by free radical attack. Abstraction of a hydrogen atom from the carbon chain of PUFA results in the formation of a carbon-centered radical (C·). This triggers the peroxidative chain reaction. Abstraction of a proton followed by oxygen taken up by the conjugated diene gives rise to the formation of peroxy-radical initiating a chain of reactions forming lipid hydroperoxides, cyclic peroxides and cyclic endoperoxides. OH·, peroxy and alkoxy-radicals have sufficient energy to initiate the process of hydrogen abstraction leading to lipid peroxidation cascade.



The biological effect of lipid peroxidation can be disastrous. This results in structural damage to the lipid bilayer making it more prone to proteolytic attack (Gutteridge, 1978). The membrane damage can result in the loss of semipermeability, release of hydrolytic enzymes, release of iron and copper complexes, all aiding in the cell dysfunction and cell death.

The Antioxidant Defense System:

The cellular machinery has evolved an efficient detoxification system rendering protection from oxidative stress. A number of enzymes and compounds are present in the cell which can protect the cellular components from the deleterious effects of activated oxygen species. The enzymatic system in mammalian cells consists of three basic enzymes: the superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). Cu/Zn-SOD is located in the cytosol where as Mn-SOD is found in the mitochondria. GSH-Px has two distinct isozymes: one found in the cytosol and one in the mitochondria. Catalase is predominantly found in the peroxisomes. The network of these defense enzymes and their detoxification action remove O₂⁻ and H₂O₂ from the biological system and prevent the iron-catalyzed Haber-Weiss reaction from occurring.

The $\text{OH}\cdot$ is by far the most reactive of all reactive species of oxygen. The antioxidant enzyme system prevents $\text{OH}\cdot$ formation. Equally important is the non-enzymatic antioxidant defense system which largely consists of small molecular weight compounds. They include β -carotene (scavenger of $^1\text{O}_2$), vitamin E (nonspecific inhibitor of lipid peroxidation), mannitol (inhibitor of $\text{OH}\cdot$), iron chelators (which bind to iron and prevent $\text{OH}\cdot$ formation), glutathione and other thiols (inhibitor of $\text{OH}\cdot$ and substrate for GSH-Px), vitamin C (scavenger of O_2^- , $\text{OH}\cdot$ and $^1\text{O}_2$) and uric acid (scavenger of $^1\text{O}_2$ and $\text{OH}\cdot$). The lipid soluble antioxidants are vitamin E, and β -carotene whereas the water soluble antioxidants are uric acid and vitamin C. Vitamin C probably acts as an antioxidant for vitamin E at the surface of lipid bilayer by scavenging the vitamin E radical and reducing it back to the original form (Barton et al; 1983). It should be emphasized that the cell responds to a given oxidative stress by orchestrating interactive enzymatic as well as nonenzymatic antioxidant defenses.

Antioxidant Defense of Tumor Cells:

A large number of studies have been performed on antioxidants in tumor cells. Tumor cells have abnormal antioxidant enzyme activities as compared to control cells. Tumor cells have been found to contain low levels of Mn-SOD activity, Cu/Zn-SOD activity, and (almost always) catalase activity. GSH-Px activity is highly variable, from very low to very high. Mn-SOD activity has been shown to be dramatically reduced in over 80 different types of human and murine neoplastic cells, whether spontaneous or oncogenically induced *in vivo* or *in vitro* by chemicals, viruses, or transplantation (Oberley et al 1979, 1982). The enzyme levels may be low in cells due to a low substrate (free radicals) levels. However, several investigations have shown that neoplastic cells have the capability to produce the superoxide radical, the substrate for SOD (Oberley, 1979, 1982). Thus it appears that SOD, an inducible enzyme, is not low in the tumor cells because O_2^- is low. This suggests that the diminished amounts of Mn-SOD activity coupled with normal levels of superoxide production in the cancer cells may be a general characteristic of tumor cells. It has been hypothesized that low SOD activity in tumors is a result of lowered

oxygen concentration (Halliwell, 1985) leading to an anoxic condition, especially at the centers of tumor foci. There is, however, considerable evidence that O_2 or O_2^- are not the reasons for low SOD activity. O_2^- causes increase in SOD activity in normal cells but not in fibrosarcoma cells in culture (Simon et al, 1981).

Tumor cells in general have a high level of GSH, although exceptions have been known (Meister and Griffith, 1979). The growth rate of human skin tumors is proportional to GSH concentration (Engin, 1976). Even though the levels of GSH are usually elevated in tumors, the activity of GSH-Px and GSH-Rx are variables. Enzymes such as SOD, CAT, GSH-Px and glutathione reductase (GSH-Rx) have been measured in a wide variety of mouse tumors as compared with normal tissue levels (Misdale et al, 1983). The above investigators found that the activities of SOD, CAT and GSH-Rx were in general lower in tumors than in normal tissues, while the activity of GSH-Px was comparable. These data are consistent with the idea that tumors generally have low SOD and CAT levels but have variable amounts of GSH-Px (Oberley et al. 1979, 1982).

Tumors of viral origin have been shown to incorporate viral insertion sequences which have the ability to "turn off" and "turn on" genes (Temin, 1980). The viral proteins have the potential to moderate gene expression. Viruses have been known to turn off Mn-SOD gene and turn on the cell proliferation gene. Alternatively, viruses may contain their own gene for cell proliferation and for alterations in the host defense enzyme status.

Abelson Murine Leukemia Virus:

Abelson-murine leukemia virus (A-MuLV) is a replication-defective transformation inducing retrovirus, which induces B-cell lymphomas *in vivo* (Potter et al 1973; Premkumar et al 1975) as well as capable of transforming cells of lymphoid and fibroblast origin *in vitro* (Rosenberg, et al. 1980). The genomic content of A-MuLV virus is a hybrid consisting of about 25% of Moloney-murine leukemia virus (M-MuLV) with an associated 3.6 kb. insert (homologous to normal mouse cell DNA) (Shields, et al 1979). Only a single translation product of the A-MuLV genome called the P120 has been identified (Witte, et al 1978) in the prototype A-MuLV

strain. P120 is a fusion protein representing the N-terminal segment of M-MuLV gag protein (30 kd) and a 90 kd protein segment of non-viral origin (Witte, et al 1978, Reynolds, et al 1978). As P120 is the only protein involved in the viral transformation, it is assumed that P120 is the transforming protein (Rosenberg, et al 1980) located as a cell surface transmembrane protein (Witte et al 1980). Anti-sera raised against the non-M-MuLV portion of the viral translation product react with the external surface of cell membranes of A-MuLV transformed cells, thereby proving that the extracellular domain of P120 is of non-viral origin (Witte et al 1979). Moreover, anti-M-MuLV sera do not react with the surface of A-MuLV transformed cells, proving thereby that the M-MuLV specific determinant of P120 is towards the cytosol. The 30 kd determinant consists of p15, p12 and a small part of p30 proteins of the gag structural genes (Witte, et al 1978). The M-MuLV unrelated extracellular component is encoded by the *V-abl*. The mouse cell homologue of the *V-abl* is called *C-abl*. *C-abl* is a gene reported to contain a large number of intervening sequences (IVS) with multiple transcripts (Eva, et al 1982). The 5' and 3' end specific probes for *C-abl* have been used to provide direct genetic evidence that only the proximal end of *V-abl* with its 5' helper viral sequence is required for fibroblast transformation (Srinivasan, et al 1982). The transmembrane P120 is a phosphoprotein, phosphorylated at a tyrosine residue (Witte et al, 1980) and in many respects resembles the sarcoma viral phosphoprotein. A-MuLV like sarcoma virus selectively transform cells of hematopoietic origin specifically the pre B cells as well as fibroblasts in culture (Scher, et al 1975). Like P120, the sarcoma viral protein, pp 60^{src}, is a membrane associated, tyrosine-specific protein kinase, whose enzymatic activity is thought to be necessary for transformation (Collett et al 1978, Hunter et al 1980). But whereas pp60^{src} is a phosphotransferase, P120 is not yet known to have that enzymatic function. Nonetheless the leukemogenic potential of A-MuLV and the sarcomagenic potential of Rous sarcoma virus suggests that there could be a common basis for the infectivity of both RNA and DNA tumor viruses.

V-abl was first described as the oncogene contained in A-MuLV and was shown to code for the transmembrane protein with tyrosine kinase activity which is essential for the transforming capacity of the virus (Prywes et al 1983). The normal cellular counterpart, the

C-abl proto-oncogene, also encodes a protein with tyrosine kinase activity (Konopka and Witte 1985). *C-abl* is a single copy gene located on the long arm of chromosome 9 in humans and on chromosome 2 in mice. This codes for the *C-abl* protein and has a molecular mass of 140 kd in humans and 150 kd in mouse (Witte et al 1979). The N-terminal region of *C-abl* protein might also be functionally important in interacting with other cellular proteins via its tyrosine kinase activity. It is known that cells respond to external stimuli via a signal transduction across the cell membrane. Surface receptors with specific ligand binding domains accomplish the task of recognizing individual stimuli. In the amplification and tight regulation of the signal transduction pathway, three kinds of protein kinases are involved. One, the Ca²⁺/phospholipid-dependent diacylglycerol-regulated protein serine kinases (protein kinase C). Second, the cAMP-dependent protein kinase system which however has not been known to be directly involved in cell growth control or transformation. Third, the growth factor receptor protein-tyrosine kinases. There are at least five growth factor receptors which have protein-tyrosine kinase activities that are stimulated several fold upon binding to their cognate ligands. They are the mitogenic epidermal growth factor receptor (EGF-R), the platelet-derived growth factor receptor (PDGF-R), the colony-stimulating factor - 1 receptor (CSF-1R) the insulin receptor (I-R) and the insulin-like growth factor - 1 receptor (IGF-1R) (Heldin and Westermark 1984; Hunter and Cooper, 1985; Sherr, et al. 1985). The structure of CSF-1R, EGF-R and that of PDGF-R are very similar although they are different from that of I-R and the IGF-1R which have (αβ)₂ structure. Nonetheless, a number of general principles apply to the growth factor receptor protein-tyrosine kinases. Enhancement of their phosphotransferase activities is very rapid following ligand binding, and in every case, an early event is auto-phosphorylation and a subsequent enhancement of receptor affinity for the ligands. This class of receptors cluster rapidly after ligand binding, internalized via coated pits into endosomes after which they elicit their mitogenic action in poorly understood ways. The importance of protein-tyrosine kinases is all the more significant, not only from the point of view of being associated with receptors that induce mitogenic growth, but also being associated with several oncogenic translation products known to implicate transformation and unrestricted growth. Many oncogenes are believed to have evolved

from growth factor receptor protein-tyrosine kinase genes. For example the *V-erb B* gene of avian erythro-blastosis virus (AEV) has been determined to have arisen from the EGF-R gene (Ullrich et al 1984) while the *V-fms* gene was probably derived from the CSF-1R gene (Sherr et al 1985) etc. All of these oncogenic proteins may transform cells by delivering a continuous and unregulated mitogenic signal which overwhelms the tyrosine phosphorylation pathway.

Epidermal Growth Factor and It's Receptor:

Epidermal growth factor (EGF) provides measurable growth-enhancing activity *in vivo* and is known to stimulate growth for a multitude of cells in culture (Carpenter and Cohen, 1979). EGF possesses a variety of biological activities. In cell culture systems EGF enhances the rate of hexose transport (Barnes and Colowick, 1976), increases DNA synthesis (Carpenter and Cohen, 1979) and stimulates rapid morphological changes (Chinkers et al., 1979, 1981). Cell surface receptors specific for EGF are found on most cell types, including cells of the epidermal origin (Carpenter and Cohen, 1979). The EGF-specific high affinity binding sites usually range from 10^4 to 10^6 /cell. Upon addition of EGF to cells, EGF binds it's receptor (EGF-R) and the EGF-EGF-R complex is internalized (Carpenter and Cohen, 1976). Post internalization events include degradation of both the receptor (Das and Fox, 1978) and the hormone (Carpenter and Cohen, 1976) by lysosomal proteases. Clustering of receptors prior to binding of EGF at the cell surface is probably required for EGF to elicit its biological response (Schechter et al., 1979, King et al., 1980a). EGF-R levels in cells treated with EGF reaches a lower steady state, indicative of a dynamic equilibrium between internalization and re-expression of EGF-R on cell surface. The replenishment event of re-acquisition of the constitutive EGF-R levels is known to take several hours (Carpenter and Cohen, 1976). The EGF-R is a 180 K polypeptide initially identified by specific labeling with a photo-reactive derivative of EGF (Das et al., 1977). Subsequently EGF-R was affinity purified from the membrane component of a human epithelioid tumor cell line A431 on an EGF affinity column (Cohen et al., 1980) and was found to have molecular weights ranging from 150-170 K. Both with the crude plasma membrane fraction as well as in its purified state this receptor protein exhibits an associated protein kinase activity that

is stimulated and enhanced with addition of EGF (Carpenter et al., 1978, 1979; Cohen et al., 1980; King et al., 1980b). The protein kinase action of EGF-R acts as an autokinase phosphorylating the receptor itself. The phosphorylated residue is determined to be at a tyrosine (Ushiro and Cohen, 1980). EGF induced phosphorylation of EGF-R is one of the distinctive biochemical reactions which occur as an early event after addition of the ligand to target cells (Hunter, et al., 1981). In cells, EGF enhances phosphorylation of EGF-R at tyrosine residue but far more predominately at serine and threonine residues as determined by phosphopeptide mapping (Hunter, et al., 1981). The phosphate acceptor sites on EGF-R enables regulation of the receptor mediated processes, including those mediating communication between EGF-R and other ligand receptor systems. To this end, there is ample evidence supporting the hypothesis for the regulatory existence of communication between hormone receptors and stimulation of mitogenesis. Platelet derived growth factor (PDGF) decreases EGF-R down-regulation occurring in response to EGF (Wrann, et al., 1980). Fibroblast growth factor (FGF) (Fox, et al., 1979), vasopressin (Rozengurt, et al., 1981), phorbol esters (tumor promotor) (Brown, et al., 1979) and diacylglycerol (DAG) (McCaffrey, 1984) modulate EGF-R affinity in mitogenically responsive cells by binding to sites other than EGF-R. This implies a mechanism in which a ligand binding to one receptor causes a second receptor to be modified. The modulation of EGF-R by PDGF, DAG, phorbol esters and FGF is shown in a cell culture system by a decreased binding of radio-labeled EGF to cultured cells. In contrast to phorbol esters and PDGF, estrogen (Mukku, et al., 1985) and transforming growth factor- β (TGF- β) (Assoian, et al., 1984) have been shown to increase EGF-R.

TNF- α , a 17 K multifunctional polypeptide hormone, secreted by activated macrophages has been known to elicit hemorrhagic necrosis of some tumors and cytotoxicity to some tumor cell lines (Williamson et al., 1983). Apart from its cytostatic and cytotoxic action, TNF- α has also been shown to be a potent growth promoting mitogen for normal human diploid fibroblasts (Vilcek et al., 1986). TNF- α stimulates the production of interleukin-1 (IL-1) (Kirstein and Baglioni, 1986) and β -interferon (Kohase et al., 1986) in human fibroblasts. Furthermore, TNF- α has also been shown to be a modulator of EGF-R expression (Palombella et al, 1987). The above

authors showed the stimulation of growth on human FS-4 fibroblasts by recombinant human TNF- α (rhTNF- α) with an increased binding of EGF to these cells. Incubation of cells with TNF- α resulted in 40-80% increase in the number of EGF-R sites with receptor protein synthesis demonstrative at around 2 to 4 hr following TNF- α treatment. Moreover, as TNF- α -induced EGF-R expression followed a dose response relationship similar to that reported for the mitogenic stimulation of FS-4 fibroblasts, it was concluded that the TNF- α -induced EGF-R expression may be causal with the mitogenic action of TNF- α on human fibroblasts.

Some of the current investigations on the health effects of TNF- α involves determining the role of the pleotropic action of the cytokine in (1) local and systemic injury under ischemia (Colletti, 1990); (2) graft vs. host disease (Clancy, 1990); (3) clinical septicemia (Casey, 1990); (4) immuno-inflammatory disorders (Bendtzen, 1990); (5) immuno-deficiency syndrome (Halliwell, 1991); (6) allergic reactions (Kyanaung et al. 1991); (7) activation of cellular oncogenes (Haliday, 1991); (8) compliment mediated immunity (Ware, 1990); and (9) further investigation in phase II clinical trials as an anti-cancer drug (Abbruzzese, 1990).

Lipid Soluble Antioxidants In Cancer Cells:

β -carotene:

Wolbach and Howe (1985) were the first investigators to discover a relationship between vitamin A and neoplasms where they found that a dietary deficiency of vitamin A in rats led to "stratified keratinizing epithelium" and restoration of vitamin A to their diet reversed the neoplastic process (Wolbach and Howe, 1985). Many subsequent studies have strongly supported a link between vitamin A and neoplastic diseases (Lippman et al., 1987). Recognition that vitamin A deficiency leads to hyperkeratosis of the skin and to squamous metaplasia opened the door for this wonder drug in treatment of cutaneous disorders. But acute exposure to vitamin A results in unacceptable toxic effects to the liver leading to hypervitaminosis A (Lippman et al., 1987). These severe side effects, primarily the hepato-toxicity, led to the search of vitamin A derivatives called "retinoids" - a term encompassing all the

natural (excluding carotenoids) and synthetic compounds having some or all of the biological activities of vitamin A (retinol). The accumulation of a vast amount of data investigating the role of retinoids for the prevention of cancer clearly demonstrate the potent differentiation-inducing and anti-proliferative effects of vitamin A and many of the newly synthesized retinoids. In general, the anti-proliferative activity of retinoids is cell-cycle specific, occurs in a broad range of transformed cell types, and is a reversible phenomenon (Lotan, 1980). In contrast to their anti-proliferative effects, retinoids tend to affect differentiation in a select group of cell types and in a highly specialized, possibly irreversible way, and the mechanism(s) by which retinoids influence differentiation may differ from that affecting proliferation (Lippman et al., 1987).

Despite extensive investigation, the complex molecular mechanism(s) of action of retinoids in causing the diverse cellular changes remains incompletely understood. Although many mechanisms have been proposed, it has only recently become possible to present a unifying hypothesis, which involves the protein kinase-C (PK-C)/phosphoinositol cascade system, to account for retinoids' varied biological effects. Several reviews detail the major actions of retinoids that have been hypothesized to modulate proliferation and differentiation (Lotan, 1980; Bollage, 1983, Tobler et al., 1986; Lippman et al., 1987) of cells. Biological actions include regulation of (1) enzyme synthesis (especially of ornithine decarboxylase, transglutaminase, and the cAMP intracellular regulatory system), (2) extracellular effects, (3) membrane function, (4) growth factors (especially epidermal growth factor, or EGF), (5) binding proteins, (6) genomic, oncogenic, and postgenomic expression, (7) immunologic activity, and finally (8) the PK-C cascade system.

PK-C, is a Ca^{2+} activated, phospholipid-dependent enzyme, which plays a critical role in the carcinogenic process (Nishizuka, 1986). PK-C has been implicated in the mediation of many phorbol ester-promoted actions, such as ornithine decarboxylase (ODC) induction and EGF receptor down-regulation (Jetten and Shirley, 1986). Membrane-bound activated PK-C may transmit to the nucleus a signal to increase gene (e.g., the ODC gene) transcription. PK-C may indirectly regulate gene expression by phosphorylating proteins such as histones and may also modulate oncogene (e.g., c-myc) expression.

In contrast, control of cell response to EGF by PK-C is accomplished directly by regulation of phosphorylation of the EGF receptor and possibly by inhibiting internalization of EGF (Jetten and Shirley, 1986).

α -Tocopherol (Vitamin E):

Wattenberg (1978,1983) has classified inhibitors of carcinogenesis into three categories depending upon their mechanistic stage of action. They include compounds that (1) prevent the formation of carcinogens from precursors; (2) inhibit carcinogens from reaching or reacting with target sites (blocking agents); and (3) suppress the expression of neoplasia by exposed cells (suppressive agents). α -Tocopherol has been studied in many model systems which reflect all these potential inhibitory roles.

In Vitro Studies: There are number of studies that reflect an active role for a-tocopherol in blocking initiation-related events. α -tocopherol inhibited benzopyrene-induced chromosomal damage in mammalian cells grown in culture (Smalls and Patterson, 1982). Shamberger et al. (1973) observed that vitamin E reduces the *in vitro* dimethylbenzo-anthracine (DMBA)-induced mutagenesis in human lymphocytes. Further, vitamin E was found to reduce the sister chromatid exchange frequencies to near control levels in CHO cells exposed to 1-2% DMSO (Patterson et al., 1981).

Vitamin E has been shown to be a very effective blocker of nitrosamine formation both *in vitro* and *in vivo* (Kamm et al., 1977; Newmark and Mergens, 1981). These studies demonstrated the importance of employing the proper form of the vitamin E molecule (i.e., free tocopherol) in order to achieve effective results.

Possible Mechanisms of Action: The major interest in α -tocopherol as a cancer-preventive agent is based on its well-recognized antioxidant free radical scavenging property. On an experimental level, it is sometimes difficult to dissociate antioxidant function from an observed effect of α -tocopherol on enzyme activity, an alkylating agent, differentiation, tumor growth, etc., since all of these events either generate or are associated with free radicals. One approach to a discussion of possible mechanisms of action is to address the observed effects of α -tocopherol by an arena of activity,

which fall into the categories of exogenous, host-mediated, and cellular effects.

a) Exogenous Effects

The protective role of vitamin E on an exogenous level is, perhaps, most quantitatively described in the studies on inhibiting nitrosamine formation. Here, α -Tocopherol participates in a redox reaction with the nitrosating agent and could stoichiometrically be oxidized to α -tocopherylquinone.

b) Host-Mediated Effects

Vitamin E may exert its effect on the immune system by modulating the release of arachidonic acid from membrane phosphatide and/or affecting prostaglandin synthesis and lipoxygenase activity. Since these biosynthetic pathways proceed via free radical mechanisms (Carpenter, 1986), the mechanism of the immuno-enhancing activity of vitamin E appears to be related to its antioxidant activity.

c) Cellular Effects

Proper cellular antioxidant status important in protecting cells against the oncogenic-, radiation- induced and chemical-induced transformation, both in the initiation and promotion phases of tumorigenesis. This underscores the idea that free radicals could play a significant role in carcinogenesis. The available evidences support the role of α -tocopherol as an anti-promoter, but the exact mechanism by which it exhibits this effect remains to be elucidated.

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CHAPTER - 1

DIFFERENTIAL CYTOTOXICITY OF TNF- α IN A MURINE LEUKEMIA VIRUS TRANSFORMATION SYSTEM

ABSTRACT

The *in vitro* cytotoxic response of murine TNF- α , obtained by immuno-purification of *E Coli* lipopolysaccharide (LPS) activated RAW 264.7 macrophage supernatant over an anti-TNF- α monoclonal antibody hydrazide column, was tested on normal murine embryonic fibroblasts (BALB/c CL.7), and its two Abelson-murine leukemia virus (A-MuLV) transformed clones (BALB/c A-MuLV 3R.1 and BALB/c A-MuLV 6R.1). The 6R.1 clone was found to be significantly more sensitive to TNF- α cytotoxicity than the CL.7 or the 3R.1 clones ($p < 0.05$). The differential TNF- α sensitivity was dose dependent and was maximal at a TNF- α concentration of 100 units/ml. Clonal TNF- α susceptibility was not only responsive to the concentration of TNF- α (0 to 100 units/ml) but also to the TNF- α incubation time (0 to 18 hrs) in an 18 hr TNF- α cytotoxicity assay. The percent cytotoxicity CL.7, 3R.1, and 6R.1 clones were 14.4 ± 0.5 , 24.1 ± 3.5 , and $49.4 \pm 2.9\%$ respectively. We conclude that transformation of a TNF- α insensitive clone with the retrovirus A-MuLV could potentially alter its susceptibility to TNF- α .

INTRODUCTION

TNF- α is a multifunctional protein that is secreted by activated macrophages and elicits hemorrhagic necrosis of certain kinds of tumors *in vivo* as well as cytotoxicity to several murine and human cell lines *in vitro* (Carswell, 1975; Haranaka et al. 1981; Williamson et al. 1983). Apart from its cytostatic, cytotoxic and null effects on several human tumor cell lines, TNF- α has also been shown to be growth promoting in normal human fibroblasts (Sugarman et al. 1985; Vilcek et al. 1986). The mechanism by which

TNF- α elicits such a diverse response is unknown. To understand the diversity in the mechanism of TNF- α action it is important to establish that a normal cell line that is insensitive to TNF- α exhibits altered sensitivity to TNF- α cytotoxicity when it is malignantly transformed. Such studies are crucial to the understanding of the differential actions of TNF- α and are lacking in the literature.

The present study was aimed at determining whether fibroblasts infected with leukemia virus were sensitized to cytolysis by TNF- α . The Abelson-Murine Leukemia Virus (A-MuLV) (which is a replication defective retrovirus with high transformation potential) has been studied extensively (Fischinger, 1980). This virus was derived during passage in mice of replication-competent Moloney-Murine Leukemia Virus (M-MuLV) and can rapidly induce leukemia *in vitro* (Scher et al. 1975; Rosenberg, N et al. 1975). A number of A-MuLV strains have been identified (Rosenberg et al. 1980); each producing a fusion protein corresponding to the N-terminal region of the *gag* gene product and a polypeptide encoded by the mouse cell derived sequences (Rosenberg et al 1980; Witte et al 1978; Reynolds et al 1978). The prototype derived from the nonproducer cell line ANN-1 (Witte et al 1979) A-MuLV strain, encodes a 120 K transmembrane protein (P120) in the infected cell. P120 possesses a protein tyrosine kinase activity which is intimately related to the transformation ability of the prototype A-MuLV virus (Witte et al. 1980). We report here the altered susceptibility of murine embryonic fibroblasts to TNF- α when transformed with A-MuLV.

MATERIALS AND METHODS

Cells: Mouse fibrosarcoma L929, normal mouse fibroblasts, CL.7, and two A-MuLV transformed mouse fibroblast cell lines, 3R.1 and 6R.1, were grown in 90% Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 10% fetal bovine serum (FBS), 10 U/ml penicillin and 10 μ g/ml streptomycin, 25 mM HEPES, pH 7.4 at 37°C in a 5% CO₂, humidified incubator. The A-MuLV immortalized mouse monocyte-macrophage cell line RAW 264.7 was grown in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% FBS for the production of mouse TNF- α . All the cell lines were procured from

the American Type Culture Collection (ATCC), Rockville, Maryland.

rhTNF- α and its Antibody: Recombinant human tumor necrosis factor- α (rhTNF- α), 5×10^7 units/mg, and monoclonal antibody against rhTNF- α , lot #5890-90, from mouse ascites (5×10^5 neutralizing units per ml) were a gift from Genentech, California.

Production of Mouse TNF- α : Mouse TNF- α was produced by activation of RAW264.7 cells with *E. coli* lipopolysaccharide (LPS) 026:B6 (Sigma Chemical Co., MO). Cells were grown to confluence in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% FBS at 37°C in a 5% CO₂ atmosphere. Before induction of TNF- α secretion, the cells were washed four times with Hank's balanced salt solution (HBSS) (Sigma Chemical Co., MO) with 25 mM HEPES, pH 7.4 to remove contaminating serum proteins. Each flask was then stimulated with 12 ml of RPMI 1640 containing 1 μ g/ml *E. coli* LPS and 50mM HEPES, pH 7.4. After incubation for 22 h, the medium was filtered through a 0.22 μ m filter twice and was frozen at -20°C until used.

Immuno-affinity Purification of Mouse TNF- α from Activated RAW 264.7 Supernatant: Anti-rh TNF- α monoclonal antibody (IgG₁) has been shown to inhibit cytotoxicity of mouse TNF- α in activated RAW 264.7 supernatants which is due to the reason that monoclonal antibody against human TNF- α is also able to bind to mouse TNF- α . The binding of anti-rhTNF- α antibody to mouse TNF- α can be used to purify mouse TNF- α from activated RAW 264.7 supernatant over an anti-rhTNF- α antibody column. This process is helpful in removal of contaminating proteins like interleukin-1 (IL-1) and other cytokines, also present in activated macrophage supernatants. The anti-rh TNF- α monoclonal antibody, purified from ascites by Genentech, CA was used to make a mouse TNF- α immunoaffinity column. Affi-Gel Hz immunoaffinity kit (Bio-Rad, CA) was used to couple the antibody to an agarose support matrix. The purified antibody was in a total protein concentration of 2 mg/ml. The coupling reaction was performed as per manufacturer's directions. Briefly, 1 mg of protein containing IgG₁, was used for coupling. Prior to coupling, the protein was desalted in an Econo-10DG column (Bio-Rad, CA) using 1X Hz coupling buffer. Column eluent (500 μ l fractions) were tested

for protein using Bradford dye binding method (Bradford, 1976). The positive fractions were pooled for immunoglobulin oxidation using sodium periodate. The desalting procedure was repeated to remove unreacted sodium periodate prior to IgG₁ coupling to the gel. One ml of gel/buffer slurry was added to 1 ml of oxidized IgG₁ and incubated for 24 hr at 25°C. After the coupling reaction, the slurry was transferred to 1 x 10 cm Econo-chromatography column (Bio-Rad). The column was equilibrated in 0.2 M Glycine-HCl pH 3.0 (elution buffer) and washed five times with application buffer composed of, RPMI-1640, with 1 mg/ml BSA (without FBS). Two mls of activated RAW 264.7 supernatant was loaded and reloaded to the column for better TNF- α binding. The fall through fraction was saved. After subsequent washing steps in high and low salt conditions, TNF- α was eluted with 4 ml of 0.2 M Glycine-HCl, pH 3.0 into a tube containing 1 ml of 5 mg/ml BSA in 1 M Tris-HCl pH 8.0 with 0.02% sodium azide. The final BSA concentration was 1 mg/ml at pH 7.3. This was called the F-2 fraction. RAW 264.7 supernatant aliquots without and with LPS activation as well as with F-1 and F-2 fractions were run in a 20% sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

TNF- α Production Assay: L929 cells were used as target cells to quantitate affinity purified murine TNF- α . The L929 cytotoxicity assay was performed as described (Larrik, et al. 1989). Briefly, serial dilutions of TNF- α were done using DMEM, 10% FBS in 96-well microtiter plates (Corning, NY) in six replicates. 1:1000 diluted stock TNF- α was plated at a subsequent dilutions of 1:4, 1:16, 1:64 and 1:128 at a volume of 50 μ l/well. L929 target cells, washed and resuspended in DMEM supplemented with 10% FBS with 1 μ g/ml actinomycin D (TNF- α sensitizer), were plated at a concentration of 1 X 10⁶ cells/ml at a volume of 50 μ l/well. After 18 hr of incubation at 37°C, and 5% CO₂, 20 μ l of a 2.5 mg/ml solution of (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT Sigma Chemical Co., MO) in 0.9% saline was added. After incubation with MTT for 4 hr at 37°C, 100 μ l were removed from each well without disturbing the crystals. The crystals were solubilized with 100 μ l dimethylsulphoxide (DMSO) and read at 550 nm using a microplate reader (Molecular Devices).

The % cytotoxicity was calculated using the formula.

$$\% \text{ cytotoxicity} = \frac{A_{\text{media}} - A_{\text{sample}}}{A_{\text{media}} - A_{\text{TX-100}}} \times 100$$

Where A_{media} , A_{sample} , and $A_{\text{TX-100}}$ are the absorbance readings of cells incubated with media, TNF- α or Triton X-100. Triton X-100 treated wells were used as positive controls (100 % killing). One unit of TNF- α was defined to be that 1/dilution which gives 50% killing. 1/dilution was plotted with % cytotoxicity to determine the 50% killing point.

Statistical Analysis : Each experiment had its own relevant control. The statistical analysis was performed by a paired t test comparison. The probability of $p \leq 0.05$ was taken to be significant. Unless otherwise mentioned the results were expressed as mean \pm S.D of six replicate readings.

RESULTS

Quantitation of TNF- α From LPS Activated RAW 264.7 Supernatant: TNF- α in *E. coli* LPS activated RAW 264.7 supernatant was quantitated using L929 cytotoxicity bioassay as described in the materials and methods section. The serial dilution was performed from a 1:1000 diluted stock. The curve of % cytotoxicity with 1/dilution of stock TNF- α supernatant was depicted in Figure-1. The % cytotoxicity was calculated as described in the materials and methods section and presented in TABLE-1. One unit of TNF- α was defined to give 50% killing of L929 cells. Under these conditions a 1:200 dilution of 1:1000 diluted activated RAW 264.7 supernatant yielded 1 unit of TNF- α . Thus the total units of TNF- α in undiluted stock was found to be 2×10^5 units/ml. At a protein concentration of 0.8 mg/ml, the specific activity of mouse TNF- α in LPS activated RAW 264.7 supernatant was 2.5×10^5 units/mg protein.

Cytotoxicity of Mouse TNF- α Inhibited by rhTNF- α Monoclonal Antibody: L929 cells, 50 μ l of 1×10^6 cells/ml, were plated in 100 μ l of media containing actinomycin D at a final concentration of 1 μ g/ml. After attachment for 4 hr, cells were incubated in six

replicates/group in five groups. The first group comprised of cells incubated with medium only. The second group comprised of cells incubated with 10 units of TNF- α (LPS activated RAW 264.7 supernatant). The third group of cells were incubated with 100 neutralizing units of rhTNF- α monoclonal antibody (mAb) and the fourth group consisted of cells incubated with 10 units of TNF- α along with 100 neutralizing units of rhTNF- α mAb. The fifth group consisted of cells incubated with TX-100 (a positive control for complete killing). The incubation was carried for 10 hr and 15 hr. The mAb was applied 2 hr prior to application of TNF- α . The % cytotoxicity with mouse TNF- α with and without the mAb against human TNF- α on L929 cells was calculated (TABLE-2). We observed that mAb against rhTNF- α was able to inhibit cytotoxicity of mouse TNF- α on a mouse fibrosarcoma cell line L929 (Figure-2). These data suggests that the mAb against rhTNF- α can bind to mouse TNF- α present in LPS activated RAW 264.7 supernatant. The above finding offered an opportunity to immunopurify mouse TNF- α from LPS activated RAW 264.7 supernatant using a rh TNF- α mAb immunoaffinity procedure.

Immunoaffinity Purification of Mouse TNF- α on an Anti-TNF- α mAb Hydrazide Immunoaffinity Column: Immunoaffinity purification of mouse TNF- α was performed as described in the materials and methods section. The fractions in the immunopurification scheme were run on a 20% SDS-PAGE and developed with silver stain, as shown in Figure-3. We observed a 17K TNF- α band present only in activated RAW 264.7 macrophage supernatant (lane 3) and was absent in unactivated supernatant (lane 2) corresponds to a band of purified rhTNF- α (Lane 6). The fall-through fraction (F-1) after loading the activated supernatant through the immunoaffinity column does not contain the 17K band (lane 4) indicating thereby that hydrazide coupled with anti-rhTNF- α mAb had specifically bound the mouse TNF- α . The antibody-specific elution fraction (F-2) contained the 17K band (lane 5) proving thereby that there was a successful elution of TNF- α from anti-TNF- α mAb column. In each case, 2 ml of supernatant was precipitated with 20% ammonium sulfate, pellet dialysed with PBS in a Pierce microdialyser system and resuspended in 100 μ l of loading buffer with tracking dye. 10 μ l

of 1:1000 dilution of a 0.5 mg/ml stock of rhTNF- α (Genetech, CA) was used as a TNF- α standard (lane 6). Lanes 1 and 7 were the molecular weight markers (12.4 K to 95.5 K, Diversified Biotech, MA).

Comparing Cytotoxic Activities of Activated RAW 264.7 Supernatant With Immunopurified Mouse TNF- α : 2 ml of activated RAW 264.7 supernatant was immunopurified on an anti-TNF- α column as described in the materials and methods section. The immunopurified TNF- α (F-2) fraction was ammonium sulfate precipitated, dialysed and resuspended in 2 ml of DMEM (without FCS). The % cytotoxicity of 1:1000 diluted stocks of activated RAW 264.7 supernatant and immunopurified mouse TNF- α is documented in TABLE-3a and graphed in Figure-4. We observed that the cytotoxicity of immunopurified mouse-TNF- α in actinomycin D primed L929 cells was not significantly different from that of the activated RAW 264.7 supernatant. We anticipated that *E. coli* LPS-activation of RAW 264.7 would give rise not only to TNF- α production, but also to interleukin-1 (IL-1) and that IL-1 could aid in TNF- α cytotoxicity synergistically (Bachwich et al. 1986). In that case, similar dilutions of TNF- α preparation from activated RAW 264.7 supernatant or the immunopurified TNF- α should have given different levels of cytotoxicity on L929 cells. The dilutions of the two TNF- α preparations were maintained equal by bringing the volume of the immunopurified TNF- α to the original column loading volume of the activated supernatant. The macrophage supernatant preparation had a 17 K TNF- α band as well as bands arising from other proteins whereas immunopurified TNF- α preparation lacked these extra bands (lanes 3 and 5, Figure-3). The protein bands that are common in lanes 4 and 5 of Figure 3 were that of BSA and its break down products. The cytotoxicity indices of the protein fractions of lanes 2, 3, 4, and 5 are presented in TABLE 3b. As shown in TABLE 3b, the fall through fraction F-1 (Lane 4) had little cytotoxicity to L929 cells. The similar cytotoxicity indices of both the TNF- α preparations (Figure 4) indicated that TNF- α was the major component of cytotoxicity to L929 cells. The immunopurified TNF- α was used in all subsequent experiments.

Differential Cytotoxicity to TNF- α Under A-MuLV Transformation: L929 murine fibrosarcoma, BALB/c CL.7 and two of its A-MuLV transformed clones 3R.1 and 6R.1 were grown on plastic coverslips under the conditions described in methods section. As shown in Figure-5, unlike CL.7, the 3R.1 and 6R.1 clones had lost contact inhibition. Morphologically 3R.1 resembles CL.7 more closely than does 6R.1. 6R.1 formed colonies (foci) in culture. A TNF- α cytotoxicity assay was performed on L929, CL.7, 3R.1 and 6R.1 cell lines. The assay was performed at a TNF- α concentration of 100 units/ml in six replicates, at a reaction volume of 100 μ l/well for 18 hr at 37°C. At the end of incubation period, 20 μ l of a 2.5 mg/ml solution of MTT in 0.9% saline was added. After incubation with MTT for 4 hr at 37°C, 100 μ l aliquotes were removed from each well without disturbing the MTT crystals. The crystals were solubilized with 100 μ l dimethylsulphoxide (DMSO) and read at 550 nm using a microplate reader. The percent cytotoxicity was calculated as described in the methods section. The calculation of percent cytotoxicity for CL.7, 3R.1, and 6R.1 cells showed a differential cytotoxicity of TNF- α to the above clones. Thus, TNF- α was found to be more cytotoxic to 6R.1 than to 3R.1 and CL.7. L929 cytotoxicity was taken as a positive control. These results are presented in Figure-6 and in TABLE-4.

The cytotoxicity assays were performed at different concentrations of TNF- α . The TNF- α dilutions were at 1:1, 1:4, 1:16, 1:32, 1:64, and 1:128 of a stock TNF- α of 100 units/ml. The cytotoxicity assay was performed in six replicates for 18 hrs. The determination of percent cytotoxicity for the CL.7, 3R.1, and 6R.1 clones showed that the differential cytotoxicity to TNF- α observed under the A-MuLV transformation was responsive to TNF- α concentration (Figure-7, TABLE-5). The percent cytotoxicity decreased with increasing TNF- α dilutions.

The cytotoxicity assay was also performed at time 0, 8, 12, and 18 hr of TNF- α incubation at a TNF- α concentration of 100 units/ml. Clonal cytotoxicity progressively increased over the range of incubation time as shown in Figure-8 and TABLE-6. The differential cytotoxicity of TNF- α was maintained over the entire

range of the TNF- α incubation. The colorimetric microtiter plates (after the MTT crystal solubilization step) at different time incubation of TNF- α were photographed using cibachrome technique to show the time dependence of L929, CL.7, 3R.1, and 6R.1 cytotoxicity visually. Figure-9 depicts the photographs of four 96 well plates for 0 hr (plate A), 8 hr (plate B), 12 hr (plate C), and 18 hr (plate D) of TNF- α incubation in a colorimetric MTT assay. We observed that L929 (plate D first row) and 6R.1 (plate D fourth row) were more susceptible to TNF- α than CL.7 (plate D second row) and 3R.1 (plate D third row). Complete killing by triton X -100 was shown in plate A (columns 7 thru 12). Thus the transformation of a TNF- α insensitive cell line CL.7 with A-MuLV resulted in a differential cytotoxicity of the transformed clones to TNF- α .

DISCUSSION

The immunopurification scheme presented in the methods section is a simple protocol to obtain a relatively clean preparation of mouse TNF- α . The activity of the TNF- α was not lost upon low pH elution as shown by the L929 bioassay (Figure 4). Furthermore our results indicate that rhTNF- α can be used to purify mouse TNF- α .

The results of this study indicated that mouse embryonic fibroblast cell line CL.7 is a TNF- α insensitive clone (Figure 7). CL.7 upon infection with the A-MuLV retrovirus became sensitized to TNF- α . However, as shown in Figure 7, the A-MuLV transformed cell 3R.1 was found to be relatively TNF- α insensitive as compared to another A-MuLV transformed clone 6R.1. Whereas 3R.1 was relatively insensitive to TNF- α , the 6R.1 clone was more susceptible to it as compared with the cytotoxicity index of the CL.7 clone ($p < 0.05$). The CL.7 clone is a normal mouse fibroblast which is TNF- α insensitive and is incapable of producing tumors in normal syngenic BALB/c mice (ATCC catalogue of cell lines and hybridomas). The A-MuLV transformed 3R.1, although found to be a TNF- α insensitive clone, is capable of producing tumors in athymic BALB/c mice (ATCC catalogue of cell lines and hybridomas). The 6R.1 clone, which was found to be highly sensitive to TNF- α , is known to be capable of producing tumors in athymic as well as normal syngenic mice (ATCC catalogue of cell lines and hybridomas). Evidently TNF- α

acted differently on the two tumorigenic cell lines studied.

TNF- α is known to possess either a growth stimulatory (as shown for normal human fibroblasts by Vilcek et al. 1986), cytostatic, or cytotoxic effects (Carswell, 1975; Haranaka et al. 1981; Williamson et al. 1983). In this investigation we focused on the cytotoxic action of TNF- α . The mechanism by which TNF- α cytotoxicity is altered *in vitro* for some A-MuLV clones and virtually remains unaltered for some others needs further investigation. It is possible that A-MuLV transformation could alter the cyto-protective protein factor(s) present in the cell culture supernatants of the transformed clones, thereby altering their sensitivity to the cytokine. It could alter the surface morphology of the cells which in turn could have an impact on the way the clones respond to the cytokines and other factors present in their extracellular environment. The A-MuLV transformation could alter the levels of TNF- α receptors on cell surfaces because of which there could be a differential toxicity to TNF- α at the level of TNF- α uptake. Transformation could modulate the constitutive levels of growth factor receptors like epidermal growth factor receptor (EGF-R) which in turn would have numerous biochemical effects including a differential TNF- α cytotoxicity. The transformation could also alter the A-MuLV specific antigen expression which in turn could modulate the biochemical status of the cells. A-MuLV transformation could alter the defense enzyme status of the cells, which could result in variations in their susceptibility to be killed by TNF- α . Thus, there could be more than one reason for the variations in the clonal susceptibility of the parent strain and the A-MuLV transformed daughter clones. However, the results of this study on the altered susceptibility of murine embryonic fibroblasts to TNF- α when transformed with the retrovirus A-MuLV provides an opportunity to further investigate the mechanisms involved in this process.

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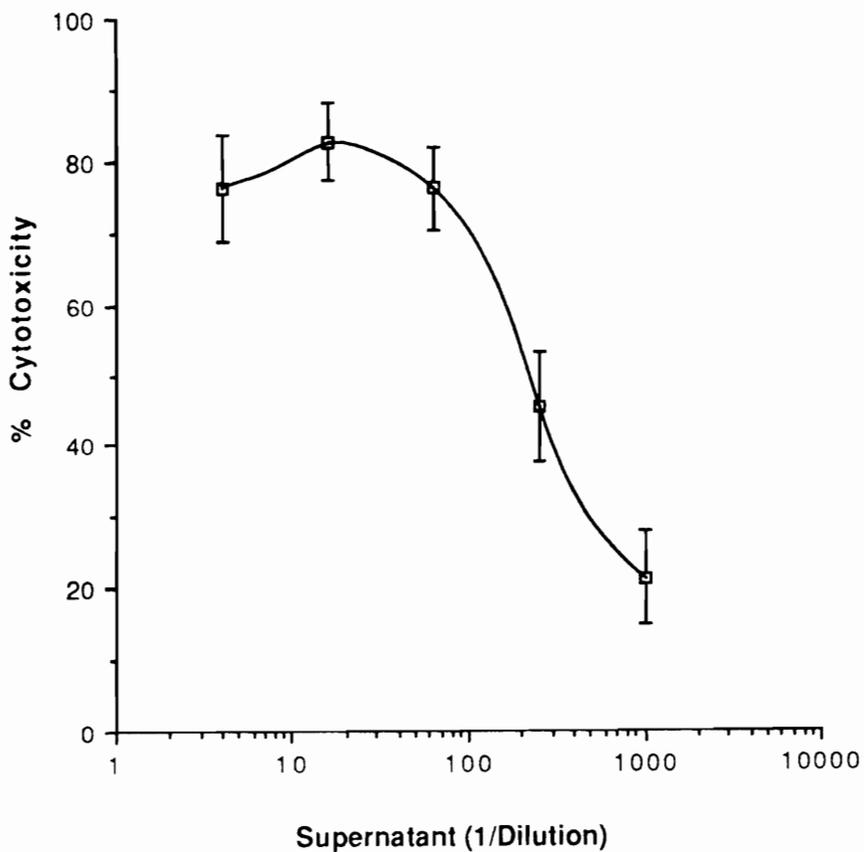


Figure 1 : Cytotoxicity Of TNF- α on L929 Cells as a Function of Activated RAW 264.7 Supernatant Dilution. 50 μ l of 1×10^6 cells/ml of L929 cells were incubated with (equal volume of) serial dilution of 1 : 1000 stock of 1 μ g/ml LPS activated RAW 264.7 supernatant for 18 hr. The % cytotoxicity (presented in TABLE-1) was determined using the MTT dye reduction assay as described in the methods section.

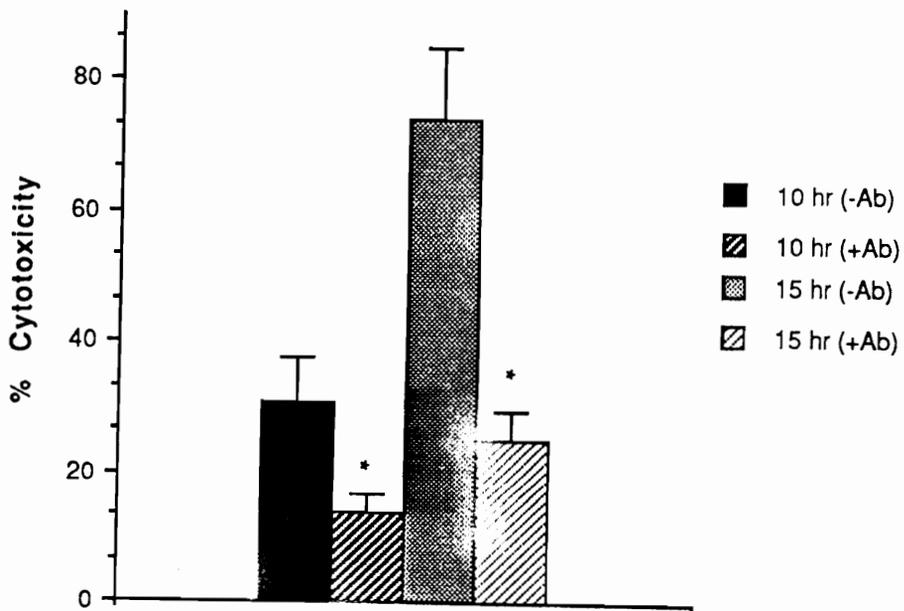


Figure 2 : Inhibition of Mouse TNF- α Cytotoxicity by rh-TNF- α Monoclonal Antibody. Incubation of L929 cells (5×10^4 cells/well) with 10 units of m TNF- α was performed with and without 100 neutralizing units of rh-TNF- α mAb for 10 and 15 hr. The rh-TNF- α mAb inhibits cytotoxicity of m TNF- α (* = $p < 0.05$) at the indicated incubation times.

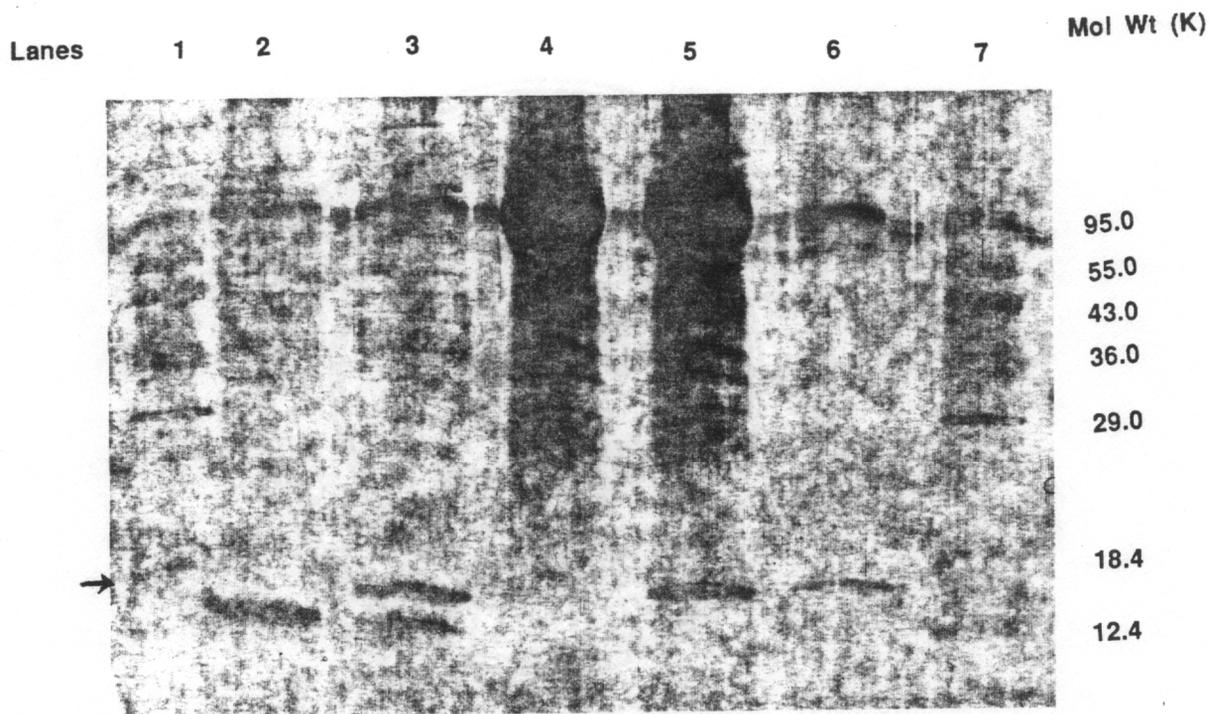


Figure 3 : A Single Step Purification of mouse TNF- α on an anti-rh TNF- α Immunoaffinity Column As Determined By 20 Percent SDS-PAGE. Lanes 1 & 7 are molecular weight markers. Lanes 2 & 3 are protein fraction of 2 ml of RAW 264.7 supernatant without and with LPS activation respectively. Lanes 4 & 5 are the F-1 & F-2 breakthrough fractions as described in the methods section. Lane 6 is rh-TNF- α marker.

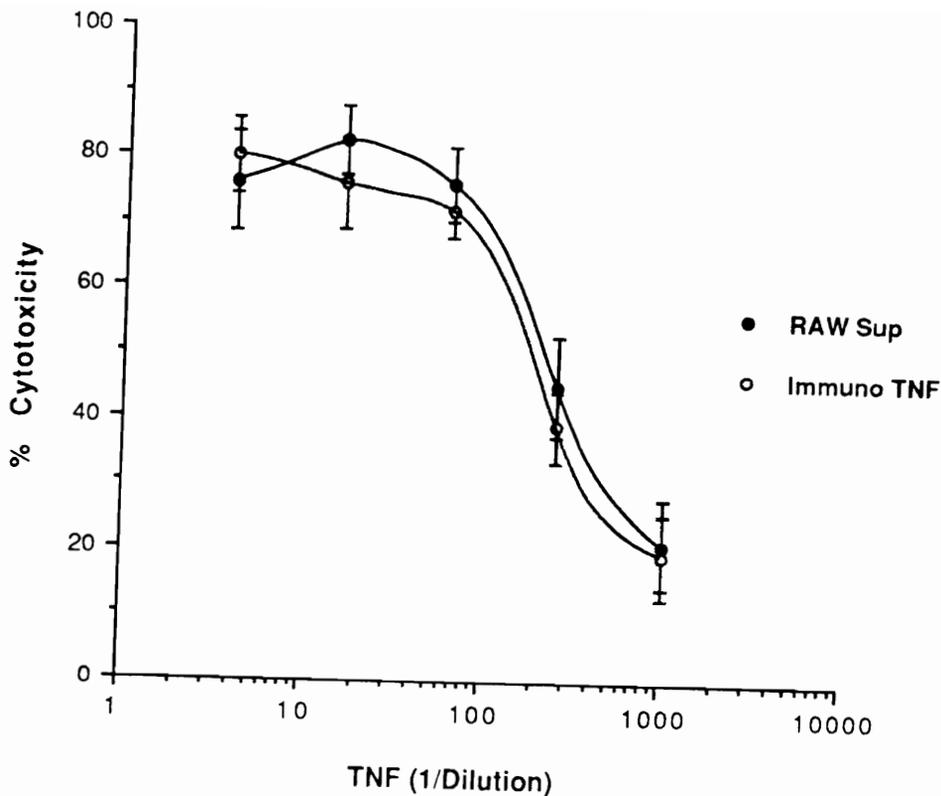


Figure 4 : Comparison of TNF- α Cytotoxicity Using Activated RAW 264.7 Supernatant And Immunopurified mouse TNF- α . Cytotoxicity of mouse TNF- α from RAW 264.7 supernatant on L929 cells as compared with that of immunopurified mouse TNF- α from an anti-rhTNF- α antibody column. The % cytotoxicity of equal dilutions (as described in the methods section) of both the mouse TNF- α preparations are not significantly different ($p < 0.05$) at the concentrations tested (TABLE-4).

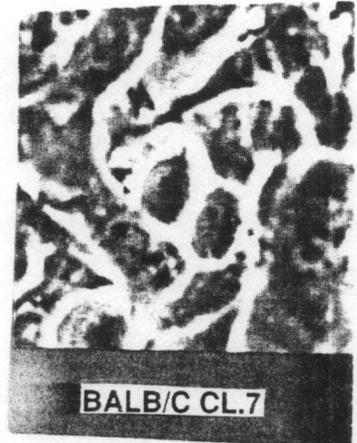


Figure 5 : Light Microscopic Study of Normal and Transformed Cells. The (a) L929 ,(b) BALB/c CL.7, (c) BALB/c A-MuLV 3R.1 and (4) BALB/c A-MuLV 6R.1 clones are shown at 100 X magnification. The 3R.1 and 6R.1 clones have lost contact inhibition. The 6R.1 clone is seen to form colonies and is morphologically distinct from the 3R.1 clone. The 3R.1 clone forms tumors in athymic BALB/c mice and is insensitive to mouse TNF- α . The 6R.1 clone forms tumors in normal syngenic mice and is sensitized to cytolysis by mouse TNF- α .

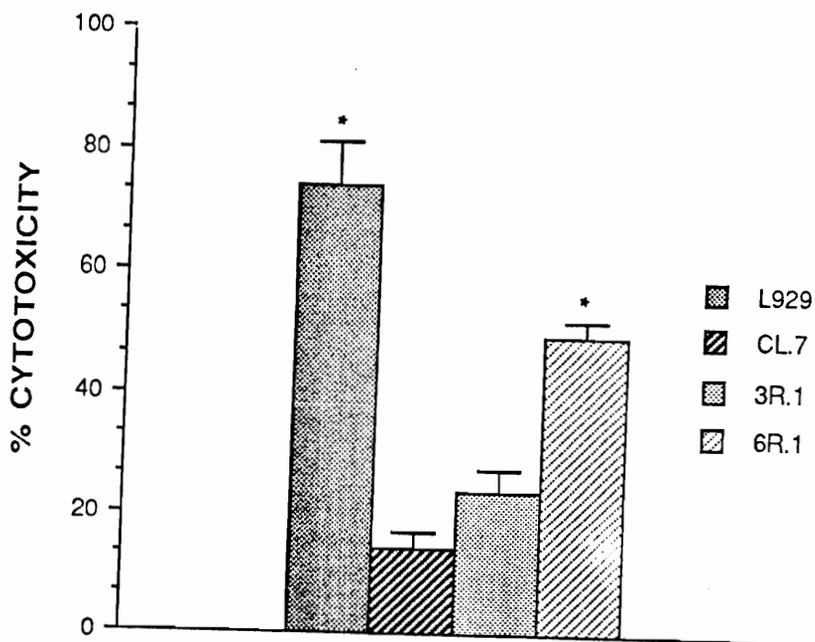


Figure 6 : TNF- α Cytotoxicity Under A-MuLV Transformation. The % cytotoxicity, presented as mean \pm S.D, of L929, CL.7, 3R.1, and 6R.1 clones to 100 units/ml (in 100 μ l reaction volume) of TNF- α is shown. The clonal toxicity of 6R.1 was significantly higher (* = $p < 0.05$) than that of CL.7 clone. The 3R.1 clone, like the CL.7 was relatively insensitive to TNF- α . The cytotoxicity assay was performed for 18 hr as described in the methods section.

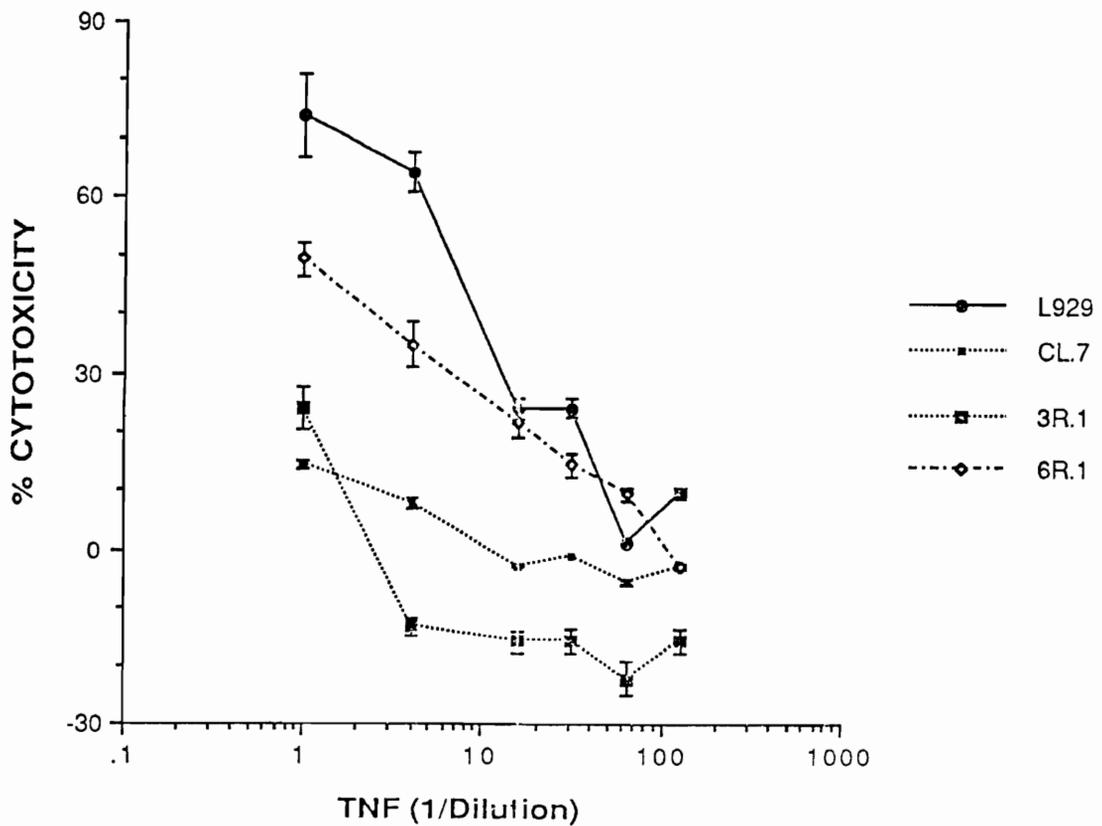


Figure 7: Percent Cytotoxicity With mouse TNF- α Dilution. The cytotoxicity at different concentrations of mouse TNF- α for L929, CL.7, 3R.1, and 6R.1 clones was responsive to serial dilution of mouse TNF- α (18hr incubation). The dilutions of mouse TNF- α for which the cytotoxicity (mean \pm S.D) of six replicate wells were tested were, 1:1 (100 units/ml), 1:4, 1:16, 1:32, 1:64, and 1:128.

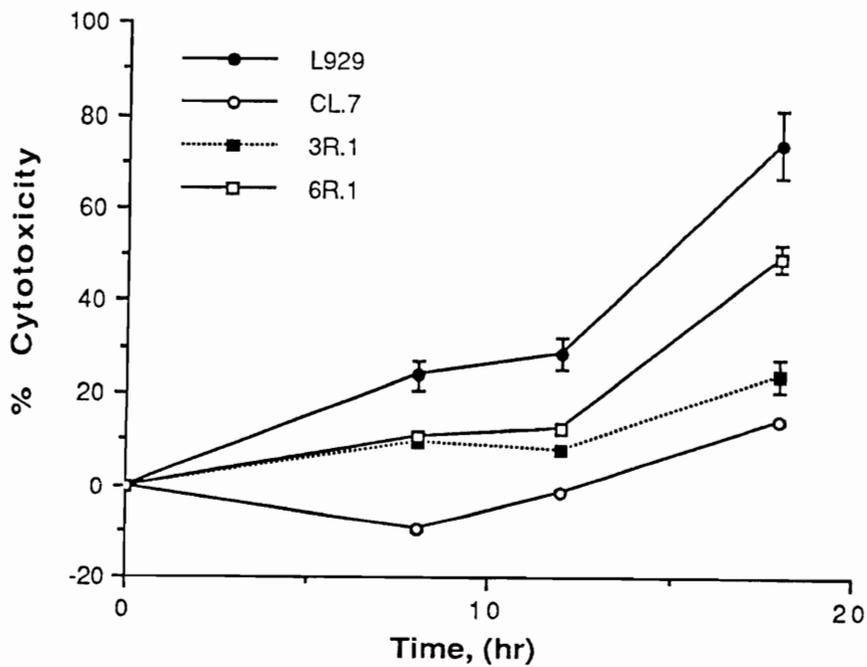


Figure 8 : The Differential Cytotoxicity of TNF- α under A-MuLV Transformation as a Function of Incubation Time. Mouse TNF- α was used at a concentration of 100 units/ml. The MTT assay was performed as described before. The TNF- α incubation time were at 0, 8, 12 and 18 hr. The results were presented as mean \pm S.D of absorbance at 550 nm.

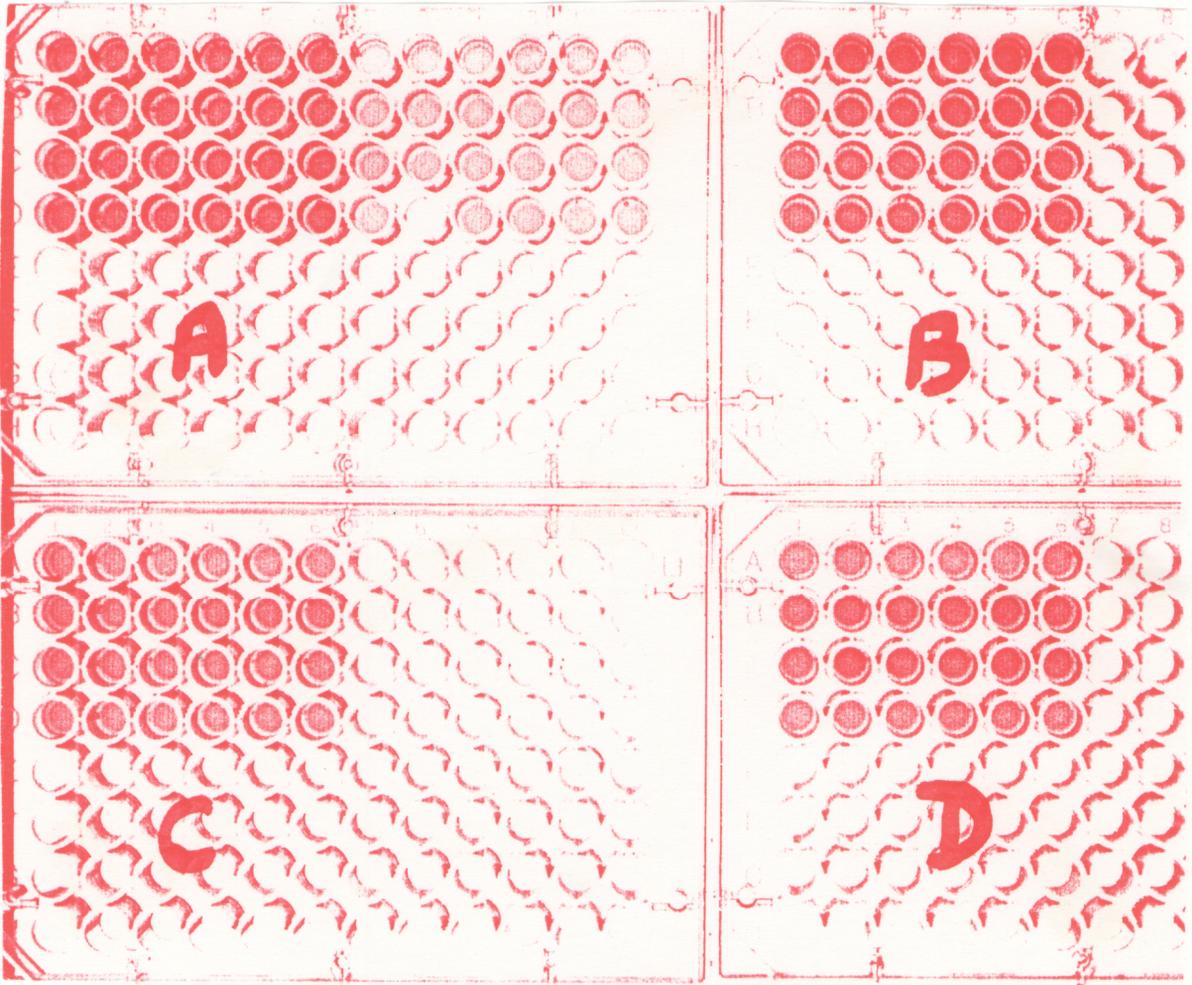


Figure 9 : A Visual Determination of Differential TNF- α Cytotoxicity. The cytotoxicity of mouse TNF- α to L929, CL.7, 3R.1, and 6R.1 clones as depicted for 0 hr (plate A), 8 hr (plate B), 12 hr (plate C), and 18 hr (plate D) of incubation. The six replicates of L929 (first row), CL.7 (second row), 3R.1 (third row) and 6R.1 (fourth row) were subjected to 100 units/ml of TNF- α . The complete killing of all the cells with 0.1 % triton-X 100 is shown in wells 7 thru 12 in plate A. The differential cytotoxicity under A-MuLV transformation is evident in plate D.

TABLE -1
CYTOTOXICITY OF E. COLI LPS ACTIVATED RAW 264.7
MACROPHAGE SUPERNATANT ON L929 CELLS

| 1/Dilution | % Cytotoxicity* |
|------------|-----------------|
| 4 | 76.1 ± 7.5 |
| 16 | 82.7 ± 5.3 |
| 64 | 76.1 ± 5.8 |
| 256 | 45.2 ± 7.8 |
| 1024 | 21.3±6.6 |

*mean ± SD of six replicate wells

TABLE 2
PROTECTION OF rhTNF- α mAb TO MOUSE TNF- α
CYTOTOXICITY ON L929 CELLS

| Treatment Group | %Cytotoxicity* |
|-----------------|----------------|
| 10 hr (Ab-) | 31.0 ± 6.6 |
| 10 hr (Ab+) | 14.1 ± 2.8 |
| 15 hr (Ab-) | 74.1 ± 10.9 |
| 15 hr (Ab+) | 25.4 ± 4.4 |

*% cytotoxicity was calculated using the formula mentioned in materials and methods section - and is represented as mean ± SD of six replicate wells.

TABLE 3a
COMPARISON OF % CYTOTOXICITY ACTIVATED RAW 264.7
SUPERNATANT WITH IMMUNOPURIFIED MOUSE TNF- α

| 1/Dilution (1:1000 stock) | % Cytotoxicity | |
|------------------------------|----------------|----------------------|
| | RAW Sup | Immuno TNF- α |
| 4 | 76.1 \pm 7.5 | 80.0 \pm 5.6 |
| 16 | 82.7 \pm 5.3 | 75.9 \pm 6.9 |
| 64 | 76.1 \pm 5.8 | 72.1 \pm 4.3 |
| 256 | 45.2 \pm 7.8 | 38.9 \pm 5.5 |
| 1024 | 21.3 \pm 6.6 | 19.4 \pm 6.6 |

TABLE - 3 b
RELATIVE TOXICITY OF SUPERNATANT FRACTIONS

| Supernatant Fraction | % Cytotoxicity |
|----------------------|----------------|
| Lane 1 | 21.6 \pm 1.1 |
| Lane 2 | 76.5 \pm 4.2 |
| Lane 3 | 27.8 \pm 2.4 |
| Lane 4 | 79.1 \pm 2.1 |

50 μ l of 1 : 1000 diluted fractions (equal dilutions were done as described in the methods section) were tested for TNF- α cytotoxicity by a L929 cytotoxicity assay. The results were presented as mean \pm S.D of six replicate readings.

TABLE - 4
CYTOTOXICITY OF TNF- α ON L929, CL.7, 3R.1
AND 6R.1 CELLS

| Cell Line | % TNF- α Cytotoxicity |
|-----------|------------------------------|
| L929 | 74.1 \pm 7.2 |
| CL.7 | 14.4 \pm 0.5 |
| 3R.1 | 24.1 \pm 3.5 |
| 6R.1 | 49.4 \pm 2.9 |

TABLE - 5
CONCENTRATION DEPENDENCE OF TNF- α CYTOTOXICITY ON
L929, CL.7, 3R.1 AND 6R.1 CELL LINES

| Dilution | % Cytotoxicity | | | |
|----------|----------------|----------------|-----------------|----------------|
| | L929 | CL.7 | 3R.1 | 6R.1 |
| Stock* | 74.1 \pm 7.2 | 14.4 \pm 0.5 | 24.1 \pm 3.5 | 49.4 \pm 2.9 |
| 1:4 | 64.3 \pm 3.3 | 7.8 \pm 0.9 | -13.2 \pm 1.7 | 34.9 \pm 4.0 |
| 1:16 | 23.9 \pm 1.8 | -2.7 \pm 0.2 | -15.8 \pm 2.0 | 21.6 \pm 2.7 |
| 1:32 | 24.2 \pm 1.6 | -1.0 \pm 0.1 | -15.7 \pm 2.4 | 14.5 \pm 1.9 |
| 1:64 | 1.4 \pm 0.1 | -5.3 \pm 0.5 | -22.2 \pm 3.0 | 9.6 \pm 1.1 |
| 1:128 | 9.5 \pm 0.9 | -2.8 \pm 0.2 | -15.7 \pm 2.3 | -2.0 \pm 0.4 |

*The stock mouse TNF- α concentration used was 100 units/ml in a 100 μ l reaction system in six replicates.

TABLE 6**DATA FOR TNF- α CYTOTOXICITY WITH TIME OF INCUBATION**

| Time (hr) | L929 | CL.7 | 3R.1 | 6R.1 |
|-----------|----------------|-----------------|----------------|----------------|
| 8 | 23.9 \pm 3.1 | -9.8 \pm 0.6 | 9.5 \pm 0.6 | 10.9 \pm 0.8 |
| 12 | 28.7 \pm 3.4 | -1.2 \pm 0.06 | 7.7 \pm 0.5 | 12.7 \pm 0.7 |
| 18 | 74.1 \pm 7.2 | 14.4 \pm 0.5 | 24.1 \pm 3.5 | 49.4 \pm 2. |

*The stock mouse TNF- α concentration used was 100 units/ml in a 100 μ l reaction system in six replicates.

CHAPTER - 2**ROLE OF CIF IN TNF- α PRODUCTION & CYTOTOXICITY UNDER A-MuLV TRANSFORMATION****ABSTRACT**

A protein factor, designated as cytotoxin-inhibiting factor (CIF), was recently shown to inhibit TNF- α production from lipopolysaccharide (LPS) activated macrophage supernatants. The detection of such protein factor(s) present in the extracellular environment of normal (CL.7), A-MuLV transformed (3R.1 and 6R.1), as well as L929 target cells in modulating their susceptibility to the cytotoxic action of tumor necrosis factor- α (TNF- α) was investigated. The cell culture supernatants (CCS) from L929, CL.7, 3R.1, and 6R.1 confluent cultures, were also tested for their ability to modulate the production of TNF- α in LPS activated rat alveolar macrophage cultures as determined by a 20 % SDS-PAGE. The CL.7 (TNF- α insensitive) as well as 3R.1 (TNF- α insensitive) CCS inhibited the TNF- α production in LPS activated macrophage cultures whereas the 6R.1 (TNF- α sensitive) CCS did not do so. The CCS derived from TNF- α insensitive CL.7 clone at 1:2 dilution, in agreement with previous findings, inhibited the cytotoxicity of TNF- α in TNF- α sensitive L929 as well as 6R.1 clones ($p < 0.05$). The heat denatured CCS from CL.7 clone however, did not show any significant protection. The CCS from A-MuLV transformed clones of CL.7, namely 3R.1 CCS as well as 6R.1 CCS showed little cytoprotective effects in both native and heat denatured forms to L929 and 6R.1 clonal susceptibility to TNF- α . Thus we conclude that the differential cytotoxicity of TNF- α under A-MuLV transformation (Chapter1) could possibly be due, in part, to the differential release of cytoprotective protein factors by these cells.

INTRODUCTION

TNF- α is a multifunctional protein that is secreted by activated macrophages (M ϕ) and elicits hemorrhagic necrosis of certain kinds of tumors *in vivo* as well as cytotoxicity to several murine and human cell lines *in vitro* (Carswell, 1975; Haranaka et al. 1981; Williamson et al. 1983). Apart from its cytostatic, cytotoxic and null effects on several human tumor cell lines, TNF- α has been shown to be growth promoting in normal human fibroblasts (Sugarman et al. 1985; Vilcek et al. 1986). The mechanism by which TNF- α elicits such a varied response is unknown. To understand the diversity in the mechanism of TNF- α action it is important to establish that a normal cell line that is insensitive to TNF- α exhibits altered TNF- α cytotoxicity when it is malignantly transformed. The transforming agent could be a chemical carcinogen, UV radiation, or a virus. The action of TNF- α is significant from the point of view of its role as an immunotherapeutic agent in cancers of viral origins. For example, clinical studies performed on leukemic patients showed a marked decrease of percent of leukemia cells in peripheral blood (Urushizaki, 1989). Studies on the cytotoxicity of leukemic cells in culture to TNF- α have shown that TNF- α insensitive murine embryonic lung fibroblasts (BALB/c CL.7) upon transformation with the replication defective Abelson-murine leukemia virus (A-MuLV) give rise to a TNF- α sensitive transformed clone BALB/c A-MuLV 6R.1 and a relatively TNF- α insensitive clone BALB/c A-MuLV 3R.1 (chapter-1). The mechanism(s) by which TNF- α exerts its differential cytotoxic actions on both the untransformed and transformed clones is not clearly understood.

Recently, it was determined that murine embryonic fibroblasts produce a factor termed cytotoxin-inhibiting factor (CIF) which inhibits the production of TNF- α as well as interleukin-1 (IL-1) in activated M ϕ (Gifford, et al. 1989). The present study was aimed at determining whether CIF production into the extracellular environment of CL.7, 3R.1 and 6R.1 clones could be implicated with the differential TNF- α cytotoxicity observed. We investigated the ability of CIF in culture supernatants from CL.7, 3R.1 and 6R.1 to modulate TNF- α production from lipopolysaccharide (LPS) activated rat alveolar M ϕ and determined their role in modulating the TNF- α .

cytotoxicity in these clones. L929 fibrosarcoma cells were used as a positive control for the cytotoxicity assays. This investigation was based on the rationale that factor(s) present in the normal and/or transformed clone supernatants could modulate both TNF- α production from activated M ϕ as well as its cytotoxicity to TNF- α sensitive cell lines.

MATERIALS AND METHODS

Cells: The L929, CL.7, 3R.1, and 6R.1 cells were grown in Dulbecco's modified Eagle's medium (DMEM), containing 10 % fetal calf serum (FCS), 10 units/ml penicillin, 10 μ g/ml streptomycin, 25 mM HEPES at pH 7.4 at 37 $^{\circ}$ C in a humidified 5% CO $_2$ incubation.

Murine TNF- α : Murine TNF- α was obtained from the supernatant of *E. Coli*-LPS-activated murine M ϕ cell line RAW 264.7. The immunopurification of murine TNF- α on an anti- TNF- α antibody column was performed (Chapter-1) and the specific activity was found to be 2.5 x 10 5 units/mg protein.

TNF- α Cytotoxicity Assay: The cytotoxicity assay for TNF- α was performed as described (Chapter-1). The concentration of TNF- α used was 50 units/ml. This concentration of TNF- α was found suitable in detecting CIF from the cell culture supernatant in a 100 μ l reaction system. The L929, CL.7, 3R.1 and 6R.1 cells were plated in 100 μ l of 10 % FCS-supplemented media at a concentration of 5 x 10 5 cells/ml. The cells were allowed to attach for 4 hr after which the media was replaced with 50 μ l of cell culture supernatants from L929, CL.7, 3R.1, and 6R.1 cells and 50 units/ml TNF- α . The cell culture supernatants used in the cytotoxicity assay were collected as follows. Media were removed from confluent cultures of L929, CL.7, 3R.1, and 6R.1 cells in 75 cc flasks (10 8 cells/flask). 12 ml of fresh media were added and left undisturbed for 48 hr. The media were collected, filtersterilized, and stored at -70 $^{\circ}$ C. The cytotoxicity assay was performed with 1 : 2 final dilution of the above supernatants for all the cell lines. Six replicate wells were incubated with either fresh media; media with 25 units/ml final concentration of TNF- α ; media with 1 : 2 final dilution of L929, CL.7, 3R.1, and 6R.1 cell culture supernatants (CCS) along with TNF- α at the same concentration; media with heat denatured CCS (85 $^{\circ}$ C

for 15 minutes) in presence of TNF- α ; and media with triton-X 100 at 0.1 % for complete killing. The percent cytotoxicity was determined as before (Chapter-1).

Harvesting Of Alveolar M ϕ , and TNF- α Production: 8-10 weeks old F344 male pathogen free rats were used to harvest alveolar M ϕ . 250 μ l of a 65 mg/ml pentobarbitol per 200 gm rat body weight was injected subcutaneously after which a tracheostomy was performed. The lung lavage with 50 ml of Hank's balanced salt solution (HBSS) was spun down at 250 g for 10 minutes. The macrophage population in the pellet was washed 2 x with HBSS and counted. 3×10^5 M ϕ were plated in 1 ml of FCS suplimented DMEM with 25 mM HEPES at pH 7.4 and were allowed to attach for 3 hr. After the attachment time the M ϕ monolayer was washed gently with DMEM (without FCS) media and TNF- α production was induced in a 1 ml final volume, with 1 μ g/ml *E Coli* LPS DMEM (without FCS), 50 mM HEPES, pH 7.4 in a 5 % CO $_2$ humidified atmosphere at 37 $^{\circ}$ C in 24 well plates. The cells were allowed to produce TNF- α for 20 hr in the presence and absence of 500 μ l of CL.7, 3R.1 and 6R.1 supernatants. As the M ϕ yeild per rat was only 6 - 8 $\times 10^5$ cells/rat, the effect of CL.7-, 3R.1-, and 6R.1- supernatants on TNF- α production was separately investigated in three different rats with appropriate control (LPS but no CSS). The 1 ml culture supernatants were filter sterilized 2x (to remove LPS) and stored at -70 $^{\circ}$ C. The culture supernatants (from an equal number of M ϕ) in 1 ml volume were ammonium sulfate precipitated, microdialized in a Pierce microdialyzer system, resuspended in 100 μ l loading buffer and electrophorized in a 20 % SDS-PAGE with 4 % stacking gel. The SDS-PAGE was run for 30 minutes at 30 mV through the stacking gel and 2 hr at 60 mV through the running gel. The gels were washed and developed using a silver staining kit (Pierce Chemical Co.).

Statistical Analysis : Each experiment had its own control. The statistical analysis was performed by a paired t test comparison. The probability of $p \leq 0.05$ was taken to be significant. Unless otherwise mentioned the results are expressed as mean \pm S.D of six replicate readings.

RESULTS

The filter sterile CCS from L929, CL.7, 3R.1, and 6R.1 cells were used to determine their cytoprotective characteristics in inhibiting TNF- α cytotoxicity in L929, CL.7, 3R.1, and 6R.1 cells. The supernatants were used in two forms, namely the native and the heat denatured forms. Heat denaturation was carried out at 85⁰ C for 15 minutes. The cytotoxicity assay were performed with 1 : 2 final dilution of the above supernatant fractions. The cytotoxicity indices were calculated as before.

The CL.7 supernatant significantly inhibited ($p < 0.05$) the cytotoxicity of TNF- α to L929 cells where as the heat denatured CL.7 supernatant had little effect in modulating its cytotoxicity (Figure-1) The native and the heat denatured supernatants of 3R.1, 6R.1, and L929 were similarly used in six replicates at a 1:2 dilution at a final volume of 100 μ l in determining their capability to modulate the L929 cytotoxicity. As presented in Figure 1, the supernatants of 3R.1, 6R.1, and L929 in native- and heat denatured-forms had little cytoprotective effects in modulation of L929 susceptibility to TNF- α .

Studies were conducted to determine whether factors present in the supernatant of the relatively TNF- α sensitive clones could modulate the susceptibility of the relatively insensitive clone CL.7. TNF- α cytotoxicity assay was also performed for CL.7 used as target cells to determine the effect of the CCS in modulating the action of TNF- α on CL.7 cells. The native and heat denatured CCS from CL.7, 3R.1, 6R.1, and L929 cells used at a final concentration of 1:2 in modulating the TNF- α action on CL.7 cells is depicted in Figure 2. The negative % cytotoxicity observed with heat denatured CCS from L929, CL.7, and 6R.1 cells is although significant ($p < 0.05$) may have its basis in the statistical effects of calculation of TNF- α cytotoxicity for a TNF- α insensitive clone.

TNF- α cytotoxicity assay was also performed for 3R.1 clone (used as target cells) to determine the effect of the CCS in modulating the action of TNF- α . . The native and heat denatured CCS from CL.7, 3R.1, 6R.1, and L929 cells used at a final concentration of

1:2 in modulating the TNF- α action on 3R.1 cells is depicted in Figure 3. The significant enhancement ($p < 0.05$) in 3R.1 % cytotoxicity observed with native and heat denatured CCS from 6R.1 cells shows that factors present in the 6R.1 CCS could, in part, be responsible for enhanced 3R.1 cytotoxicity. Similar results were obtained for L929 supernatant. The enhancement in the cytotoxicity of 3R.1 cells in presence of its own heat denatured CCS is although significant ($p < 0.05$) may have its basis in the statistical effects of calculation of TNF- α cytotoxicity for a TNF- α insensitive clone.

The CL.7 supernatant significantly inhibited the cytotoxicity of 6R.1 cells ($p < 0.05$) whereas the denatured CL.7 supernatant had little effect in modulating its cytotoxicity (Figure-4). The native and the heat denatured supernatants of 3R.1, 6R.1, and L929 were similarly used in six replicates at a 1:2 dilution at a final volume of 100 μ l in determining their capability to modulate the 6R.1 cytotoxicity. The results of Figure 4 indicated that the supernatants of 3R.1, 6R.1, and L929 in native and heat denatured forms had little cytoprotective effects in modulation of 6R.1 susceptibility to TNF- α .

The effect of CL.7, 3R.1, and 6R.1 clone supernatants on the LPS-activated TNF- α production in rat alveolar macrophages was studied in a 20 % SDS-PAGE and is presented in Figure-5. Lanes 1, 3, and 7 are LPS activated macrophage supernatant protein fractions (ammonium sulfate precipitate) from 3×10^5 M ϕ activated in 1 ml of culture volume in 1 μ g/ml LPS from three different rat alveolar M ϕ preparations. Lanes 2, 4, & 8 represent protein fraction from LPS activated M ϕ supernatant in presence of CL.7, 3R.1, and 6R.1 clone CCS (500 μ l CCS in 1ml reaction volume). Lane 5 was the molecular weight standard. Comparing lanes 1 and 2 shows that the production of TNF- α (17K band) was inhibited in presence of CL.7 supernatant. A similar result was obtained with 3R.1 supernatant (lanes 3 and 4). 6R.1 supernatant had little ability to inhibit TNF- α production (lanes 7 & 8). These results show that CL.7 and 3R.1 clones (which are TNF- α insensitive) produce protein factor(s) in their supernatant which inhibit TNF- α production in rat alveolar M ϕ . The 6R.1 clone has lost these factor(s) in its CCS as evidenced from a lack of cytoprotective effect of 6R.1 CCS against TNF- α cytotoxicity to L929 cells (Figure-1). Further, 6R.1 CCS has also lost its ability to

inhibit TNF- α production from activated M ϕ . Thus, it seems possible that cells not susceptible to TNF- α could protect themselves from the cytotoxic action of TNF- α , in part, via protein factor(s) released in the extracellular environment.

DISCUSSION

The results of Chapter 1 indicate that CL.7 and 3R.1 are TNF- α insensitive cell lines whereas 6R.1 is a TNF- α sensitive clone. The hypothesis that protein factor(s) present in the supernatants of TNF- α insensitive cell lines could, in part, be cytoprotective against TNF- α action was tested. Cytotoxin inhibitor factor (CIF), a protein factor, produced in fibroblast supernatants is known to inhibit TNF- α production in LPS activated M ϕ (Gifford, et al.1989). CIF is known to be produced by normal murine embryonic fibroblasts and also by some tumor cells (Gallily, et al. 1987). It has been postulated that CIF secreted by tumor cells could, in part, be responsible in protecting tumor cells against the lytic effect of TNF- α . In the present investigation we showed that a protein factor(s) in CCS of the CL.7 (TNF- α insensitive clone) cell line inhibits TNF- α production (Figure-5) as well as suppresses the cytotoxicity of TNF- α to TNF- α -sensitive L929 (Figure-1) and 6R.1 (Figure-4) cell lines. The results of the study indicate that CL.7 supernatant could possibly have a protein factor which loses its activity upon heat denaturation, but can inhibit the cytotoxicity of TNF- α .

The protein factor seems to be lost upon A-MuLV transformation. This is evidenced from the following results : (1) an equal volume of CCS from 3R.1 and 6R.1 clones in native as well as denatured forms had little modulatory effect on the cytotoxicity of L929 cells (Figure-1), (2) the 6R.1 cells supernatant in native and denatured forms however increased the 3R.1 cytotoxicity significantly ($p < 0.05$) as depicted in Figure-3, and (3) the cytoprotective factor seems however, to be missing in the 6R.1 clone. The 6R.1 and the 3R.1 cell lines are A-MuLV transformed clones of CL.7. It is possible that the normal TNF- α insensitive clone (CL.7) under A-MuLV transformation would be insensitive to TNF- α as long as it retains its ability to produce extracellular protein factor(s) which are not only cytoprotective to the toxic

action of TNF- α but also inhibit its production from the LPS activated M ϕ . The 6R.1 clone on the other hand is TNF- α sensitive which could be, in part, due to a loss of cytoprotective protein factor(s) in its CCS. Unlike CL.7-CCS, the 6R.1-CCS failed to protect L929 cells from TNF- α killing. The 3R.1 clone is capable of producing tumors only in athymic BALB/c mice whereas the 6R.1 clone produces tumors in both athymic as well as normal syngenic mice. The results of the above study indicate that the tumorigenic 3R.1 cells, that are found to be TNF- α insensitive, could escape from the lytic effects of TNF- α by producing cytoprotective protein factor(s) including CIF in their extracellular environment. The determination of the mechanism of action of such protein factor(s) including CIF in modulation of TNF- α production from activated M ϕ as well as its modulation of TNF- α cytotoxicity needs further investigation.

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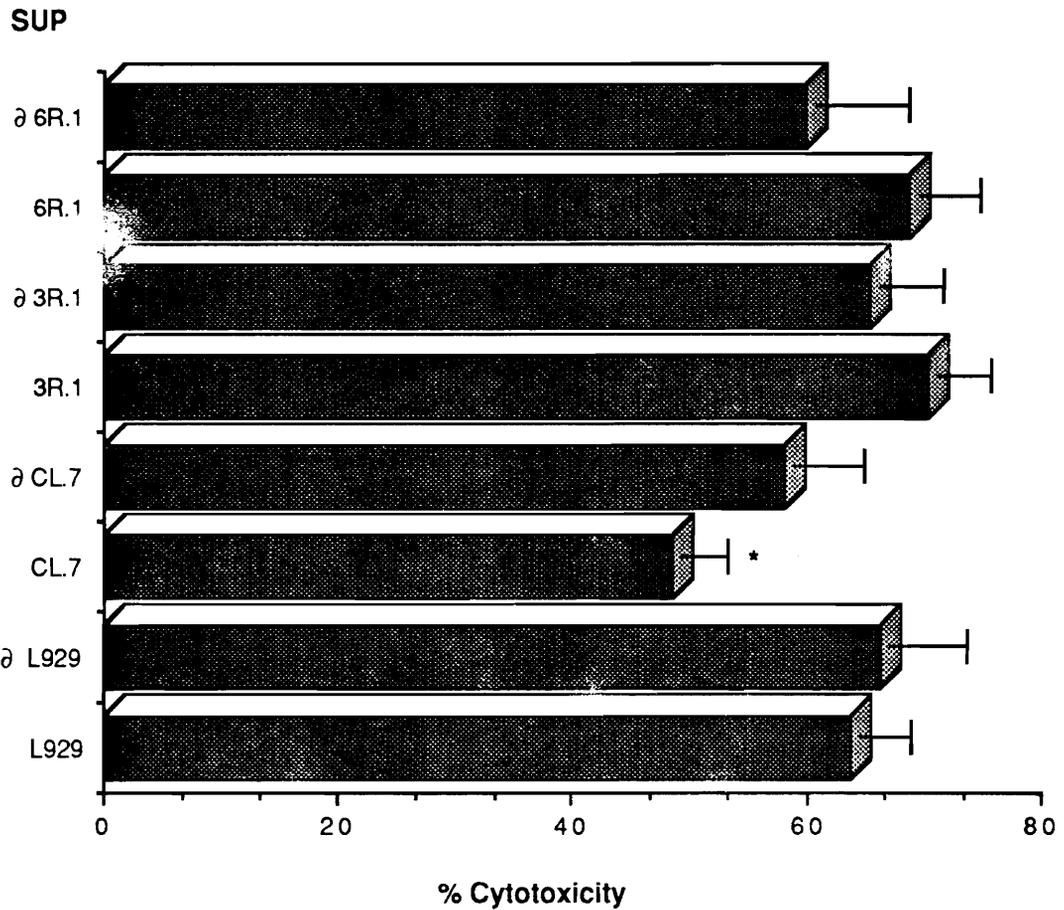


Figure 1 : Role of Cytotoxin Inhibitor (TNF- α antagonist) in Cell Culture Supernatants in Modulating TNF- α Cytotoxicity on L929 Cells. The cytotoxicity assay for mouse TNF- α (50 units/ml) in 100 μ l reaction volume was carried out as described in the methods section. The cell culture supernatants were present in native and heat denatured (∂) form (80⁰ C for 15 minutes) as a 1 : 2 final dilution. The data was presented as mean \pm S.D of six replicate wells. The CL.7 supernatant in the native form was shown to inhibit the TNF- α induced cytotoxicity.

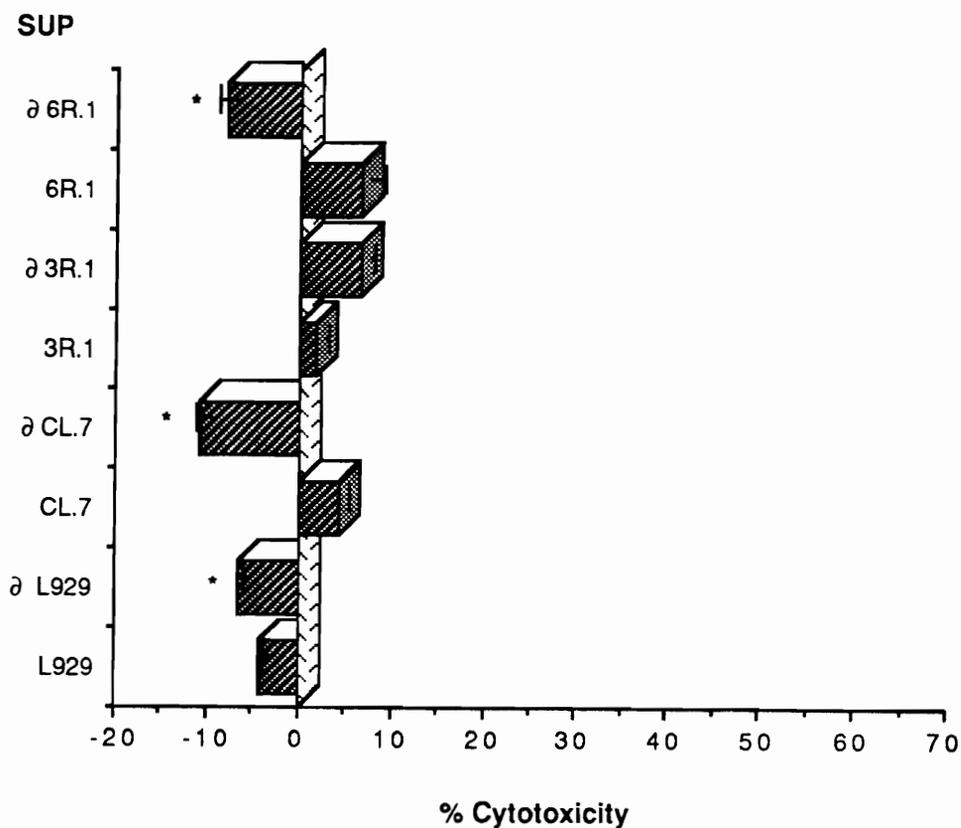


Figure 2 : Modulation of TNF- α Cytotoxicity on CL.7 Cells and Role of Cytotoxin Inhibitor (TNF- α antagonist) in Cell Culture Supernatants. The cytotoxicity assay for mouse TNF- α (50 units/ml) in 100 μ l reaction volume was carried out as described in the methods section. The cell culture supernatants were present in native and heat denatured (∂) form (80⁰ C for 15 minutes) as a 1 : 2 final dilution. The data was presented as mean \pm S.D of six replicate wells.

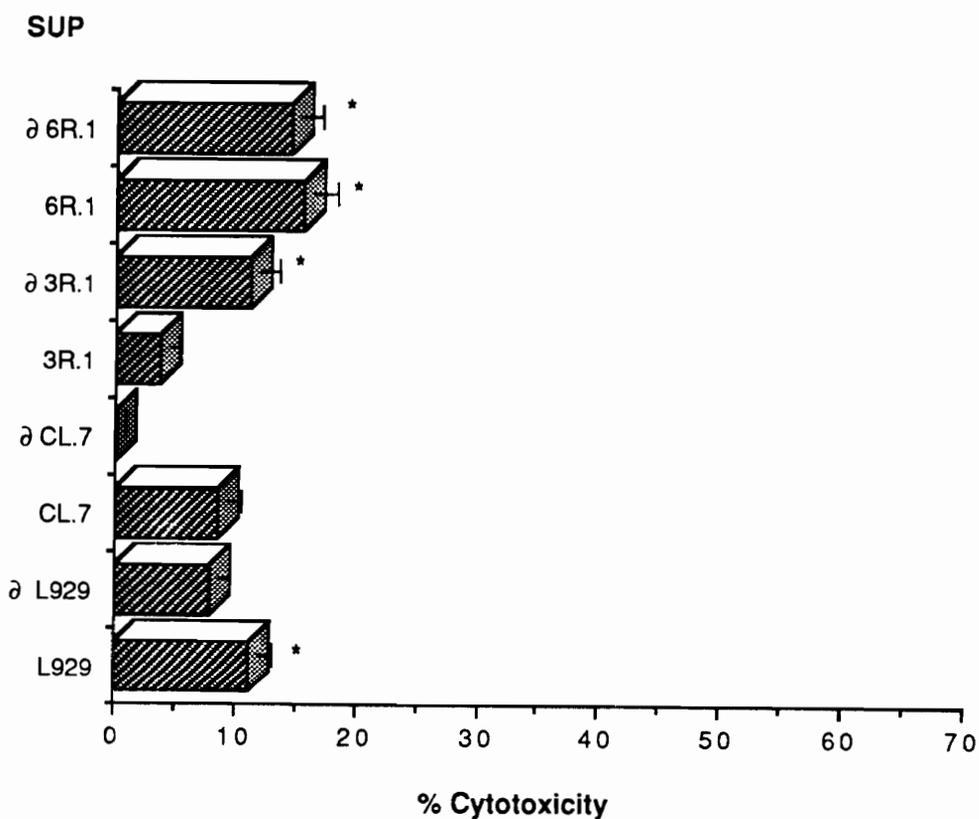


Figure 3 : Modulation of TNF- α Cytotoxicity on 3R.1 Cells: Role of Cytotoxin Inhibitor (TNF- α antagonist) in Cell Culture Supernatants. The cytotoxicity assay for m TNF- α (50 units/ml) in 100 μ l reaction volume was carried out as described in the methods section. The cell culture supernatants were present in native and heat denatured (∂) form (80⁰ C for 15 minutes) as a 1 : 2 final dilution. The data was presented as mean \pm S.D of six replicate wells.

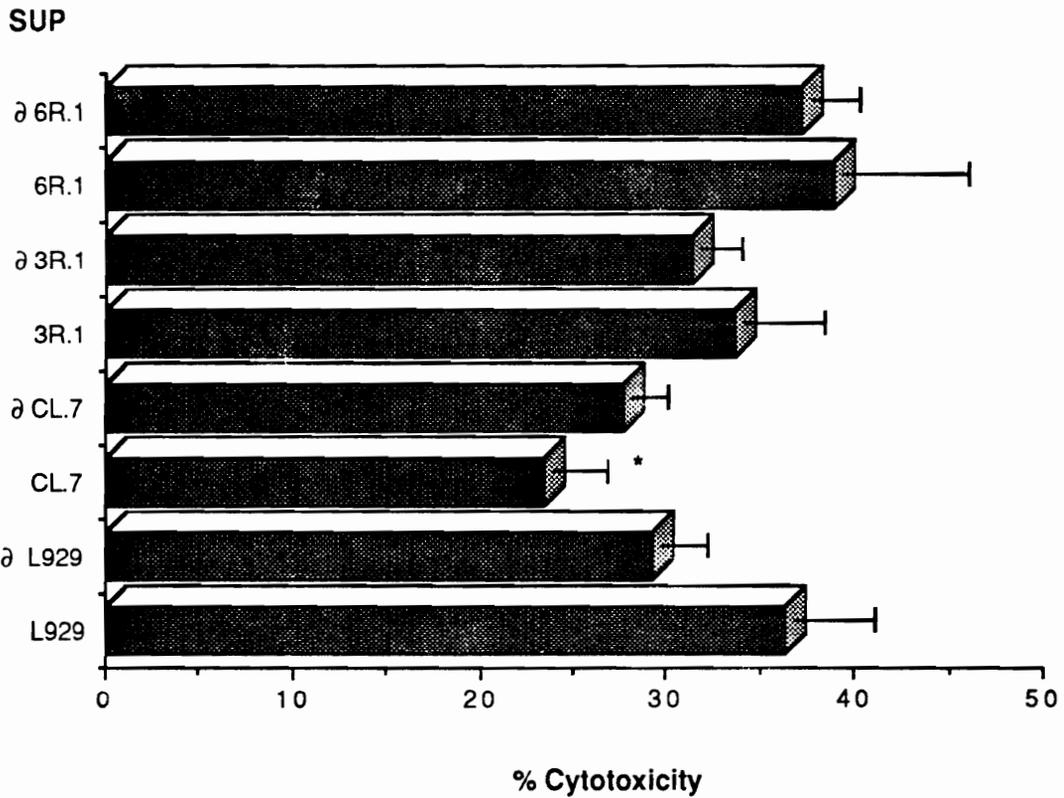


Figure 4 : Modulation of TNF- α Cytotoxicity on 6R.1 Cells: Role of Cytotoxin Inhibitor (TNF- α antagonist) in Cell Culture Supernatants. The cytotoxicity assay for mouse TNF- α (50 units/ml) in 100 μ l reaction volume was carried out as described in the methods section. The cell culture supernatants were present in native and heat denatured (∂)form (80 $^{\circ}$ C for 15 minutes) as a 1 : 2 final dilution. The data was presented as mean \pm S.D of six replicate wells.

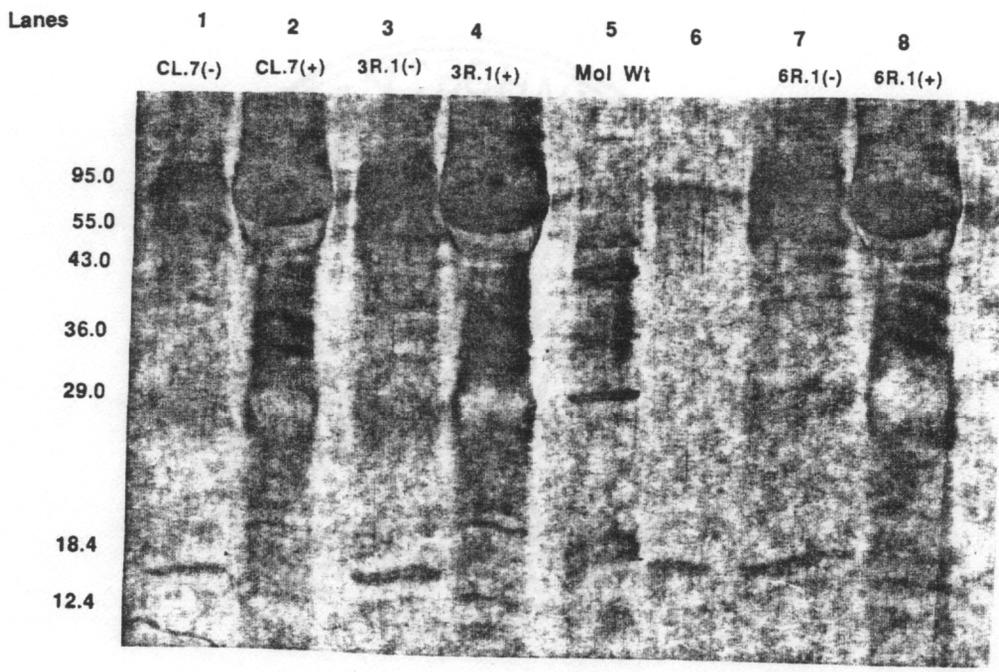


Figure 5 : SDS - PAGE Analysis of TNF- α Production. Role of CIF in CCS in modulation of TNF- α production in the supernatants of LPS activated alveolar M \emptyset . The lanes 1, 3, and 7 are LPS activated M \emptyset supernatant protein fraction from three different rat alveolar preparations. Lanes 2, 4, and 8 are the protein fractions as in lanes 1, 3, and 7 except that the M \emptyset activation with LPS was done in presence of CL.7, 3R.1, and 6R.1 supernatants. Lane 5 was the molecular weight standard.

CHAPTER - 3**FLOW CYTOMETRIC AND ULTRASTRUCTURAL ANALYSIS OF
DIFFERENTIAL TNF- α CYTOTOXICITY UNDER A-MuLV
TRANSFORMATION****ABSTRACT**

Flow cytometric forward angle light scatter (FALS) and 90° light scatter (LI90) single and double parameter statistics were used to distinguish between normal (CL.7) and Abelson-murine leukemia virus (A-MuLV) transformed tumorigenic 3R.1 and 6R.1 clones. The A-MuLV transformed cells were distinctively smaller (as determined by FALS) and have smaller nuclear to cytoplasmic volume ratio (as determined by LI90) than the untransformed CL.7 cells. A rapid flow cytometric assay was developed to monitor TNF- α cytotoxicity on a cell by cell basis on the above clones. The assay was established in the TNF- α sensitive murine L929 fibrosarcoma cell line. Cell numbers per channel, as a function of DNA bound propidium iodide (PI) fluorescence intensity (a measure of cell permeability) were plotted on a single parameter histogram for different TNF- α incubation times. With increasing incubation time of TNF- α there was an enhanced penetration of PI and hence an enhanced PI red fluorescence. The PI fluorescence intensity curve was represented in terms of number of cells, and was taken as a measure of TNF- α cytotoxicity. The percent cytotoxicity indices of normal (CL.7) and two A-MuLV transformed clones as determined by the flow cytometric assay were comparable to those obtained by the tetrazolium dye reduction assay. As TNF- α is known to alter the surface morphology of some cell types (specially the endothelial cells), we investigated whether the differential cytotoxicity of TNF- α under A-MuLV transformation was reflected in the alterations of the surface morphology of the normal and the A-MuLV clones. An electron microscopic investigation was carried out to observe changes in surface characteristics under the differential

cytotoxicity of TNF- α . The scanning electron microscopic (SEM) results seem to suggest that TNF- α sensitive cells (L929 and 6R.1) tend to lose their surface morphology (blebs, microspikes, and lamellipodia) as compared to the TNF- α insensitive CL.7 and A-MuLV cell lines. Thus we concluded that the differential cellular permeability and the selective changes in the cellular morphology of the normal and the A-MuLV transformed clones under TNF- α cytotoxicity are signatures of the differential susceptibility of these clones to TNF- α .

INTRODUCTION

Tumor necrosis factor- α (TNF- α) is a macrophage/monocyte derived pleotropic cytokine known for its wide range of biological activities. TNF- α modulates cellular functions both *in vivo* as well as *in vitro* (Beutler et al., 1989). Effort to elucidate the post receptor binding action of TNF- α revealed that TNF- α mediated cell injury is associated with fragmentation of cellular DNA which was speculated to play a major role in destruction of TNF- α sensitive cancer cells (reviewed, Beutler et al. 1989). Further, TNF- α has been known to induce hemorrhagic necrosis of certain tumors *in vivo* via its vascular effects. Among the primary TNF- α effects on vascular endothelium are: the down regulation of expression of thrombomodulin, release of interleukin-1 (IL-1), enhanced adhesion of neutrophils to vascular endothelium, cellular reorganization of endothelial cells and changes in endothelial cell morphology (Beutler, et al. 1989). The addition of more than 20 units/ml of TNF- α on confluent primary endothelial cell culture causes the cells to become elongated, to rearrange their actin filament, to have an overlapping morphology and lose their stainable fibronectin (Stolpen, et al., 1986). Similarly, the incubation of mouse TNF- α with human endothelial cells have been known to inhibit proliferation of the cells (Sato, et al. 1986).

Investigations of the myriad TNF- α effects have led to the development of many assay systems for the determination of TNF- α cytotoxicity. The most popular system is to measure the cell viability spectrophotometrically by monitoring the reduction of a tetrazolium dye (Larrik, et al. 1989). Potential assay systems for monitoring TNF- α cytotoxicity could be based on the increase of

membrane fluidity and permeability (Anghileri, et al., 1987), DNA fragmentation and lipid peroxidation (Zimmerman, et al. 1989) as well as dimethylsulfoxide (DMSO)-dependent methane production (Yamauchi, et al. 1989). The TNF- α -induced changes in membrane permeability as well as its ability to induce changes in cell surface morphology could be used to study the effects of TNF- α in normal (relatively TNF- α insensitive) as well as transformed (relatively TNF- α sensitive) cells.

Recently we determined that mouse TNF- α exhibited differential toxicity under Abelson-murine leukemia virus (A-MuLV) transformation (Chapter 1). The TNF- α insensitive cell line BALB/c CL.7 when transformed with A-MuLV gave two clones one of which (A-MuLV 3R.1) was TNF- α insensitive and the other (A-MuLV 6R.1) was TNF- α sensitive. The present investigation was aimed at determining whether differential TNF- α cytotoxicity under A-MuLV transformation led to a TNF- α induced differential change in membrane permeability with its associated changes in cell surface morphology. The membrane permeability was monitored fluorometrically by measuring the ability of propidium iodide (PI), a viable cell impermeant, to bind the DNA of a TNF- α treated cell. The fluorometric assay was first established on a known TNF- α sensitive cell line (L929 mouse fibrosarcoma) and was performed using a flow cytometer which has been in use to study a variety of cell functions, ranging from gross cellular functions such as cell division to highly specific metabolic functions of subcellular origin. The present study was aimed at monitoring TNF- α cytotoxicity in cells in culture using flow cytometric determination of PI fluorescence. In particular, an assay system for TNF- α cytotoxicity was established in a TNF- α sensitive murine fibrosarcoma cell line L929. The assay was tested to determine the cytotoxicity of TNF- α to a normal murine fibroblast cell line (BALB/c CL.7) along with two of its Abelson murine leukemia virus transformed cell lines (A-MuLV 3R.1 and A-MuLV 6R.1). The morphological effects of TNF- α on the normal and the transformed cells as well as on L929 cells were performed using SEM at 360X and 1500X.

MATERIALS AND METHODS

Cells: L929, CL.7, 3R.1 and 6R.1 cells were procured from American Type Culture Collection (ATCC) Rockville, MD. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) with 25 MM HEPES, 10 units/ml penicillin and 10 µg/ml streptomycin at pH 7.4.

Cytokine: Murine TNF- α was prepared by immunopurification of, *E.Coli* lipopolysaccharide (LPS) activated, leukemia virus immortalized, murine macrophage cell line RAW 264.7. The purification was done on an anti-TNF- α antibody column (Chapter 1). The specific activity of TNF- α was found to be 6×10^5 units/mg protein by a tetrazolium dye reduction bioassay (Larrik et al 1989).

Flow Cytometry: Propidium iodide (PI) (Calbiochem, San Diego, CA) was used at 50µg/ml in 3.8 mM sodium citrate for monitoring DNA content distribution as described (Krinshan, 1975). This staining method eliminates the cell fixation and RNase treatment.

TNF- α cytotoxicity Assay: L929 cells were plated at 5×10^5 cells/well in 100 µl/ml streptomycin, 25 mM HEPES with 1 µg/ml actinomycin D. Actinomycin D is known to enhance TNF- α cytotoxicity. After attachment to the 96 well flat bottom plate the cells were incubated in 100 µl of (100 U/ml) TNF- α for 0, 1, 4, 13, and 16hr. After the incubation time the cells were scraped using a flattened pipet tip and to them was added 20 µl of PI (0.05 mg/ml) prior to analysis by flow cytometry. PI, being a cell impermeant dye, will be incorporated only into those cells that have lost their semi-permeability due to the TNF- α cytotoxicity. After incorporation, PI will bind to the double stranded DNA and can be quantitated using flow cytofluorometry. The area under the DNA curve, presented in terms of number of cells within the area, will be a measure of cytotoxicity of TNF- α . If the flow cytometer counts a fixed number of cells, (10,000 cells in our investigation), then the area under the curve (minus the control area) would directly represent percent cytotoxicity. This process was repeated for CL.7, 3R.1 and 6R.1 clones. Prior to running the cells on the flow cytometer, the cells were filtered through a 37 µm nylon mesh. For

the single and the double parameter plots the forward angle light scatter was collected on a linear integral mode and the 90° light scatter was collected on a log integral mode. Cell numbers per channel, as a function of fluorescence intensity, were collected on a single parameter histogram with 256 channels. The total number of counts per each test was 1×10^5 cells.

Scanning Electron Microscopy: L929, CL.7, 3R.1 and 6R.1 cells were incubated in 75 mm² flasks with and without 100 units/ml murine TNF- α for 15 h. After the incubation time the cells were scrapped and washed in Hank's balanced salt solution (HBSS). The cell pellets were resuspended in 5% glutaraldehyde, 3% formaldehyde and 2.75% picric acid in 0.1M sodium cacodylate buffer, pH 7.3, overnight. The cells were washed twice in 0.1M sodium cacodylate, pH 7.3 and post fixed in 1% osmium tetroxide in the same buffer for 1 hour. After the osmium tetroxide fixation step, the cells were washed in the buffer twice for 10 minutes each. After washing, the cells were dehydrated in graded alcohol at 15, 30, 50, 70, 95 and 100% for 15 minutes each. The dehydrated tissue culture samples were then critically point dried (LADD Res. Ind.), mounted on scanning electron microscope (SEM) stubs, sputter coated with 200 Å gold and observed at 360X or 1500X in a JEOL JSM35C SEM.

Statistical Analysis : Each experiment had its own control. The statistical analysis was performed by a paired t test comparison. The probability of $p \leq 0.05$ was taken to be significant. Unless otherwise mentioned the results are expressed as mean \pm S.D of six replicate readings.

RESULTS

The forward angle light scatter (FALS) and 90 degree light scatter (LI90) single parameter statistics for CL.7, 3R.1 and 6R.1 were shown in Figure-1. FALS for CL.7 showed a normal distribution of cells. A reduction of FALS mean channel number was observed for 3R.1 and 6R.1 cells showing that these cells were smaller and had distinct alteration in cell shape as well as surface to volume ratios (compared to the CL.7 cells). The 90° light scatter (LI90) for 3R.1 and 6R.1 clones were significantly smaller than that for CL.7. A higher mean channel number for LI90 was known to be associated with a higher ratio of nuclear to cytoplasmic volume (Shapiro,

1988). Therefore CL.7 had a higher nuclear to cytoplasmic volume than the A-MuLV transformed clones. From the above data we observed that A-MuLV transformed clones had reduced mean channel number for FALS as well as LI90. There was a general loss of homogeneity of all population upon transformation with A-MuLV. Study of LI90 for CL.7 and its two A-MuLV transformed clones showed that there was a bimodality of distribution for 3R.1 and 6R.1 clones which was absent in the untransformed clones. The percentage of cells in the second quadrant was 87.4% for CL.7 as compared to 40.5% and 47.4% for 3R.1 and 6R.1 clones respectively. A reduction of cells in the second quadrant was taken to be a major feature observed under A-MuLV transformation. The analysis of population statistics of normal and transformed clones enabled a distinction to be made between the two clones by virtue of FALS and LI90. Thus flow cytometry could help in distinguishing between normal and leukemic cell populations. A two dimensional plot of FALS with LI90 for the normal cell CL.7 as well as the two A-MuLV transformed clones was shown in Figure-2. Quadrant statistical analysis showed that upon transformation with A-MuLV, CL.7 clone shifted towards the quadrant origin. This was indicative of the changes in the cellular morphology associated with transformation.

A rapid flow cytometric assay was developed to monitor TNF- α cytotoxicity. The assay was established in the murine L929 fibrosarcoma cell line as described in the methods section. Cell numbers per channel, as a function of PI fluorescence intensity were plotted on a single parameter histogram for different TNF- α incubation time as shown in Figure-3. The Figure-3 showed that with increasing incubation time of TNF- α there was an enhanced penetration of PI and hence an enhanced PI red fluorescence intensity. The fluorescence intensity curve was represented in terms of number of cells, and was a measure of TNF- α cytotoxicity.

$$\% \text{ Cytotoxicity} = \frac{A_{(TNF^+)} - A_{(TNF^-)}}{10,000} \times 100\%$$

where $A_{(TNF^+)}$ is the area under the PI curve (in terms of number of cells) for cells incubated with TNF- α ; $A_{(TNF^-)}$ is the area under the PI curve for the control group (in terms of number of cells). The difference gave the number of cells affected by TNF- α treatment.

The percent cytotoxicity of TNF- α to L929 cells as a function of time was shown in Figure-4. This curve resembled a classical cytotoxicity curve of TNF- α on the TNF- α sensitive L929 cells. The cytotoxicity of TNF- α to L929 cells at 16h were compared with that for the clones CL.7, 3R.1 and 6R.1 and was shown in Figure-5. The Figure-5 demonstrated that TNF- α susceptibility was more in 6R.1 than in CL.7 or 3R.1. A comparison of differential TNF- α cytotoxicity under A-MuLV transformation was shown using flow cytometry and tetrazolium dye reduction assay as presented in Figure-6. The cytotoxicity assay using flow cytometry yielded similar result as compared with the tetrazolium dye reduction assay. We concluded that the effect of TNF- α in modulating membrane permeability could be used to advantage in monitoring its cytotoxicity using flow cytometry on a cell by cell basis.

The addition of TNF- α to endothelial cell culture caused an alteration in their cellular morphology. The cells became elongated, rearranged their actin filaments, had an overlapping morphology, and lost their their stainable fibronectin (Beutler et al., 1989). To determine the morphological changes associated with TNF- α action in an A-MuLV transformation we investigated the surface morphological effects on these cells using scanning electron microscopy (SEM). A typical SEM micrograph of a fibroblast in culture with its associated blebs, microspikes, and lamellipodia was as shown in Figure 7. The surface morphology of our fibroblast cultures of CL.7, 3R.1 and 6R.1 using SEM were depicted at 1500x in Figure 8(a). Figure 8(b) showed the same cells but incubated with TNF- α for 15hr. In Figure 8(a) we observed that 3R.1 as well as 6R.1 cells had lost contact inhibition. The CL.7 cells were flattened fibroblastic cells with little surface morphology. The retraction fibers, microspikes and lamillipodia were few. The 3R.1 cells were similar to the CL.7 cells apart from the fact that they grew in colonies and had lost contact inhibition. The 3R.1 had a larger number of microspikes on their surface than CL.7. The 6R.1 cells had a large variety of surface morphology including lamellipodia and microspikes and surface blebs. Figure 8(b) showed the CL.7, 3R.1 and 6R.1 cells after treatment with TNF- α (100 units/ml for 15 h). It was observed that CL.7 and 3R.1 cells with characteristic fewer lamellipodia and their cellular morphology were not affected by

TNF- α whereas 6R.1 cells seemed to lose a lot of surface morphology including the blebs, lamellipodia and microspikes upon treatment with TNF- α . The loss of surface morphology in relatively TNF- α sensitive 6R.1 clone was very similar to that for L929 cells as shown in figure-9a (at 360X) and 9b(at 1500X). The L929 cells were characterized by a lot of surface blebs. The surface blebs disappeared with time incubation of TNF- α and the cells in general lost surface morphology; became flattened and were fewer in number in the SEM field of view. The affinity of cells to attach to culture dish was not an artifact in our system as the cells were pelleted first before being fixed and processed for SEM. It was concluded that TNF- α cytotoxicity could in part result in loss of surface morphology and that such a loss could be monitored using SEM.

DISCUSSION

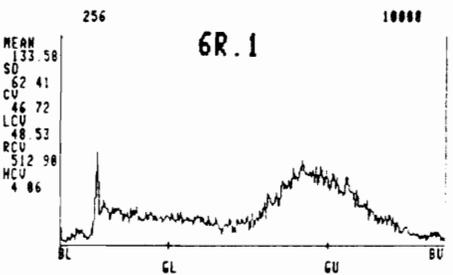
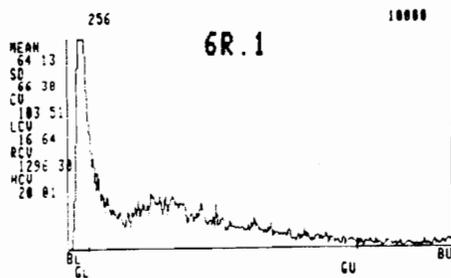
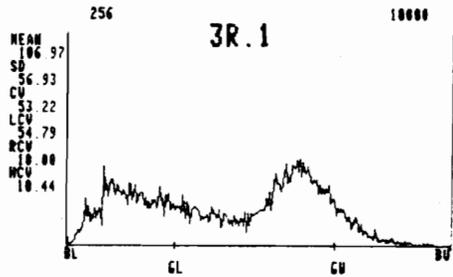
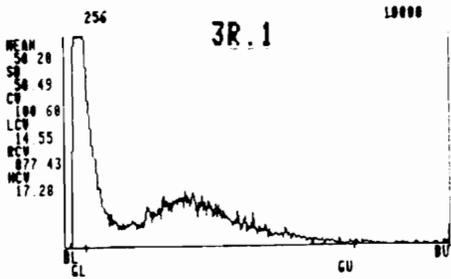
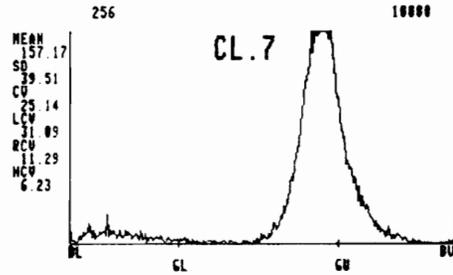
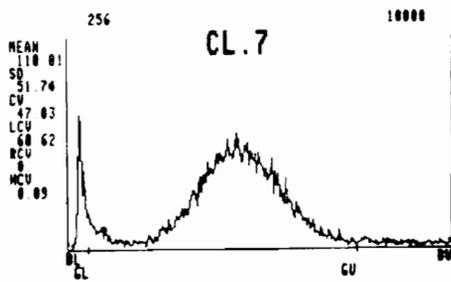
A quick and reliable TNF- α cytotoxicity assay system was established using PI fluorescence in flow cytometer on a cell by cell basis. The assay system was very sensitive and highly reproducible. This assay was used to verify the differential cytotoxicity of normal murine fibroblasts CL.7 and its leukemic clones 3R.1 and 6R.1 (Figure 6). The flow cytometer quadrant statistics was applied to identify the differences between the normal population of cells from those of the leukemic ones. The differences in the quadrant statistics for the normal and leukemic cells could be in part due to the clonal nature of the transformed cells associated with a variation in their surface morphology. As TNF- α is known to alter the surface morphology of some cell types, especially endothelial cells (Beutler et al., 1989), it could contribute in altering the surface morphology of normal and A-MuLV transformed cells. An electron microscopic investigation was carried out to observe changes in surface characteristics under the differential cytotoxicity of TNF- α . The SEM results suggested that TNF- α sensitive cells lost their surface morphology as compared with the relative TNF- α insensitive cell lines. The lack of surface morphology (blebs, microspikes, and lamellipodia) of TNF- α insensitive cells (CL.7, 3R.1) was characteristically different from those of TNF- α sensitive cells (L929 and 6R.1). L929 (mouse

fibrosarcoma) as well as 6R.1 (leukemic mouse embryonic fibroblast), although have differences in their origin, behaved identically in their response to TNF- α . Both the clones were sensitized to TNF- α , and both of them lost their surface morphology when treated with TNF- α . This was evident from the disappearance of the surface blebs and appearance of a flattened morphology when the cells were subjected to TNF- α treatment. The loss of microspikes, known to act as sensory devices (enable the cells to detect features of its surroundings), with TNF- α treatment indicates that the cytotoxic action of TNF- α included its ability to desensitize the cell before actually carrying out its lethal effect. The reasons for the differences in the surface morphology of CL.7, 3R.1, and 6R.1 as determined by FALS and LI90 could be a basis for their differential morphologic alterations under TNF- α action. Thus, it appears that the differential cellular permeability and the selective changes in the cellular morphology of the normal and the A-MuLV transformed clones were signatures of the differential susceptibility of these clones to TNF- α .

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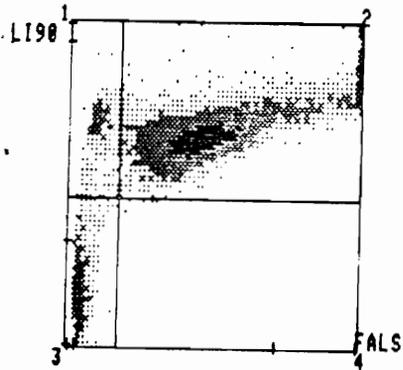
ONE PARAMETER STATISTICS



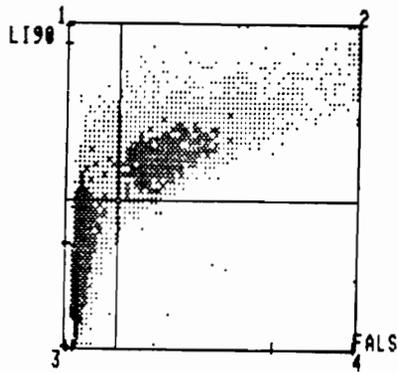
FALS

LI90

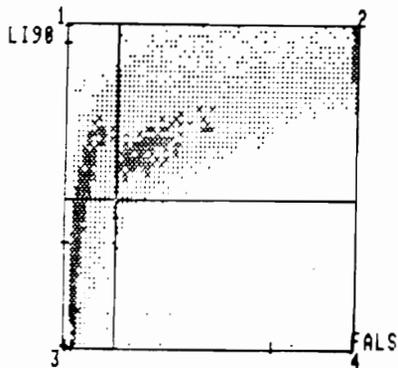
Figure 1 : Single Parameter Flow Cytometric Analysis of Untransformed and A-MuLV Transformed Cells. FALS and LI90 of CL.7, 3R.1, and 6R.1 cells Using Flow Cytometric Analysis as described in the methods section.



| QUAD | PERCENT | PEAK POS | PEAK HT | AREA |
|------|---------|----------|---------|------|
| 1 | 3.98 | 6, 45 | 17 | 398 |
| 2 | 87.35 | 29, 40 | 88 | 8737 |
| 3 | 8.59 | 2, 3 | 54 | 859 |
| 4 | 0.08 | 18, 29 | 2 | 8 |



| QUAD | PERCENT | PEAK POS | PEAK HT | AREA |
|------|---------|----------|---------|------|
| 1 | 7.77 | 3, 30 | 22 | 777 |
| 2 | 48.49 | 19, 35 | 32 | 4858 |
| 3 | 51.02 | 2, 3 | 159 | 5183 |
| 4 | 0.72 | 16, 29 | 8 | 72 |



| QUAD | PERCENT | PEAK POS | PEAK HT | AREA |
|------|---------|----------|---------|------|
| 1 | 16.51 | 3, 30 | 31 | 1651 |
| 2 | 47.44 | 63, 62 | 42 | 4745 |
| 3 | 35.84 | 1, 6 | 193 | 3585 |
| 4 | 0.21 | 13, 29 | 3 | 21 |

Figure 2 : Double Parameter Quadrant Statistical Analysis Using LI90 vs. FALS under A-MuLV transformation.

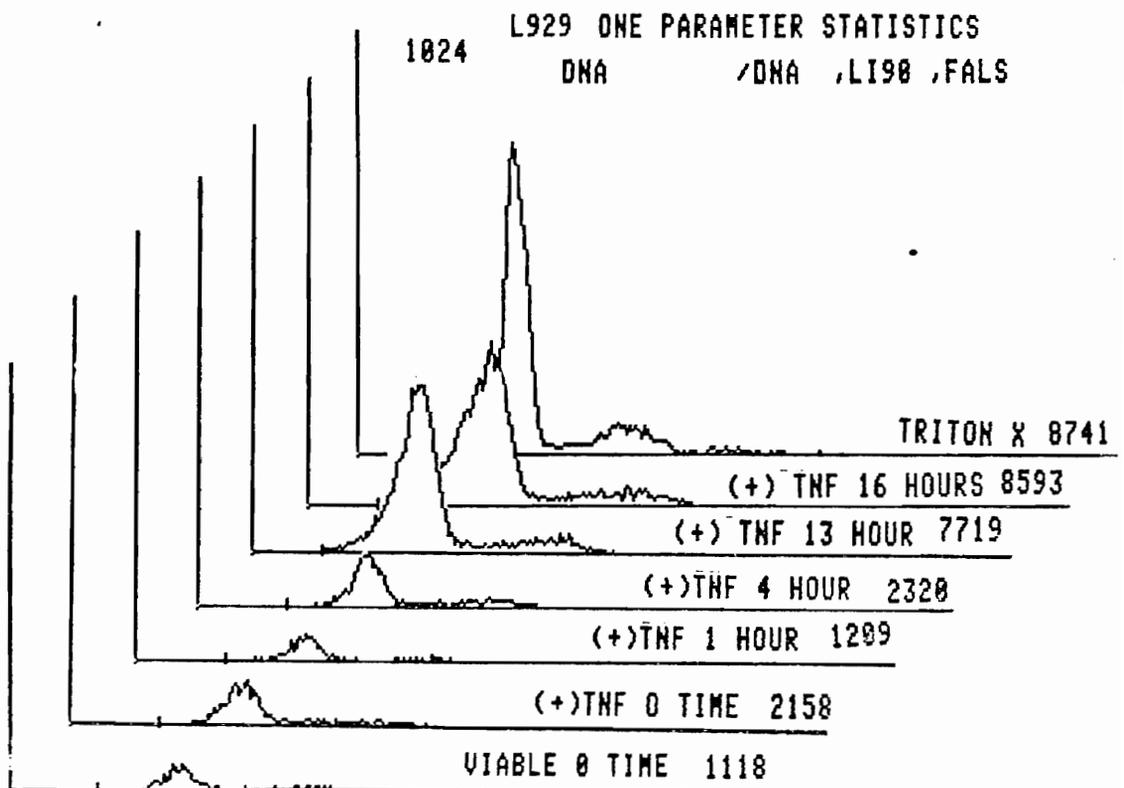


Figure 3 : Single Parameter Histogram of PI Fluorescence as a Measure Of TNF- α Cytotoxicity. PI fluorescence for L929 cells treated with 100 units/ml TNF- α were shown for indicated times. Each run counted 10,000 cells. The number at the right of each figure represented the number of cells under the PI fluorescence curve. This number was a measure of TNF- α cytotoxicity as PI is excluded from the viable cells and only the dead cells allow PI to enter and bind its DNA. PI excitation was at 488 nm and emission was between 513 to 520 nm.

TNF Cytotoxicity On L929 Cells (Flow Cytometric DNA Fluorescence Assay)

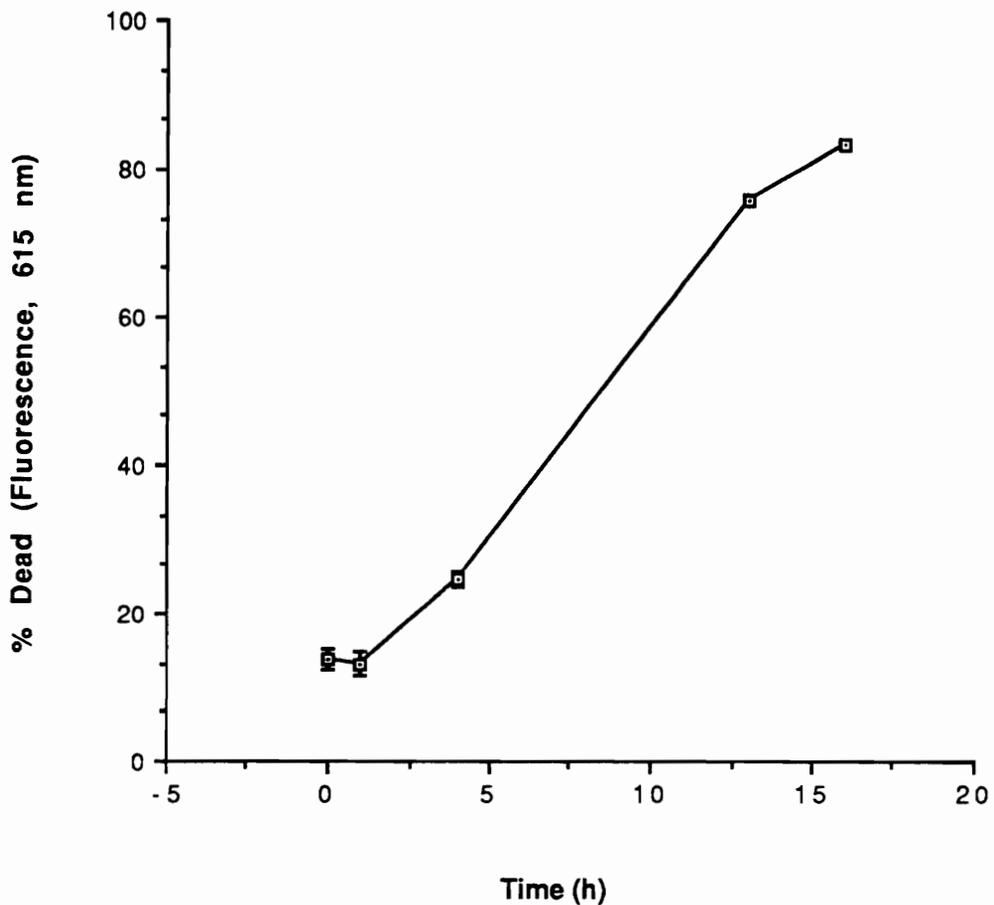


Figure 4 : Flow Cytometric Analysis of TNF- α Cytotoxicity as a Function of TNF- α Incubation Time. 10,000 L929 cells were counted by flow cytometry from a cell population (in 24 well plates) exposed to different time incubation of TNF- α . The number of cells under the PI fluorescence curve was a measure of percent cytotoxicity as described in the methods section.

TNF Cytotoxicity Under A-MuLV Transformation

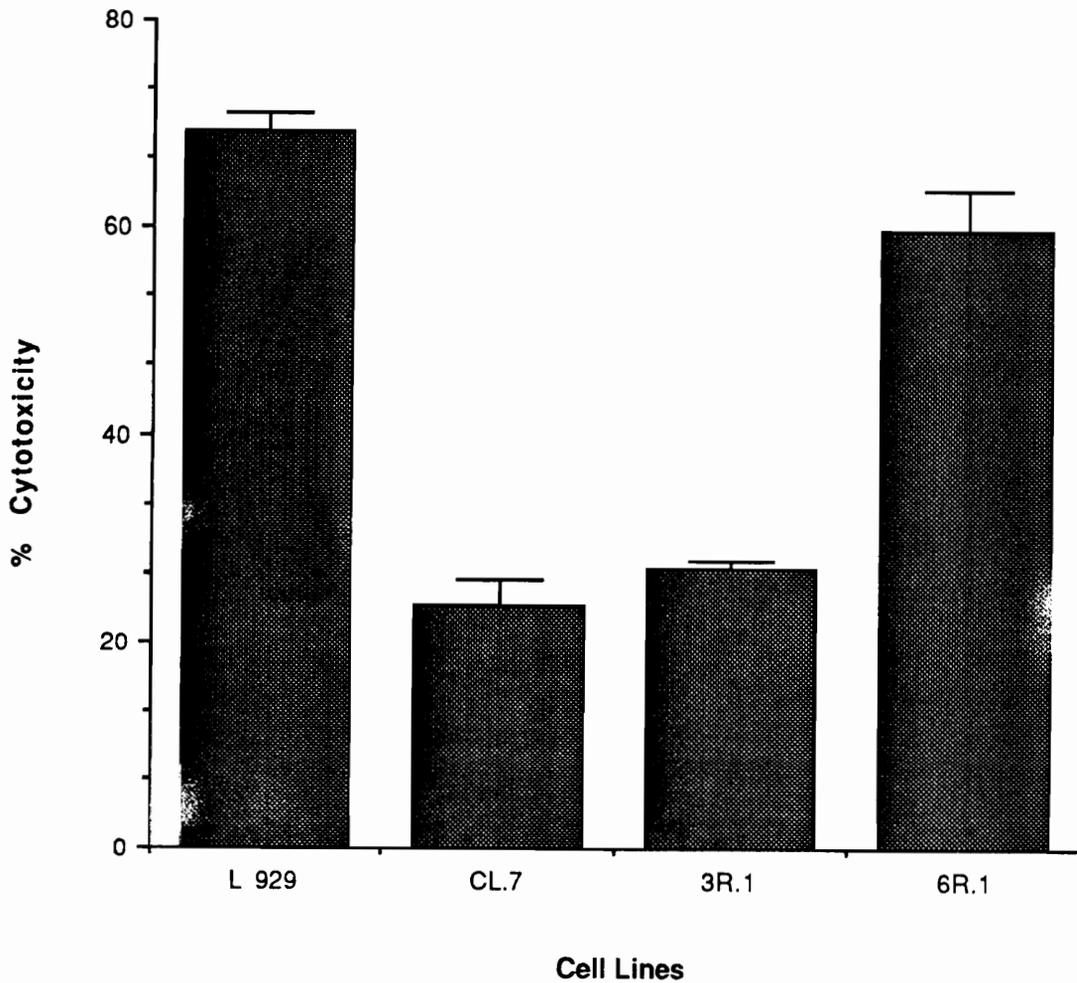


Figure 5 : Flow Cytometric Determination of TNF- α Cytotoxicity. Percent cytotoxicity of L929, CL.7, 3R.1 , and 6R.1 cells were measured Using PI fluorescence Of triplicate 10,000 cells from triplicate 24 well confluent cultures subjected to 100 units/ml of TNF- α for 15 hr. The details of the experiment were as given in the methods section.

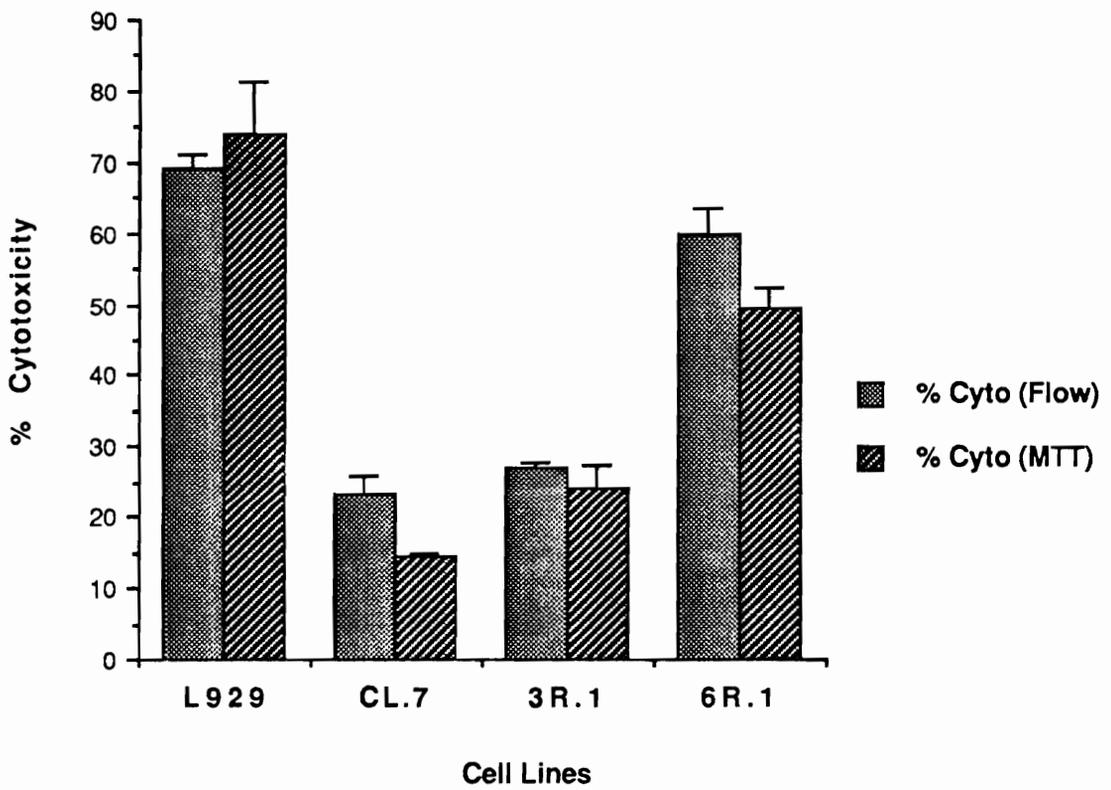


Figure 6: Comparison of TNF- α Cytotoxicity by Flow Cytometry And MTT reduction Assay.

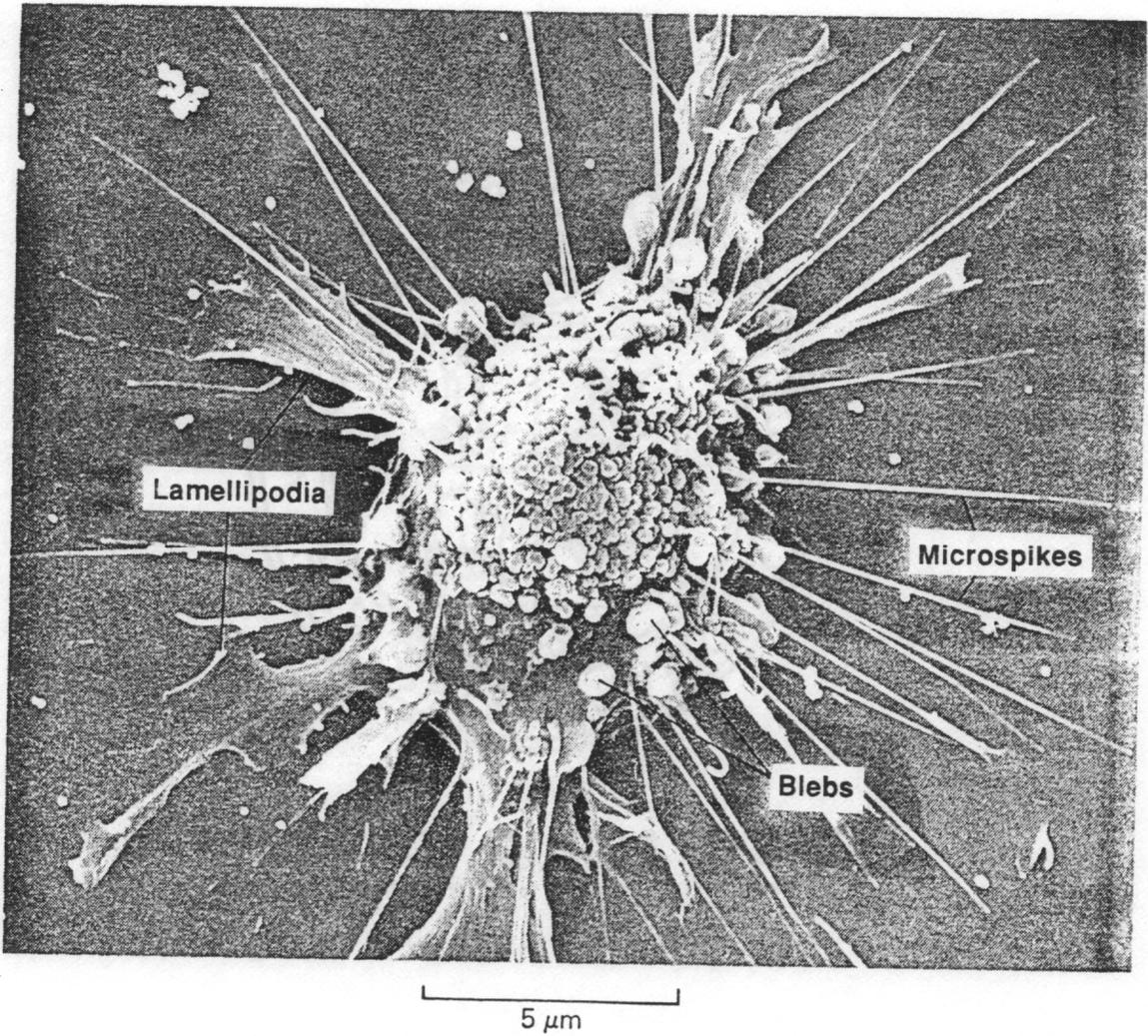
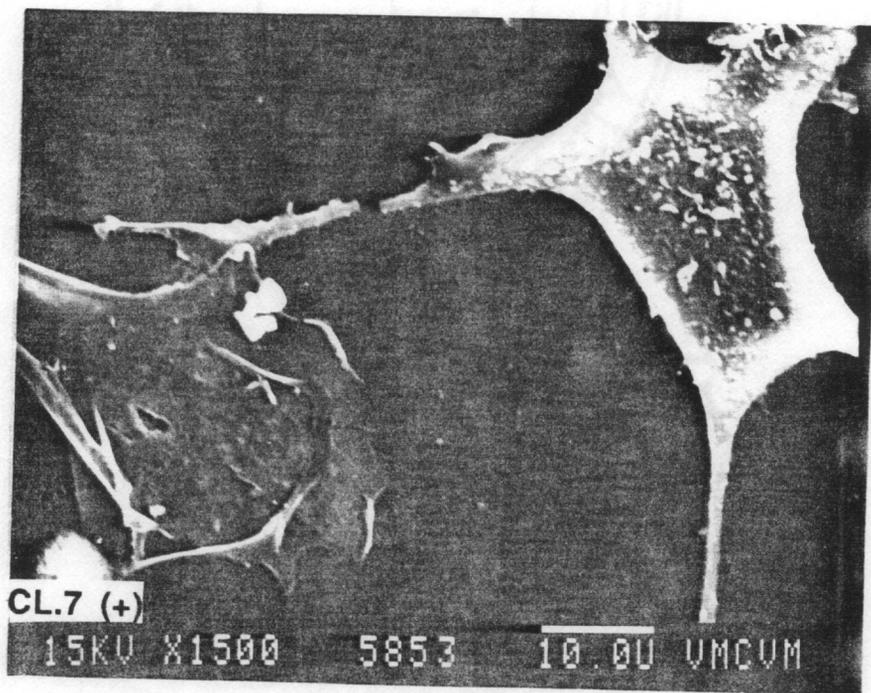
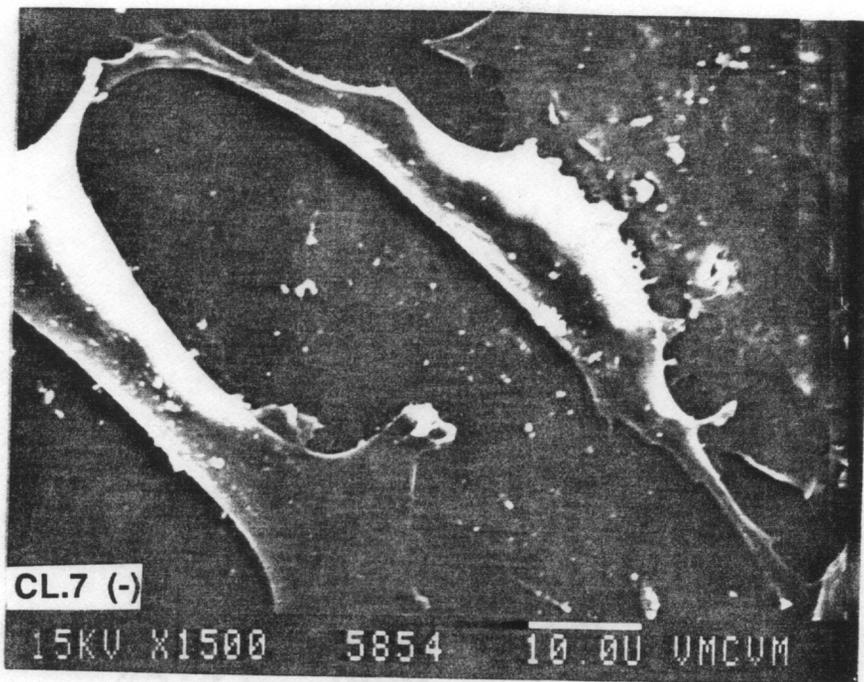
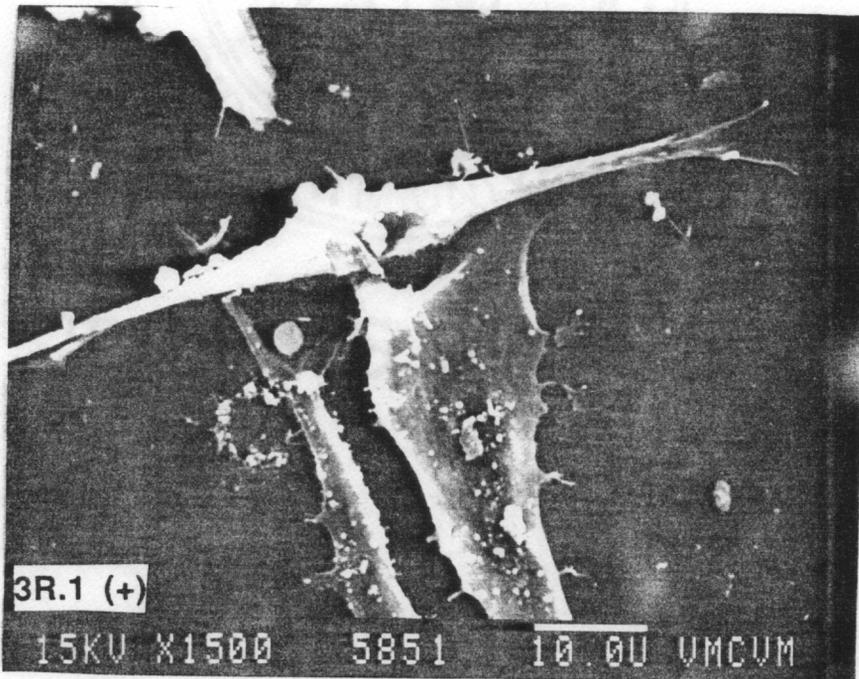
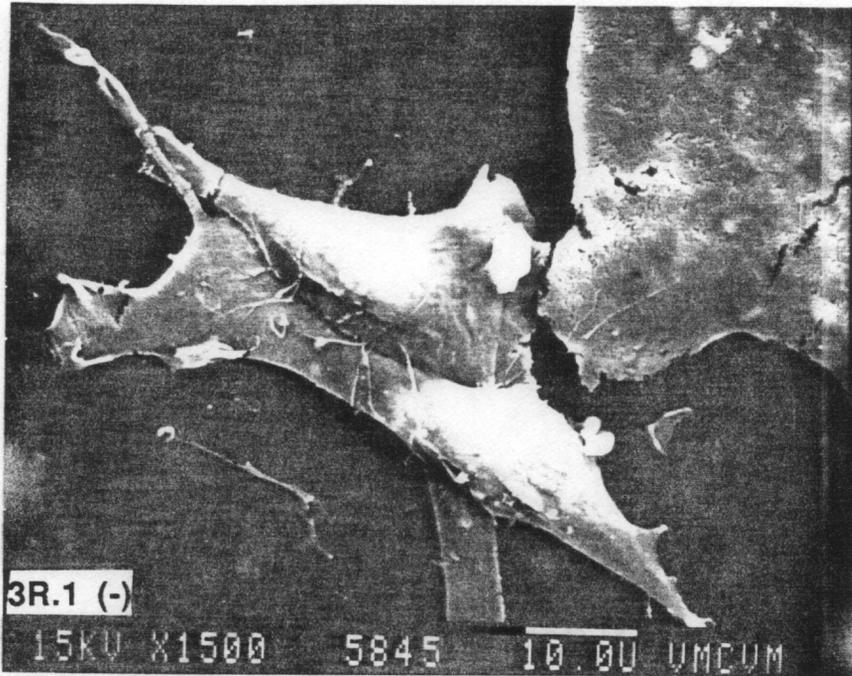


Figure 7 : Scanning Electron Micrograph of a Fibroblast Settling Onto a Culture Dish. Extending from the cell surface are three distinct types of protrusions : blebs, flattened sheets (or lamellipodia), and microspikes.

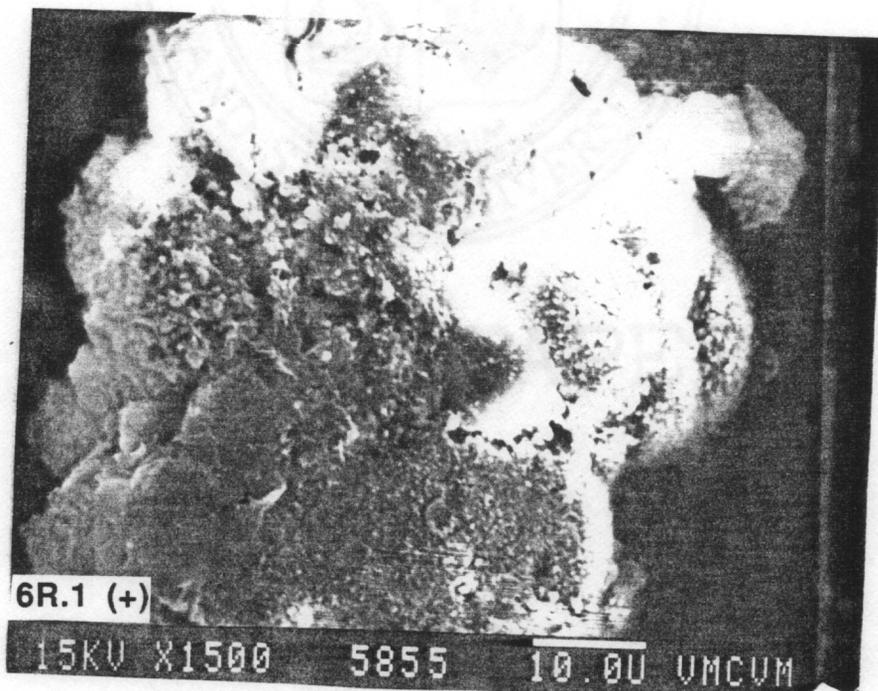
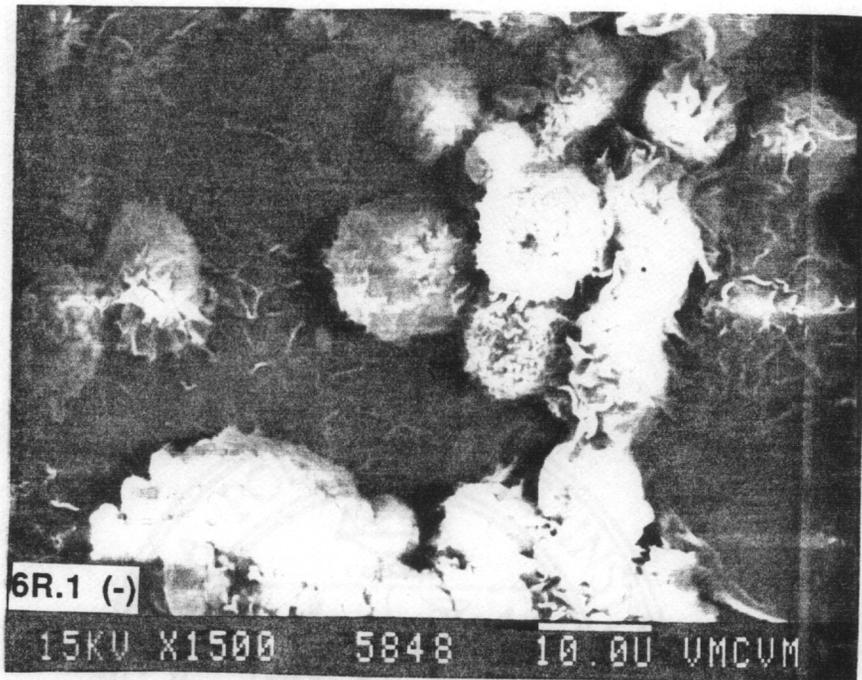
Figure 8: Surface morphology of CL.7, 3R.1, and 6R.1 Cells Using Scanning Electron Microscopy (SEM). The cell samples for SEM were processed as described in the methods section. (a) CL.7 (\pm TNF- α), (b) 3R.1(\pm TNF- α), and (c) 6R.1(\pm TNF- α) cellular morphology with(+)/without (-) TNF- α exposure. 3R.1 and 6R.1 (the A-MuLV transformed cells) clones had lost contact inhibition. The 6R.1 possessed a large number of microspikes and lamellipodia on their cell surface. When subjected to 100 units/ml TNF- α for 15 hr the CL.7 and 3R.1 clones had unchanged surface morphology whereas the 6R.1 clone had lost a lot of surface morphology including blebs, lamellipodia, and microspikes.



(a)

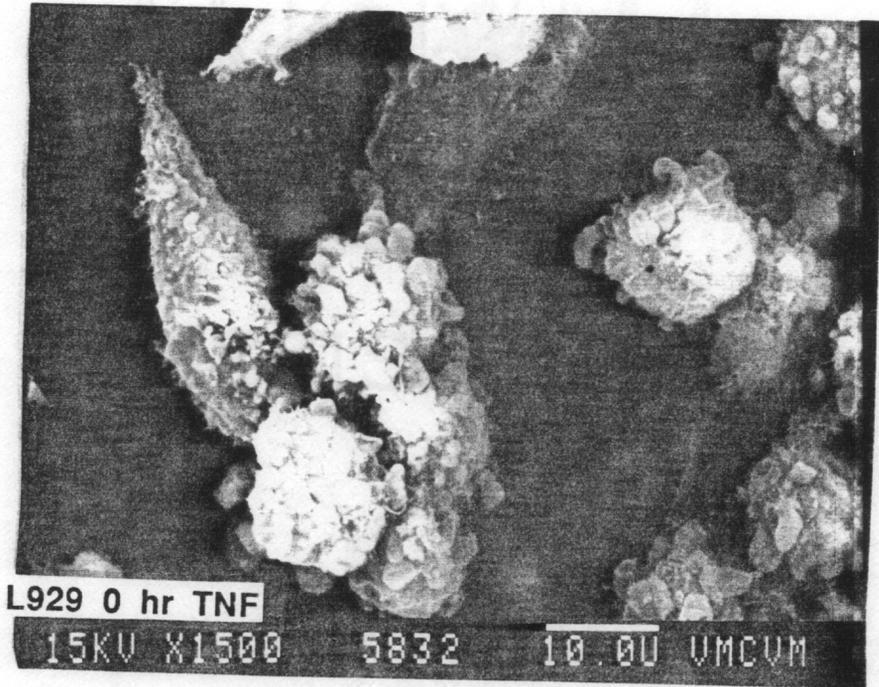
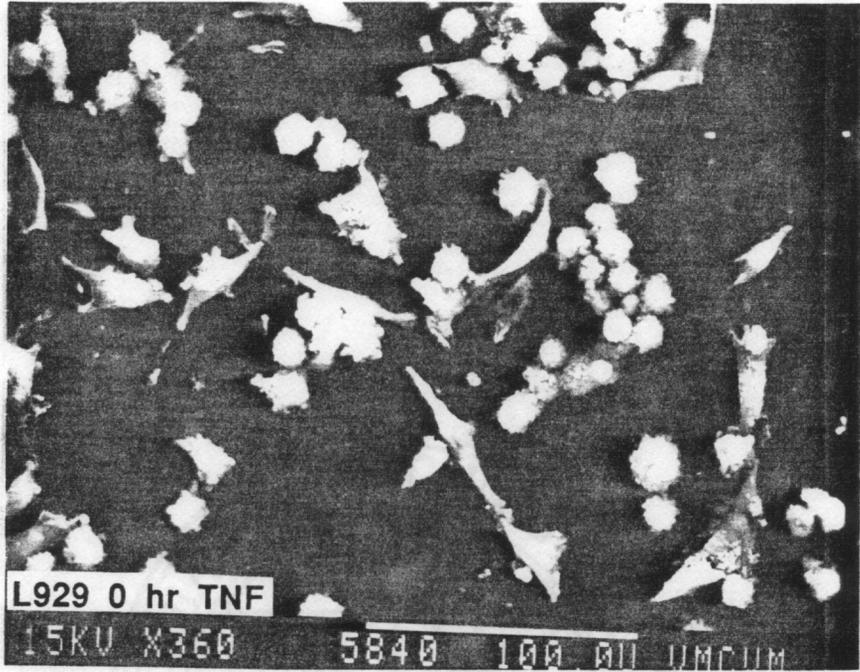


(b)

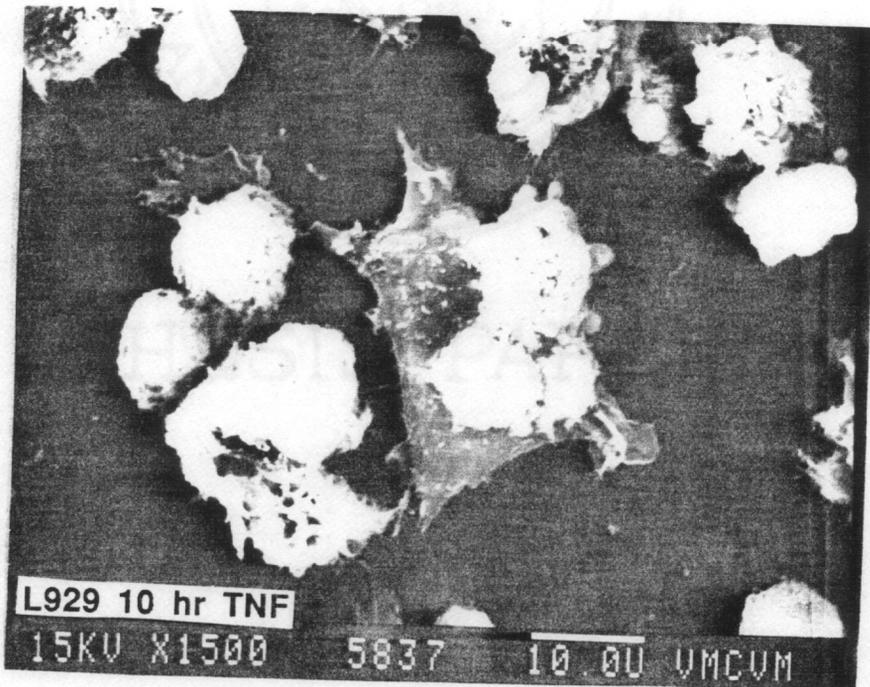
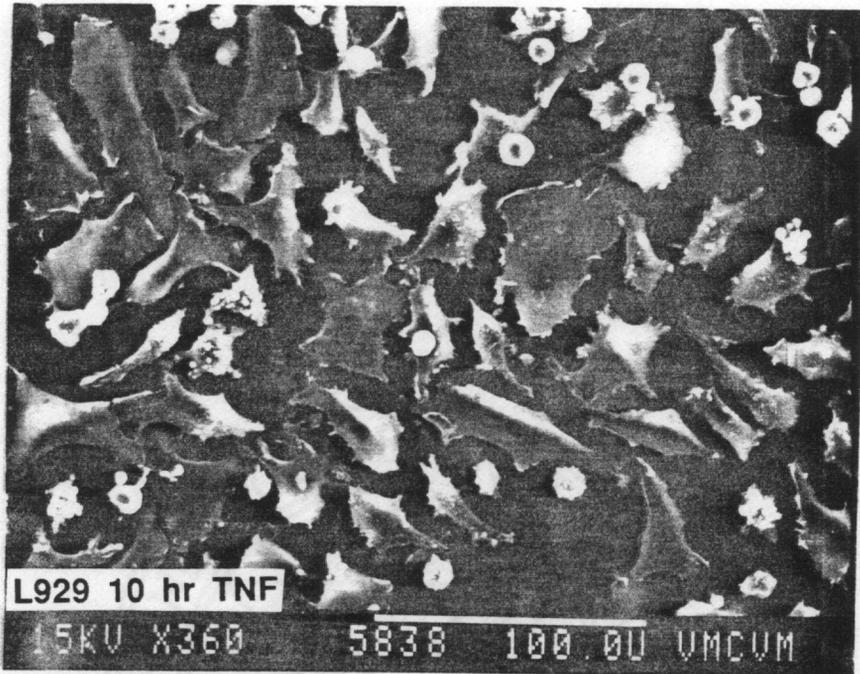


(c)

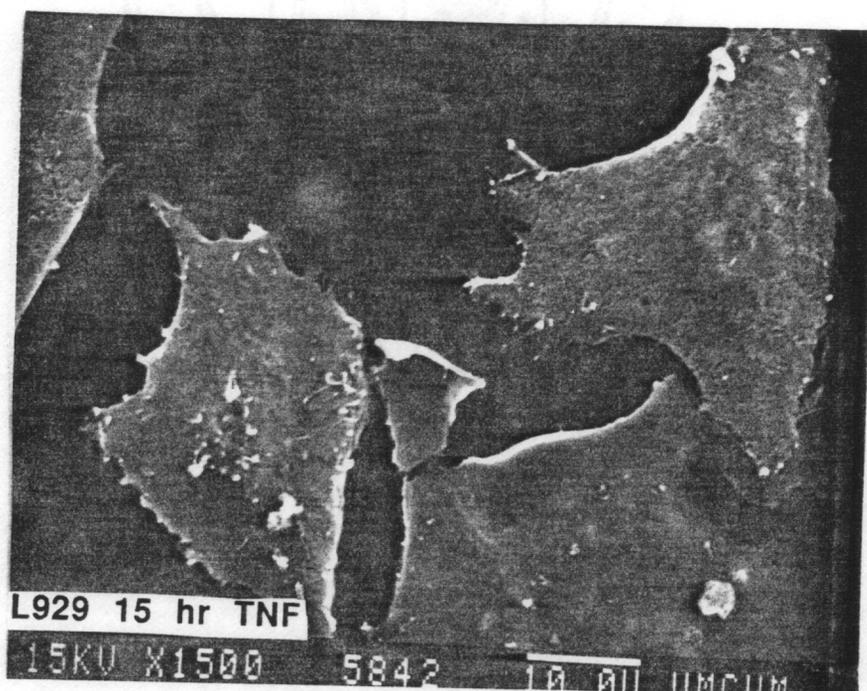
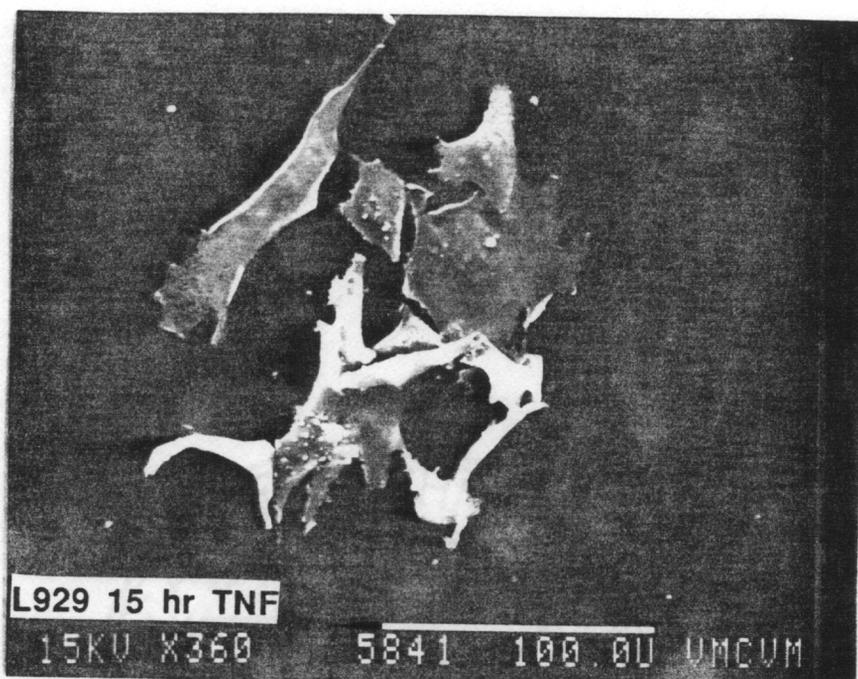
Figure 9 : Surface morphology of L929 Cells At 0, 10, And 15 hr Of TNF-a Incubation At (a) 0 hr TNF at 360 X and 1500 X and (b) 10 hr TNF at 360 X and 1500 X and (c) 15 hr TNF at 360 X and 1500 X magnification using SEM. Surface blebs as well as the number of L929 cells in the field of view decrease with TNF-a incubation time.



(a)



(b)



(c)

CHAPTER - 4**INDUCTION OF TNF-R EXPRESSION UNDER A-MuLV
TRANSFORMATION****ABSTRACT**

The cell surface expression of TNF-R is essential for binding and eliciting of TNF- α cellular response. However, constitutive TNF-R levels have not been correlated with the biological responsiveness of TNF- α . We investigated whether the relatively differential responsiveness of normal and A-MuLV transformed cells to TNF- α (Chapter 1) could be in part due to a differential TNF-R expression on these clones. This objective was based on the hypothesis that the A-MuLV transformation could potentially alter the TNF-R content of the transformed clones. Such alterations could potentially lead to a differential TNF- α response at the level of ligand uptake. The TNF- α binding and internalization assays were performed by using radio-labeled recombinant human (rh) TNF- α at a specific activity of 5.6×10^4 cpm/ng protein. The quantitative binding assay was performed at 4⁰ C for 2 hr. The internalization of TNF- α was monitored at 37⁰ C for 0 to 5 hrs. The total TNF- α binding and internalization kinetics of CL.7 (the TNF- α insensitive clone) and 6R.1 (the TNF- α sensitive clone) were identical. The TNF- α insensitive A-MuLV clone 3R.1 had a higher TNF-R content than the CL.7 or the 6R.1 clone ($p < 0.05$). Interestingly the binding of TNF- α to 3R.1 enhanced its cell surface TNF-R content, which further increased the TNF- α internalization with incubation time at 37⁰ C. The TNF- α -induced cell surface TNF-R modulation had been shown only for macrophages. The current report is the first instance where TNF- α -induced TNF-R modulation was shown for a fibroblastoid cell line.

INTRODUCTION

TNF- α is a polypeptide hormone with a molecular weight of 17000 daltons. Structure of this protein from rabbit, mouse and human sources show a remarkable interspecies conservation of its amino acid residues (Fransen et al., 1985; Pennica et al., 1984). Human TNF- α is expressed as a 233 amino acid prohormone which is then cleared during biological processing to form the 157 amino acid active TNF- α protein (Beutler et al., 1987). The 76 additional amino acids in the prohormone sequence are attached to the N-terminus of the mature protein with an unknown biological activity. The mature protein has been known to exist as dimers, trimers or pentamers in solution (Beutler et al., 1986). It is a relatively hydrophobic protein containing one intrachain disulfide bond (Aggarawal, et al., 1985). Upon heating, it is irreversibly denatured and also it is known to lose biological activity upon freezing and thawing.

Specific receptors for TNF- α has been found in a wide variety of cell types (Baglioni et al., 1985; Aggarawal et al., 1985, Tsujimoto, et al., 1985). Cultured adipocytes 3T3-L1 have been shown to have approximately 3,000 receptors per cell, with a dissociation constant (Kd) of 3×10^{-9} M (Beutler, B. et al., 1985). Murine fibrosarcoma L929 cells, which are sensitive to cytotoxic effects of TNF- α , have 2,200 receptors per cell with a Kd of 6.1×10^{-10} M (Tsujimoto et al., 1985), while TNF- α -resistant fibroblasts FS-4 are known to have 7,500 receptors per cell with a Kd of 3.2×10^{-10} M. This evidence has been used to argue against the correlation of the biological responsiveness of a cell type to TNF- α . A maximal biological response is elicited by occupancy of as few as 5% of the receptors by TNF- α (Tsujimoto, et al., 1986). Studies indicate that TNF- α like other polypeptide cytokines elicit their cellular response after binding to specific cell surface receptors. Investigations on TNF- α receptors (TNF-R) have been made possible by use of highly purified rhTNF- α (Baglioni et al., 1985). Different post receptor binding events include internalization of the TNF- α -receptor complex via the classical receptor-mediated endocytic pathway (Mosselmans, et al., 1988). Inhibition of internalization and degradation of receptor bound TNF- α by agents such as colchicine

and chloroquine have been shown to effectively inhibit TNF- α cytotoxicity (Ruff et al., 1981). Expression of TNF- α membrane receptors is necessary, but not sufficient to determine the responsiveness of a given target cell (Kull et al., 1985; Scheerich et al., 1986; Sugarman et al., 1985). With increasing knowledge of the diversity of cellular responses to TNF- α (Le. et al, 1987) there is an overall lack of understanding of the mechanisms by which diverse TNF- α responses are controlled. Regulating mechanisms could occur at levels of receptor expression, affinity of ligand to receptor, efficiency of ligand-receptor complex internalization and heterogeneity of the receptor, in contributing to the differential response pattern of distinctive cell types. It has been known with other cytokines that magnitude of a particular biological response is proportional to the quantity of ligand receptor interactions thereby making the number of expressed membrane receptors a critical criterion in determination of cellular sensitivity to the cytokine in question. For example, gamma-interferon response on tumor cells depend on levels of its receptor levels (Berkovic, et al., 1986). In the case of TNF-R the assumption of constitutive expression of the receptor in cancer cells (Sugarman, et al., 1985) have been modified to include the myriad positive and negative regulatory mechanisms controlling the membrane TNF-R levels. For example, TNF-R have been known to be reversibly induced in T cells and expressed in a stimulus dependent manner (Scheurich et al., 1987). Similarly membrane expression of TNF-R in activated T cells can be rapidly modulated by activators of protein kinase-C resulting in loss of TNF- α binding capacity (Scheurich et al., 1986). Phorbol myristate acetate (PMA) and oleylacetyl glycerol (OAG) have both been known to down regulate TNF- α binding capacity of both normal and malignant cells (Ungulab et al., 1987). Differences in the constitutive expression of TNF-R have been reported for cells undergoing differentiation. For example, TNF-R found on immature mouse myeloid leukemic cells were enhanced 2 fold upon differentiation into macrophages and 5 fold when these were treated with γ -interferon (Michishita et al., 1990).

In this study we investigated whether A-MuLV transformation leads to an induction of TNF-R. In particular we determined the TNF-R levels in normal BALB/c CL.7 mouse embryonic fibroblast and

two of its A-MuLV transformed clones, namely BALB/c A-MuLV 3R.1 and BALB/c A-MuLV 6R.1. The underlying motivation to the present study stemmed from our previous finding that under A-MuLV transformation there is a differential susceptibility to TNF- α . We investigated the causative factor involved in the relative differential susceptibility of TNF- α in untransformed and A-MuLV transformed clones from the point of view of constitutive TNF-R levels, TNF- α binding, and TNF- α -TNF-R complex internalization.

MATERIALS AND METHODS

Cells: Mouse fibrosarcoma L929, normal mouse fibroblasts, BALB/c CL.7, and two A-MuLV transformed mouse fibroblast cell lines, BALB/c A-MuLV 3R.1 and BALB/c A-MuLV 6R.1, were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 10 % fetal bovine serum (FBS), 10 units/ml penicillin and 10 μ g/ml streptomycin, 25 mM HEPES, pH 7.4 at 5% CO₂ in a humidified 37°C incubator. The A-MuLV immortalized mouse monocyte-macrophage cell line RAW 264.7 was grown in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% FBS for the production of mouse TNF- α . All the cell lines were procured from the American Type Culture Collection (ATCC), Rockville, Maryland.

TNF- α Cytotoxicity Assay: L929 cells were used as target cells to quantitate the affinity purified murine TNF- α . The L929 cytotoxicity assay was performed as described (Larrik, et al. 1989). Briefly, serial dilutions of TNF- α were performed using DMEM, 10% FBS in 96-well microtiter plates (Corning, NY) in six replicates. Starting with a 1:1000 dilution stock, we plated at a further dilution of 1:4, 1:16, 1:64 and 1:128 dilution in a volume of 50 μ l/well. L929 target cells, washed and resuspended in DMEM 10% FBS and 1 μ g/ml actinomycin D at a density of 10⁶ targets/ml, were plated at a concentration of 50 μ l (5 x 10⁴) cells per well. After 18 hr of incubation at 37°C, and 5% CO₂, we added 20 μ l of a 2.5 mg/ml solution of a freshly prepared (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), obtained from Sigma Chemical Co., MO in 0.9% saline. After incubation with MTT for 4 hr at 37°C, 100 μ l were removed from each well without disturbing the

crystals. The crystals were solubilized with 100 μ l dimethyl sulphoxide (DMSO) and read at 550 nm using a microplate reader (Molecular Devices). The percent cytotoxicity was calculated using the formula

$$\% \text{ cytotoxicity} = \frac{A_{\text{media}} - A_{\text{sample}}}{A_{\text{media}} - A_{\text{TX-100}}} \times 100$$

Where A_{media} , A_{sample} , and $A_{\text{TX-100}}$ were the absorbance readings of cells incubated with media, TNF- α or triton X-100. One unit of TNF- α was defined to be that 1/dilution which gave 50% killing. 1/dilution was plotted with % cytotoxicity to determine the 50% killing point.

Radio-iodination of TNF- α :

Murine TNF- α immunopurified from LPS activated RAW 264.7 supernatant (Chapter 1) was used for all cytotoxicity assays and rhTNF- α was used for receptor binding assays. To quantitate TNF- α binding sites, rhTNF- α was radio-iodinated using Na¹²⁵I. 15 μ g of rhTNF- α in PBS (generous gift from Genentech, CA) was used for labeling with Na¹²⁵I by the chloramine T method (McConahey et al., 1980). Briefly, 5 μ g of rh TNF- α in 10 μ l volume was mixed with 5 μ l of 0.5 μ g/ml chloramine T (Sigma Chemical Co., MO) in 0.05 M potassium phosphate buffer (KPi), pH 7.5 and 250 μ Ci of Na¹²⁵I (New England Nuclear, MA) for 15 minutes. The reaction was stopped by using 20 μ l of (0.5 μ g/ml) sodium metabisulphite (MCB). 35 μ l reaction mixes from three reaction vials were made up to 500 μ l in 0.05 M KPi, pH 7.5. After another addition of 2 ml of the above buffer and 1 ml of 1% BSA in the same buffer a total of 3.5 ml was loaded on to an anion exchange column, made from analytical grade anion exchange resin 1-X8, 50-100 mesh chloride form (Bio-Rad), washed successively with 2 ml of each of 0.05 M KPi (pH 7.5) 0.5 M KPi (pH 7.5) 0.05 M KPi with 1% BSA and 0.05 M KPi. The last wash was done three times. The eluent was collected in a tube containing 1 ml of 1% BSA in 0.05 M KPi, pH 7.5. The specific count obtained was 5.6 X 10⁴ cpm/ng TNF- α .

Estimation of Surface TNF-R and Internalization of ¹²⁵I-TNF- α :

Surface binding and internalization of TNF- α were measured as described (Tsujimoto et al., 1985). Briefly cells were plated (10^5 cells/ml, 1 ml/well) in 24 well plates, in six replicates, in DMEM with 10% FCS, 25 mM HEPES, pH 7.4, with 10 U/ml penicillin and 10 μ g/ml streptomycin for 48 hr. Cells were transferred to 4°C for 1 hr prior to labeling with ¹²⁵I-rhTNF- α at a concentration of 1 nM (17 ng/ml). The radioactive TNF- α was directly added to the culture medium and incubated for 2 hr at 4°C. At this point triplicate wells were washed 4 times with Hank's balanced salt solution (HBSS) (500 μ l/wash) at 4°C, and solubilized with 1 ml of 0.1 N NaOH with 0.1% Triton X 100. Radioactivity was counted on a Beckman 5500 gamma-counter to measure the amount of TNF- α internalized. A second triplicate of wells were further incubated, now at 37°C, for different times (0 to 300 minutes) after which the cells were placed back on ice and washed 4 times with ice cold HBSS, 500 μ l/wash, to remove excess unbound ¹²⁵I-TNF- α . To make a quantitative distinction between cell surface bound and internalized TNF- α cells were incubated with ice cold 50 mM glycine/HCl, 100 mM NaCl, pH 3 for 5 minutes at 4°C. This incubation step is aimed at stripping cells off any surface bound TNF- α . The internalized TNF- α was still retained inside the cells. After incubation, the media was removed from the cells. Cells were then lysed with 1 ml of 0.1 N NaOH with 0.1% Triton X 100 after which they were read in the Beckman 5500 gamma counter. Non-specific binding was determined by incubating ¹²⁵I-TNF- α in presence of 200 fold excess TNF- α in triplicates.

Statistical Analysis : Each experiment had its appropriate control. The statistical analysis was performed by a paired t test comparison. The probability of $p \leq 0.05$ was taken to be significant. Unless otherwise mentioned the results were expressed as mean \pm S.D of six replicate readings.

RESULTS

The quantitative estimate of binding and internalization of TNF- α was performed as described in the materials and methods section. Cells in six replicates were transferred to 4°C for 1 hr prior to labeling with ¹²⁵I-rhTNF- α (17 ng/ml). Radioactivity was counted on a Beckman 5500 gamma-counter.

The total TNF- α binding and internalization kinetics at 37°C for L929 cells was as depicted in Figure 1. The Figure 1 showed that the binding and internalization kinetics go through a point of inflection at around 1 hr. The early binding and internalization rates were significantly higher than that after 1 hr. The binding and internalization of the ¹²⁵I-rhTNF- α for CL.7, 3R.1 and 6R.1 cell lines were as depicted in Figures 2, 3, and 4, respectively. In all the Figures the internalization curves were significantly depressed than the total TNF- α binding curves suggesting that the total TNF-R present were higher than the amount of receptors internalized. It was shown that CL.7 and 6R.1 clones had very similar TNF- α binding and internalization kinetics. 3R.1 had a high level of TNF- α binding as well as internalization (Figure 3). As CL.7 and 6R.1 had similar TNF- α binding and internalization kinetics it was concluded that the TNF-R expression need not be critical in explaining the differential cytotoxicity of TNF- α under A-MuLV transformation.

The internalization kinetics of TNF- α -TNF-R complex presented as cpm/ μ g protein were traced for all the four cell lines as shown in Figure 5. The internalization of TNF- α , was determined by incubating ¹²⁵I-rhTNF- α at 4°C for 2 h followed by stringent washes and the uptake of ¹²⁵I-rhTNF- α was monitored, and compared for L929, CL.7, 3R.1, and 6R.1 following a further incubation at 37°C. The Figure 5 showed that the 3R.1 clone had a higher level of TNF- α internalized than the other clones studied ($p < 0.05$). The TNF- α binding kinetics was distinctly different for 3R.1 clone than for CL.7 or 6R.1 cell lines. The CL.7 and 6R.1 cell lines showed a saturable binding of TNF- α . From the binding and internalization kinetics of

TNF- α for 3R.1 cells it was evident that binding of TNF- α to its receptor induced a higher level of TNF-R on the cell surface leading to still higher incorporation of ^{125}I -rhTNF- α with time. The induction process seems to start around 2 hr (Figure 3) by which time the binding was saturable. Post saturation binding at around 2 hr, there is a further increase in ^{125}I -rhTNF- α incorporation possibly due to TNF-R induction by TNF- α (Figure 5). The kinetics of ^{125}I -rhTNF- α binding and internalization for 3R.1 are depicted in Figure 3. These data could possibly mean that TNF-R levels not only get induced to a higher level, but that a greater internalization rate occurred in the A-MuLV transformed 3R.1 clone. Residual TNF-R levels were calculated by subtracting total TNF-R from the level of TNF-R internalized. The residual expression of TNF-R on cell surface post TNF- α internalization, shown in figure 6, furthers the evidence that there was an induction of TNF-R with time incubation of TNF- α for the 3R.1 clone. The higher expression of TNF-R on 3R.1 than on the 6R.1 clone could possibly due to the differences in the incorporation of the A-MuLV genetic material in the cellular genome.

DISCUSSION

A TNF- α cytotoxicity assay on L929, CL.7, 3R.1 and 6R.1 cell lines showed a differential response to murine TNF- α (Chapter 1). TNF- α susceptibility was comparatively more for 6R.1 than for 3R.1 and CL.7 (Figure 6 of Chapter 1). The nature of differential cytotoxicity to TNF- α under A-MuLV transformation was responsive both to TNF- α concentration (Figure 7, Chapter 1) as well as TNF- α incubation time (Figure 8, Chapter 1). As susceptibility of TNF- α at the cellular level is mediated by ligand-receptor binding, we investigated the binding and internalization of ^{125}I -labeled rhTNF- α in these cell lines.

Murine fibrosarcoma L929 cells, which were sensitive to cytotoxic effects of TNF- α , have 2,200 receptors per cell (Tsujimoto et al., 1985), and the TNF- α -resistant FS-4 fibroblasts are known to have 7,500 receptors per cell (Tsujimoto et al., 1985). This evidence is against the correlation of the biological responsiveness of a cell type to TNF- α . A maximal biological response is elicited by occupancy of as few as 5% of the receptors

by TNF- α (Tsujiimoto, et al., 1986). The expression of TNF-R on the cell surface facilitated TNF- α binding. The binding of TNF- α to its receptor was necessary for eliciting of TNF- α response. The levels of TNF-R found in the cell type as discussed above have not been correlated with the type of TNF- α response. The cytotoxicity indices of the normal control (CL.7) and the A-MuLV transformed clones (3R.1 and 6R.1) can not be explained on the basis of their TNF-R content. The CL.7 clone which was TNF- α insensitive possessed receptor numbers comparable to that of the TNF- α sensitive 6R.1 clone. Further, the 3R.1 clone which is also TNF- α insensitive has a higher TNF-R ($p < 0.05$) than CL.7 or 6R.1. This is in agreement with the evidence against the correlation of the biological responsiveness of a cell type to TNF- α and its TNF-R expression. Interestingly the binding of TNF- α to 3R.1 enhanced its cell surface TNF-R content, which further increased the TNF- α uptake with time at 37 $^{\circ}$ C. TNF-R levels in cells treated with TNF- α reaches a lower steady state (for CL.7, and 6R.1), indicative of a dynamic equilibrium between internalization and re-expression of TNF-R on cell surface. TNF-R levels in 3R.1 cells treated with TNF- α reached a high level, indicative of an interactive internalization and re-expression of 3R.1 TNF-R on cell surface. The TNF- α induced cell surface TNF-R modulation has been known to be operative for macrophages. This however is the first instance where TNF- α induced TNF-R modulation has been demonstrated for a fibroblast cell line.

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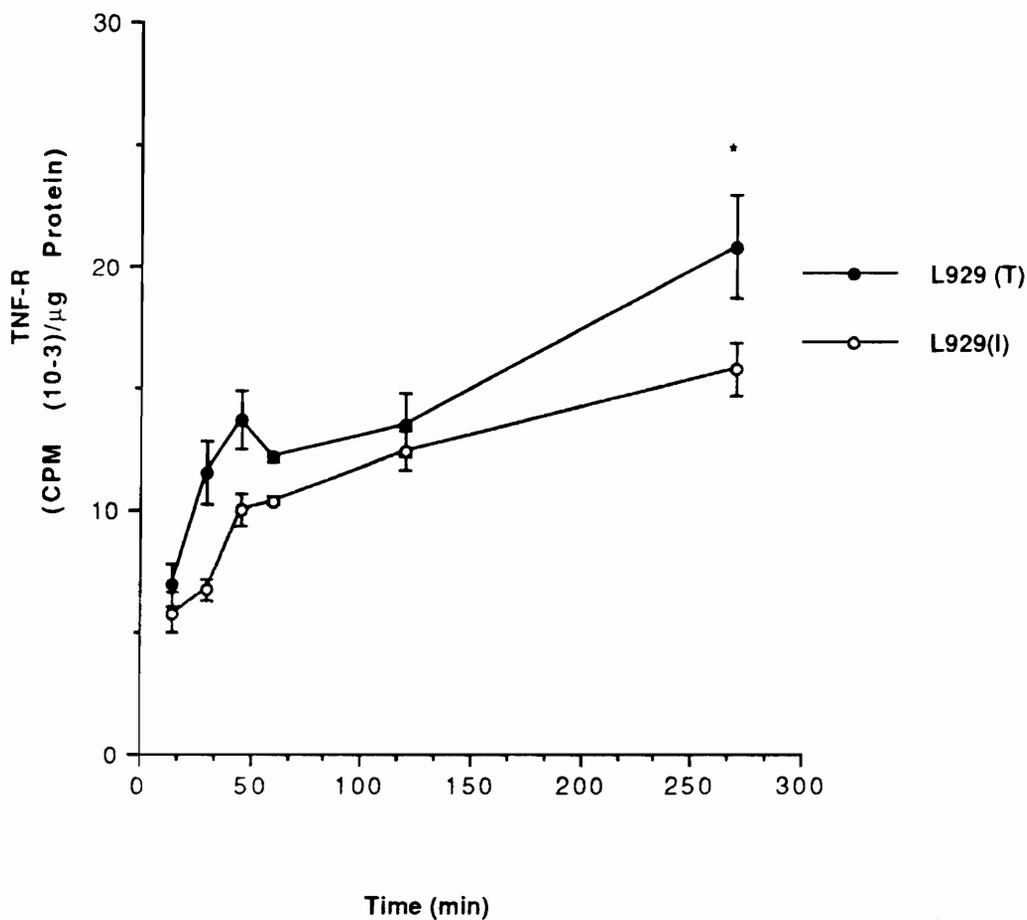


Figure 1: TNF- α Binding and Internalization in L929 Cells. Total binding and internalization experiments were carried out as described in the methods section. 17 ng/ml of 125 I rh-TNF- α was used per 10^5 cells /well to determine total binding (T) and amount internalized (I) in terms of cpm/ μ g protein. The total TNF- α binding was significantly higher than the TNF- α internalized throughout the incubation time. (* = $p < 0.05$). 10^4 cpm of 125 I-TNF- α = 10.5 f moles.

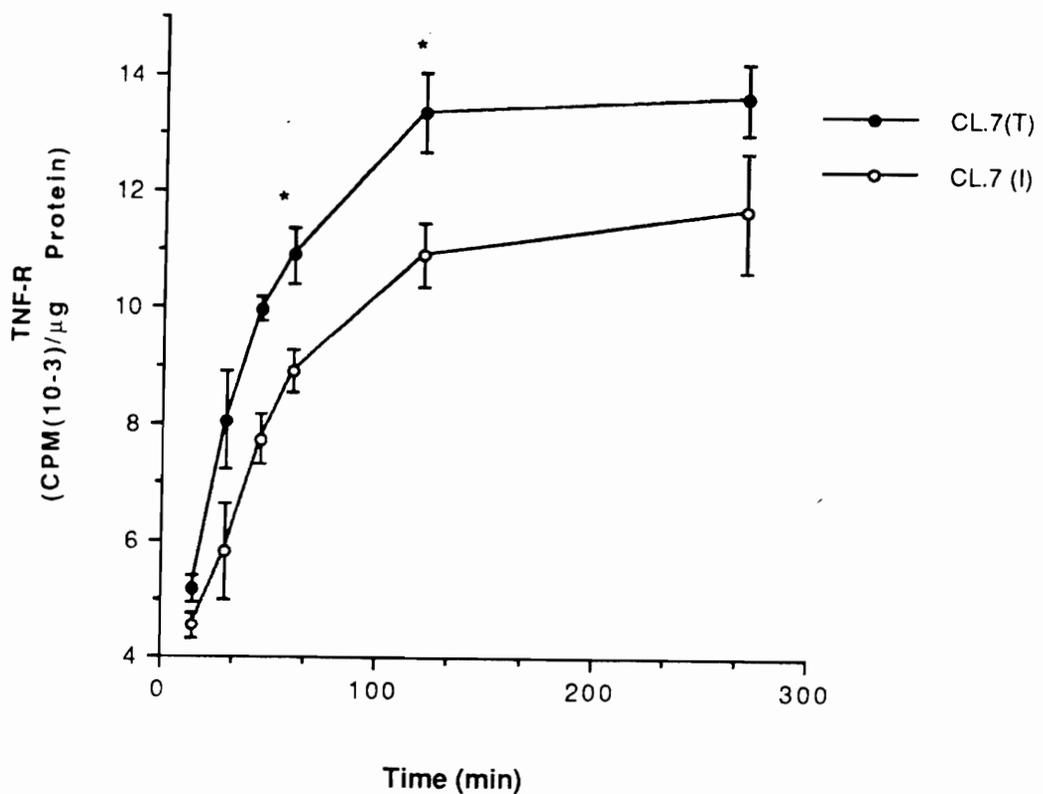


Figure 2: TNF- α Binding and Internalization in CL.7 Cells. Binding and internalization experiments were carried out as described in the methods section. 17 ng/ml of 125 I rh-TNF- α was used per 10^5 cells/well to determine total binding (T) and amount internalized (I) in terms of cpm/ μ g protein. (* = $p < 0.05$). 10^4 cpm of 125 I-TNF- α = 10.5 f moles.

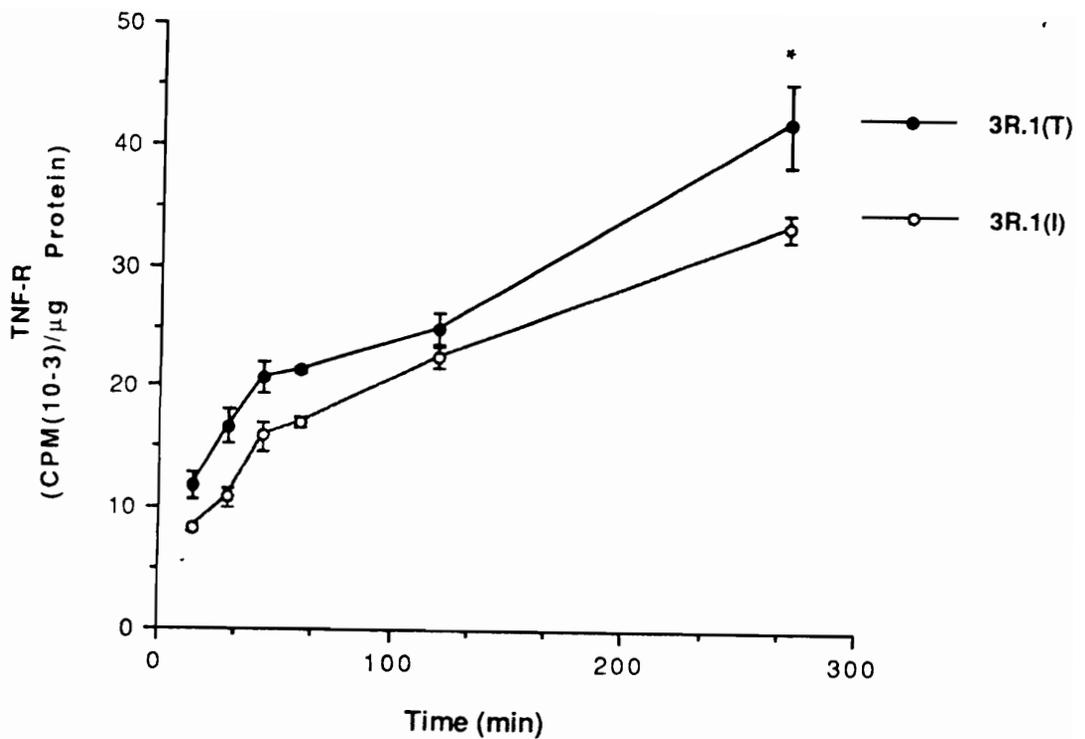


Figure 3: TNF- α Binding and Internalization in 3R.1 Cells. Binding and internalization experiments were carried out as described in the methods section. 17 ng/ml of 125 I rh-TNF- α was used per 10^5 cells/well to determine total binding (T) and amount internalized (I) in terms of cpm/ μ g protein. (* = $p < 0.05$). 10^4 cpm of 125 I-TNF- α = 10.5 f moles.

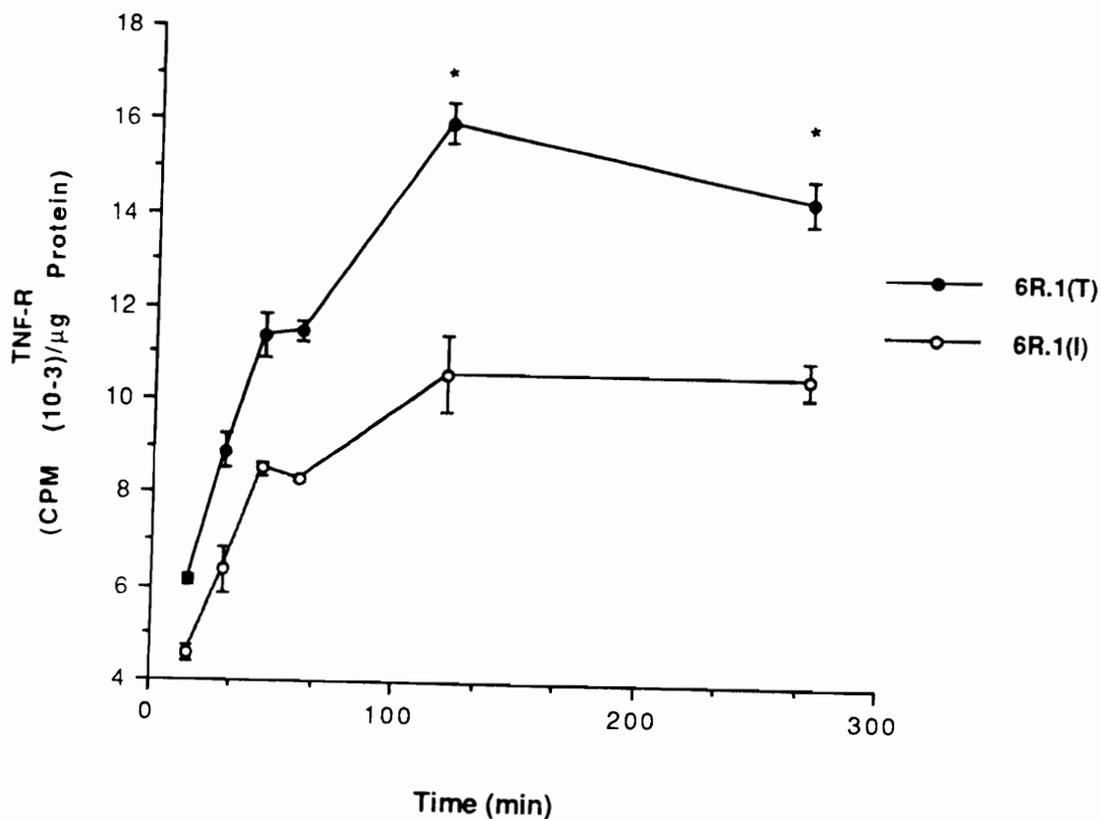


Figure 4: TNF- α Binding and Internalization in 6R.1 Cells. Binding and internalization experiments were carried out as described in the methods section. 17 ng/ml of 125 I rh-TNF- α was used per 10^5 cells/well to determine total binding (T) and amount internalized (I) in terms of cpm/ μ g protein. (* = $p < 0.05$). 10^4 cpm of 125 I-TNF- α = 10.5 f moles.

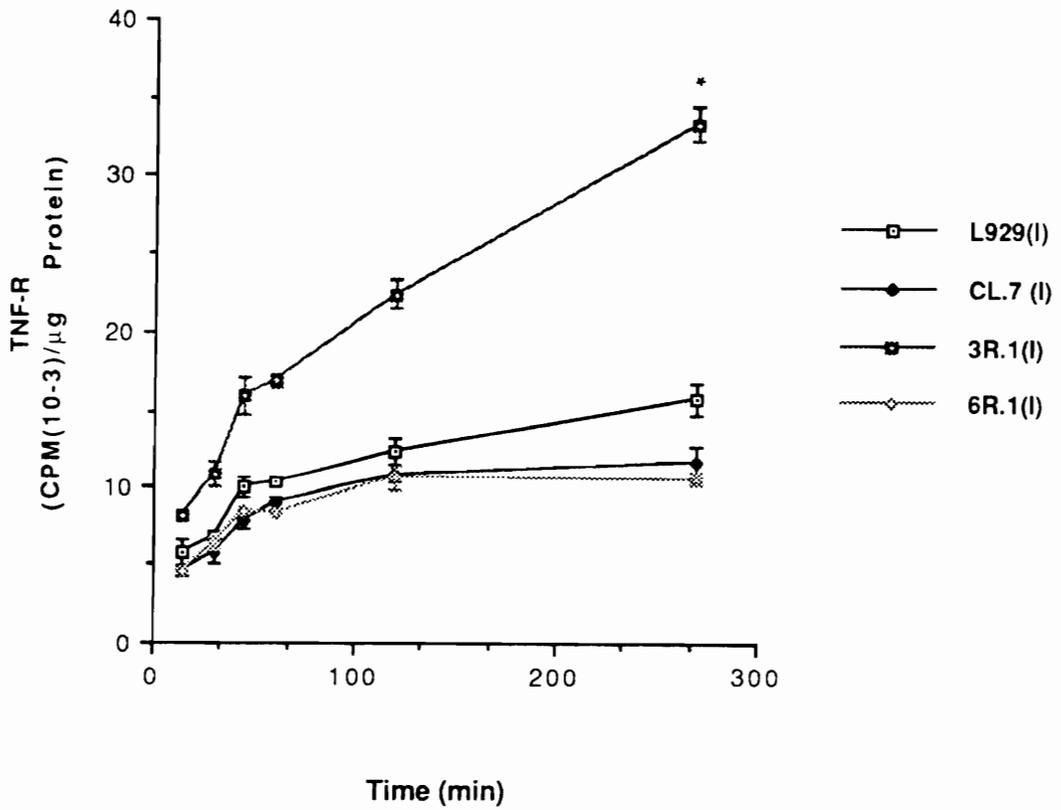


Figure 5 : Comparative TNF- α Internalization Under A-MuLV Transformation. (* = $p < 0.05$). 10^4 cpm of ^{125}I -TNF- α = 10.5 f moles.

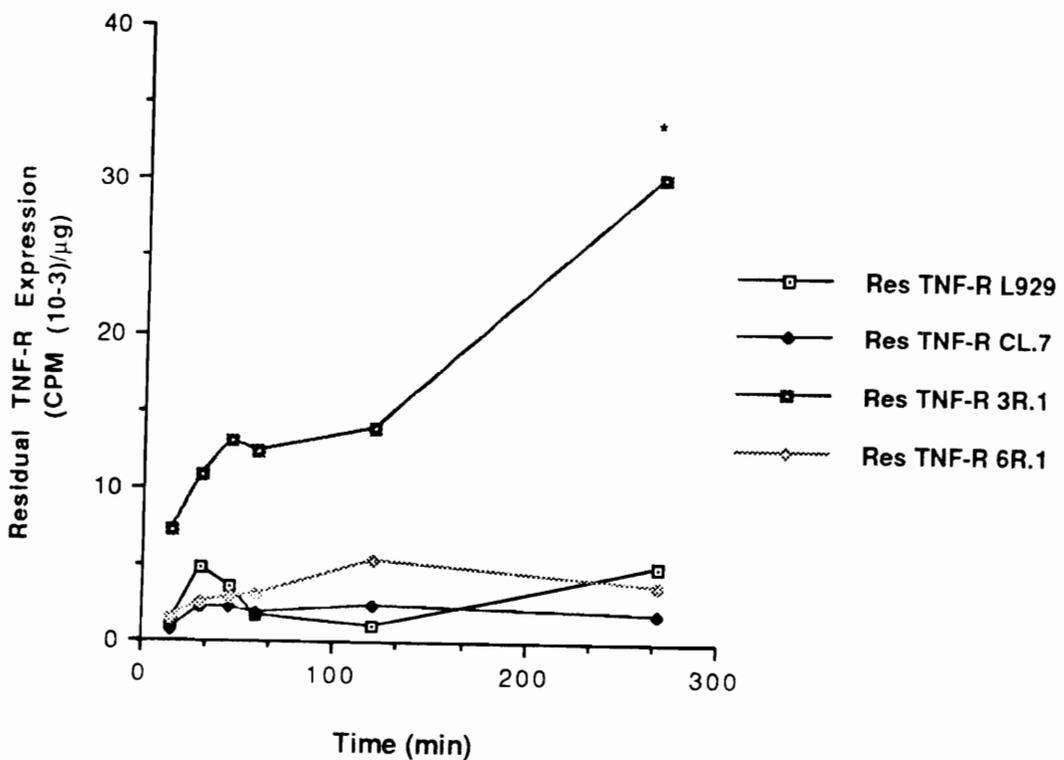


Figure 6 : TNF-Receptor Expression Post TNF- α Internalization. The residual binding of TNF- α to its cell surface receptor was determined for the L929, CL.7, 3R.1, and 6R.1 clones from $(\text{cpm (t)} - \text{cpm (l)})/\mu\text{g}$ protein in triplicate wells. The 3R.1 clone residual TNF-R levels increased with incubation time showing a TNF- α inducible TNF-R expression in this clone. (* = $p < 0.05$). 10^4 cpm of ^{125}I -TNF- α = 10.5 f moles.

CHAPTER - 5**TUMOR NECROSIS FACTOR- α CYTOTOXICITY AND EPIDERMAL GROWTH FACTOR RECEPTOR EXPRESSION UNDER A-MuLV TRANSFORMATION****ABSTRACT**

TNF- α is known to be a modulator of epidermal growth factor - receptor (EGF-R) expression in normal human fibroblasts. Recently we determined that TNF- α was differentially cytotoxic to normal murine embryonic fibroblast (CL.7) and its Abelson-murine leukemia virus (A-MuLV) transformed clones (Chapter 1). The current investigation was based on the hypothesis that TNF- α could elicit a differential response in control of EGF-R expression for normal untransformed and A-MuLV transformed cells. The A-MuLV, a murine RNA tumor virus, was shown in this investigation to be capable of altering the expression of EGF-R. The A-MuLV transformation suppressed the EGF-R levels for 3R.1 clone and induced the expression of EGF-R in the case of the 6R.1 cell line. We observed that our results were in partial contradiction with the results of Blomberg, et al (1980). These authors claimed that the transformation with A-MuLV leads to a loss of EGF-binding sites. We however presented evidence of both loss of EGF-binding site (for 3R.1) and its enhanced expression in the case of the clone 6R.1 as compared with the normal, untransformed clone BALB/c CL.7. The constitutive EGF-R levels were determined in terms of cpm 125 I-EGF bound/ μ g protein in six replicates for L929, CL.7, 3R.1, and 6R.1 clones. Whereas the clones CL.7 and 3R.1 had lower levels of EGF-R, the clone 6R.1 expressed a higher level of the receptor ($p < 0.05$). The rates of induction of CL.7 and 3R.1 clonal EGF-R with TNF- α incubation time were found to be similar in the time span of 0 to 17 hrs. The induction of EGF-R for 6R.1 cell line was higher ($p < 0.001$) than the CL.7 or the 3R.1 clones. The 6R.1 clone thus not only possessed a high level of EGF-R but also had a greater potential to further its EGF-R induction in presence of TNF- α . The induction of

EGF-R for 3R.1 and 6R.1 with TNF- α was responsive to TNF- α concentration. The EGF-R content of the clones were linearly associated with their susceptibility to TNF- α . The transformation with A-MuLV thus may be different from transformation by RNA sarcoma viruses with respect to the abolition of EGF-binding sites. Cytotoxicity of the clones to TNF- α versus it's EGF-R content showed that those clones (CL.7 and 3R.1) that were less sensitive to TNF- α had fewer EGF-R than the clone (6R.1) which had a higher susceptibility to TNF- α and a higher level of EGF-R.

INTRODUCTION

Epidermal growth factor (EGF) provides measurable growth-enhancing activity *in vivo* and is known to stimulate the growth for multitude of cells in culture (Carpenter and Cohen, 1979). EGF possesses a variety of biological activities. In cell culture systems EGF enhances the rate of hexose transport (Barnes and Colowick, 1976), increases DNA synthesis (Carpenter and Cohen, 1979) and stimulates rapid morphological changes (Chinkers et al., 1979, 1981). Cell surface receptors specific for EGF are found on most cell types including cells of the epidermal origin (Carpenter and Cohen, 1979). The EGF-specific high affinity binding sites usually range from 10^4 to 10^6 /cell. Upon addition of EGF to cells, EGF binds it's receptor (EGF-R) and the EGF-EGF-R complex is internalized (Carpenter and Cohen, 1976). Post internalization events include degradation of both the receptor (Das and Fox, 1978) and the hormone (Carpenter and Cohen, 1976) by lysosomal proteases. Clustering of receptors prior to binding of EGF at the cell surface is probably required for EGF to elicit its biological response (Schechter et al., 1979, King et al., 1980a). EGF-R levels in cells treated with EGF reach a lower steady state, indicative of a dynamic equilibrium between internalization and re-expression of EGF-R on cell surface. The replenishment of EGF-R levels is known to take several hours (Carpenter and Cohen, 1976). The EGF-R is a 180 K polypeptide initially identified by specific labeling with a photoreactive derivative of EGF (Das et al., 1977). Subsequently EGF-R was affinity purified from the membrane component of a human epithelioid tumor cell line A431 on an EGF column (Cohen et al.,

1980) and had molecular weights ranging from 150-170 K. Both with the crude plasma membrane fraction as well as in its purified state this receptor protein exhibits an associated protein kinase activity that is stimulated and enhanced with addition of EGF (Carpenter et al., 1978, 1979; Cohen et al., 1980; King et al., 1980b). The protein kinase action of EGF-R acts as an autokinase phosphorylating the receptor itself. The phosphorylated residue is determined to be at a tyrosine (Ushiro and Cohen, 1980). EGF induced phosphorylation of EGF-R is one of the distinctive biochemical reactions which occur as an early event after addition of the ligand to target cells (Hunter, et al., 1981). In cells, EGF enhances phosphorylation of EGF-R at tyrosine residue but far more predominantly at serine and threonine residues as determined by phosphopeptide mapping (Hunter, et al., 1981). The phosphate acceptor sites on EGF-R enables regulation of the receptor mediated processes, including those mediating communication between EGF-R and other ligand receptor systems. To this end, there is ample evidence supporting the hypothesis for the regulatory existence of communication between hormone receptors and stimulation of mitogenesis. Platelet derived growth factor (PDGF) decreases EGF-R down regulation occurring in response to EGF (Wrann, et al., 1980). Fibroblast growth factor (FGF) (Fox, et al., 1979), vasopressin (Rozengurt, et al., 1981), phorbol esters active in tumor promotion (Brown, et al., 1979) and diacylglycerol (DAG) (McCaffrey, 1984) modulate the EGF-R affinity in mitogenically responsive cells by binding to sites other than EGF-R. A mechanism in which a ligand binding to one receptor causes a second receptor to be modified is implied. The modulation of EGF-R by PDGF, DAG, phorbol esters and FGF is shown in a cell culture system by a decreased binding of radiolabeled EGF to cultured cells. In contrast to phorbol esters and PDGF, estrogen (Mukku, et al., 1985) and transforming growth factor- β (TGF- β) (Assoian, et al., 1984) have been shown to increase EGF-R.

TNF- α , a 17 K multifunctional polypeptide hormone, secreted by activated macrophages has been known to elicit hemorrhagic necrosis of some tumors and cytotoxicity to some tumor cell lines (Williamson et al., 1983). Apart from its cytostatic and cytotoxic action TNF- α has also been shown to be a potent growth promoting mitogen for normal human diploid fibroblasts (Vilcek et al., 1986).

TNF- α stimulates the production of interleukin-1 (IL-1) (Kirstein and Baglioni, 1986) and β -interferon (Kohase et al., 1986) in human fibroblasts. Apart from the above effects, TNF- α has also been shown to be a modulator of EGF-R expression (Palombella et al, 1987). The above authors showed a stimulation of growth on human FS-4 fibroblasts by recombinant human TNF- α (rh TNF- α) with an increased binding of EGF to these cells. Incubation with TNF- α resulted in 40-80% increase in the number of EGF-R sites with receptor protein synthesis demonstratable at around 2-4 hr following TNF- α treatment. Furthermore since TNF- α induced EGF-R expression followed a dose response relationship similar to that reported for the mitogenic stimulation of FS-4 fibroblasts it was concluded that the TNF- α induced EGF-R expression may be causal with the mitogenic action of TNF- α on human fibroblasts.

The objective of the present study was based on the hypothesis that the transformation induced with Abelson-murine leukemia virus (A-MuLV) a murine RNA tumor virus, had a potential to alter cellular growth factor regulatory systems. Transformation of murine fibroblast BALB/c CL.7 with A-MuLV resulted in the production of two transformed clones namely, BALB/c A-MuLV 3R.1 and BALB/c A-MuLV 6R.1. The 3R.1 and 6R.1 clones were malignantly transformed cells and were capable of producing tumors in immunodeficient and immunodeficient as well as normal syngenic mice respectively (Patek, et al., 1978). It has been known that many tumors, originating as epidermoid carcinomas, fibrosarcomas and astrocytomas, possessed a high level of EGF-R on their cell surface (Fabricant et al., 1977). The A-MuLV induced malignantly transformed clones of CL.7, namely 3R.1 and 6R.1, being capable of producing fibrosarcoma in mice might possess altered expression of EGF-R on their cell surface. We determined the EGF-R levels in the normal and A-MuLV transformed clones and investigated whether constitutive expression of EGF-R in each of the cell lines have any bearing with the susceptibility of the cells to TNF- α .

MATERIALS AND METHODS

Materials:

Mouse TNF- α was purified on an anti-TNF- α antibody column from activated RAW 264.7 supernatant and had a specific activity of 2.5×10^5 units/mg protein (Chapter 1). EGF was procured from Sigma Chemical Co., MO. Na¹²⁵I was purchased from New England Nuclear.

Radio-iodination of EGF:

EGF was iodinated by a modification of method described (Carpenter et al., 1976). Briefly 250 μ Ci of Na¹²⁵I was mixed with 10 μ l of (0.5 μ g/ml) EGF in 0.05 M potassium phosphate buffer (kPi), pH 7.4 along with 5 μ l of 0.5 μ g/ml chloramine-T (Sigma Chemical Co., MO). This soft labelling was made to proceed for 1-5 minutes after which the reaction was stopped with 20 μ l of (0.5 mg/ml in 0.05 M kPi, pH 7.5) sodium metabisulphite (MCB Co.). To the reaction system, 2.5 ml of kPi, pH 7.5 and 1 ml of 1% BSA was added. The mixture was loaded onto a pre-equilibrated Ag1-X8 anion exchange column and the eluate was collected in a tube containing 1 ml of 1% BSA in kPi, pH 7.5. EGF was iodinated to a specific activity of $5.9-6 \times 10^4$ cpm/ng protein.

Cell culture:

Mouse fibrosarcoma L929, normal mouse fibroblasts, BALB/c CL.7, and two A-MuLV transformed mouse fibroblast cell lines, BALB/c A-MuLV A.3R.1 and BALB/c A-MuLV A.6R.1, were grown in Dulbecco's modified Eagle's medium with 4.5 g/L glucose 90%, fetal bovine serum (FBS) 10%, 10 units/ml penicillin and 10 μ g/ml streptomycin, 25 mM HEPES, pH 7.4 at 5% CO₂ in a humidified 37°C incubator. The A-MuLV immortalized mouse monocyte-macrophage cell line RAW 264.7 was grown in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% FBS for the production of mouse TNF- α . All the cell lines were procured from the American Type Culture Collection (ATCC), Rockville, Maryland.

TNF- α cytotoxicity assay:

The L929 cytotoxicity assay was performed as described (Larrik, et al. 1989). Briefly, serial dilutions of TNF- α were performed using DMEM, 10% FBS in 96-well microtiter plates (Corning, NY) in six replicates. Starting with a 1:1000 dilution of stock, we plated at 1:4, 1:16, 1:64 and 1:128 dilution at a volume of 50 μ l/well. L929 target cells, washed and resuspended in DMEM, 10% FBS, 1 μ g/ml actinomycin D at a density of 10^6 targets/ml, were plated in 50 μ l containing 5×10^4 cells per well. After 18 hr of incubation at 37 $^\circ$ C, 5% CO $_2$, we added 20 μ l of a 2.5 mg/ml solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), obtained from Sigma, MO made in 0.9% saline. After incubation with MTT for 4 hr at 37 $^\circ$ C, 100 μ l were removed from each well without disturbing the crystals. The crystals were solubilized with 100 μ l of DMSO and read at 550 nm using a microtiter plate reader (Molecular Devices).

125 I-EGF binding assay:

Cells were seeded in 24-well plates (1×10^5 cells/well in 1 ml medium) and incubated in a humidifier CO $_2$ incubator for 24 hr. Cells were incubated in 1 ml of 1:2000 dilution of 2×10^5 units/ml stock containing 100 units TNF- α /ml for 0, 5, 9, 13, and 17 hours in DMEM supplemented with FCS. After the incubation time, cells were washed twice with DMEM medium, 25 mM HEPES with 1 mg/ml BSA for 10 minutes at 37 $^\circ$ C. After washing, cells were incubated with 500 μ l of ice cold DMEM supplemented with 25 mM HEPES and 5 mg/ml BSA containing 125 I-EGF at 15 ng/ml for 4 hr at 4 $^\circ$ C. Subsequently the plates were washed 4 times with the above ice cold medium. The radioactive washes were disposed in the appropriate container. After washing off the unbound 125 I-EGF, cells were solubilized in 1 ml of 0.1 N NaOH with 0.1% triton X-100 (Sigma Chemical Co., MO) and radioactivity was counted using a Beckman 5500 gamma counter.

Statistical Analysis : The statistical analysis was performed by a paired t test comparison. The probability of $p \leq 0.05$ was taken to be significant. Unless otherwise mentioned the results are expressed as mean \pm S.D of six replicate readings.

RESULTS

Constitutive EGF-R levels were determined as cpm ^{125}I -EGF bound/ μg protein in six replicates and were presented (Figure 1) for L929 fibrosarcoma, CL.7 fibroblast, 3R.1 (leukemia virus transformed CL.7) and 6R.1 (leukemia virus transformed CL.7) clones. Whereas the CL.7 and 3R.1 clones had lower levels of EGF-R, the clone 6R.1 expressed a significantly higher level ($p < 0.05$) of the receptor. Incidentally, the EGF-R in L929 cells were found to be fewer than that for CL.7 ($p < 0.05$). The inducibility of the EGF-R with TNF- α for normal and the retrovirally transformed clones were determined. The cells were incubated with TNF- α at 100 units/ml as a function of time and subsequently EGF-R were quantitated as described in the methods section.

TNF- α induced the expression of EGF-R in L929, in CL.7, in 3R.1, and in 6R.1 clones (Figure 2). The rates of induction of CL.7 and 3R.1 clonal EGF-R levels with TNF- α incubation time were found to be similar in the range of 0 to 17 hrs. TNF- α induced EGF-R levels in the CL.7 clone was however higher than that for 3R.1 ($p < 0.05$). The induction of EGF-R for 6R.1 cell line was higher ($p < 0.001$) than the CL.7 or the 3R.1 clones. The 6R.1 EGF-R expression followed a hyperbolic curve throughout the incubation time of 0 to 17 hr. The 6R.1 clone thus not only possessed a very high level of EGF-R but also had a relatively higher potential to further its EGF-R induction in presence of TNF- α .

The induction of EGF-R at 8 hr of TNF- α incubation was performed as a function of TNF- α concentration for the normal and the transformed clones. The ^{125}I -EGF retained on the surface of the cell, indicative of the EGF-R content, was measured for cells incubated with TNF- α for 8 hr at TNF- α concentrations of 0, 100, and 1000 units/ml. The cpm/ μg protein as a function of TNF- α concentration is depicted in TABLE - 1. The induction of EGF-R for 3R.1 and 6R.1 with TNF- α was responsive to TNF- α concentration. The induction of EGF-R levels for CL.7 however decreased at a higher TNF- α concentration.

The percent cytotoxicity of CL.7, 3R.1, and 6R.1 clones along with their constitutive EGF-R and TNF-R contents were presented in TABLE-2. As discussed in Chapter 4, the percent cytotoxicity of the clones did not associate with their TNF-R levels. In contrast, the EGF-R content of a clone was linearly associated with the clonal susceptibility to TNF- α . The association of constitutive EGF-R with TNF- α cytotoxicity under A-MuLV transformation is presented in Figure-3.

DISCUSSION

The transformation induced by A-MuLV, a murine RNA tumor virus was shown in this investigation to be capable of altering the expression of cellular growth factor receptors like the EGF-R. The A-MuLV transformation not only suppressed the EGF-R levels for the 3R.1 clone, it also was capable of inducing the constitutive clonal expression of EGF-R (in the case of 6R.1 cell line). We observed that our results were in partial contradiction with the results of Blomberg 1980 (Nature, Volume 286, 504-507). The above authors claimed that the transformation with A-MuLV lead to a loss of EGF-binding sites. We however present evidence of both loss of EGF-binding site (for 3R.1) and its enhanced expression in the case of the clone 6R.1 as compared with the untransformed clone BALB/c CL.7. The transformation with A-MuLV thus may be different from transformation by RNA sarcoma viruses with respect to the abolition of EGF-binding sites. The A-MuLV transformed BALB/c embryonic fibroblasts, capable of inducing tumors in BALB/c mice, have been shown to possessed a differential sensitivity to TNF- α (Chapter 1). TNF- α is known to be cytotoxic to some transformed cell *in vitro*. It has been shown to be a modulator of cell surface EGF-R expression (Palombella et al. 1987) The modulation of EGF-R expression of normal and A-MuLV transformed cells with TNF- α was investigated. TNF- α enhanced the EGF-R expression in all the clones. Cytotoxicity of the clones to TNF- α versus it's EGF-R content (Figures 3) showed that those clones that were less sensitive to TNF- α had a lesser content of EGF-R than the clone (6R.1) which had a higher susceptibility to TNF- α and a higher level of EGF-R.

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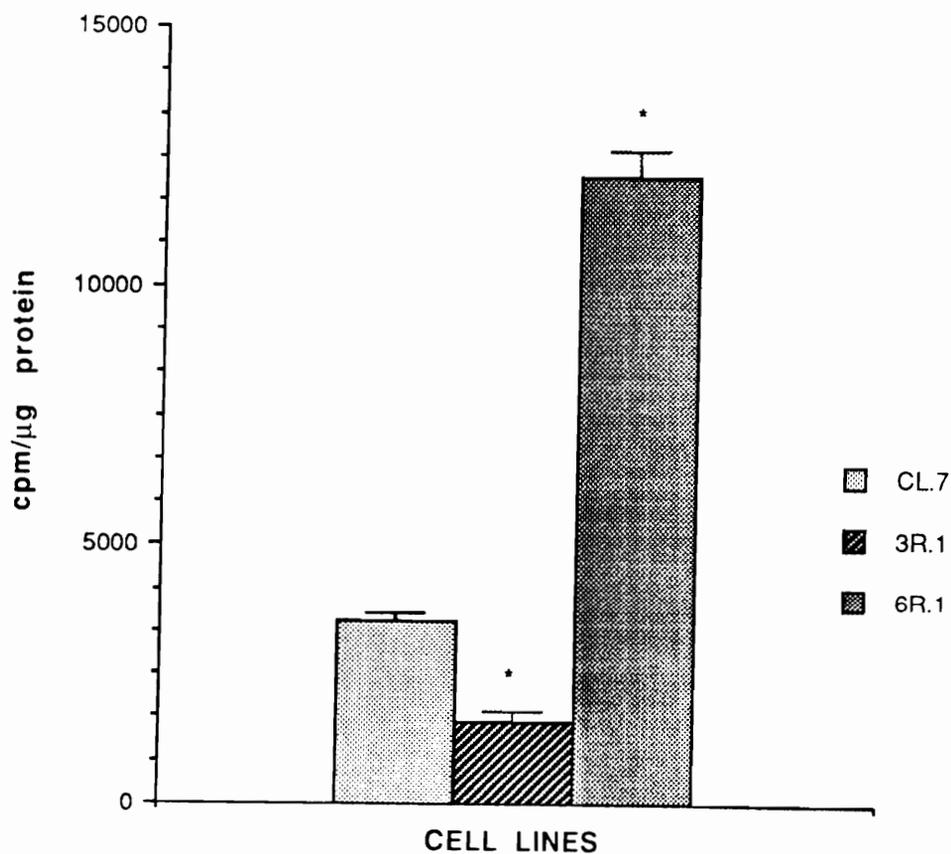


Figure 1 : EGF-R Expression Under A-MuLV Transformation. 125 I-EGF (15 ng/ml) in 1 ml reaction volume were incubated for 4 hr at 4 ° C. The EGF binding for L929, CL.7, 3R.1 and 6R.1 cells were determined as described. (* = $p < 0.05$)

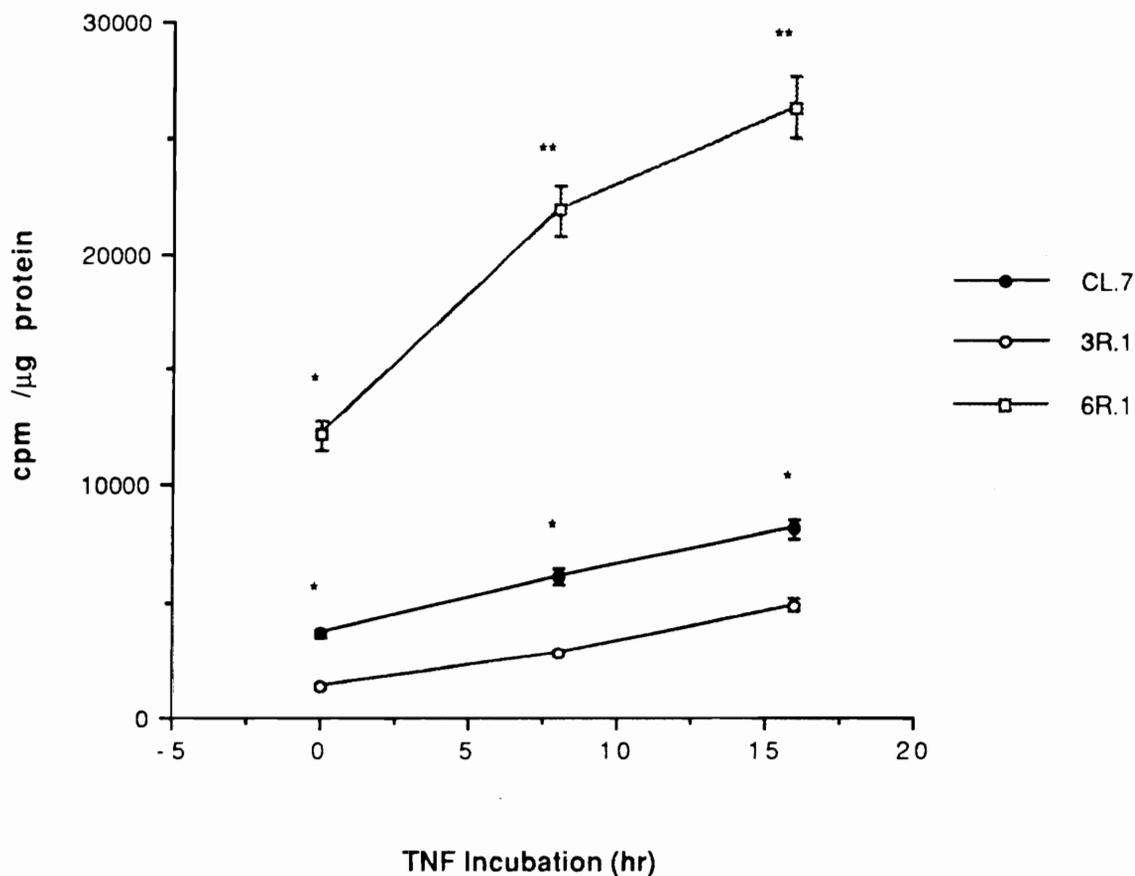


Figure 2: The Induction of EGF-R With TNF- α Under A-MuLV Transformation. The TNF- α sensitive 6R.1 EGF-R induction was prominently higher than the normal CL.7 or even the A-MuLV 3R.1 clone at a TNF- α concentration of 100 units/ml. The results were presented as mean \pm S.D of six replicate wells. (* = $p < 0.05$)

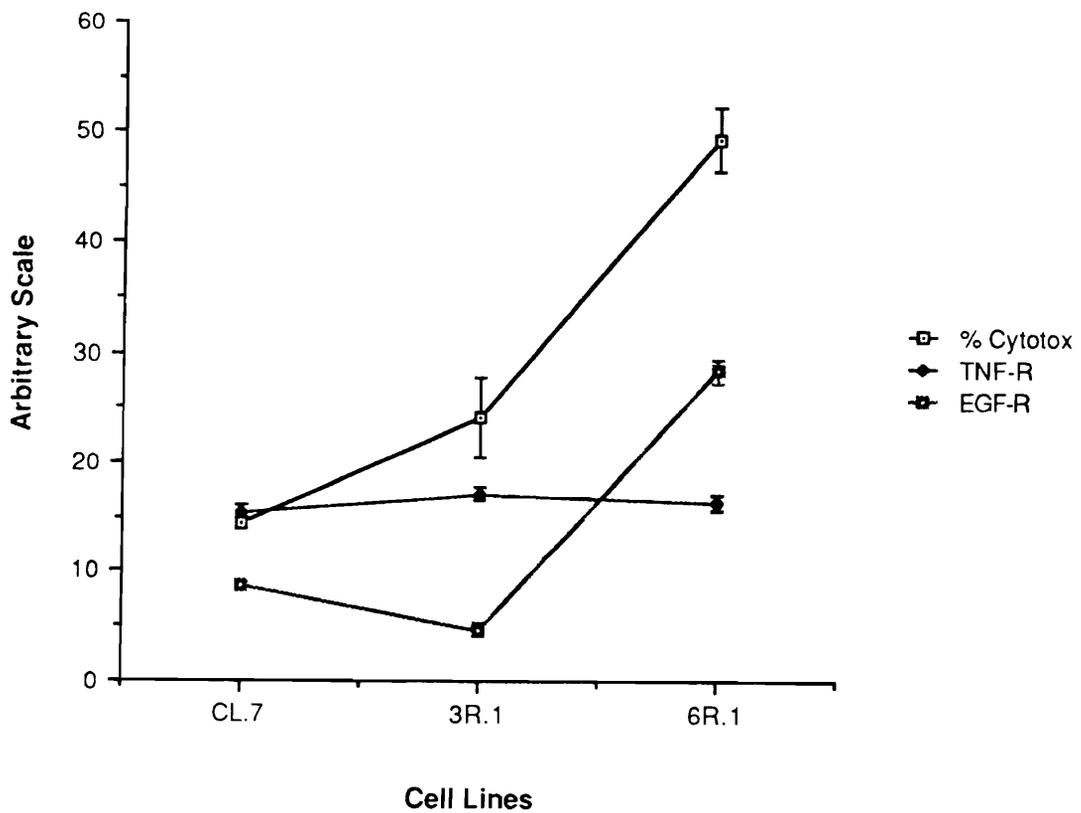


Figure 3 : Constitutive EGF-R Expression, TNF-R Levels, And TNF- α Cytotoxicity. Higher sensitivity of a clone to TNF- α cytotoxicity was associated with a higher EGF-R expression. (* = $p < 0.05$)

TABLE - 1**EGF-R Expression With TNF- α Dilution**

| Cell Line | TNF- α (Units/ml) | | |
|-----------|------------------------------|------------------|------------------|
| | EGF-R (cpm/ μ g protein) | | |
| | 0 | 100 | 400 |
| CL.7 | 3,723 \pm 145 | 6,513 \pm 159 | 5,785 \pm 191 |
| 3R.1 | 1,330 \pm 22 | 2,952 \pm 155 | 3,079 \pm 149 |
| 6R.1 | 11,956 \pm 573 | 22,433 \pm 464 | 24,236 \pm 783 |

TABLE - 2**Relationship Between Clonal TNF- α Cytotoxicity, TNF-R And EGF-R**

| Cell Line | TNF- α % Cytotoxicity | TNF-R cpm (10^{-3})/ μ g | EGF-R cpm (10^{-3})/ μ g |
|-----------|---------------------------------|-------------------------------------|-------------------------------------|
| CL.7 | 14.4 \pm 0.5 | 15.5 \pm 0.6 | 8.7 \pm 0.4 |
| 3R.1 | 24.1 \pm 3.5 | 17.2 \pm 0.6 | 4.6 \pm 0.6 |
| 6R.1 | 49.4 \pm 2.9 | 16.4 \pm 0.8 | 28.4 \pm 1.1 |

Chapter - 6**ROLE OF MODULATORS OF THE ARACHIDONIC ACID CASCADE AND PROTEIN KINASE C IN TNF-R AND EGF-R EXPRESSION****ABSTRACT**

Events in the cellular biochemical pathway in modulating the TNF- α and EGF binding to their respective cell surface receptors were investigated. The study was conducted in a leukemia virus transformation model system (chapter 1) comprising of the normal (CL.7) and the leukemic (3R.1 and 6R.1) clones. The cell surface binding and internalization kinetics were studied with radio-labelled TNF- α and EGF. The investigation was to look at the effect of modulators of arachidonic acid cascade and PK-C in governing the uptake and internalization of TNF- α and binding of EGF under the leukemia virus transformation. EGF binding to EGF-R was inhibited by dexamethasone (phospholipase A₂ inhibitor), indomethacin (cyclooxygenase inhibitor), SKF-525A (cytochrome P450 inhibitor) and PMA (protein kinase C activator) for the leukemic clone 6R.1, showing that the phospholipase A₂ activated cyclooxygenase pathway, an active cytochrome P450, and protein kinase C, all have the capability to modulate the binding of EGF to its cell surface receptor. SKF-525A and PMA were shown to inhibit EGF binding for CL.7, 3R.1 as well as 6R.1 clones. TNF- α binding to its receptor was enhanced by inhibition of cyclooxygenase pathway for CL.7, 3R.1 and 6R.1 ($p < 0.05$). Indomethacin as well as dexamethasone however inhibits TNF- α binding to TNF-R for L929 cells. The inhibitors of the arachidonate pathway known to reduce TNF- α cytotoxicity (Suffy et al. 1987) were shown to modulate TNF- α action at the level of binding of the ligand to its receptor. The activators of PK-C like PMA and OAG are known to mediate down regulation of TNF- α binding capacity in both normal and malignant cells (Ungraub, et al. 1987). In our leukemia transformation model only PMA had a non-specific suppression of TNF- α binding in CL.7, 3R.1 and in 6R.1 clones. OAG

(PK-C activator) suppresses TNF- α binding for CL.7 but significantly ($p < 0.05$) enhances its binding for 3R.1 and 6R.1 clones. This could possibly be due to the difference in PK-C activation of the normal and leukemia virus transformed clones as compared with that for OAG. PMA induced suppression of TNF- α binding was however accompanied with an enhanced TNF- α internalization for L 929 cells (TABLE 1). PMA inhibited the binding of EGF and TNF- α to all the cell lines studied whereas OAG did not do so. This showed that the regulation of hormone and growth factor ligands binding to their receptors by PK-C activation need not be a general cellular growth surveillance property. SKF-525A inhibited the binding of EGF as well as TNF- α to all the cells. The decrease in TNF- α cytotoxicity by the cytochrome P450 inhibitor SKF-525A could be, in part, at the level of TNF- α binding and internalization. The mechanism of this inhibition is however not clearly understood. Thus early membrane events, revealed by use of inhibitors of arachidonic acid cascade and modulators of PK-C, could modulate the binding and internalization of EGF and TNF- α and thereby control the physiologic future of the cell.

INTRODUCTION

Myriad intracellular pathways are triggered by the binding of the polypeptide hormone tumor necrosis factor- α (TNF- α) and epidermal growth factor (EGF) to their cell surface receptors. Whereas EGF binding to its cell surface receptor (EGF-R) produces mitogenic action, binding of TNF- α to its cell surface receptor (TNF-R) could either be mitogenic, as in normal human fibroblasts, or be cytotoxic to growing tumor cells (Larrik et al 1988). The binding of the ligands to their cell surface receptors is essential for eliciting their mitogenic or cytotoxic action. It is believed that second messengers could be involved in orchestrating this response. For example, oleylacetyl glycol (OAG) and phorbol maristate acetate (PMA), the known activators of protein kinase-C have been determined to mediate down regulation of TNF- α binding capacity in both normal and malignant cells (Unglaub, et al. 1987; Scheurich et al. 1986). Binding of TNF- α to HL60 cells provokes an increase in the binding of GTP to G protein (Imamura, et al 1988). G proteins in turn serve as signal transducers, linking extracellular receptors to

enzyme systems such as adenylate cyclase, phospholipase C (PLC), and phospholipase A₂ (PLA₂), which generate intracellular second messengers. As for TNF-R, TNF- α binding causes a rapid increase in cytoplasmic cAMP with a subsequent rise in cAMP-dependent kinase activity (Zhang, et al. 1988). Role of TNF- α in activating the initiation of arachidonate metabolism, in conjunction with the central role of PLA₂, can't be ruled out. Inhibitors of the arachidonate pathway reduce TNF- α cytotoxicity (Suffys, et al 1987). Dexamethasone, a potent corticosteroid, inhibits the release of both the arachidonic acid and TNF- α mediated cytotoxic effects. PLA₂-activating protein is induced by action of TNF- α on endothelial cells (Clark et al 1988). Melitin (PLA₂ activator) activates the cytotoxicity and mitogenic response of TNF- α whereas quinacrine and dexamethasone inhibit these responses (Palombella, et al 1989). PLA₂-induced release of arachidonic acid and its metabolites is prevalent only in TNF- α susceptible cells (Neale et al 1988).

Metabolism of arachidonate via the lipoxygenase or the cyclooxygenase pathways have not been implicated in blocking TNF- α cytotoxicity. For example, indomethacin (cyclooxygenase inhibitor) which effectively blocks prostaglandin generation and nordihydroguaiaretic acid (NGDA), an inhibitor of the lipoxygenase pathway, do not modulate the TNF- α mediated cytotoxicity. It has also been hypothesized that phosphatidylinositol pathway mediates the protective actions against TNF- α . PLC-mediated hydrolysis biproducts are diacylglycerol (DAG), PK-C activator, and inositol 1,4,5-triphosphate [Ins(1,4,5)P₃], Ca²⁺ mobilizer, which act as second messengers. These second messengers might not be important from the point of view of TNF- α cytotoxicity as PLC inhibitors have not been implicated with inhibition of TNF- α cytotoxic pathway. Another mechanism of regulation of cellular biochemistry is via phosphorylation of proteins by protein kinases. Protein kinase C, known to be activated by biproducts of PLC induction namely DAG and Ca²⁺, has been shown to be involved in regulation of TNF-R. Thus the TNF- α cytotoxicity cascade is controlled in part by activation of G-protein coupled second messenger system as well as via modulation of PK-C.

Interaction of EGF with cell surface receptors leads to an

activation of a cyclic nucleotide-independent protein kinase (Carpenter et al 1979). This protein kinase was specific for tyrosine residue (Ushiro, et al 1980) and was later shown to be the receptor itself (Buhrow et al 1982). Like TNF- α -TNF-R complex, EGF-EGF-R complex is internalized and found in the lysosomes upon subcellular fractionation and like TNF- α , EGF binding induces a half maximal mitogenic response at a concentration at which 10% of its sites are occupied.

The present study was aimed at investigating the early events in the cellular biochemical pathway in modulating TNF- α and EGF binding to their respective cell surface receptors. The study was conducted in a leukemia virus transformation model system (chapter 1) comprising of the normal (CL.7) and the leukemic (3R.1 and 6R.1) clones. The binding and internalization were studied with radio-labelled TNF- α and EGF. The investigation was to look at the effect of modulators of arachidonic acid cascade and PK-C in governing the uptake and internalization of TNF- α and binding of EGF under the leukemia virus transformation.

MATERIALS AND METHODS

Materials: Recombinant human TNF- α (rhTNF- α) was procured from Genentech, CA. EGF was from Sigma Chemical Co. Na¹²⁵I was purchased from New England Nuclear. Dexamethasone, indomethacin, phorbol, 12-myristate, 13-acetate (PMA), N-(6-aminohexyl)-5-chloro-1 naphthalene -sulfonamide, HCl (W-7), 1-oleoyl-2-acetyl- glycerol (OAG), 1-(5-isoquinoline sulfonyl)-2-methyl-piperazine (H-7), 5,8,11-eicosatrinoic acid (ETI), and SKF-525 A (proadifen) were purchased from Calbiochem, CA.

Cells: L929, CL.7, 3R.1, and 6R.1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) containing 10 units/ml penicillin, 10 μ g/ml streptomycin, 25 mM HEPES, at pH 7.4, in a humidified incubator at 37 ° C.

Radio-iodination of EGF: EGF was iodinated by a modification of a described method (Carpenter et al 1976). Briefly 250 μ Ci of Na¹²⁵I

was mixed with 10 μ l (5 μ g) of EGF in 0.05 M KPi, pH 7.4 along with 5 μ l of 0.5 μ g/ml chloramine T (Sigma Chemical Co.). This soft labelling proceeded for 5 minutes after which the reaction was stopped with 20 μ l of (0.5 mg/ml in 0.05 M KPi, pH 7.4) sodium metabisulphite (MCB Co.). To the reaction system we further added 2.5 ml of the above buffer, and 1 ml of 1 % BSA in the same buffer. The mixture was loaded on to a pre-equilibrated Ag 1-X8 anion exchange column and the eluate collected in a tube containing 1 ml of 1 % BSA in 0.05 M KPi, pH 7.4 . EGF was iodinated to a specific activity of 6.5×10^4 cpm/ng EGF.

Radio-iodination Of TNF- α : rh-TNF- α was used for all receptor binding assays and was radio-labelled using Na¹²⁵I by the chloramine T method as previously described. The specific activity of iodinated TNF- α was determined to be 5.6×10^4 cpm/ng TNF- α .

¹²⁵I-EGF Binding Assay: Cells were seeded in 24 well plates (1 X 10⁵ cells/well in 1 ml medium) and incubated in a humidified incubator. The cells were washed in media without fetal calf serum and pre-incubated at 37^o C for 30 minutes in 500 μ l of; OAG (10 μ M); PMA (10 ng/ml); H-7 (50 μ M); H-7 (50 μ M) + PMA (10 ng/ml); dexamethasone (0.2 μ M); dexamethasone (0.2 μ M) + PMA (10 ng/ml); dexamethasone (0.2 μ M) + OAG (10 μ M); indomethacin (5 μ M); ETI (10 μ M); W-7 (25 μ M); and SKF-525A (10 μ M). W-7 incubation was performed for 1 hr prior to ¹²⁵I-EGF labelling. At the end of the incubation time the plates were transferred to 4^oC for 15 minutes prior to labelling with 500 μ l of ¹²⁵I-EGF at a concentration of 15 ng/ml for 4 hr at 4^o C. After the incubation time the plates were washed 4 x in cold with media without FCS, solubilized with 0.1 N NaOH with 0.1 % triton X-100 and samples were counted for radioactivity in a Beckman 5500 gamma counter and quantitated for protein content using a Shimadzu UV-Visible spectrophotometer. Radio-labelled EGF binding was expressed as cpm/ μ g protein for triplicate samples as mean +/- S.D.

¹²⁵I-TNF- α Binding And Internalization Assay : Surface binding and internalization of TNF- α were measured as described (Tsujiimoto et al. 1985). The binding and internalization assays were performed in

triplicate wells preincubated with inhibitors whose concentrations were similar to that used for ^{125}I -EGF binding assay.

Statistical Analysis : The statistical analysis was performed by a paired t test comparison. The probability of $p \leq 0.05$ was taken to be significant. Unless otherwise mentioned the results were expressed as mean \pm S.D of six replicate readings.

RESULTS

The binding of ^{125}I -EGF to the cell surface EGF-R was determined for CL.7, 3R.1, 6R.1 and L929 cells. Compared to the CL.7 clone the binding of radio-labelled EGF, expressed as cpm/ μg protein, was depressed for 3R.1 and greatly enhanced for 6R.1 clone (Figure 1). L929 cells exhibited little EGF binding. The ^{125}I -EGF binding for the above cell lines preincubated with dexamethasone (phospholipase A_2 inhibitor); indomethacin (prostaglandin synthesis inhibitor via blockade of cyclooxygenase), ETI (5- and 12-lipoxygenase inhibitor but does not inhibit cyclooxygenase), and SKF-525 A (inhibitor of cytochrome P450) were shown in Figure 1. SKF-525 A at 10 μM concentration significantly ($p < 0.05$) inhibited the binding of radio-labelled EGF to CL.7, 3R.1, and 6R.1 clones.

The role of phospholipase A_2 inhibitor in conjunction with modulators of protein kinase C (PK-C) including PMA (PK-C activator), OAG (PK-C activator), and W-7 (calmodulin antagonist) for ^{125}I -EGF binding was shown in Figure 2. Clearly Dexamethasone alone as well as in conjunction with PMA inhibited the EGF binding in 6R.1 clone. Effect of activators and inhibitors of PK-C alone in modulation of ^{125}I -EGF binding were depicted in Figure 3. It was observed that PMA (PK-C activator, Figure 3) and SKF-525 A (cytochrome P 450 inhibitor, Figure 1) decrease the ^{125}I -EGF binding for CL.7, 3R.1 and 6R.1 cells ($p < 0.05$). H-7, a cGMP dependent protein kinase as well as PK-C inhibitor, enhanced the binding of EGF to its cell surface receptor in the L929 as well as 6R.1 clones but did not do so for either CL.7 or 6R.1 clones (Figure 3). Preincubation with dexamethasone as well as ETI inhibited the ^{125}I -EGF binding only in the 6R.1 clone. Phospholipase A_2 activated lipoxygenase pathway was implicated for enhanced binding of EGF in this clone.

Indomethacin had little effect in modulation of EGF binding. Activation of PK-C by PMA resulted in decreased binding of ^{125}I -EGF whereas using activator of PK-C (OAG) did not affect its binding to CL.7, 3R.1, and 6R.1 cells showing thereby that these two activation systems have different characteristics. We concluded that the PMA activation of PK-C inhibited the EGF binding irrespective of the viral transformation. For the 6R.1 clone however a PLA₂ activated lipooxygenase pathway facilitated the EGF binding.

Binding and internalization of ^{125}I -TNF- α were determined as mentioned in the methods section. Activator of PK-C (OAG) at 10 μM enhanced the TNF- α binding to its receptor in 6R.1 clone ($p < 0.05$, Figure 4). OAG enhancement of TNF- α binding was higher than control levels in 3R.1 cells but not significant at $p < 0.05$ level (Figure 4). PMA at 10 ng/ml, another PK-C activator, lowered the TNF- α binding for all cell lines studied ($p < 0.05$, Figure 4). Thus PMA modulation of TNF- α binding was independent of the viral transformation. H-7 (PK-C inhibitor) had little effect on TNF- α binding. Binding of TNF- α to TNF-R could be, in part, influenced by modulators of PK-C. OAG enhanced TNF- α internalization significantly for 3R.1 clone ($p < 0.05$) and had little effect on TNF- α internalization for other cell lines studied. PMA however, inhibited the TNF- α internalization for CL.7, 3R.1 and 6R.1 clones (Figure 5). Effect of preincubation with inhibitors of arachidonic acid cascade for TNF- α binding to TNF-R were shown in Figures 6 a and 6 b. Preincubation with 10 μM SKF-525A inhibited TNF- α binding for all cell lines studied ($p < 0.05$). Dexamethasone, indomethacin, and ETI enhanced the binding of TNF- α ($p < 0.05$) in the 3R.1 clone as compared with its control TNF- α levels. The binding of TNF- α to its receptor was enhanced ($p < 0.05$) in 6R.1 clone in the presence of indomethacin. OAG (PK-C activator) increased the binding of TNF- α in CL.7, 3R.1 and 6R.1 cell lines in presence of dexamethasone. PMA, in presence of dexamethasone, reversed this effect significantly only in the 6R.1 clone ($p < 0.05$). SKF-525A inhibited the internalization of TNF- α in CL.7, 3R.1 and 6R.1 clones ($p < 0.05$, Figure 7 a). Dexamethasone, indomethacin, and ETI not only enhanced the binding of TNF- α to TNF-R in 3R.1 clone (Figure 6a) but also increased TNF- α internalization ($p < 0.05$, Figure 7a). PMA inhibited

the internalization of TNF- α in presence of dexamethasone for CL.7 and 6R.1 cell lines (Figure 7b) but had little effect on that for 3R.1 clone. We concluded that PMA activated PK-C inhibited TNF- α binding as well as internalization independent of the leukemia virus transformation. This was based on the finding that PMA inhibited TNF- α binding in 3R.1 and 6R.1 cells (Figure 4) and TNF- α internalization in CL.7, 3R.1, and 6R.1 clones (Figure 5). Further SKF-525A inhibited the TNF- α binding and internalization ($p < 0.05$) for all the clones studied. Dexamethasone and indomethacin enhanced the binding and internalization of TNF- α in 3R.1 clone ($p < 0.05$) showing thereby that PLA₂ activated cyclooxygenase pathway could possibly be involved in the binding and internalization of TNF- α .

DISCUSSION

The overall results of the EGF and TNF- α radio-labelled cell surface binding and internalization study were presented in TABLE-1. EGF binding to EGF-R was inhibited by dexamethasone, ETI, SKF-525A and PMA for 6R.1 cell line showing there by that phospholipase A₂ activated lipooxygenase pathway, an active cytochrome P450, and protein kinase C all had the capability to modulate the binding of EGF to its receptor. SKF-525A and PMA were shown to inhibit EGF binding for CL.7, 3R.1 as well as 6R.1 clones ($p < 0.05$).

TNF- α binding to its receptor was enhanced by inhibition of cyclooxygenase pathway for CL.7, 3R.1 and 6R.1 ($p < 0.05$). Indomethacin and dexamethasone inhibited TNF- α binding to TNF-R for L929 cells. The inhibitors of the arachidonate pathway known to reduce TNF- α cytotoxicity (Suffy et al. 1987) were shown to modulate TNF- α action at the level of binding of the ligand to its receptor. The activators of PK-C have been known to mediate down regulation of TNF- α binding capacity in both normal and malignant cells (Unghaub, et al. 1987). In our leukemia transformation model only PMA had a non specific suppression of TNF- α binding in CL.7, 3R.1 and 6R.1 clones. OAG (PK-C activator) suppressed TNF- α binding for CL.7 but enhanced its binding for 3R.1 and 6R.1 clones ($p < 0.05$). This could possibly be due to the difference in PK-C activation of the normal and leukemia virus transformed clones as compared with

that for OAG. PMA induced suppression of TNF- α binding was however accompanied with an enhanced TNF- α internalization for L 929 cells (TABLE 1).

PMA inhibited the binding of EGF and TNF- α to all the cell lines studied whereas OAG activated PK-C did not do so. This showed that the regulation of hormone and growth factor ligands binding to their receptors by PK-C activation need not be a general cellular growth surveillance property. SKF-525A inhibited the binding of EGF as well as TNF- α to all the cells ($p < 0.05$). The decrease in TNF- α cytotoxicity by the cytochrome P450 inhibitor SKF-525A could be at the level of TNF- α binding. The mechanism of this inhibition is however not clear. Thus early membrane events, revealed by use of inhibitors of arachidonic acid cascade and modulators of PK-C, could modulate the binding and internalization of ligands like EGF and TNF- α and thereby control the physiologic future of the cell.

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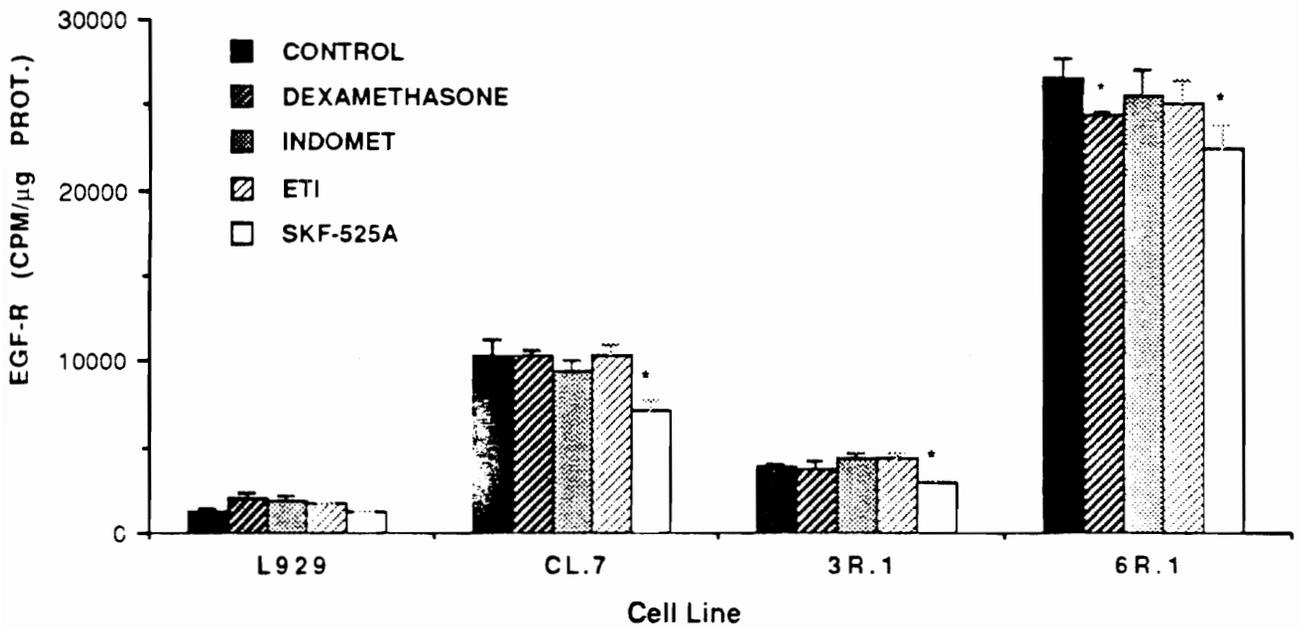


Figure 1 : Role of Inhibitors of Phospholipase A₂ , Cyclooxygenase, Lipooxygenase, And Cytochrome P 450 In ¹²⁵I- EGF Binding Under A-MuLV Transformation. The EGF labelling and the cell surface binding was in absence and presence of the inhibitors as described in the methods section. The data is presented as mean ± S.D of triplicate cultures. (* = p<0.05). 10⁴ cpm/μg protein equals 29.9 f moles/μg protein.

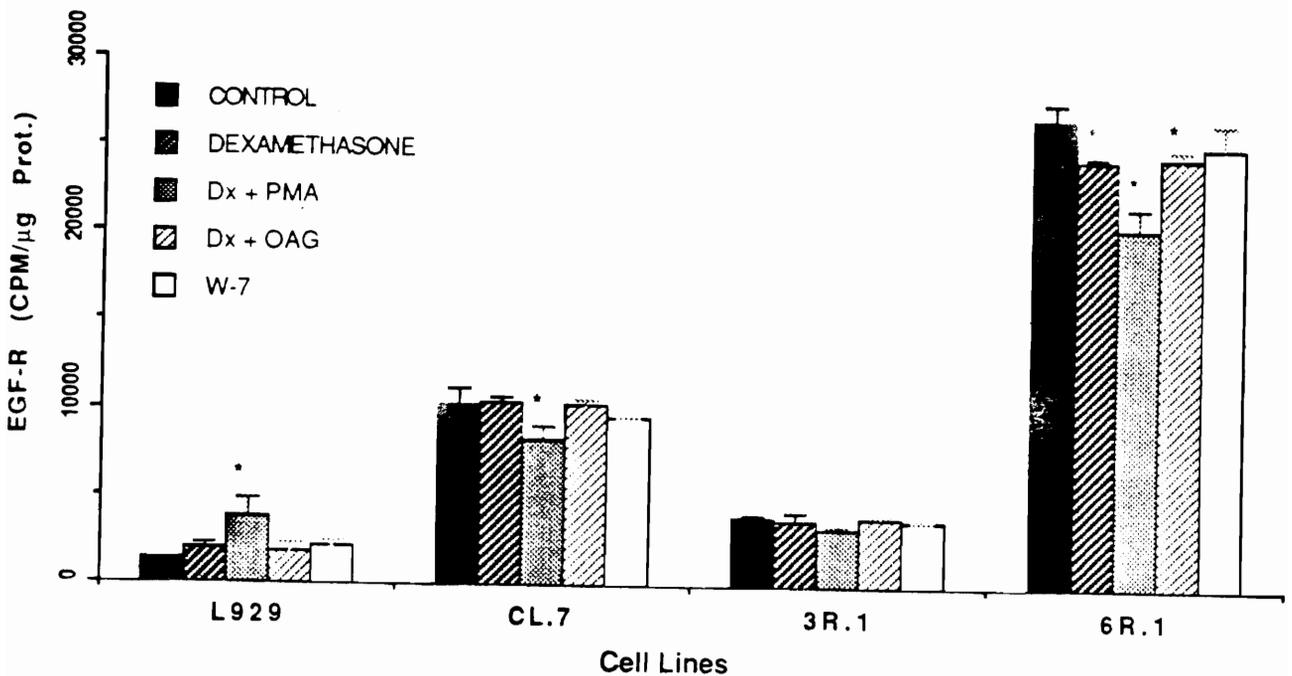


Figure 2 : Role of Inhibitors of Phospholipase A₂ in Conjunction With PK-C Modulators, PMA and OAG as well as Calmodulin Antagonist W-7 In Modulation Of EGF Binding To EGF-R. The EGF labelling and the cell surface binding was in absence and presence of the inhibitors as described in the methods section. The data is presented as mean \pm S.D of triplicate cultures. (* = p<0.05). 10⁴ cpm/ μ g protein equals 29.9 f moles/ μ g protein.

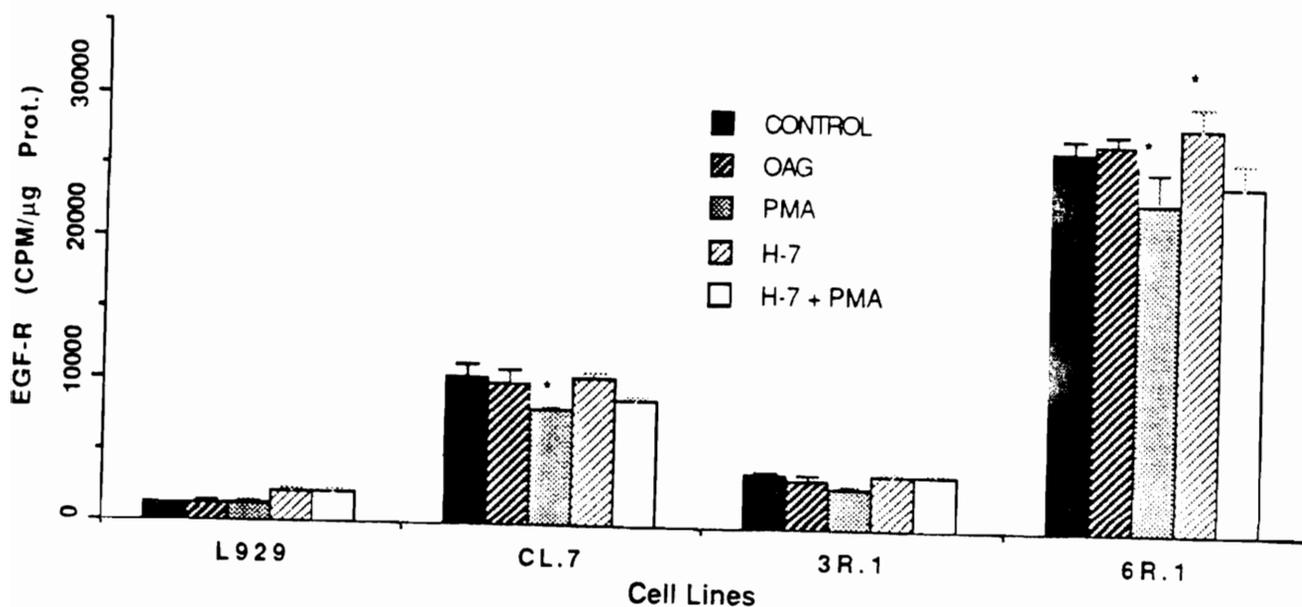


Figure 3 : The Effect of Activators And Inhibitors of PK-C On EGF-R Modulation Under A-MuLV Transformation. The EGF labelling and the cell surface binding was in absence and presence of the inhibitors as described in the methods section. The data is presented as mean \pm S.D of triplicate cultures. (* = $p < 0.05$). 10^4 cpm/ μ g protein equals 29.9 f moles/ μ g protein.

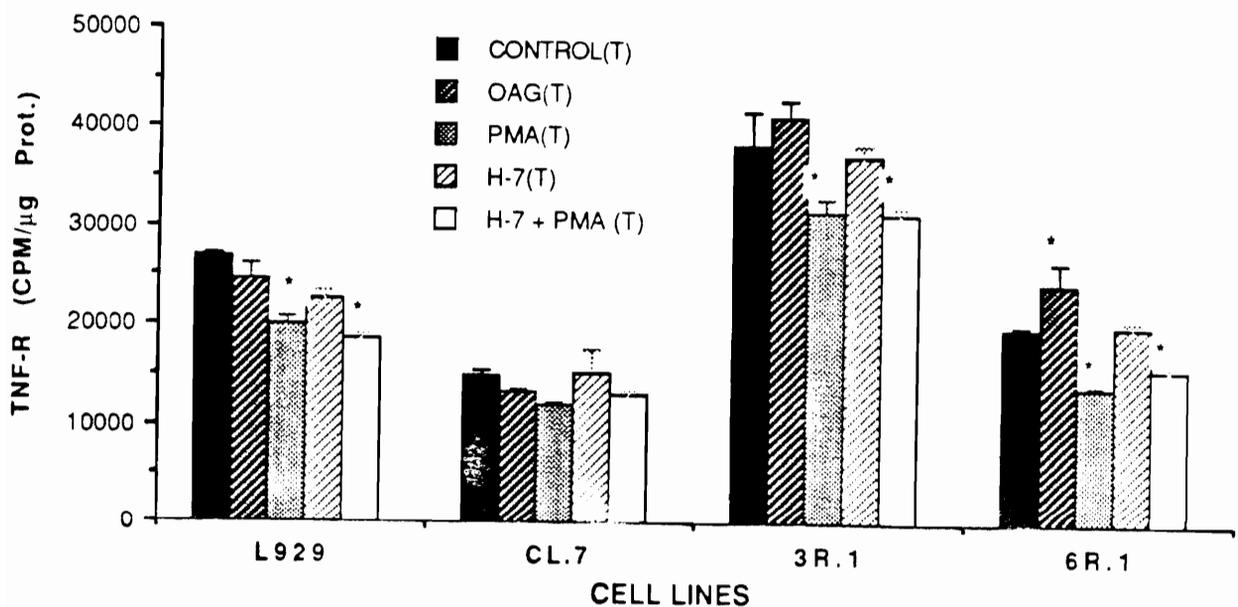


Figure 4 : The Effect of Activators And Inhibitors of PK-C On TNF- α Binding Under A-MuLV Transformation. The TNF- α labelling and the cell surface binding was in absence and presence of the inhibitors as described in the methods section. The data is presented as mean \pm S.D of triplicate cultures. (* = $p < 0.05$). 10^4 cpm/ μ g protein equals 10.5 f moles/ μ g.

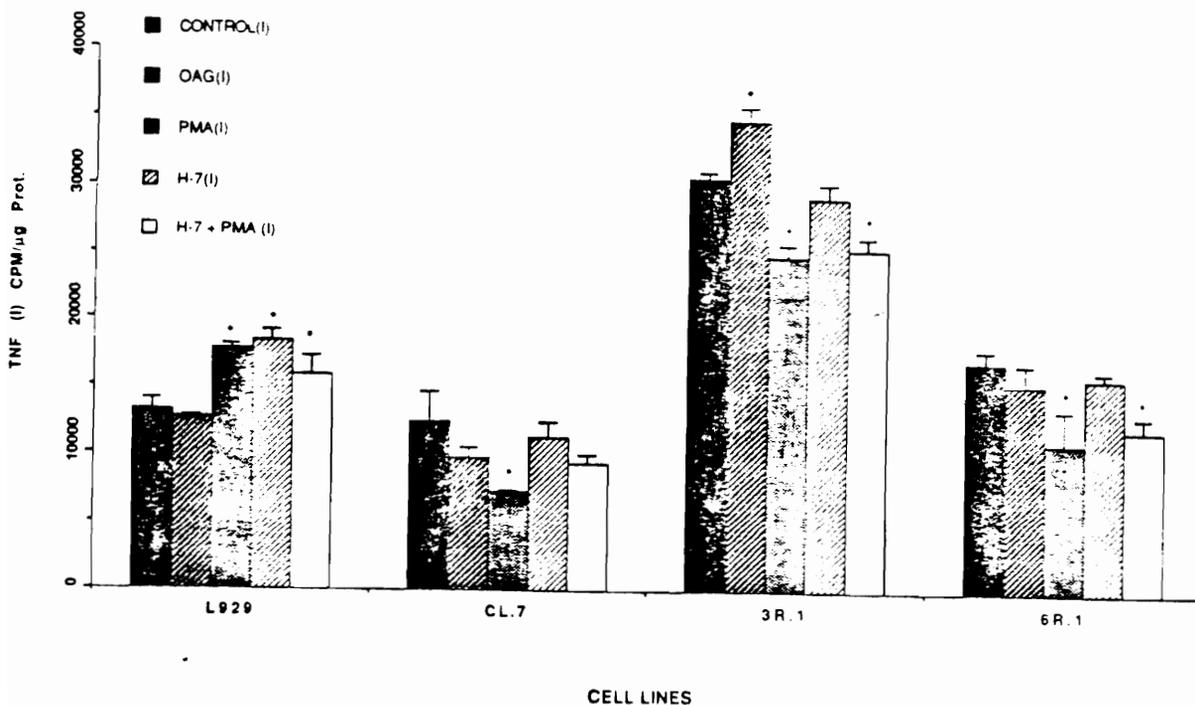


Figure 5 : The Effect of Activators And Inhibitors of PK-C on TNF- α Internalization Under A-MuLV Transformation. The TNF- α labelling and the internalization assay was in absence and presence of the inhibitors as described in the methods section. The data is presented as mean \pm S.D of triplicate cultures. (* = $p < 0.05$). 10^4 cpm/ μ g protein equals 10.5 f moles/ μ g.

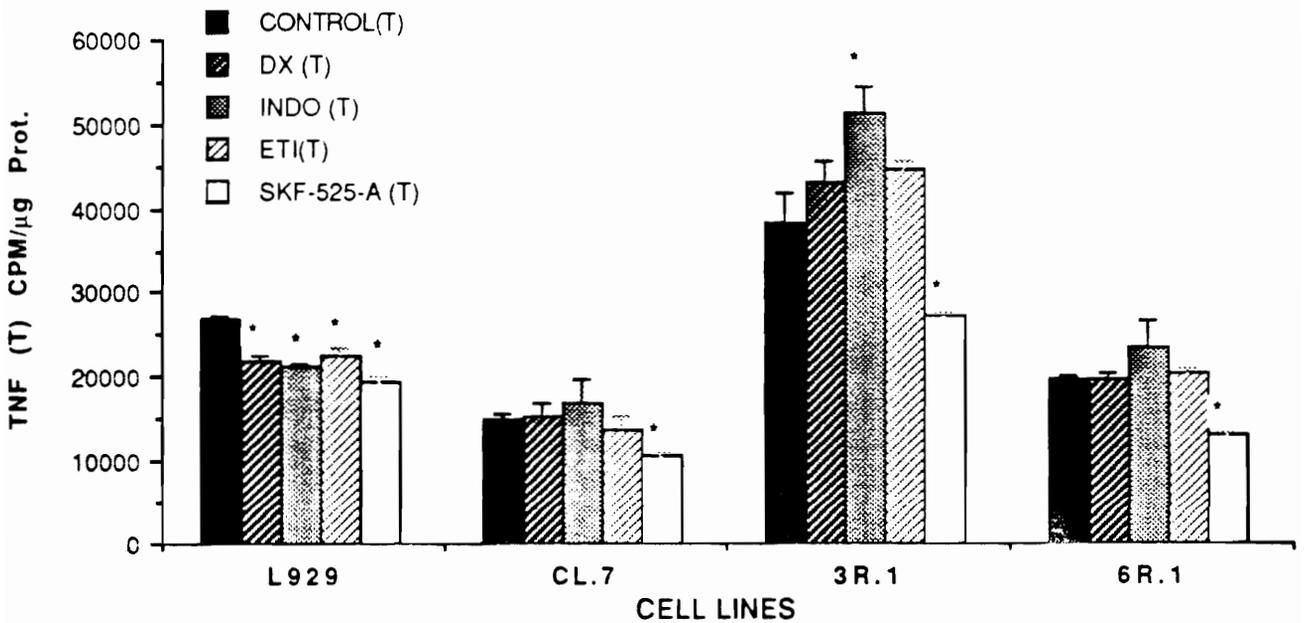


Figure 6 (a) : TNF-R Binding Modulation By Inhibitors of Arachidonic Acid Cascade. The TNF- α labelling and the cell surface binding was in absence and presence of the inhibitors as described in the methods section. The data is presented as mean \pm S.D of triplicate cultures. (* = $p < 0.05$). 10^4 cpm/ μ g protein equals 10.5 f moles/ μ g.

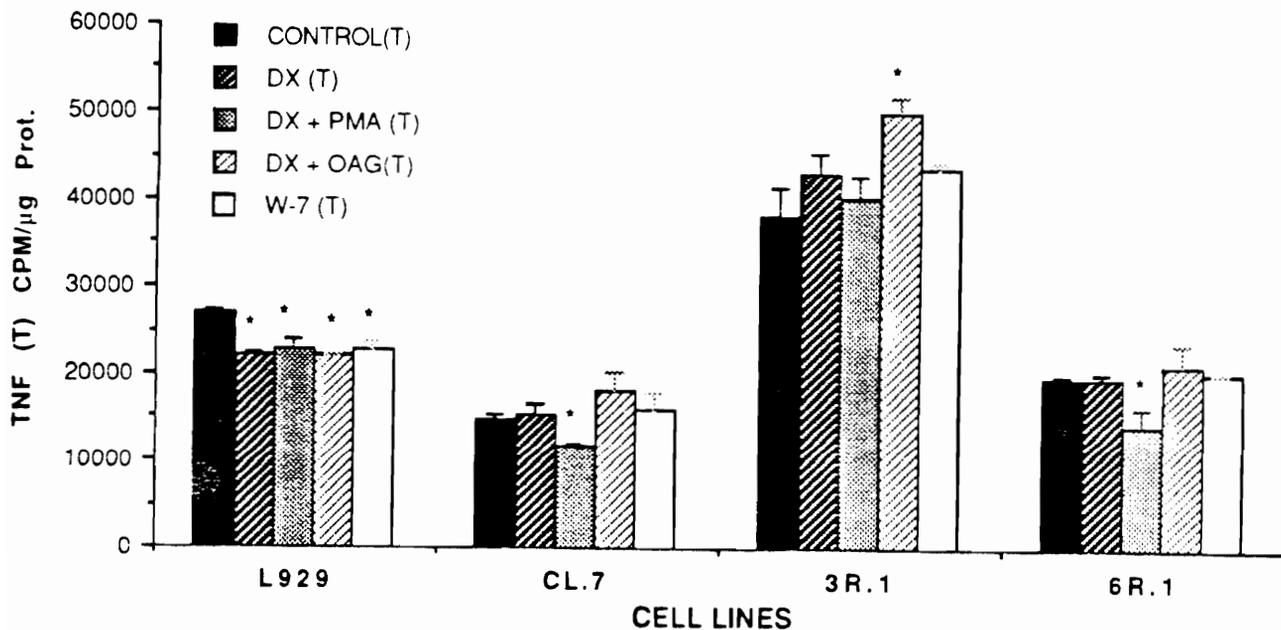


Figure 6 (b) : TNF-R Binding Modulation By Inhibitors of Arachidonic Acid Cascade And PK-C The TNF- α labelling and the cell surface binding was in absence and presence of the inhibitors as described in the methods section. The data is presented as mean \pm S.D of triplicate cultures. (* = $p < 0.05$). 10^4 cpm/ μ g protein equals 10.5 f moles/ μ g.

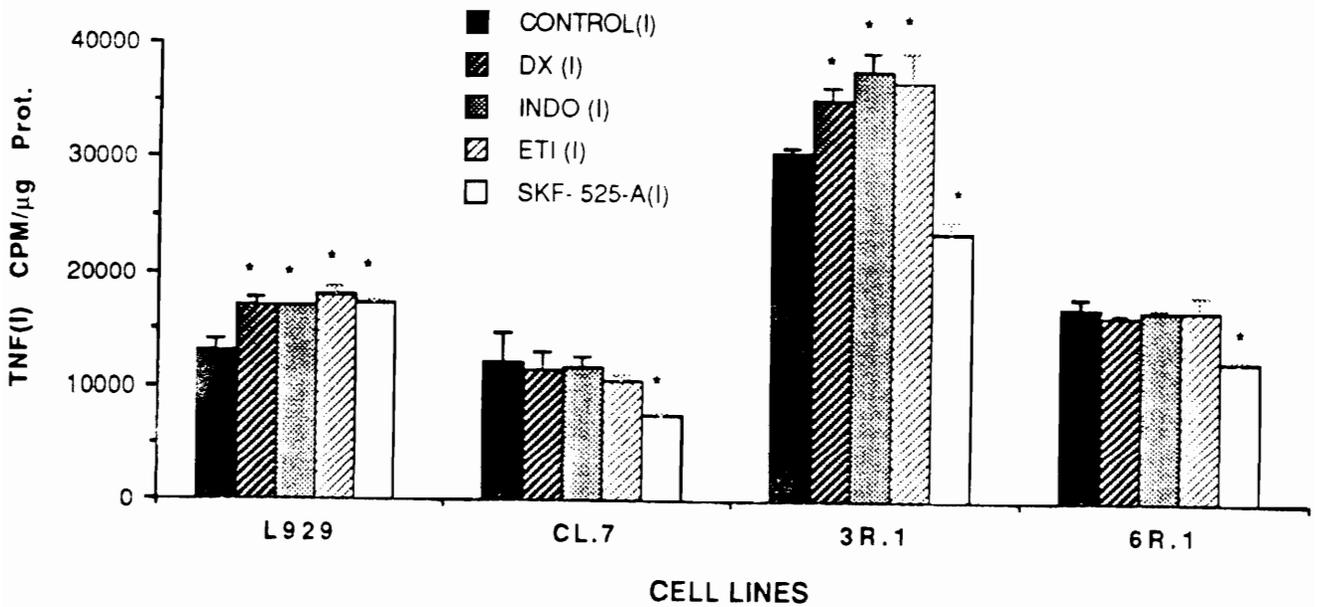


Figure 7 (a) : Role of Arachidonic Acid Cascade in TNF- α Internalization Under A-MuLV Transformation. An Inhibitor Study. The TNF- α labelling and the internalization assay was in absence and presence of the inhibitors as described in the methods section. The data is presented as mean \pm S.D of triplicate cultures. (* = $p < 0.05$). 10^4 cpm/ μ g protein equals 10.5 f moles/ μ g.

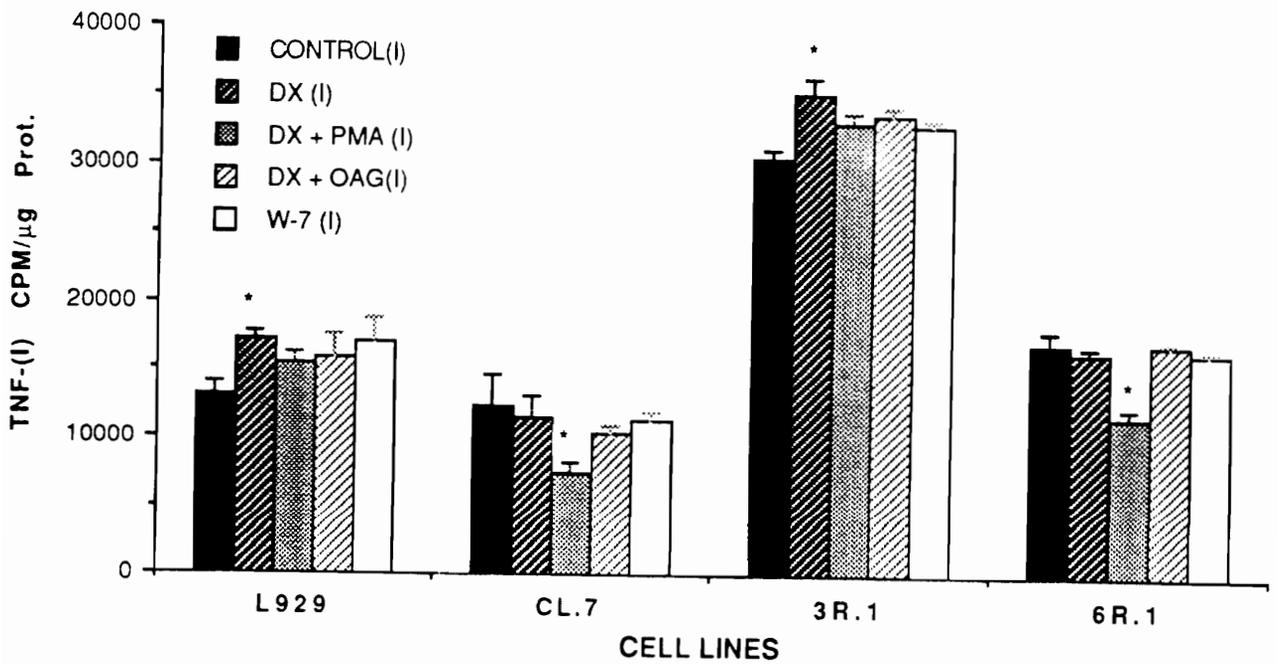


Figure 7 (b) : Role of Arachidonic Acid Cascade And PK-C Modulators In TNF- α Internalization Under A-MuLV Transformation. An Inhibitor Study. The TNF- α labelling and the internalization was in absence and presence of the inhibitors as described in the methods section. The data is presented as mean \pm S.D of triplicate cultures. (* = $p < 0.05$). 10^4 cpm/ μ g protein equals 10.5 f moles/ μ g.

TABLE-1
EGF & TNF- α Binding With Modulators Of Arachidonic Acid
Cascade And Protein Kinase - C

| GROUP | EGF | | | | TNF | | | | | | | |
|-----------|-----|---|---|---|-------|---|---|---|--------------|---|---|---|
| | | | | | TOTAL | | | | INTERNALIZED | | | |
| | 9 | 7 | 3 | 6 | 9 | 7 | 3 | 6 | 9 | 7 | 3 | 6 |
| DEXAMET | ↑ | | | ↓ | ↓ | | ↑ | | ↑ | | ↑ | |
| INDOMET | | | | | ↓ | ↑ | ↑ | ↑ | ↑ | | ↑ | |
| ETI | | | | ↓ | ↓ | | ↑ | | ↑ | | ↑ | |
| SKF-525A | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↑ | ↓ | ↓ | ↓ |
| OAG | | | | ↓ | ↓ | ↑ | ↑ | | | | ↑ | |
| PMA | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↑ | ↓ | ↓ | ↓ |
| H-7 | ↑ | | | ↑ | ↓ | | | | ↑ | | | |
| H-7 + PMA | ↑ | | | ↑ | ↓ | | | | ↑ | | | |
| DEX + PMA | ↑ | ↓ | | ↓ | ↓ | ↓ | | ↓ | ↑ | ↓ | ↑ | ↓ |
| DX + OAG | | | | ↓ | ↓ | ↑ | ↑ | ↑ | ↑ | | ↑ | |
| W-7 | | | | ↓ | | ↑ | | ↑ | ↑ | | ↑ | |

The cell lines were renumbered as follows : 9 : L929; 7 : CL.7; 3 : 3R.1; and 6 : 6R.1 respectively. The arrows represent significant differences over control at $p < 0.05$.

CHAPTER - 7**EFFECT OF LIPID SOLUBLE ANTIOXIDANTS ON MODULATION OF TNF- α INDUCIBLE, EGF-R EXPRESSION UNDER A-MuLV TRANSFORMATION****ABSTRACT**

The ability of cytokines like TNF- α and lipid soluble antioxidants like α -tocopherol, β -carotene, and dimethyl sulfoxide (DMSO) in modulation of EGF-R expression under a malignant transformation system was studied. In our transformation system, murine embryonic fibroblast BALB/c CL-7 was the untransformed cell line and BALB/c A-MuLV 3R.1 and BALB/c A-MuLV 6R.1 were the transformed clones. The objective of this investigation was to determine whether the TNF- α inducible EGF-R could be modulated using the antioxidants like vitamin A, vitamin E and DMSO. The EGF-R was expressed in terms of the amount of radio-labelled EGF retained on the cells as cpm/ μ g protein. Examination of protective effects of lipid soluble antioxidants on cytotoxicity of TNF- α showed that, Vitamin A (1 mM) and Vitamin E (0.1 mM and 1.0 mM) reduced the TNF- α cytotoxicity to L929 cells ($p < 0.05$). Vitamin A (1 mM) suppressed the EGF-R expression both in presence and absence of TNF- α for CL.7 and 3R.1 clones ($p < 0.05$). In the 6R.1 clone vitamin E (1 mM) enhanced the TNF- α inducible EGF-R expression ($p < 0.05$). This enhancement was synergistic with TNF- α induction of EGF-R. Vitamin A suppressed the TNF- α inducible 6R.1 EGF-R significantly ($p < 0.05$). This investigation showed that lipid soluble antioxidant vitamin A elicited an EGF-R suppression, independent of the A-MuLV transformation-induced repression or enhancement and the TNF- α - induced EGF-R expression. This finding was promising as it established the potential of vitamin A, under a complex interactive picture, as an anticancer agent. Vitamin E on the other hand was found to facilitate the binding of EGF to EGF-R. Although vitamin A and vitamin E previously had been shown to be potential anticancer

agents, vitamin A, due to its EGF-R suppressing property, could be an appropriate therapeutic agent for epidermoid cancers known to possess a higher level of EGF-R. Thus modulation of EGF-R in A-MuLV transformed clonal fibroblasts by vitamin A and vitamin E suggests a potential role of such lipid soluble antioxidants in prevention of progression of forms of cancers that depend on a highly expressed EGF-R levels on their cell surface.

INTRODUCTION

EGF and Its Receptor:

Epidermal growth factor (EGF) provides measurable growth-enhancing activity in vivo and is known to stimulate growth of multitude of cells in culture (Carpenter and Cohen, 1979). EGF possesses a variety of biological activities. In cell culture systems EGF enhances the rate of hexose transport (Barnes and Colowick, 1976), increases DNA synthesis (Carpenter and Cohen, 1979) and stimulates rapid morphological changes (Chinkers et al., 1979, 1981). Cell surface receptors specific for EGF are found on most cell types including cells of the epidermal origin (Carpenter and Cohen, 1979). The EGF-specific high affinity binding sites usually range from 10^4 to 10^6 /cell. Upon addition of EGF to cells, EGF binds its receptor (EGF-R) and the EGF-EGF-R complex gets internalized (Carpenter and Cohen, 1976). Post internalization events include degradation of both the receptor (Das and Fox, 1978) and the hormone (Carpenter and Cohen, 1976) by lysosomal proteases. Clustering of receptors prior to binding of EGF at the cell surface is probably required for EGF to elicit its biological response (Schechter et al., 1979, King et al., 1980a). EGF-R levels in cells treated with EGF reach a lower steady state, indicative of a dynamic equilibrium between internalization and re-expression of EGF-R on cell surface. Replenishment of the constitutive EGF-R levels after EGF mediated internalization is known to take several hours (Carpenter and Cohen, 1976).

The EGF-R is a 180 K polypeptide initially identified by specific labeling with a photoreactive derivative of EGF (Das et al., 1977). Subsequently EGF-R was affinity purified from the membrane component of a human epithelioid tumor cell line A431 on an EGF column (Cohen et al., 1980) and had molecular weights ranging from

150-170 K. The EGF receptor in the crude plasma membrane fraction as well as in its purified state exhibits an associated protein kinase activity which is stimulated with the addition of EGF (Carpenter et al. 1978, 1979; Cohen et al. 1980; King et al. 1980 b). EGF-R acts as an autokinase, phosphorylating the receptor itself. The phosphorylated residue is determined to be at a tyrosine (Ushiro and Cohen, 1980). EGF induced phosphorylation of EGF-R is one of the distinctive biochemical reactions which occur as an early event after addition of the ligand to target cells (Hunter et al., 1981). In cells, EGF enhances phosphorylation of EGF-R at tyrosine residue but far more predominately at serine and threonine residues as determined by phosphopeptide mapping (Hunter, et al., 1981). The phosphate acceptor sites on EGF-R enables regulation of the receptor mediated processes, including those mediating communication between EGF-R and other ligand receptor systems. To this end, there is ample evidence supporting the hypothesis for the regulatory existence of communication between hormone receptors and stimulation of mitogenesis. Platelet derived growth factor (PDGF) decreases EGF-R down regulation occurring in response to EGF (Wrann et al., 1980). Fibroblast growth factor (FGF) (Fox et al., 1979), vasopressin (Rozengurt, et al., 1981), phorbol esters active in tumor promotion (Brown, et al., 1979) and diacylglycerol (DAG) (McCaffrey, 1984) reduce EGF-R affinity in mitogenically responsive cells by binding to sites other than the EGF-R. Thus ligand binding to one receptor causes a second receptor to be modified. The modulation of EGF-R by PDGF, DAG, phorbol esters and FGF is shown in a cell culture system by a decreased binding of radiolabeled EGF to cultured cells. In contrast to phorbol esters and PDGF, estrogen (Mukku, et al., 1985) and transforming growth factor- β (TGF- β) (Assoian, et al., 1984) have been shown to increase EGF-R expression. The modulators of EGF-R could regulate the receptor mediated communication between EGF-R and other ligand receptor systems.

Tumor Necrosis Factor- α (TNF- α):

TNF- α , a 17 K polypeptide hormone, secreted by activated macrophages, has been known to elicit hemorrhagic necrosis of some tumors and cytotoxic to some tumor cell lines (Williamson et al., 1983). Apart from its cytostatic and cytotoxic action, TNF- α has

also been shown to be a potent growth promoting mitogen for normal human diploid fibroblasts (Vilcek et al., 1986). TNF- α stimulates the production of interleukin-1 (IL-1) (Kirstein and Baglioni, 1986) and β -interferon (Kohase et al., 1986) in human fibroblasts. Apart from the above effects TNF- α has also been shown to be a modulator of EGF-R expression (Palombella et al., 1987). The above authors showed a stimulation of growth of human FS-4 fibroblasts by recombinant human TNF- α (rh-TNF- α) with a concomitant increase in binding of EGF to these cells. Incubation with TNF- α resulted in 40-80% increase in the number of EGF-R sites with receptor protein synthesis demonstrable at around 2-4 hr following TNF- α treatment. Furthermore as TNF- α induced EGF-R expression followed a dose response relationship similar to that reported for the mitogenic stimulation of FS-4 fibroblasts it was concluded that the TNF- α induced EGF-R expression may be causal with the mitogenic action of TNF- α on human fibroblasts.

Production of malignant transformation at the cellular level manifests in the formation of tumors at the tissue levels. Studies from tumor registries show that approximately 90% of all tumors arise from epithelial cells (Savage et al., 1972; Matrisian et al., 1982). Such tumors of epithelial origin have altered cellular growth characteristics via modulation of cell surface growth factor receptor proteins like EGF-R. In fact, it has been known that many tumors, originating as epidermoid carcinomas, fibrosarcomas and astrocytomas, possess a high level of EGF-R on their cell surfaces (Fabricant et al., 1977). Agents that can alter EGF-R expression either at the transcription, translation or at post translational level have the potential to bring about changes in the growth properties, be it the normal process of self-renewal or the abnormal process of transformation. A lipid soluble antioxidant like vitamin A (β -carotene), has been known to suppress EGF-R via a protein kinase-C (PK-C) dependent mechanism (Jetten et al., 1986). Thus, vitamin A, via possibly, its antioxidant property (quencher of singlet oxygen 1O_2) and growth factor modulating property, could elicit anticancer effects. Yet another lipid soluble antioxidant, a nonspecific terminator of lipid peroxidative process, known for its anticancer properties is vitamin E (α -tocopherol). To date little is known about the effect of vitamin E on the cell surface expression of EGF-R. Both

vitamin A and vitamin E have been identified as important nutritional components with anticancer characteristics. Another anticancer agent is the immuno-modulatory pleotropic cytokine, TNF- α . TNF- α induces the expression of EGF-R (Palombella et al., 1987) via a PK-C independent pathway (Bird et al., 1989). It is important for one to appreciate that these myriad growth factor regulatory agents, act interactively and elicit their overall response in a very complex fashion.

The objective of the present study is to determine the overall effect of cytokines like TNF- α and lipid soluble antioxidants like α -tocopherol (scavenger of lipid radicals), β -carotene (scavenger of 1O_2) and dimethyl sulfoxide (DMSO) (scavenger of $\cdot OH$ radical) in modulation of EGF-R expression under a malignant transformation system. The transformation is effected with Abelson-murine leukemia virus (A-MuLV), a RNA tumor virus, which has been known to down regulate the EGF-R expression in its malignantly transformed clones (Blomberg et al., 1980). In our transformation system, murine embryonic fibroblast BALB/c CL-7 is the untransformed cell line and BALB/c A-MuLV 3R-1 and BALB/c A-MuLV 6R-1 are transformed clones capable of producing tumors in, immunodeficient, and immunodeficient and normal syngenic mice respectively (Patek et al., 1978). Unlike the claim of Blomberg et al., 1980, we found that the A-MuLV transformation does not necessarily result in lowering of the EGF-R expression. Whereas the clone 3R.1 had repressed EGF-R levels the clones 6R-1 had an enhanced EGF-R constitutive expression (chapter 3). TNF- α was able to induce EGF-R in all the three cell lines (chapter 3). The purpose of this investigation was to determine whether the TNF- α inducible EGF-R could be transmodulated using the antioxidants like vitamin A, vitamin E and DMSO.

MATERIALS AND METHODS

Cell Culture:

Mouse fibrosarcoma L929, normal mouse fibroblast CL-7, and two A-MuLV transformed mouse fibroblast cell lines 3R-1 and 6R-1 were grown in Dulbeccos modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) with 10 units/ml penicillin and 10 $\mu g/ml$

streptomycin with 25 mM HEPES at pH 7.4 at 5% CO₂ in a humidified 37°C incubator.

Reagents:

Mouse TNF- α was produced from the LPS activated mouse monocyte-macrophage cell line RAW 264.7 and immunopurified on an anti TNF- α antibody column as described (chapter 1). The specific activity of mouse TNF- α was determined by a L929 cytotoxicity bioassay (Larrik et al 1989) to be 5×10^5 units/mg protein. EGF was procured from Sigma Chemical Co. Na¹²⁵I was purchased from New England Nuclear.

Radio-Iodination of EGF:

EGF was iodinated by a modification of the method described (Carpenter et al., 1976). Briefly 250 μ Ci of Na¹²⁵I was mixed with 10 μ l of (0.5 mg/ml) EGF in 0.05 M kPi, pH 7.4 along with 5 μ l of 0.5 mg/ml chloramine-T (Sigma Chemical Co.). This soft labelling was allowed to proceed for 1.5 minutes after which the reaction was stopped with 20 μ l of (0.5 mg/ml in 0.05 M kPi, pH 7.5) sodium metabisulphite (MCB Co.). To the reaction system 2.5 ml of kPi, pH7.5, was added to 1 ml of 1% BSA. The mixture was loaded onto a pre-equilibrated AG1-X8 anion exchange column and the eluate collected in a tube containing 1 ml of 1% BSA in kPi, pH 7.4 EGF was determined to be iodinated to a specific activity of 6×10^4 cpm/ng protein.

¹²⁵I-EGF Binding Assay:

Cells were seeded in 24 well plates (1×10^5 cells/well) in six replicates in complete medium. Controls and treatment groups containing TNF- α (100 units/ml), α -tocopherol (0.5 mM and 1.0 mM), β -carotene (0.1, 0.5 and 1.0 mM) and DMSO (100 and 500 mM) were washed with ice cold DMBM and incubated with 15 ng/ml ¹²⁵I-EGF for 4 h at 4°C. Subsequently the plates were washed 4 X with ice cold DMEM. The cells were solubilized in 1 ml of 0.1 N NaOH with 0.1% Triton-X 100 and radioactivity counted using a Beckman 5500 gamma counter. The protein levels in the sample were determined using the Bradford dye binding assay (Bradford, 1976).

TNF- α Cytotoxicity Assay:

The TNF- α cytotoxicity assay was performed as described (Larrik et al., 1989).

Statistical Analysis : The statistical analysis was performed by a paired t test comparison. The probability of $p \leq 0.05$ was taken to be significant. The results were expressed as mean \pm S.D of six replicate readings.

RESULTS

TNF- α cytotoxicity assay was performed in 96 well plates using L929 cells. 50 μ l of 1×10^6 cells/ml was plated with 50 μ l media or 50 μ l media supplemented with vitamin A (0.1 and 1.0 mM final concentration), vitamin E (0.1 and 1.0 mM final concentration), or DMSO (50, 100, and 500 mM final concentration). The cytotoxicity assay was performed for 18 hr in the presence of 1 μ g/ml actinomycin D (sensitizer) with 100 units/ml immunopurified mouse TNF- α . Examination of protective effects of lipid soluble antioxidants on cytotoxicity of TNF- α on L929 cells showed that, among vitamin E, vitamin A and DMSO, at the concentrations tested, vitamin E was the most important agent in blocking the cytotoxic action of TNF- α (Figure 1). Vitamin A (1 mM) and Vitamin E (0.1 mM and 1.0 mM) reduced the TNF- α cytotoxicity ($p < 0.05$). The data showed that both vitamin A and vitamin E at 1 mM final concentration block the TNF- α cytotoxicity ($p < 0.05$). DMSO inhibited the toxic effects TNF- α but the inhibition was not significant at $p < 0.05$.

As vitamin A and vitamin E at 1 mM final concentration were shown to inhibit the TNF- α cytotoxicity to L929 cells, it was hypothesized that these lipid soluble antioxidants could possibly have a modulating effect on the TNF- α induced EGF-R induction (Pallombella et al. 1987). TNF- α was shown to induce EGF-R expression in CL-7, 3R-1 and 6R-1 cell lines (Chapter 3). Effect of vitamin A, vitamin E and DMSO on inducibility of EGF-R by TNF- α were determined (TABLE 1). The constitutive (control) expression of EGF-R in L929, CL.7, 3R.1, and 6R.1 showed that the clonal EGF-R in

3R.1 cells was depressed whereas its levels in 6R.1 cells was enhanced. L929 cells had little expression of EGF-R. Incubation of the clones with 100 units/ml TNF- α significantly enhanced the EGF-R in all the clones. TNF- α (100 units/ml) incubation with vitamin E (0.1 and 1.0 mM) resulted in an increase in ^{125}I -EGF binding in 6R.1 as well as CL.7 clones. TNF- α (100 units/ml) incubation with vitamin A (0.1 and 1.0 mM) resulted in a marked inhibition of ^{125}I -EGF binding in all the clones. DMSO at 500 mM markedly inhibited the TNF- α EGF-R in 6R.1, 3R.1, and L929 cells. As shown in Figure 2, vitamin E (1 mM) had a little enhancing effect on the TNF- α induction of EGF-R in the CL.7 clone, whereas vitamin A (1 mM) suppressed the EGF-R expression both in presence and absence of TNF- α (TABLE 1, $p < 0.05$). Similar results were obtained for 3R.1 clone (Figure 3). In the 6R.1 clone (Figure 4) vitamin E (1 mM) enhanced the TNF- α inducible EGF-R expression ($p < 0.05$). Vitamin A on the other hand suppressed the TNF- α inducible EGF-R ($p < 0.05$) in 6R.1 clone. From TABLE 1 it was clear that in 6R.1 clone, vitamin E (0.5 mM) enhanced the EGF binding to its receptor ($p < 0.05$). This enhancement was synergistic with TNF- α induction of EGF-R. Vitamin A (0.5 mM and 1 mM) in presence of TNF- α , suppressed EGF binding significantly in CL.7, 3R.1 and 6R.1 clones. However in L929 cells, vitamin A surprisingly enhanced the binding of ^{125}I -EGF in presence and absence of TNF- α . DMSO had little effect on ^{125}I -EGF binding at 100 mM concentration, however, at 500 mM, it inhibited EGF binding significantly in all the cell lines.

DISCUSSION

It is known that vitamin A suppresses EGF-R levels via phosphorylation of EGF-R (Jetten et al., 1986). TNF- α induces EGF-R levels in human FS-4 fibroblasts via a PK-C independent pathway. The present investigation showed that the lipid soluble antioxidants like vitamin A elicit their response of EGF-R suppression, independent of A-MuLV transformation induced repression or enhancement and/or cytokine, like TNF- α , induced EGF-R expression. This finding is promising because this establishes the potential of vitamin A, under a complex interactive picture, as an anticancer agent. Vitamin A could, possibly via growth factor receptor expression modulatory property, elicit its anticancer effects on

those tumors that express an enhanced EGF-R. Vitamin E on the other hand was found to facilitate binding of EGF to EGF-R. Although vitamin A and vitamin E have been shown to be potential anticancer agents, vitamin A could particularly be useful against cancers of epidermoid origin, which incidently have been known to possess a higher level of EGF-R levels. Thus, in summary, lipid soluble antioxidants could possibly have a modulating effect on the TNF- α induced EGF-R induction and modulation of EGF-R in A-MuLV transformed clonal fibroblasts by vitamin A and vitamin E suggests a potential role of these vitamins in prevention of progression of forms of cancers that depend on a highly expressed EGF-R levels on their cell surface.

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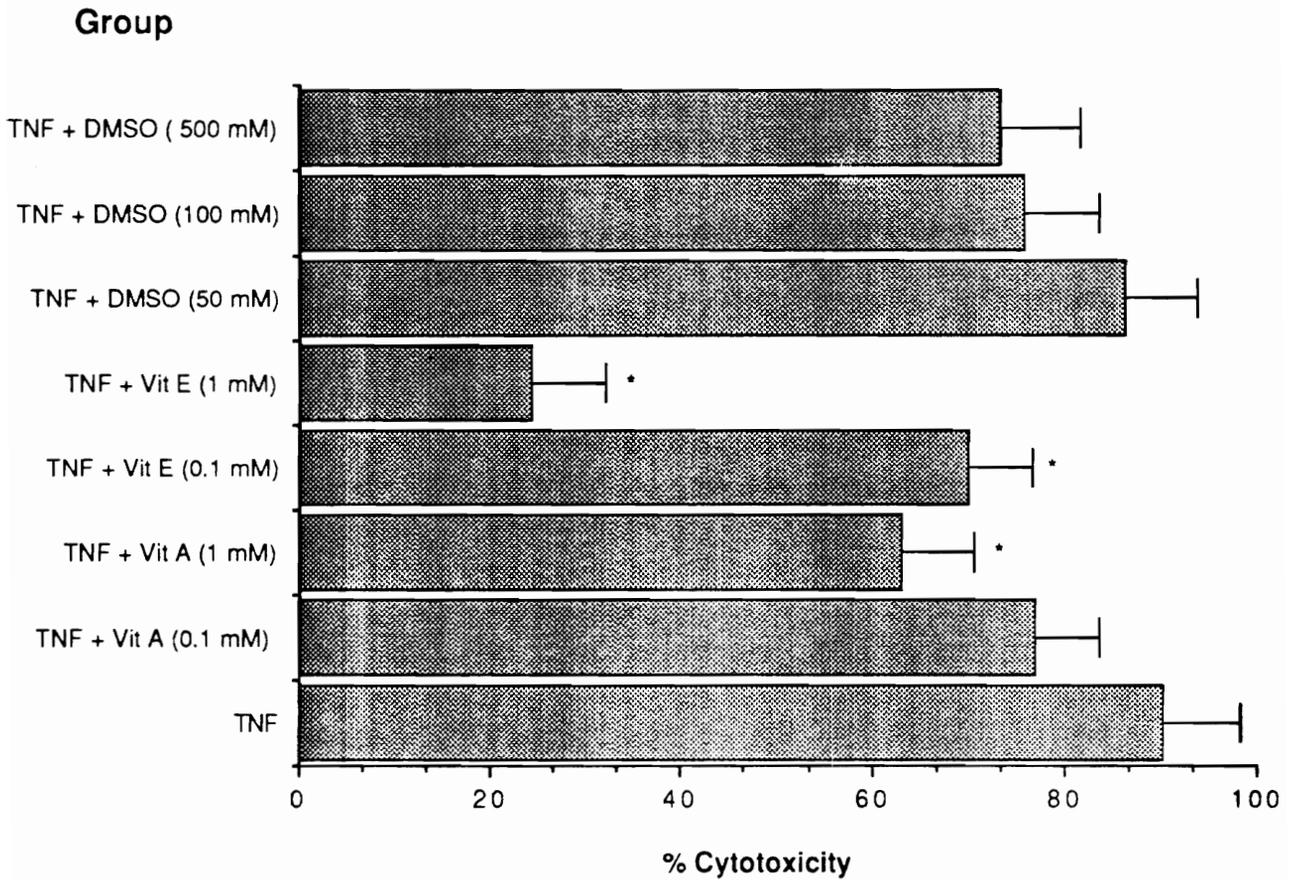


Figure 1 : Protection From TNF- α Cytotoxicity To L929 Cells By Lipid Soluble Antioxidants. The Cytotoxicity assay was performed in presence of vitamin E, vitamin A, and DMSO at the above mentioned concentrations. Vitamin E as well as vitamin A at 1 mM concentration inhibited the TNF- α cytotoxicity significantly ($p < 0.05$). The % cytotoxicity is presented as mean \pm S.D of six replicate well readings. (* = $p < 0.05$)

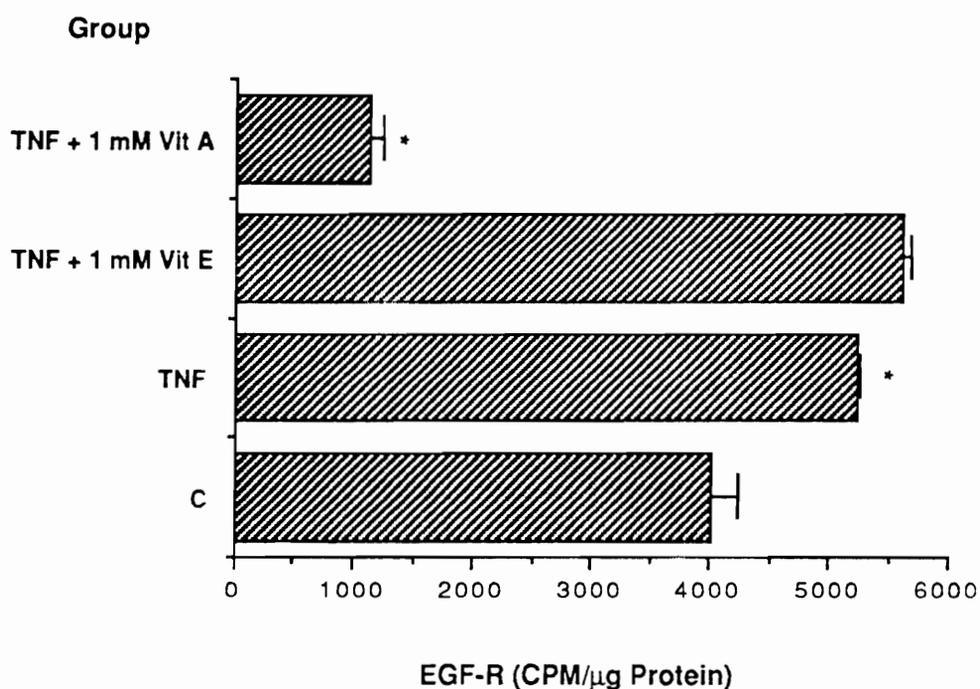


Figure 2 : EGF-R Modulation of CL.7 by TNF- α in Conjunction With vitamin A and vitamin E. The binding of 125 I- EGF was performed after treatment with TNF- α (100 units/ml) in presence or absence of vitamin A and vitamin E at the above concentrations. The results are expressed as cpm/ μ g protein of six replicates. TNF- α induces the EGF-R expression significantly ($p < 0.05$). Vitamin E induces EGF-R levels synergistically with TNF- α whereas vitamin A inhibits TNF- α inducible EGF-R expression. (* = $p < 0.05$)

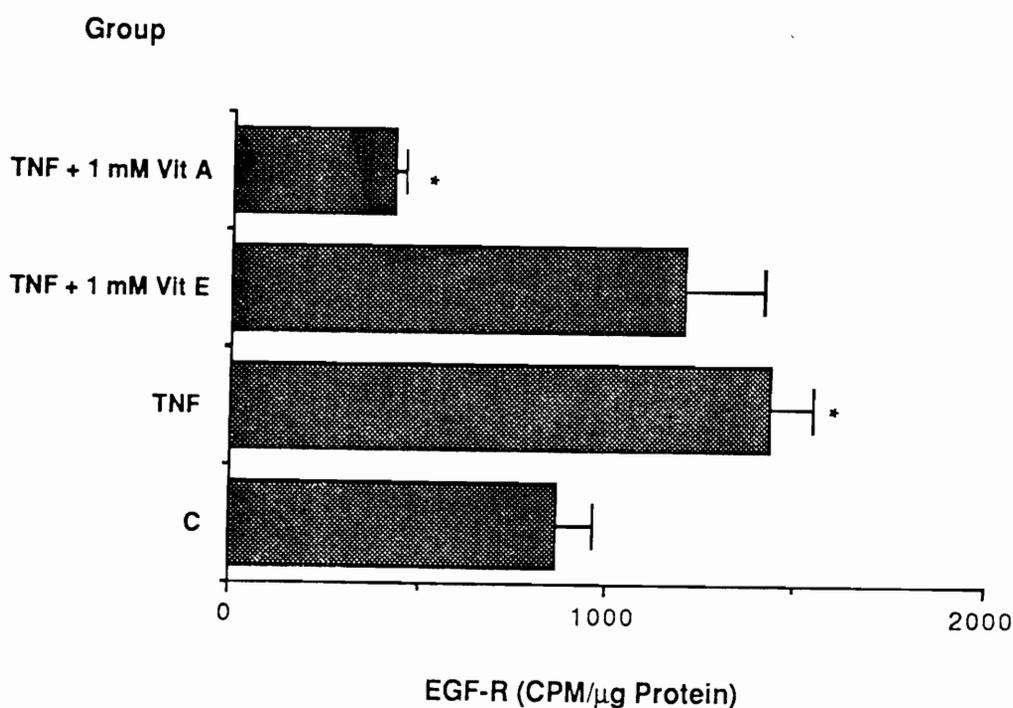


Figure 3 : EGF-R Modulation of 3R.1 by TNF- α in Conjunction With vitamin A and vitamin E. The binding of 125 I- EGF was performed after treatment with TNF- α (100 units/ml) in presence or absence of vitamin A and vitamin E at the above concentrations. The results are expressed as cpm/ μ g protein of six replicates. TNF- α induces the EGF-R expression significantly ($p < 0.05$). Vitamin E induces EGF-R levels synergistically with TNF- α whereas vitamin E inhibits TNF- α inducible EGF-R expression. (* = $p < 0.05$)

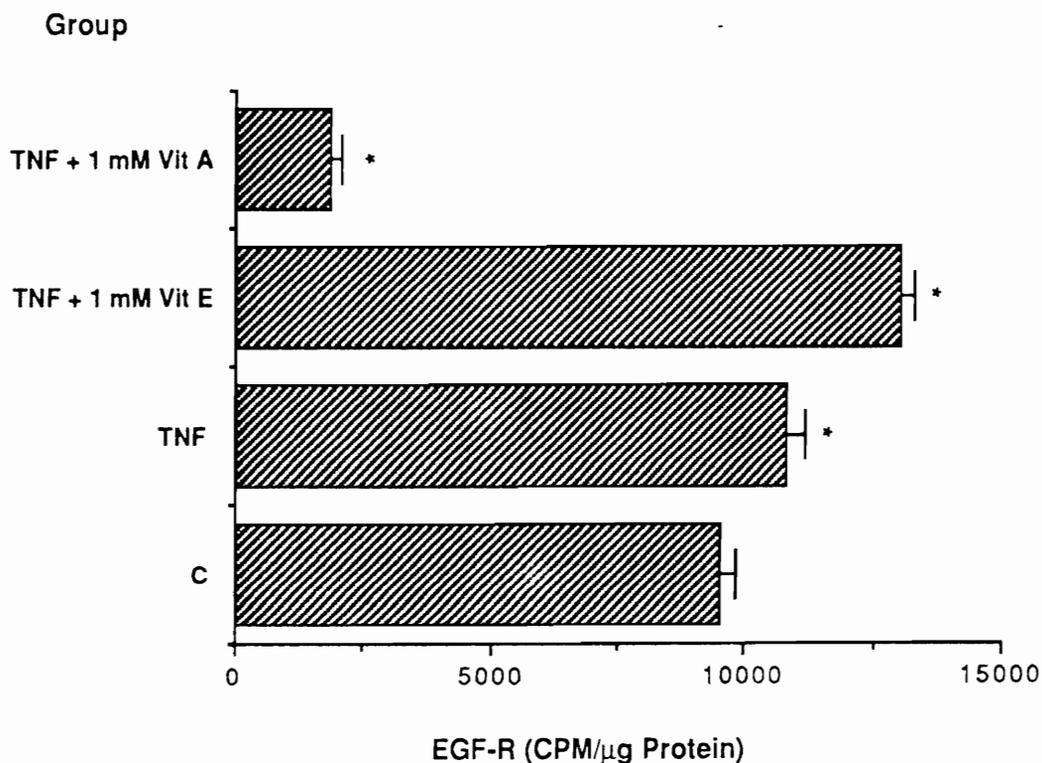


Figure 4 : EGF-R Modulation of 6R.1 by TNF- α in Conjunction With vitamin A and vitamin E. The binding of 125 I- EGF was performed after treatment with TNF- α (100 units/ml) in presence or absence of vitamin A and vitamin E at the above concentrations. The results are expressed as cpm/ μ g protein of six replicates. TNF- α induces the EGF-R expression significantly ($p < 0.05$). Vitamin E induces EGF-R levels synergistically with TNF- α whereas vitamin E inhibits TNF- α inducible EGF-R expression. (* = $p < 0.05$)

TABLE - 1

Modulation Of EGF-R Expression With TNF- α And Lipid Soluble Antioxidants

| Group | EGF-R (CPM μ g Protein) | | | |
|--------------------|-----------------------------|----------------|----------------|-----------------|
| | L929 | CL.7 | 3R.1 | 6R.1 |
| C | 665 \pm 39 | 4020 \pm 216 | 867 \pm 100 | 9530 \pm 300 |
| TNF | 705 \pm 44 | 5239 \pm 38 | 1431 \pm 117 | 10803 \pm 381 |
| C + 0.5 mM Vit E | 697 \pm 40 | 4035 \pm 40 | 679 \pm 93 | 11518 \pm 141 |
| TNF + 0.5 mM Vit E | 687 \pm 20 | 5018 \pm 170 | 1046 \pm 76 | 12639 \pm 467 |
| TNF + 1.0 mM Vit E | 678 \pm 104 | 5629 \pm 56 | 1201 \pm 210 | 13027 \pm 269 |
| C + 0.1 mM Vit A | 1332 \pm 178 | 3952 \pm 111 | 808 \pm 50 | 8114 \pm 306 |
| TNF+ 0.1 mM Vit A | 1501 \pm 36 | 5906 \pm 186 | 793 \pm 23 | 8812 \pm 135 |
| TNF + 0.5 mM Vit A | 1723 \pm 47 | 1746 \pm 99 | 535 \pm 10 | 2896 \pm 263 |
| TNF + 1.0 mM Vit A | 3031 \pm 204 | 1123 \pm 122 | 428 \pm 25 | 1867 \pm 200 |
| C + 100 mM DMSO | 598 \pm 5 | 3552 \pm 85 | 1219 \pm 116 | 9902 \pm 155 |
| TNF + 100 mM DMSO | 608 \pm 53 | 4024 \pm 111 | 1025 \pm 126 | 9654 \pm 309 |
| TNF + 500 mM DMSO | 587 \pm 37 | 2633 \pm 51 | 820 \pm 84 | 7137 \pm 164 |

10⁴ cpm / μ g protein = 27.6 f moles EGF/ μ g protein.

CHAPTER - 8**TNF- α MODULATES TYROSINE PHOSPHORYLATION OF
A-MuLV SPECIFIC CELL SURFACE ANTIGEN****ABSTRACT**

V-abl, a viral oncogene contained in the, replication-defective, transformation inducing, Abelson-murine leukemia virus (A-MuLV) codes for the transmembrane protein p 120 with tyrosine kinase activity. The p 120 polyprotein is known to be essential for the transforming capacity of the virus. Recently we determined that tumor necrosis factor- α (TNF- α), is not cytotoxic to BALB/c mouse embryonic fibroblast CL.7 and it's A-MuLV transformed clone 3R.1 but is cytotoxic to another A-MuLV clone of CL.7 namely 6R.1 (Chapter 1). TNF- α , shown to modulate the tyrosine phosphorylation of EGF-R, was investigated whether the differential toxicity of TNF- α to the A-MuLV transformed clones was due to differences in the content of the *V-abl* protein product, p120, on the cell surface and whether TNF- α elicited its response by a differential transmodulation of tyrosine phosphorylation of oncogenic protein p120. Antibodies raised against the membrane domains of p120 (ie. against the gag gene products p15 and p30) were used along with antibody against the triton-disrupted virus to quantitate the cell surface viral antigen. Binding of control, anti-p15, anti-p30 and anti-A-MuLV (triton-disrupted) goat antibodies to CL.7, 3R.1 and 6R.1 cells grown on glass coverslips showed that the binding of anti-triton-disrupt A-MuLV antibody was higher for transformed cells than the normal (CL.7) cells ($p < 0.05$). Cellular phosphotyrosine levels were determined by use of radio-labelled anti-phosphotyrosine antibody. Quantitation of phosphotyrosine levels for CL.7, 3R.1 and 6R.1 cells with TNF- α incubation showed that CL.7 and 3R.1 had non-inducible phosphotyrosine levels, whereas cellular phosphotyrosine levels for the TNF- α sensitive cell line (6R.1) was inducible with incubation time of TNF- α , maximally induced around 8

hours. The expression of A-MuLV gene product remained unaltered with TNF- α incubation time. The expression of equivalent amount of A-MuLV surface antigen and its non-inducibility with TNF- α for 3R.1 and 6R.1 cell lines, taken together with the fact, that cellular phosphotyrosine levels were inducible in 6R.1 cell line but were not inducible for 3R.1 cell line suggested that 3R.1 would be a p120 kinase mutant whereas 6R.1 had a p120 kinase activity. The level of, p120 specific, kinase activity was determined by an antigen capture assay. The inducibility of p120 phosphotyrosine was determined with TNF- α incubation time. The 6R.1 clone possessed an inducible phosphotyrosine whereas 3R.1 had a transient phosphotyrosine induction found to be maximal around 2hr of TNF- α incubation. CL.7 and 3R.1, that were not sensitive to TNF- α , had little induction of p120 phosphotyrosine whereas 6R.1 (the TNF- α sensitive cell line) had an inducible phosphotyrosine observed to be maximal around 8hr of TNF- α incubation. The p120 tyrosine phosphorylation, not known to be sensitive to any ligand, was shown in this investigation to be sensitive to the cytokine TNF- α .

INTRODUCTION

Abelson murine leukemia virus (A-MuLV) is a replication-defective transformation inducing retrovirus, which induces B-cell lymphomas in vivo (Potter et al 1973; Premkumar et al 1975) and is capable of transforming cells of lymphoid and fibroblastic origin in vitro (Rosenberg, et al. 1980). The genomic content of A-MuLV virus is a hybrid consisting of about 25% of Moloney-murine leukemia virus (M-MuLV) with an associated 3.6 kb. insert (homologous to normal mouse cell DNA) (Shields, et al 1979). Only a single translation product of the A-MuLV genome called p120 has been identified (Witte, et al 1978) in the prototype A-MuLV strain. The p120 fusion protein consists of the N-terminal segment of M-MuLV gag protein (30 kd) and a 90 kd oncoprotein of non-viral origin (Witte, et al 1978, Reynolds, et al 1978). As p120 is the only protein involved in the viral transformation, it is assumed that p120 is the transforming protein (Rosenberg, et al 1980). It is a transmembrane protein located on the cell surface (Witte et al 1980). Anti-sera raised against the non-M-MuLV portion of the viral translation product react with the external surface of the membrane of A-MuLV transformed cells, thereby proving that the extracellular

domain of p120 is of nonviral origin (Witte et al 1979). Moreover anti-M-MuLV sera do not react with the surface of A-MuLV transformed cells suggesting that the M-MuLV specific determinant of P120 is towards the cytosol. The 30 kd determinant consists of p15, p12 and a small part of p30 proteins of the gag structural genes (Witte, et al 1978). The M-MuLV unrelated extracellular component is encoded by the *abl* gene. The mouse cell homologue of the V-*abl* is called *C-abl*. *C-abl* is a gene reported to contain a large number of intervening sequences (IVS) with multiple transcripts (Eva, et al 1982). 5' and 3' end specific probes for *C-abl* have been used to provide direct genetic evidence that only the proximal end of *abl* gene with its 5' helper viral sequence is required for fibroblast transformation (Srinivasan, et al 1982). The transmembrane p120 is a phosphoprotein, phosphorylated at a tyrosine residue (Witte et al, 1980) and in many respect resembles the sarcoma viral phosphoprotein. The A-MuLV, like sarcoma virus, selectively transform cells of hematopoietic origin specifically the pre B cells as well as fibroblasts in culture (Scher, et al 1975). Like p120, the sarcoma viral protein, pp 60^{src}, is a membrane associated, tyrosine-specific protein kinase, whose enzymatic activity is thought to be necessary for transformation (Collett et al 1978, Hunter et al 1980). But whereas pp60^{src} is a phosphotransferase, p120 is not yet known to have that enzymatic function. Nonetheless the leukemogenic potential of A-MuLV and the sarcomagenic potential of Rous sarcoma virus suggest that there could be a common basis for the infectivity of both RNA and DNA tumor viruses.

V-*abl* was first described as the oncogene contained in A-MuLV and was shown to code for the transmembrane protein with tyrosine kinase activity which is essential for the transforming capacity of the virus (Prywes et al 1983). The normal cellular counterpart, the *C-abl* proto-oncogene, also encodes a protein with tyrosine kinase activity (Konopka and Witte 1985). *C-abl* is a single copy gene located on the long arm of chromosome 9 in humans and on chromosome 2 in mice and codes for the *C-abl* protein having a molecular mass of 140 kd in humans and 150 kd in mouse (Witte et al 1979). The N-terminal region of *C-abl* protein might also be functionally important in interacting with other cellular proteins (Figure-1) via it's tyrosine kinase activity. It is known that cells respond to external stimuli via a signal transduction across the cell membrane. Surface receptors with specific ligand binding domains

accomplish the task of recognizing individual stimuli. In the amplification and tight regulation of the signal transduction pathway, three kinds of protein kinases are involved. One, the Ca^{2+} /phospholipid-dependent diacylglycerol-regulated protein serine kinases (protein kinase C). Second, the cAMP-dependent protein kinase system which however has not been known to be directly involved in cell growth control or transformation. Third, the growth factor receptor protein-tyrosine kinases. There are at least five growth factor receptors which have protein-tyrosine kinase activities that are stimulated several fold upon binding their cognate ligands. They are the epidermal growth factor receptor (EGF-R), the platelet-derived growth factor receptor (PDGF-R), the colony-stimulating factor-1 receptor (CSF-1R), the insulin receptor (I-R), and the insulin-like growth factor-1 receptor (IGF-1R) (Heldin and Westermark 1984; Hunter and Cooper, 1985; Sherr, et al. 1985). The structure of CSF-1R, EGF-R and that of PDGF-R are very similar although they are different from that of I-R and the IGF-1R which are known to possess $(\alpha\beta)_2$ structure. Nonetheless, a number of general principles apply to the growth factor receptor protein-tyrosine kinases. Enhancement of their phosphotransferase activities is very rapid, following ligand binding, and in every case an early event is auto-phosphorylation and a subsequent enhancement of receptor affinity for the ligands. This receptor class clusters rapidly after ligand binding, is internalized via coated pits into endosomes, and then elicits its mitogenic action in not-so-well understood ways. The importance of protein-tyrosine kinases is all the more significant not only from the point of view of being associated with receptors that induce mitogenic growth but also being associated with several oncogenic translation products known to implicate transformation and unrestricted growth. Many oncogenes are believed to have evolved from growth factor receptor protein-tyrosine kinase genes. The *V-erb* B gene of avian erythroblastosis virus (AEV) has been determined to have arisen from the EGF-R gene (Ulrich et al 1984) while the *V-fms* gene was probably derived from the CSF-1R gene (Sherr et al 1985) etc. All of these oncogenic proteins may transform cells by delivering a continuous and unregulated mitogenic signal which overwhelms the tyrosine phosphorylation pathway. There seems to be a number of principles at work in the oncogenic activation of growth factor receptor genes. The loss of the putative N-terminal extracellular binding domain is

common to the *V-erb B*, *V-ros* and *trk* proteins. This necessarily makes the receptors unable to respond to ligand, although it is unclear whether the absence of a binding domain will put the receptor protein-tyrosine kinase into an activated or simply an unregulated state. The loss of C-terminal sequences is observed for the *V-erb B* protein. This loss removes the major auto-phosphorylation site, and thereby possibly relieves the inhibitory effect of this C-terminal domain. In contrast to the other receptor-related oncogene products, the *neu* oncogene product is very similar in structure to its normal cellular counterpart. In this case a simple point mutation which introduces a charged residue into the transmembrane domain appears to activate the protein-tyrosine kinase, possibly by mimicking ligand occupancy.

Despite the strong indications that the oncogenic protein products induce transformation through an unregulated tyrosine phosphorylation, there exists little proof to substantiate the above statement. Without a knowledge of the critical substrates for the growth factor receptor protein-tyrosine kinases, we cannot determine whether these proteins are constitutively phosphorylated in the transformed cells. The same rule applies to another series of oncogenic proteins which have protein-tyrosine kinase activity, namely the *V-src*, *V-yes*, *V-fgr*, *V-fps /fes* and last but not least, *V-abl* proteins. These all have unregulated activities, which might be able to phosphorylate proteins normally phosphorylated by growth factor receptor protein-tyrosine kinases which are involved in modulation of growth of both normal and transformed cells.

The 17 k polypeptide hormone tumor necrosis factor α (TNF- α) has been extensively studied due to its ability to modulate growth of both normal and tumor cells in culture (Rosenblom et al 1989; Goeddel et al 1986). Several recent studies of human fibroblasts have shown a relationship between TNF- α action and the synthesis, binding affinity and phosphorylation of the epidermal growth factor receptor (EGF-R) (Palombella, et al 1987, Bird et al, 1989). EGF-R is expressed as a cell surface 170 k transmembrane protein with an associated protein-tyrosine kinase (Cohen, et al, 1982). Treatment of normal fibroblasts in culture with TNF- α enhanced the expression of EGF-R as determined by ^{125}I -labeled EGF (Palombella et al, 1987.) Recent studies on TNF- α sensitive and TNF- α resistant cell lines

have shown that TNF- α modulates protein-tyrosine kinase activity of EGF-R on tumor cell lines which were sensitive to the cytotoxic action of TNF- α but did not alter the EGF-R tyrosine-kinase activity in TNF- α resistant cell lines (Donato, et al, 1989).

Recently we have determined that TNF- α was not cytotoxic to BALB/c mouse embryonic fibroblast CL-7 and one of its A-MuLV transformed clone 3R.1 but was cytotoxic to another A-MuLV transformed CL.7 clone namely 6R.1 (Chapter 1). Further, TNF- α is known to modulate the tyrosine phosphorylation of EGF-R (Bird et al, 1989). The object of the present investigation was to determine whether the differential toxicity of TNF- α to the A-MuLV transformed clones was due to differences in the content of the viral antigen p120 on the cell surface and to investigate whether TNF- α elicited its differential response by a differential modulation of tyrosine phosphorylation of oncogenic protein p120.

MATERIALS AND METHODS

Cell Lines: CL.7, 3R.1 and 6R.1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS), 10units/ml penicillin and 10 μ g/ml streptomycin, 25mM HEPES pH7.4 at 37°C in a humidified incubator maintained at 5% CO₂ level. The cells were procured from American type culture collection (ATCC), Rockville, Maryland.

Antisera Against A-MuLV Protein:

Goat control serum, goat antiserum against triton-X disrupted A-MuLV virus, and goat antiserum against leukemia virus proteins RLV-p15 and RLV-p30 were purchased from Biological Carcinogenesis Branch, National Cancer Institute, Bethesda, MD.

Immunoaffinity Purification of Goat Antibodies on an Anti-Immunoglobulin Column:

Rabbit anti-goat IgG coupled to agarose beads were purchased from Sigma Chemical Company, MO. 4 ml of agarose beads slurry were transferred to make a 2 ml column. The column was washed

with 20 ml of phosphate buffered saline (PBS). 2 ml of antiserum was loaded on to the column and the fallthrough fraction was applied twice. Column was washed and equilibrated with 20 ml of PBS. The goat antibodies were eluted from the column with 5 ml of 0.1M glycine, 0.15 M NaCl, pH2.4 into a tube containing 500 μ l of 1M Tris (pH 8.0). The antibody fraction was assayed for protein content (Bradford, 1976). The column was washed and then regenerated by equilibrating with a twenty bed volume of PBS before applying the next antiserum.

Radio-iodination of Antibodies Using Chloramine-T:

The goat polyclonal antibodies were iodinated using a modification of the method described (Carpenter et al 1976). Briefly 10 μ l of protein in a total volume of 100 μ l was used for the immunopurified normal goat, MLV Triton-X, RLV-p15 and RLV-p30 antibodies. To the antibody vials, 6 μ l of Na¹²⁵I (250 μ Ci) were added and the iodination was initiated using 10 μ l of 0.5mg/ μ l of chloramine-T (Sigma Chemical Co.). This soft labelling proceeded for 1.5 minutes after which the reaction was stopped with 20 μ l of (0.5 mg/ml in 0.05 M KPi pH7.4) sodium metabisulphite (MCB Co.). After stopping the reaction 1 ml of 1% BSA was added to the sample. The sample was loaded into a pre-equilibrated Ag1-X8 anion exchange column, and the eluate was collected in a tube containing 1 ml of 1% BSA in KPi, pH 7.4. Two ml of PBS was added into the reaction vial and the wash was added the column. The final volume of the radioactive material collected was fixed at 5 ml. The mouse monoclonal anti-phosphotyrosine antibody (Boehringer Mannheim Biochemicals, IN) was radio-labelled with Na¹²⁵I using 10 μ l sample (10 μ g), 3 μ l of Na¹²⁵I (125 μ Ci), 5 μ l chloramine-T (0.5 mg/ml), and the reaction was stopped with 20 μ l sodium metabisulphite (0.5 mg/ml). Then the radio labelled antibody was separated from the unreacted Na¹²⁵I by using Ag1-X8 column. The final reaction volume was fixed at 5 ml. 5 μ l of each sample was used to determine the specific activity of the labelled antibodies presented as cpm per mg protein.

Specificity of Binding of Radio-Labelled Antibodies to Cells: Quantitation of Cell Surface A-MuLV Antigen:

CL.7, 3R.1 and 6R.1 cells were grown in 24 well plates on

coverslips (10^5 cells/well) in DMEM with 10% FCS. Cells were allowed to attach and reach confluency (10^6 cells) in 18 hours. Media were then removed, the cells were washed with PBS and then the coverslips were rinsed with large volume of 50% methanol and 50% acetone for 2 minutes. This exposed the antigenicity of membrane bound proteins. Coverslips were washed 4 times with PBS prior to the antibody binding assay. 40 ng each of normal (5.2×10^7 cpm/ μ g), RLV-p15 (4.7×10^7 cpm/ μ g) and triton-X disrupted A-MuLV (6.1×10^7 cpm/ μ g) goat antibodies were incubated with triplicate coverslips of CL.7, 3R.1 and 6R.1 cells in 500 μ l of DMEM containing 1% BSA (BSA reduces nonspecific binding). The incubation was carried for 2 hours at room temperature in a humidified atmosphere. After the incubation time the cells were washed 4 times with PBS and then the coverslips counted in a Beckman 5500 gamma-counter. The quantity of antibody to be applied to the coverslip was saturable as determined by a previous experiment.

Cytokine: TNF- α was obtained by immunopurification of lipopolysaccharide (LPS) activated RAW 264.7 macrophage supernatant on an anti-TNF- α antibody column (Chapter 1).

Quantitation of Cellular Phosphotyrosine Under TNF- α Cytotoxicity:

Cells were grown on cover slips in 24 well plates (typically 10^6 cells/coverslip) and incubated without and with 100 units/ml TNF- α for 0, 2, 4, 8 and 16h. After the incubation time cells were washed 2 times with PBS and rinsed for 2 minutes with 50% methanol and 50% acetone. The coverslips were then washed 4 X with PBS prior to incubating with radio-labelled anti-phosphotyrosine antibody to quantitate the phosphotyrosine levels. Coverslips were incubated with 1 ml of 1:1000 dilution (1 μ g/ml) of radio-labelled anti-phosphotyrosine antibody for 2 hrs in a humidified atmosphere at room temperature. The coverslips were rinsed 4 X with PBS prior to counting in Beckman 5500 gamma-counter. The radioactivity retained (cpm/million cells) were plotted against time of TNF- α incubation.

A-MuLV Specific Phosphotyrosine Modulation With TNF- α :

A-MuLV specific phosphotyrosine content was determined using an antigen capture assay. Anti-phosphotyrosine antibody was labelled with Na¹²⁵I. The specific activity of labelling was determined to be 1×10^7 cpm/ μ g protein. CL.7, 3R.1 and 6R.1 cells were grown to confluency in ten 75 c.c flasks (10^7 cells/flask). Duplicate flasks of CL.7, 3R.1 and 6R.1 cells were incubated with 100 units/ml TNF- α for 0, 2,4,8 and 16 hours. At the end of the incubation period cells were washed with DMEM with 1% BSA. The cell monolayer was then incubated with 1.5 ml of digitonin release buffer [250mM sucrose; 17mM 3-N-morpholine propane sulfonic acid (MOPS); pH 7.4, 25 mM EDTA; 0.2 ng of pepstatin (Sigma Chemical Co) per ml; 1 mM phenylmethylsulfonyl fluoride and 0.8 mg per ml digitonin] at room temperature for 2 minutes for the release of cytosolic fraction as described (Mackall et al 1979). The treatment was stopped by diluting with 3 ml of release buffer without digitonin. After removal of the medium the perforated cell monolayers were washed with DMEM. The post washing steps included the removal of the lipid bilayer by treatment of the perforated cells with excess 50% methanol and 50% acetone for 2 minutes. The cell monolayer was then washed with DMEM without FCS, scraped with a cell scraper and resuspended in 500 μ l volume of DMEM 50 μ l of this suspension then corresponded to membrane fraction of 10^6 cells and was called the "antigen preparation."

To 8 well strip enzyme immuno assay (EIA) plates (Costar, MA), 50 μ l of 30 μ g/ml of antibody control (normal goat immunoglobulin) or goat antibody against triton-X disrupted A-MuLV virus was applied and incubated at room temperature for 2 hours in a humidified atmosphere. Wells were washed 2 times with DMEM and then blocked by incubating with 3% BSA in DMEM with 0.02% sodium azide (Sigma Chemical Co., MD) by filling to the top of the wells. After incubating in a humidified atmosphere for 2 hours the wells were washed in DMEM. Then 50 μ l of antigen solution was applied to the wells for 1 hours in a humidified atmosphere. The wells were washed again with DMEM to remove unbound antigen. At this point it is essential to determine the amount of second antibody required to get saturable binding. The logic for quantitating A-MuLV specific phosphotyrosine levels by antigen capture assay was as follows.

First the A-MuLV polyclonal antibody was bound to the EIA plate. Then allowing the cell surface antigen (p120) to incubate with the BSA blocked antibodies, selected for retaining the A-MuLV protein on the EIA plate. The background binding was determined by allowing the A-MuLV antigen to bind nonspecific goat antibody in a similar EIA well. To quantitate the phosphotyrosine levels, post antigen binding, appropriate amount of (previously determined) ^{125}I -labeled anti-phosphotyrosine antibody was added. The radioactivity retained after stringent washing reflected the amount of the phosphotyrosine present. The advantage of polyclonal antibody as the first antibody ensures that A-MuLV specific antibody binding does not inhibit phosphotyrosine specific anti-phosphotyrosine antibody binding in the antigen capture assay.

To determine the amount of ^{125}I -labeled anti-phosphotyrosine antibody required to get saturable binding, duplicates of 1st antibody and antigen bound EIA wells were incubated with 1:10,000, 1:5000, 1:1000 and 1:500 dilution of anti-phosphotyrosine antibody (stock:10 $\mu\text{g}/10\mu\text{l}$). Radio-labelled anti-phosphotyrosine antibody (1:500 stock dilution) was used at 1:20, 1:10, 1:2 and 1:1 of this dilution to achieve the above mentioned dilutions. Saturable dilution was determined and the concentration of antibody was kept fixed for the rest of the assay. 1st antibodies used were: control goat immunoglobulin and anti-Triton-X disrupt A-MuLV goat immunoglobulin. The antigens were membrane protein fraction (50 μl) of CL.7, 3R.1 and 6R.1 cells incubated with 12 ml of media with and without 100 units/ml TNF- α for 0, 2, 4, 8 and 16 h. The 2nd antibody was a 1:2 dilution of ^{125}I -labeled anti-phosphotyrosine antibody (1:500 dilution of original stock). After the 2nd antibody was incubated in the 1st antibody bound antigen coated EIA plate for 2hr in a humidified atmosphere, it was subjected to a stringent wash in DMEM before reading in Beckman 5500 gamma-counter.

Statistical Analysis : The statistical analysis was performed by a paired t test comparison. The probability of $p \leq 0.05$ was taken to be significant. The results were expressed as mean \pm S.D of six replicate readings.

RESULTS

Binding of control, anti-p15, anti-p30 and anti-A-MuLV (triton-disrupted) goat antibodies onto CL.7, 3R.1 and 6R.1 cells grown on glass coverslips in 24 well plate is shown in Figure 2. The binding of anti-triton-disrupted A-MuLV antibody was significantly higher for transformed cells than the normal (CL.7) cells ($p < 0.05$). Binding of anti-p30 was significantly higher for 3R.1 and 6R.1 cells as compared to CL.7 ($p < 0.05$). A possible reason as to why anti-triton-disrupt A-MuLV antibody gave a higher binding in CL.7 cells than other antibodies could be due to the very nature of p120. Since the transmembrane p120 is a hybrid construct of a viral component p30 located on the cytosolic side and the cellular component p90 (*C-abl* product) towards the extracellular side, any polyclonal antisera raised against this protein will have a considerable amount of antibodies directed against the cellular component "the *C-abl* product."

Since the normal cells express the membrane located *c-abl* product p 90, the radio-labelled polyclonal antibodies against triton disrupted A-MuLV, containing antibodies directed against the p 90 portion of the p 120 poly protein, would bind to it and give a high reading even for the A-MuLV untransformed normal clone CL.7. Anti-A-MuLV antibodies could still be used to quantitate the A-MuLV specific surface antigen. As seen in Figure 2, 3R.1 and 6R.1 both had equivalent amount of A-MuLV surface antigen. A possible reason for anti-p30 antibody showing low retentivity than the triton disrupted anti-A-MuLV, for the transformed cells in comparison to the normal cells, could be due to an incomplete removal of cellular lipids by 50% methanol 50% acetone treatment.

Cellular phosphotyrosine levels were determined exactly as described in the materials and methods section. Quantitation of phosphotyrosine levels for CL.7, 3R.1 and 6R.1 cells with incubation of TNF- α was shown in Figure 3 a, b and c. The TNF- α insensitive lines, CL.7 and 3R.1, had non-inducible phosphotyrosine levels, whereas cellular phosphotyrosine levels for the TNF- α sensitive cell line 6R.1 was inducible with the incubation time of TNF- α with a maximal induction around 8 hours. Cell surface A-MuLV antigen was quantitated by using anti-triton disrupted A-MuLV antibody. There

was no change in the expression of A-MuLV gene product with incubation time of TNF- α (Figure-6). The expression of equivalent levels of A-MuLV surface antigen (Figure-2) and its non-inducibility (Figure-4) with TNF- α for 3R.1 and 6R.1 cell lines, taken together with the fact that cellular phosphotyrosine levels were inducible in 6R.1 cell line (Figure-3) but were not inducible for 3R.1 cell line (Figure 3 b) suggested a likely possibility that 3R.1 would be a p120 kinase mutant whereas 6R.1 had an active p120 kinase activity.

To check this hypothesis we determined the p120 specific kinase activity by an antigen capture assay as described in the materials and methods section. The amount of ^{125}I -labelled anti-phosphotyrosine antibody (2nd antibody) required to give saturable counts for predetermined amounts of 1st antibody as well as the cell antigens was shown in Figure 5. We chose 1:1000 dilution as the concentration of the second antibody for all the EIA wells. The 1st antibody being specific for p120 antigen, the phosphotyrosine levels determined in this assay were also specific, i.e., the counts would reflect the p120 phosphotyrosine levels. The inducibility of p120 phosphotyrosine was determined with TNF- α incubation time (Figure-6). As shown, 6R.1 had inducible phosphotyrosine levels, maximally induced around 8hr, whereas 3R.1 had a transient induction (around 2hr of TNF- α incubation). It was observed that both CL.7 and 3R.1 that were not sensitive to TNF- α had low and hardly inducible p120 phosphotyrosine whereas 6R.1 (the TNF- α sensitive cell line) had a progressively inducible phosphotyrosine level, maximally induced around 8hr, with TNF- α incubation. The detection of phosphotyrosine levels were confirmed by performing the second antibody incubation with an increasing concentration (0 to 100 $\mu\text{g/ml}$) of O-phospho-L-tyrosine (Boehringer Mannheim, Biochemicals, IN). It was found that the binding of the radioactive second antibody progressively decreased with increasing concentration of O-phospho-L-tyrosine for the 6R.1 sample incubated for 8 hr with TNF- α (Figure-7). This substantiated that the P120 specific phosphotyrosine levels were measured in the antigen capture assay.

DISCUSSION

TNF- α , known to regulate the EGF-R phosphotyrosine levels in some TNF- α sensitive cell lines as compared to the non-regulation of EGF-R phosphotyrosine levels in TNF- α insensitive cell line (Donatte et al 1989) was established to do the same for A-MuLV oncoprotein p120. As was demonstrated previously 3R.1 was an A-MuLV transformed cell line that was insensitive to TNF- α as was its parent cell line BALB/c CL.7, whereas another A-MuLV clone of BALB/c CL.7 namely 6R.1 was TNF- α sensitive (Chapter 1). In this investigation it was demonstrated that TNF- α could regulate the p120 phosphotyrosine levels of 6R.1 but did not do so for the TNF- α insensitive cell line 3R.1. That p120 kinase mutants were TNF- α insensitive whereas those A-MuLV transformed clones with kinase activity would be TNF- α sensitive merits further investigation. We do however prove a very important point, i.e., p120 tyrosine phosphorylation, not known to be sensitive to any ligand, was shown to be sensitive to the cytokine TNF- α . It is important to determine whether cytokines like TNF- α could regulate tyrosine phosphorylation of other membrane bound oncoproteins like C-src and growth factor receptors like PDGF, FGF, I-R, and CSF-1R all of which are known to possess protein tyrosine kinase activity.

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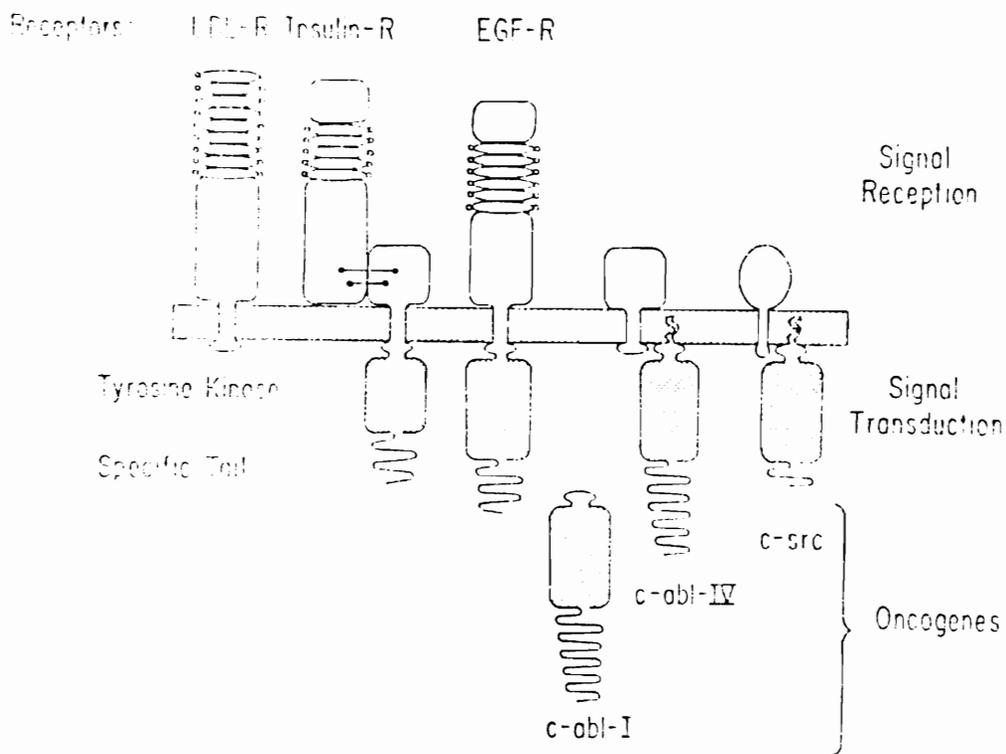


Figure 1 : Receptors And Protein - Tyrosine Kinase Oncogenes. The hatched box represents the plasma membrane separating the extracellular space (above) from the cytoplasm (below). The protein kinase domain is *stippled*. *C-abl* is the cellular counterpart of the A-MuLV viral oncogene *V-abl*. Reproduced from "Oncogenes and growth control" (1986). Khan, P & Graf, T (Ed), pp 108, Springer Verlag, New York

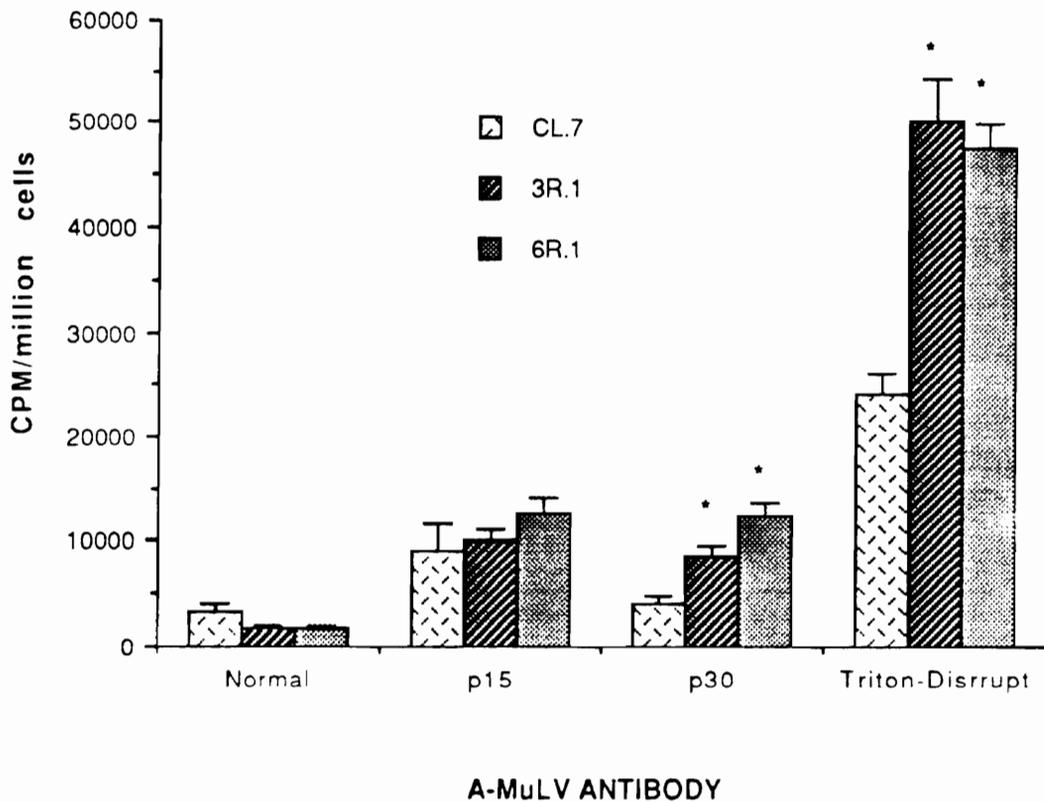


Figure 2 : Quantitation of Cell Surface Antigen Using Radio-labeled Anti-A-MuLV Goat Antibodies. The purification, radio-labeling, and cell surface binding of goat IgG were performed as described. The cpm/million cells of ^{125}I - IgG retained on cell surface for six replicate coverslips are presented as mean \pm S.D. The binding of anti-p 30 and anti-triton disrupted A-MuLV goat IgG are significantly higher ($p < 0.05$) for A-MuLV transformed clones than the normal untransformed clone CL.7. (* = $p < 0.05$).

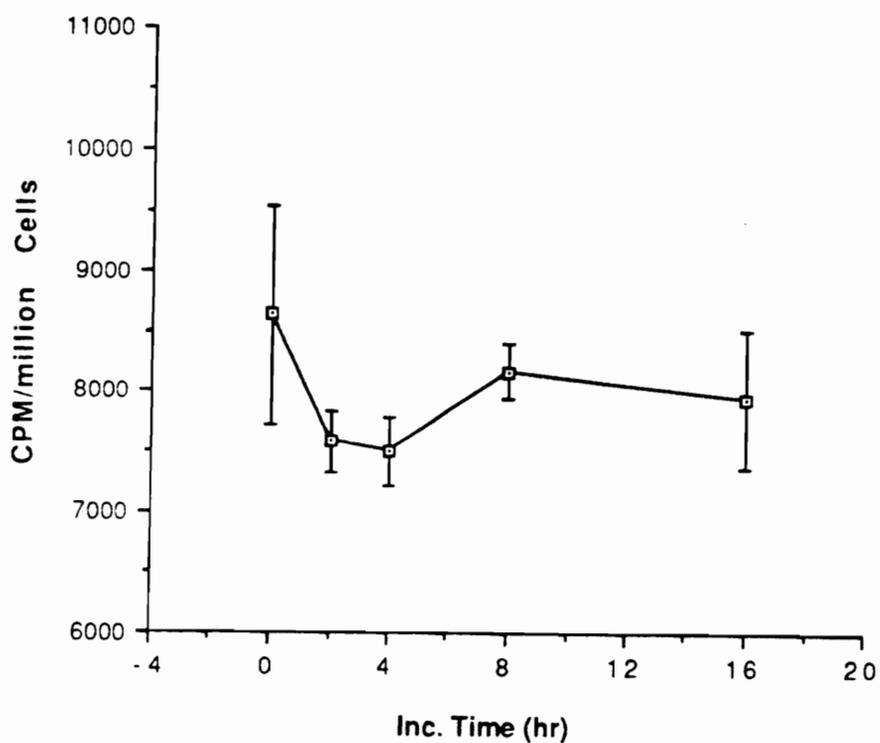


Figure 3 (a): The quantitation of Total Cellular Phosphotyrosine Levels For CL.7 Cells. The cpm/million cells of 125 I-anti-phosphotyrosine antibody retained on coverslips are plotted as a function of tumor necrosis factor $-\alpha$ (TNF- α) incubation time.

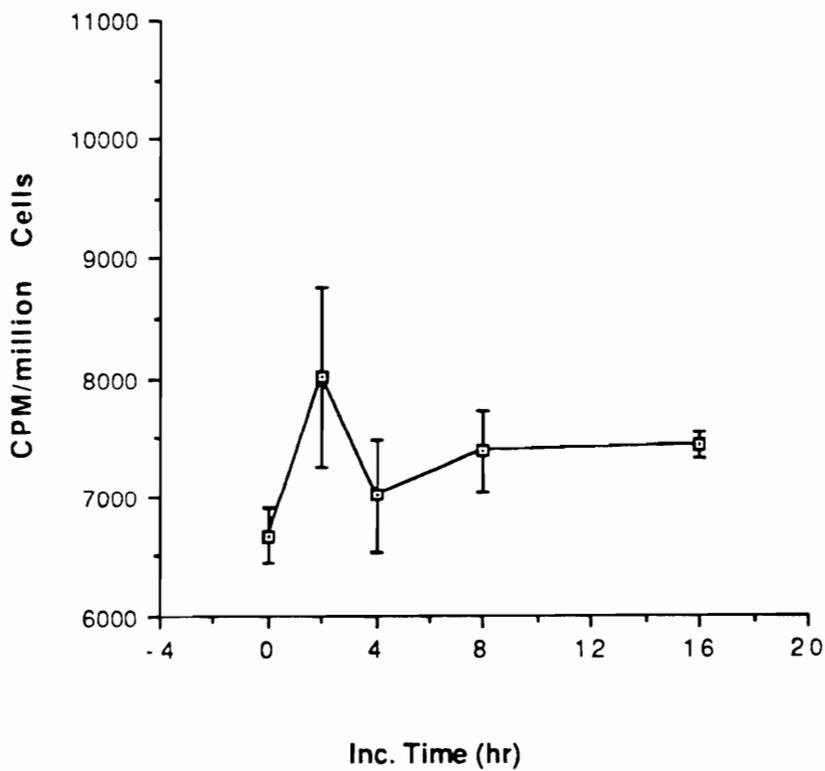


Figure 3 (b): The quantitation of Total Cellular Phosphotyrosine Levels For 3R.1 Cells. The cpm/million cells of 125 I-anti-phosphotyrosine antibody retained on coverslips are plotted as a function of tumor necrosis factor α (TNF- α) incubation time.

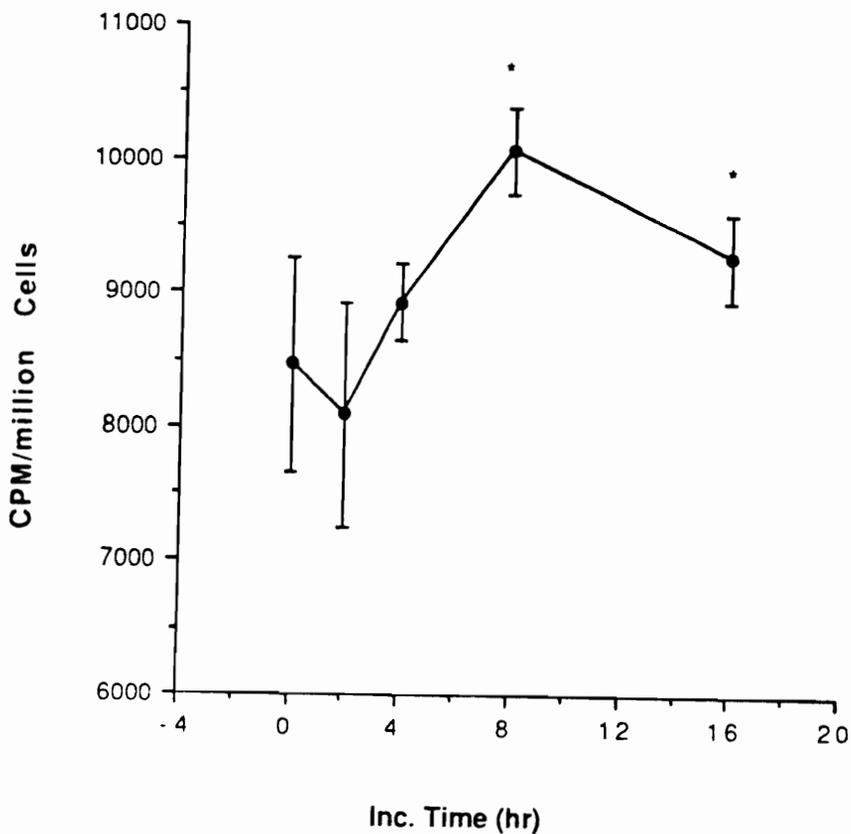


Figure 3 (c): The quantitation of Total Cellular Phosphotyrosine Levels For 6R.1 Cells. The cpm/million cells of ^{125}I -anti-phosphotyrosine antibody retained on coverslips are plotted as a function of tumor necrosis factor $-\alpha$ (TNF- α) incubation time and shows a distinct induction pattern. (* = $p < 0.05$).

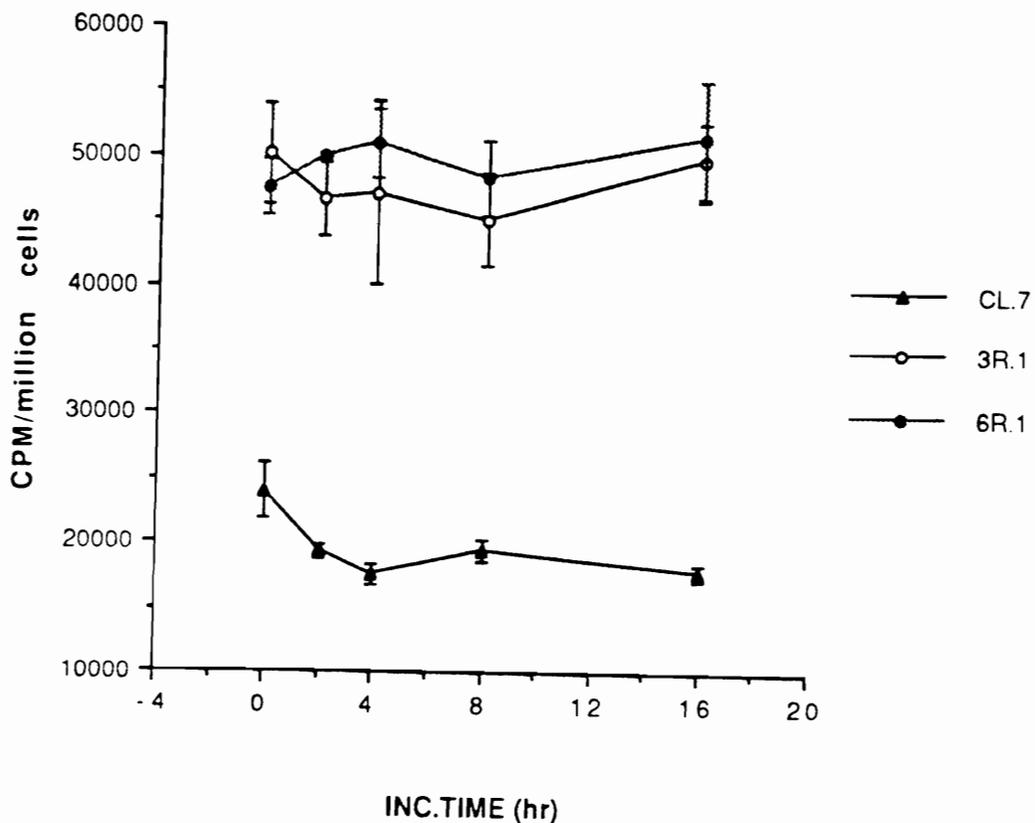


Figure 4 : Effect of tumor necrosis factor - α (TNF- α) on A-MuLV Specific Surface Antigens. Six replicate coverslips of CL.7, 3R.1, and 6R.1 cells incubated for different times with TNF- α were assessed for their A-MuLV specific surface antigen by using 125 -I labelled anti-triton X disrupted goat IgG. The experiment was performed as described in the methods section.

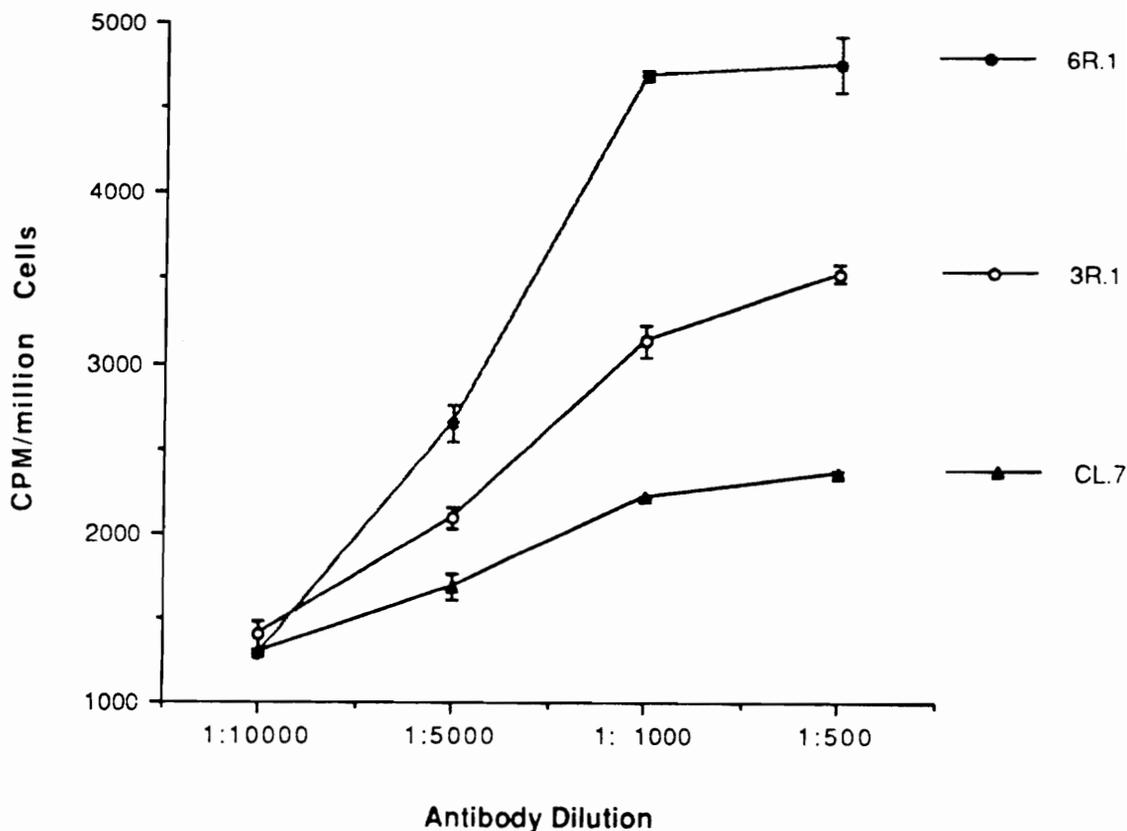


Figure 5 : Saturation Binding of Anti-Phosphotyrosine Antibody as a Function of Antibody Dilution. ^{125}I - anti-phosphotyrosine antibody (1×10^7 cpm/ μg protein) at a stock concentration of $1 \mu\text{g}/\mu\text{l}$ was used in an antigen capture assay, in duplicate, as described in the methods section. The 1 : 1000 dilution of the radio-labeled antibody was determined to give saturation binding.

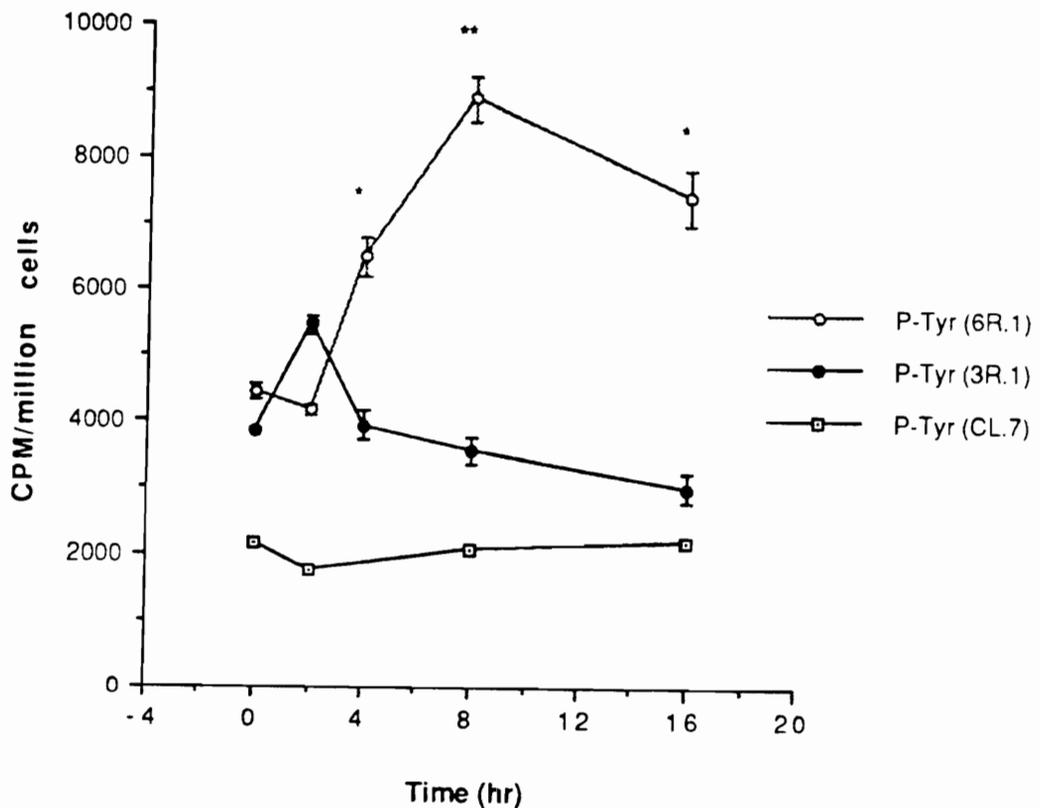


Figure 6 : A-MuLV Specific Phosphotyrosine Induction With TNF- α . The experimental protocol was similar to as in Figure 5. Control IgG or anti-(triton disrupted A-MuLV) IgG were incubated (50 μ l of 30 μ g/ml) in triplicate EIA wells, blocked with BSA and coated with 50 μ l of antigen preparation. The 125 I-labelled second antibody at 1 : 1000 dilution were applied for quantitating the A-MuLV p 120 specific phosphotyrosine as a function of TNF- α incubation time. The results are presented as mean \pm S.D. (* = p<0.05), (** = p<0.001).

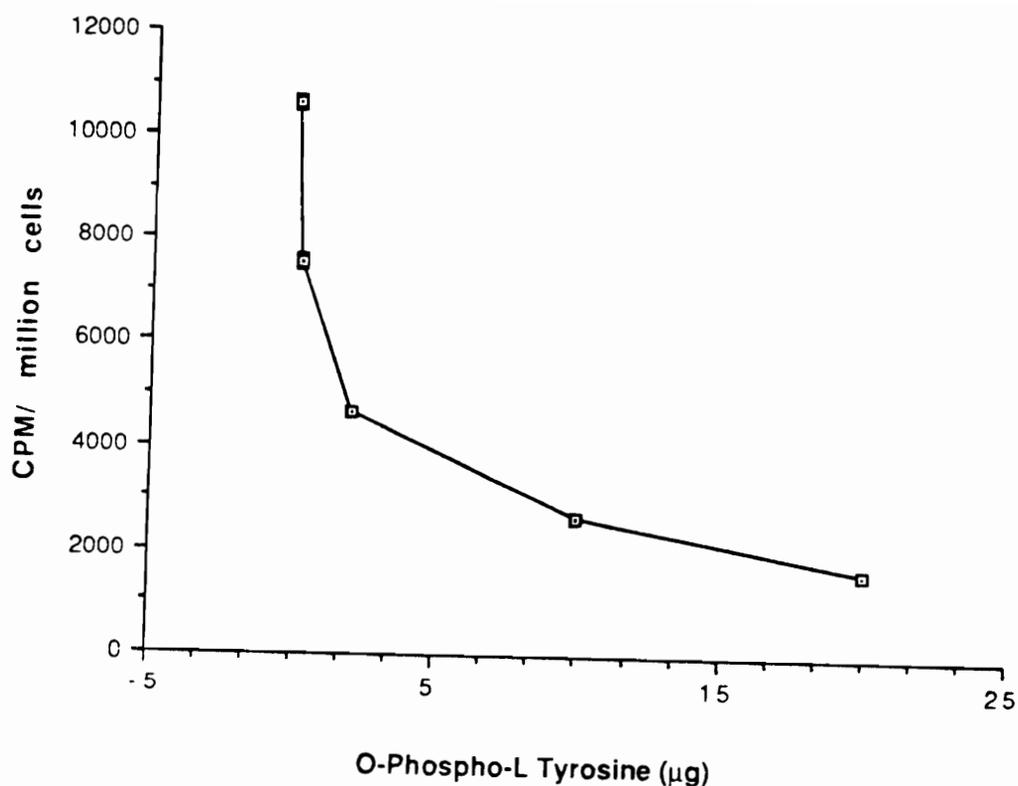


Figure 7 : Inhibition of Binding of Anti-Phosphotyrosine Ab By O-phospho-L tyrosine. Duplicate EIA plates coated with the first antibody and the 6R.1 antigen (from the 8 hr TNF- α preparation) were incubated with ^{125}I -labelled anti-phosphotyrosine Ab in presence of an increasing concentration of O-phospho-L tyrosine. The reduction of second antibody retention in the EIA well signifies that O-phospho-L tyrosine competitively inhibited the second Ab binding to the p 120 phosphotyrosine.

CHAPTER - 9**MONITORING MITOCHONDRIAL TNF- α
CYTOTOXICITY USING EPR SPECTROMETRY****ABSTRACT**

In studying the mechanism of cytotoxicity of TNF- α on L929 cells it was found that the mitochondrion was one of the earliest organelles to be affected (Matthews et al 1983). The mitochondrial toxicity affects the electron transport chain of the inner mitochondrial membrane which results in the accumulation of reducing equivalents. Potentially any such accumulation can reduce a cationic species provided of course that the redox potentials are matched. The overall objective of this investigation was to determine whether it was possible to monitor the toxicity to mitochondria of cells (for eg L929 fibrosarcoma) known to be susceptible to TNF- α by monitoring the mitochondrial reduction of EPR signal height of a cationic spin probes 4-(N,N-dimethyl-N-hexadecyl) ammonium 2,2,6,6-tetramethyl piperidine-1-oxyl, iodide (CAT 16). Further, the reduction of CAT 16, taken as a measure of cytotoxicity to intact mitochondria, was used to investigate whether differential cytotoxicity to TNF- α under A-MuLV transformation in clones CL.7, 3R.1 and 6R.1 was manifested at the level of mitochondrial toxicity.

The spectrum of the paramagnetic species CAT 16 was a 1:1:1 triplet EPR signal. This spin label was found to be heavily quenched when incubated with normal L929 mitochondria. When the L929 cells were incubated with TNF- α , with increasing incubation time there was a gradual loss of ability of the mitochondria from L929 cells to reduce the CAT 16 signal. This loss in the ability of mitochondria, from cells subjected to cytotoxic action of TNF- α , to quench CAT16 was taken as a measure of TNF- α toxicity. The decay of CAT 16 EPR signal when incubated with mitochondria from BALB/c CL.7, and its two A-MuLV transformed clones 3R.1 and 6R.1

subjected to different times of TNF- α incubation illustrated that the percent inhibition of CAT 16 quenching with time was different for different cell lines. When the slope of the decay of CAT 16 EPR signal quenching curves was plotted with percent cytotoxicity of the cell lines with TNF- α as determined by MTT reduction assay it was observed that the susceptibility of a cell line to TNF- α correlated with the rate of reduction inhibition of the cationic paramagnetic probe CAT 16 with the corresponding mitochondrial preparation as monitored by EPR spectrometry. We confirmed the differential cytotoxicity of TNF- α under A-MuLV transformation to manifest at the level of differential mitochondrial toxicity of the same cell lines. Specifically the normal cell line CL.7 and one of its A-MuLV transformed clone having been found to be less sensitive to TNF- α had mitochondria with a higher ability to quench CAT 16 with TNF- α incubation time than the mitochondria from one of the CL.7 transformed clone, 6R.1, which was more susceptible to TNF- α . It was concluded that if a cell line was susceptible to TNF- α its mitochondria lost the ability to quench CAT 16. The inability of mitochondria from a TNF- α sensitive cell line to quench CAT 16 could be a very sensitive method to compare relative cytotoxicity of different cell lines to TNF- α using EPR.

INTRODUCTION

TNF- α is a macrophage/monocyte-derived anticancer cytokine (Carswell, et al. 1975, Matthews, 1978, Niitsu, et al. 1985) which exhibits a strong cytotoxic response to tumor cells in vitro (Niitsu et al 1988, Watanabe et al 1985, 1988 a, b, Helson et al 1975, Matthews et al, 1978 and Ruff et al 1981). The mechanism of the cytotoxic action of TNF- α remains largely obscure although some studies have suggested the involvement of lysosomal enzymes (Kull et al 1981, Watanabe et al 1988 c) and hydroxyl radicals (Watanabe et al 1988 c, Suffys et al 1987, Matthews et al 1987 Yamauchi, et al. 1989 a) based on the observation that TNF- α cytotoxicity is inhibited in the presence of hydroxyl radical scavengers like dimethyl sulfoxide (DMSO) and promethazine and in the presence of desferal (iron chelator) an inhibitor of hydroxyl radical. Hydroxyl

radical formation, in the cytotoxic pathway of rhTNF- α on mouse tumorigenic fibroblast L-M cells, was detected in presence of DMSO by the evolution of methane gas (Yamauchi et al 1989). There are many indications of involvement of oxygen radicals in TNF- α action. For example, the expression of interleukin-2 (IL-2) receptors on T-lymphocytes, necessary for T cell proliferation, may depend on free radicals and on TNF- α . Further evidence suggests that free radicals might provide a positive signal for the release of TNF- α (Clark et al 1987).

Free radicals produced by the action of TNF- α can cause damage to cell organelles, cell membranes, cellular DNA etc. In studying the direct cytolysis of L929 cells by TNF- α it has been found that there is a delay of several hours before the cells died and that the mitochondria were the first organelles to be damaged. The mitochondria have swollen appearance and have reduced number of cristae (Matthews et al 1983, Matthews et al 1987b). Mitochondria being one of first sites known to be affected by TNF- α , it is important to monitor this event as a measure of susceptibility of a cell to TNF- α . The swelling of the mitochondria could potentially affect the electron transport chain of the inner mitochondrial membrane. This electron transport chain consists of a series of redox components. During steady-state respiration (also known as state IV respiration), the level of reduction of the various components of the electron transport chain depends on the position of the redox component in relation to the final acceptor O_2 . In an anaerobic state, the electron transport components become fully reduced. During steady-state respiration, reducing equivalents are accumulated at various "sites" along the electron transport pathway. When uncoupled (state III respiration), this accumulation of reducing equivalents is decreased in most components of the chain except for cytochrome oxidase (i.e. cytochrome a and a_3). Potentially any such accumulation can reduce an added test substance, provided of course that the redox potentials of the reductant and the test substance are appropriately related. The test substance could be a reporter molecule with interpretable spectroscopic properties detectable with spectroscopic techniques, such as fluorescence, nuclear magnetic resonance (NMR) or electron paramagnetic resonance (EPR).

In terms of sensitivity, fluorescence is the method of choice, by which fluorochrome concentrations of 10^{-9} M can be detected. Fluorescence detection at the cellular level could go down to as low as several angstroms spatially and to nanoseconds in the time scale of events. But fluorochromes are rather large molecules and can create considerable perturbation when inserted into a membrane and their localization in the membrane is rather uncertain. This can be further complicated by the presence of chromophores and quenchers in the membrane.

NMR on the other hand had great advantages with respect to fluorescent and paramagnetic probes. The use of protein or ^{13}C magnetic resonance offers the most precise information in an unperturbed system. However, NMR sensitivity is still very low when the experimental times are long. The time resolution of NMR is between 10^{-4} and 10^{-6} seconds.

EPR of nitroxyl radicals is apparently a good compromise between fluorescence and NMR. It's sensitivity can go down at best to 10^{-6} M and it can be employed to resolve time events in the nanosecond range. EPR, further, is not affected by scattering, turbidity of the sample or presence of fluorochromes or chromospheres associated with the membrane. Insertion of spin labels into membranes produce certain degree of perturbation, but, due to their relatively small size, the perturbation is certainly smaller than for fluorescent probes.

One of the major disadvantages of the nitroxyl group as a spin label is that it can be easily reduced, and therefore could result in a severe loss of EPR signal after introduction of the spin labels into biological membrane, especially when provided with electron transport components as in the case of respiring mitochondria. The spin label as a reporter can be used to convey it's own reduction (Tinberg et al 1972, Torres-Pereira et al 1974, Schweier et al 1975, Bal dassare et al 1976 and Azzi et al. 1973). Choice of appropriate spin labels, it's interaction with electron transport chain in presence and absence of various uncouplers have been studied (Alexandre et al 1977). Alexandre et al 1977 investigated the relative rates of reduction of several spin labelled molecules that partition differently across hydrophobic-interface of inner

membrane in rat liver mitochondria. They determined that EPR signals from cationic spin labels like cetyltrimethylammonium bromide (CTAB) are rapidly lost (in mole/mg protein/min). This loss of signal was determined to be due to reduction and not destruction of the label. The label reduction was concluded to arise from channeling of the reducing equivalents of mitochondrial electron transport to the membrane interface. The interaction of the cationic spin probe 4-(N, N-dimethyl-N-dodecyl)-ammonium-2,2,6,6-tetramethyl-piperidine-1-oxyl (Cat12) with intact mitochondria (Hashimoto et al 1984) resulted in a reduction of EPR signal. This signal reduction was inhibited by 10mM $K_3Fe(CN)_6$. The response of the probe was determined to be (sensitive to the membrane potential and not to the surface charge. Another cationic probe 4-(N,N-dimethyl-N-hexadecyl) ammonium -2,2,6,6-tetramethyl piperidine-1-oxyl, iodide (CAT 16) has been used in many applications including detection of structural alterations and fluidity of mitochondrial membrane (Herman et al 1984, Mutet et al 1984). CAT 16 is similar in action as CAT12 with respect to its reduction in intact mitochondria.

The purpose of this investigation was to determine whether it was possible to monitor the toxicity to mitochondria of cells (for eg L929 fibrosarcoma) known to be susceptible to TNF- α , by monitoring the reduction of EPR signal height of cationic spin probes like CAT 16. Further, if the reduction of CAT 16 could be taken as a measure of cytotoxicity to the intact mitochondria, the possibility that the differential cytotoxicity to TNF- α under A-MuLV transformation in clones CL.7, 3R.1, and 6R.1 could manifest at the level of mitochondrial toxicity was investigated.

MATERIALS AND METHODS

Cells - Mouse fibrosarcoma L929, normal mouse fibroblast BALB/c CL.7, and two A-MuLV transformed mouse fibroblast cell lines, BALB/c A-MuLV 3R.1 and BALB/c A-MuLV 6R.1 were grown in Dulbecco's modified Eagle's medium with 4.5 g/l glucose 90%, fetal bovine serum (FBS) 10%, 10 units/ml penicillin and 10 μ g/ml streptomycin, 25mM HEPES pH7.4 in 5% CO₂ in a humidified 37 $^{\circ}$ c incubator. All the cell lines were procured from the American Type

Culture Collection (ATCC), Rockville, Maryland.

Cytokine: TNF- α was obtained by activation of an A-MuLV immortalized mouse monocyte-macrophage cell line RAW 264.7 with *E. coli* lipopolysaccharide (LPS). The TNF- α so obtained in the cell culture supernatant was filter sterilized (2X) and then purified over an anti-TNF- α antibody column. The specific activity of TNF- α obtained was 2.5×10^5 units/mg protein as determined by tetrazolium dye reduction assay (Larrik et al. 1989).

TNF- α Cytotoxicity: Two 75 cc confluent flasks per treatment was incubated in 10 ml of media with and without 100 units/ml of TNF- α for 0,4,8,12 and 16 hours. The incubation medium did not contain any TNF- α sensitizers like γ -interferon or actinomycin D. The cell lines used for the investigation of mitochondrial cytotoxicity were L929, CL7, 3R1 and 6R1.

Preparation of Mitochondria-Enriched Fraction:

After the predetermined incubation time with and without the TNF- α , cells (two 75 cc flasks/group) were washed with Hanks balanced salt solution (HBSS) at 37°C. The cell monolayers were incubated with 1.5 ml of digitonin release buffer (250 mM sucrose; 17 mM 3-(N-morpholino) propane sulfonic acid (MOPS); pH 7.4; 2.5 mM EDTA, 0.02 mg of pepstatin (Sigma Chemical Co, MO) per ml; 1 mM phenylmethylsulfonyl fluoride and 18 mg/ml digitonin at room temperature for 2 minutes for the release of the cytosolic fraction as described (Mackall et al 1979). The treatment was stopped by diluting with 3 ml of release buffer without digitonin. After removal of the medium the "perforated" cell monolayers remaining on the two culture flasks were scraped with 500 μ l of release buffer without digitonin, pooled, and homogenized twice by 10 passages of tight-fitting teflon pestle at 4°C. The homogenate was diluted with 1 volume of release buffer without digitonin. The resulting suspension was centrifuged at 1,500 x g for 15 minutes at 4°C to remove intact cells, cell debris and nuclei (which forms the pellet). The supernatant was centrifuged at 15,000 xg for 15 minutes at 4°C, yielding a pellet which is the mitochondria enriched fraction. The pellets were resuspended in 180 μ l of release buffer without digitonin and their protein content determined using dye

binding method of Bradford (Bradford, 1976). 10 μ l of 0.1 mg/ml pellet was mixed with 80 μ l of HBSS and 10 μ l of CAT16 (0.02 mM final reaction concentration) for monitoring EPR signal.

EPR Settings: The EPR measurements were made on a Bruker D-200 X-band spectrometer using a magnetic field modulation frequency of 100 KHz. The EPR parameters were as follows: Receiver gain (RG) : 5×10^5 , time constant(TC) : 640 ms, scan time (ST) : 200 sec, modulation amplitude (MA) : 1G, phase (PH) : 270, center field (CF) : 3480 G, and sweep width (SW) : 100 G. In the kinetic studies, the EPR spectrometer was set at a field setting to monitor the last line of the EPR signal trio as a function of time.

RESULTS

The 1:1:1 EPR spectrum of CAT 16 (0.02mM final concentration), in an aqueous medium at the above mentioned EPR setting, is shown in Figure-1. Figure 2-a depicts the spectra of CAT 16 when incubated with equal amount of mitochondrial preparations from L929 cells incubated for different times of incubation with TNF- α . The enrichment of mitochondria from cells in culture was done as described in the methods section. The above figure showed the progressive inability of the spin label to quench CAT 16 with TNF- α incubation time. CAT 16 is observed to be heavily quenched in presence of normal L929 mitochondria. Figures 2 b to 2 e are the CAT 16 spectra in presence of 0.01 mg/ml mitochondria from cells incubated for 4,8, 12 and 16 hr with 100 units/ml of TNF- α . The CAT 16 spectrum was reduced within two minutes of incubation with the mitochondria. As can be seen from the Figure 2, with increasing incubation time there was a gradual loss of ability of the mitochondria from L929 cells to reduce the CAT 16 signal. This loss of ability of mitochondria, from cells subjected to cytotoxic action of TNF- α , to quench CAT16 can be taken as a measure of TNF- α toxicity. The mitochondrial pellet from control cells are able to quench CAT16 signal (Figure 2a) whereas those from cells treated with TNF- α can't quench CAT16. The experiment was repeated for the same times of incubation with BALB/c CL.7, and it's two A-MuLV transformed clones 3R.1 and 6R.1. The result of the decay of CAT 16

EPR signal with TNF- α incubation time is shown in Figure 3. This figure illustrates that % inhibition of CAT 16 quenching with time is different for different cell lines. The % quenching inhibition kinetic plot had the highest slope for the cell line that was most sensitive to TNF- α (ie. L929). The mitochondria from the TNF- α insensitive cells had comparatively a smaller quenching inhibition slope. In other words, mitochondria from TNF- α sensitive cells rapidly lost the ability to quench CAT 16 when the cells were incubated with TNF- α . This loss was sensitive to the TNF- α incubation time. The mitochondria from TNF- α insensitive cells however lost their ability to quench CAT 16. When the slope of the above quenching curves (Figure 4 a thru 4 d) was plotted with percent cytotoxicity of the same cell lines with TNF- α , as determined by MTT reduction assay (Figure-5) it was noticed that the susceptibility of a cell line to TNF- α does indeed correlate with the rate of reduction inhibition of the cationic paramagnetic probe CAT 16 when incubated with the corresponding mitochondrial preparations as monitored by EPR spectrometry.

DISCUSSION

The mitochondrion is known to be the first site of TNF- α cytotoxicity. The effect of TNF- α action must some how alter the mitochondrial respiration leading to an alteration in the ability of the mitochondria to quench a cationic spin label like CAT 16. Whereas normal mitochondria readily quench CAT 16, mitochondria from cells exposed to TNF- α lost their ability to quench CAT 16 provided of course the cell line was susceptible to TNF- α . A TNF- α sensitive cell line like L929 fibrosarcoma when exposed to TNF- α had mitochondria which steadily lost their ability to quench CAT 16 with TNF- α incubation time (Figure 2). We confirmed the differential cytotoxicity of TNF- α under A-MuLV transformation to manifest at the level of differential mitochondrial toxicity of the same cell lines. Specifically, the normal cell line, CL.7, and one of its A-MuLV transformed clone having been found to be less sensitive to TNF- α had their mitochondria enriched fraction possessing a higher ability to quench CAT 16 with TNF- α incubation time than the

mitochondria from one of the CL.7 transformed clone, 6R.1, which is more susceptible to TNF- α (Figure-5). The reason as to why TNF- α is more susceptible to one cell line than any other is not yet known. However our study confirms that if a cell line is susceptible to TNF- α , its mitochondria lost the ability to quench CAT 16. This inability of mitochondria from a TNF- α sensitive cell line to quench CAT 16 can be taken as an index of cytotoxicity. Specifically, inhibition of the rate of reduction of CAT 16 probe could be a very sensitive method to compare relative cytotoxicity of different cell lines to TNF- α .

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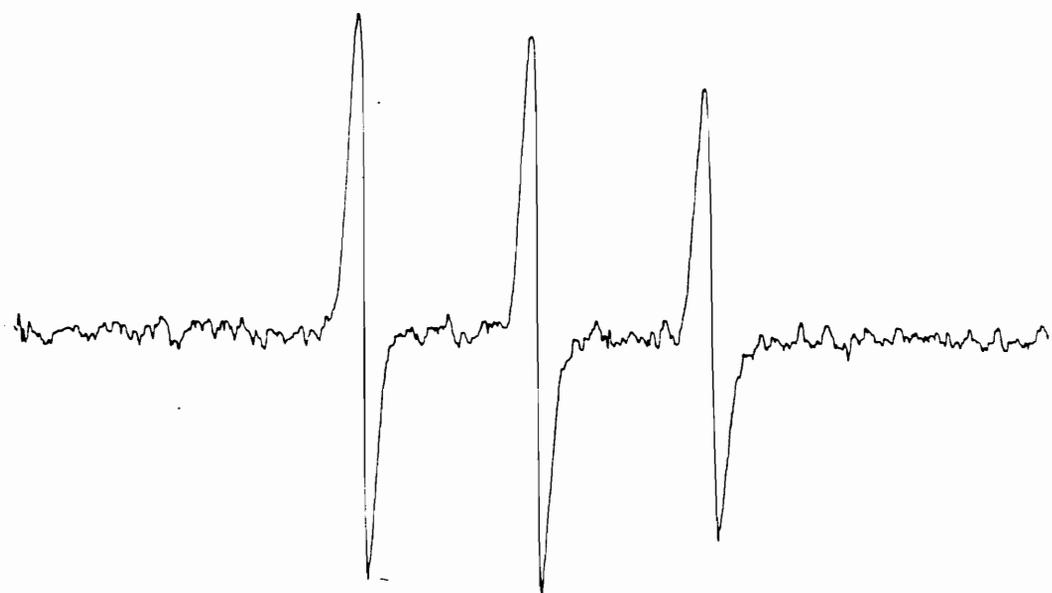


Figure 1: The CAT 16 1 : 1: 1 EPR spectrum at 0.02 mM final concentration in HBSS. The EPR settings were as mentioned in the methods section.

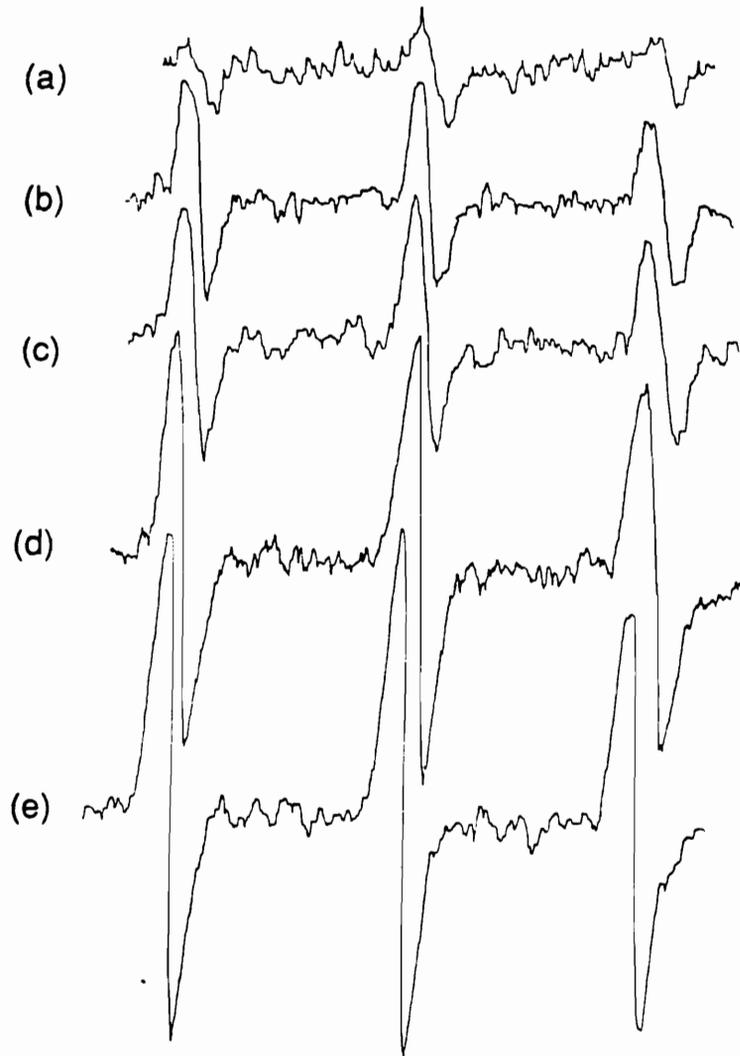


Figure 2: Loss of CAT 16 quenching ability of mitochondria of L929 cells incubated with TNF- α as a function of incubation time. Control mitochondria quench the CAT 16 signal (at 0.02 mM final concentration) as shown in (a). The mitochondria from cells incubated with 100 units/ml TNF- α for 4 hr (b); 8 hr (c); 12 hr; (d); and 16 hr (e) have lost their ability to quench CAT 16 signal.

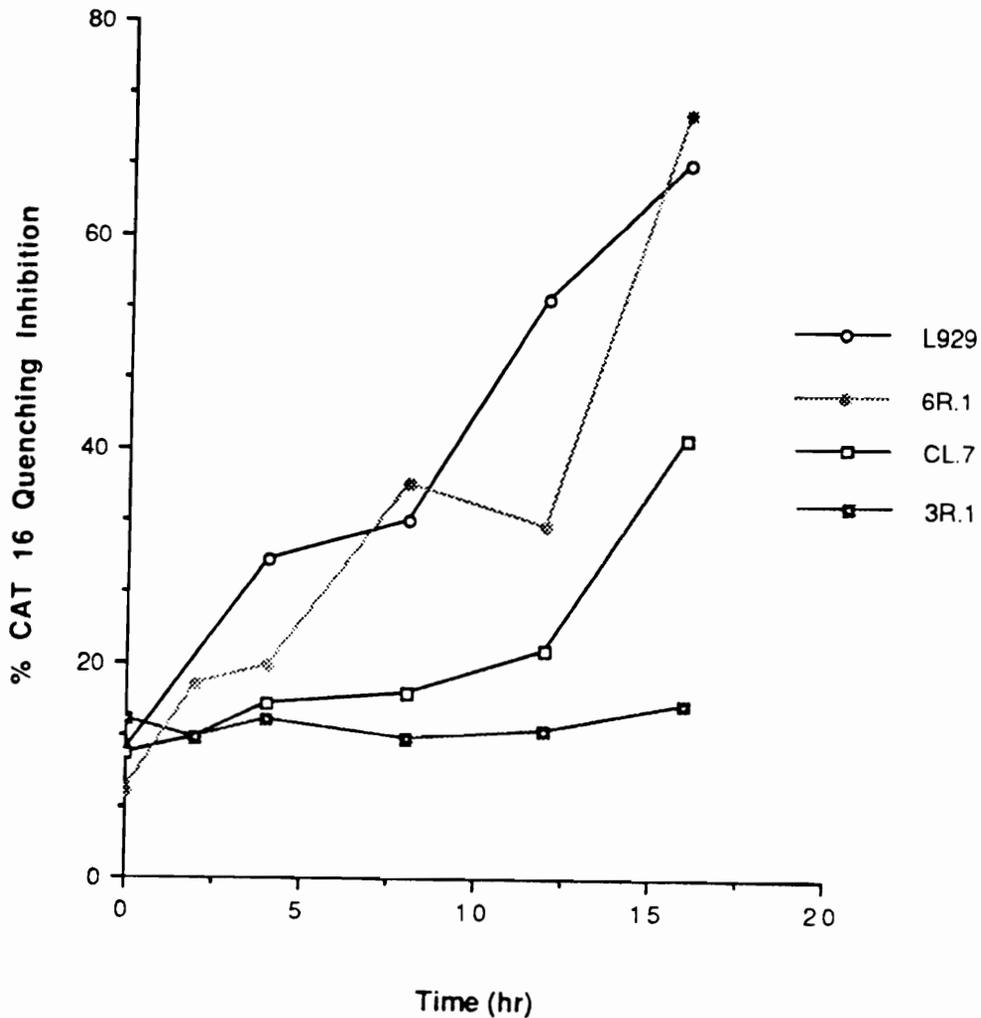


Figure 3 : The Differential Mitochondrial Toxicity To TNF- α Under A-MuLV Transformation. The % inhibition of CAT 16 quenching for mitochondrial preparations from L929, CL.7, 3R.1, and 6R.1 cells exposed to 100 units/ml TNF- α for different incubation times is shown. The % inhibition of quenching is determined from the formula % inhibition = (quenched height/control height) X 100.

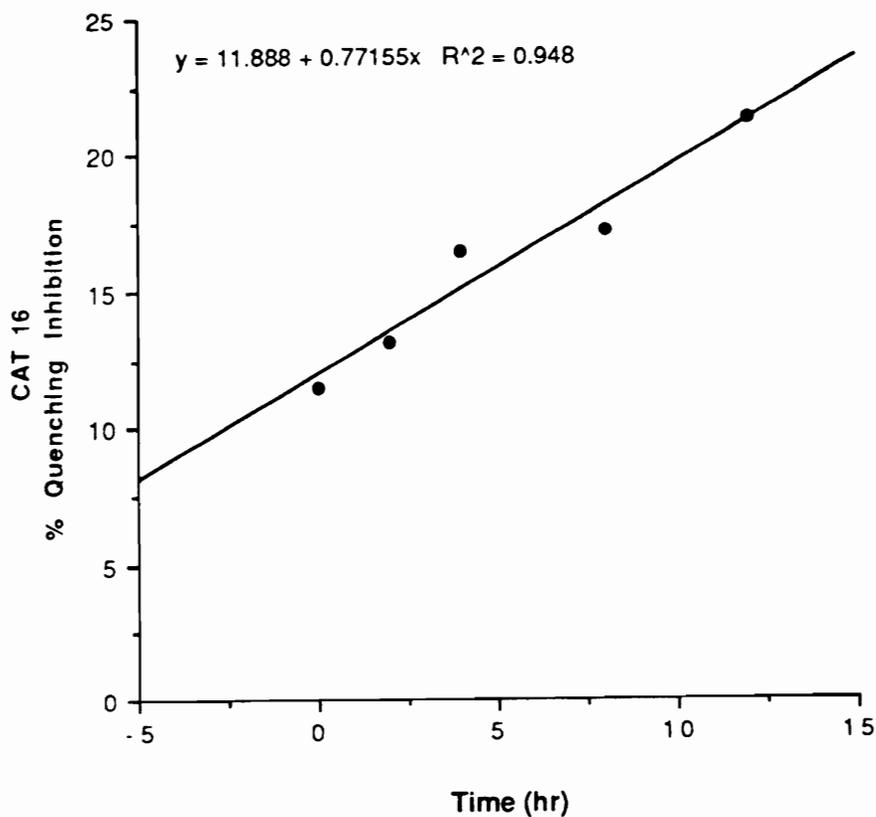


Figure 4(a) : Mitochondrial CAT 16 Quenching With TNF- α Incubation Time. The normal mitochondria quench CAT 16 signal. The TNF- α treated mitochondria do not quench CAT 16 signal. This inhibition of CAT 16 quenching rates are plotted for CL.7 mitochondria with the TNF- α incubation time.

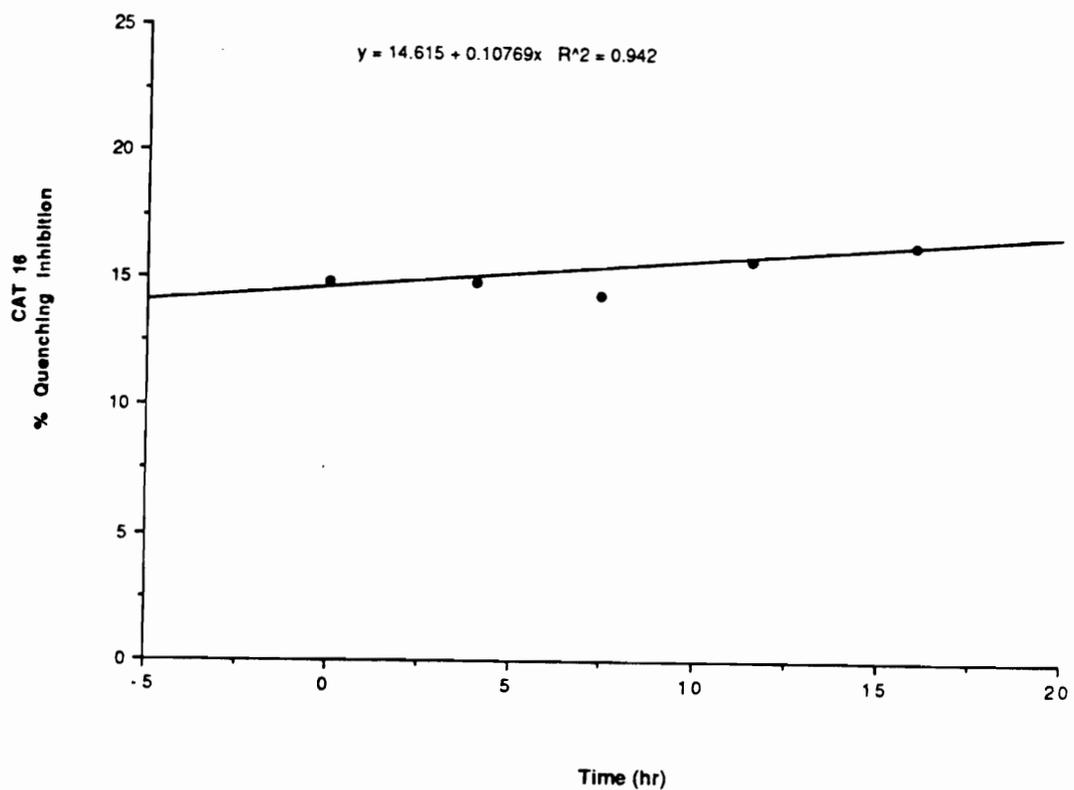


Figure 4(b) : Mitochondrial CAT 16 Quenching With TNF- α Incubation Time. The normal mitochondria quench CAT 16 signal. The TNF- α treated mitochondria do not quench CAT 16 signal. This inhibition of CAT 16 quenching rates are plotted for 3R.1 mitochondria with the TNF- α incubation time.

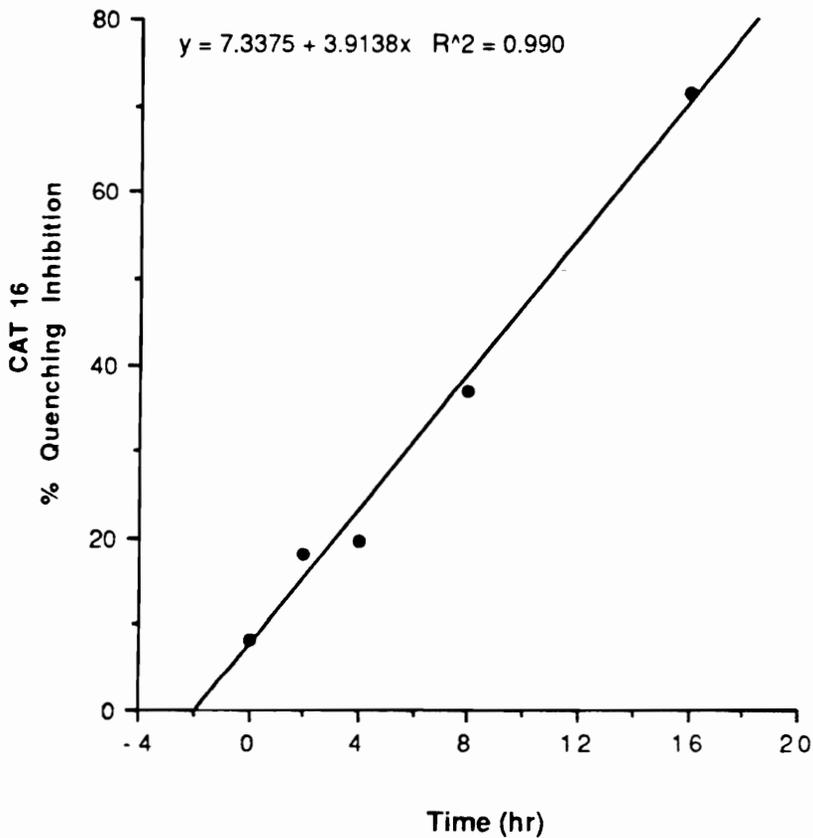


Figure 4(c) : Mitochondrial CAT 16 Quenching With TNF- α Incubation Time. The normal mitochondria quench CAT 16 signal. The TNF- α treated mitochondria do not quench CAT 16 signal. This inhibition of CAT 16 quenching rates are plotted for 6R.1 mitochondria with the TNF- α incubation time.

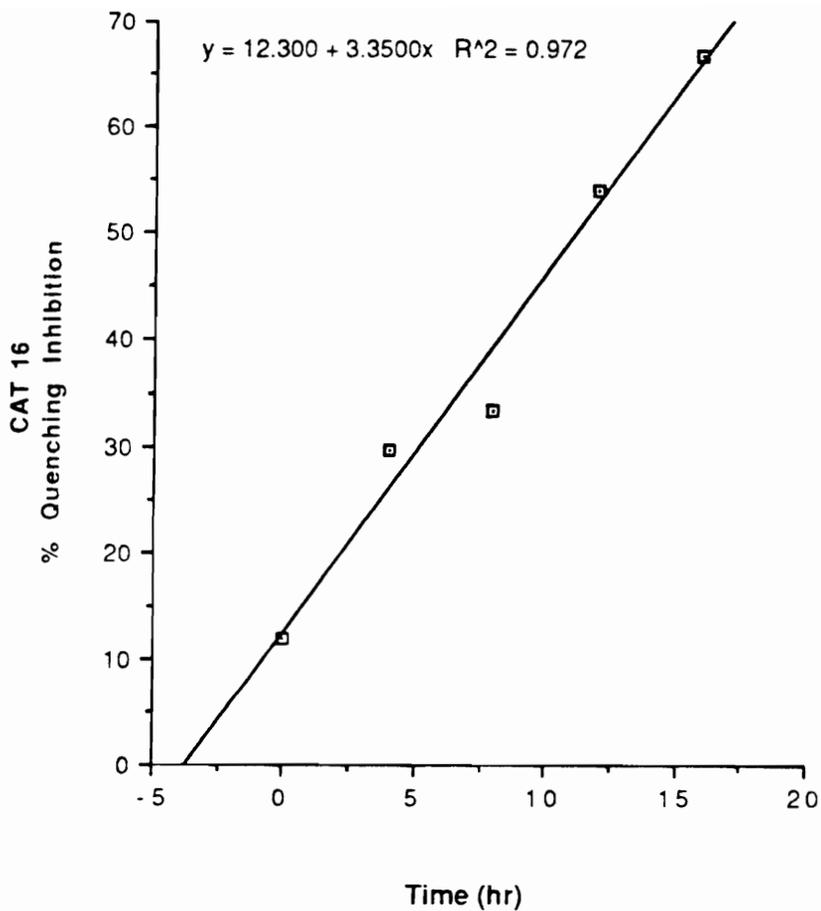


Figure 4(d) : Mitochondrial CAT 16 Quenching With TNF- α Incubation Time. The normal mitochondria quench CAT 16 signal. The TNF- α treated mitochondria do not quench CAT 16 signal. This inhibition of CAT 16 quenching rates are plotted for L929 mitochondria with the TNF- α incubation time.

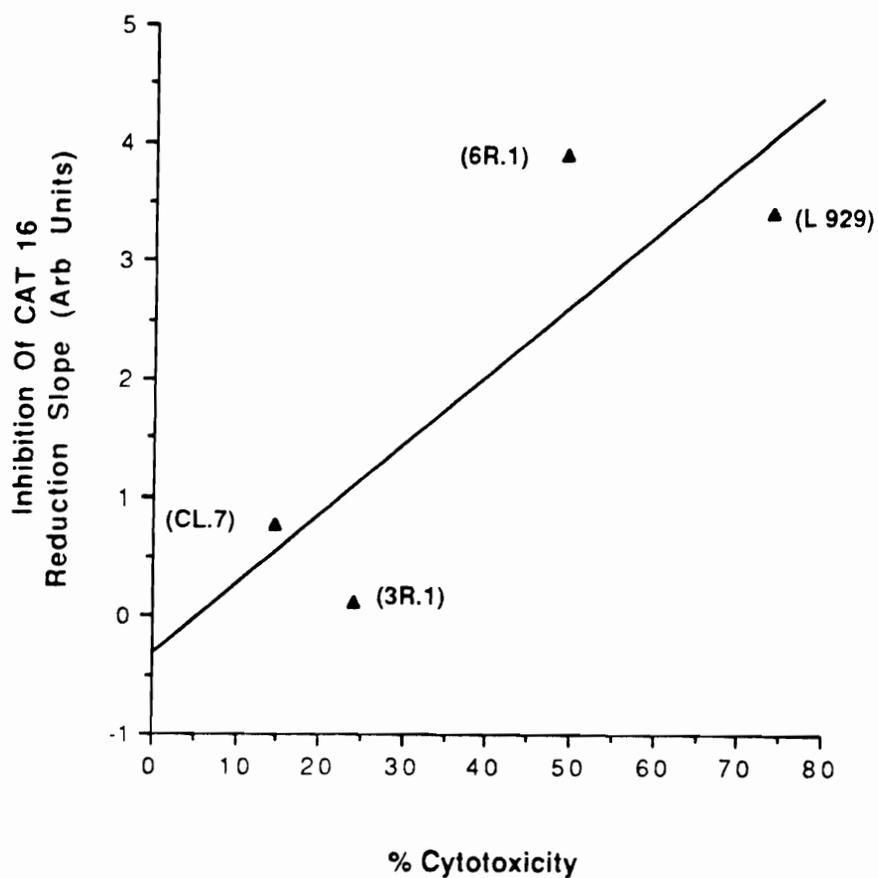


Figure 5 : Correlation of TNF- α Cytotoxicity With The Rate of Mitochondrial Reduction of CAT 16. The values of % cytotoxicity were taken from chapter 1. The mitochondrial quenching Inhibition Slopes Were Derived From Figure 4 (a) thru 4 (d).

CHAPTER - 10**DEFENSE ENZYME STATUS IN TNF- α CYTOTOXICITY
UNDER A-MuLV TRANSFORMATION****ABSTRACT**

Tumor necrosis factor- α (TNF- α), a macrophage derived polypeptide hormone has been known to elicit its cytotoxic effect via an oxidative process. This oxidant attack manifests at the organelle level by inducing mitochondrial toxicity, fragmentation of cellular DNA, release of lysosomal enzymes and peroxidation of membrane lipids. Recent investigations have determined that cytotoxicity of TNF- α could be mediated by production of free radical species. To counteract and cope with the adverse effects of this oxidative stress (ie to oxygen and it's reactive intermediates), the cellular machinery developed an array of defense enzymes. Nonenzymatic antioxidants also confer protective effects against such oxidative stress. The overall objective of the current investigation was to determine whether the differential susceptibility of normal (CL.7) and A-MuLV transformed clones (3R.1 and 6R.1) to killing by TNF- α might be influenced by its content of cellular antioxidants such as Cu/Zn- superoxide dismutase (SOD), Mn-SOD, glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rx), total glutathione (GSH), glucose-6-phosphate dehydrogenase (G-6-PDH), and cytochrome P450 reductase. As viral transformation has the potential to alter the antioxidant status of a cell, it is possible that differential susceptibility to TNF- α could, in part, be due to an altered antioxidant enzymes status. Thus, differences in the antioxidant enzymes of the above cells could make them differentially capable of handling the reactive species of oxygen implicated to be produced as a consequence of intracellular metabolism of TNF- α . The present study is aimed at determining not only the defense status of CL.7, 3R.1 and 6R.1 clones but also to determine the alterations in their antioxidant levels in response to the action of TNF- α as a function of time. Cu/Zn- SOD, Mn-SOD,

GSH-Px, GSH-Rx, total glutathione (GSH), G-6-PDH, and Cytochrome P450 levels were determined in control (no TNF- α) and TNF- α treated (100 units/ml) CL.7, 3R.1, and 6R.1 cells at incubation times ranging from 0 to 16 hrs by use of specific microtiter assay systems. The 3R.1 clone had a significantly higher ($p < 0.05$) TNF- α noninducible Cu/Zn-SOD and Mn-SOD levels than the CL.7 and the 6R.1 clones. The Cu/Zn-SOD and Mn-SOD in CL.7 clone were however inducible with TNF- α incubation time. The constitutive cytochrome P450 levels were found to be higher in 3R.1 clone than in CL.7 or 6R.1 clones. The cytochrome P450 in 3R.1 and CL.7 clones unlike in 6R.1 were inducible with TNF- α incubation time. The A-MuLV transformed clones, unlike CL.7, do not induce their glutathione reductase levels with TNF- α incubation time. GSH-Px was found to be significantly higher in 3R.1 than in CL.7 or 6R.1 clones. The GSH-Px was found to be inducible with TNF- α incubation time in CL.7 as well as 3R.1 cell lines. From the above data we concluded that the protection of CL.7 clone from TNF- α mediated cytotoxicity could be in part due to its otherwise low levels of defense enzymes inducible with exposure to TNF- α . The protection of 3R.1 clone from TNF- α mediated cytotoxicity could in part be due to its high levels of defense enzymes found to be non-inducible with TNF- α incubation time. The susceptibility of 6R.1 clone to TNF- α mediated cytotoxicity could be in part due to its low levels of defense enzymes found to be non-inducible with TNF- α incubation time. Thus induction of defense enzyme activity in CL.7 could protect it from the deleterious oxidant effects of TNF- α making CL.7 TNF- α insensitive. The 3R.1 clone although did not induce its defense enzymes upon TNF- α action however protected itself from a TNF- α mediated oxidative damage via a high level of constitutive defense status. The 6R.1 clone on the other hand neither had high levels of antioxidant enzymes nor were the enzymes inducible with TNF- α incubation. Thus 6R.1 clone was not prepared to cope with the oxidative stress induced upon TNF- α action.

INTRODUCTION

Oxidant Damage by TNF- α :

The macrophage is known to be the principal mediator of the effect of endotoxic challenge (Torti, et al 1985). It elicits this response by production of a low molecular weight 17 K protein factor called tumor necrosis factor- α (TNF- α) (Beutler, et al. 1985). TNF- α is responsible for orchestrating the hemorrhagic necrosis of transplantable tumors *in vivo* (Carswell et al. 1975). TNF- α shows diverse biological effects on different cell types. A summary of different TNF- α effects are shown in Figure 1. As seen in Figure 1, binding of TNF- α to its cell surface specific receptor (TNF-R) is required to elicit the pleotropic effect of TNF- α . TNF- α has an overall growth enhancing effect, null effect and cytotoxic effect on various murine and human cell lines (Sugarman et al. 1985), Creasey et al, 1987, Ruggerio et al. 1987, TABLE 1). These differences have been investigated at the level of TNF- α binding to TNF-R and it was determined that different responsiveness to TNF- α cannot be attributed to differences in affinity of TNF- α to TNF-R. At present it is not at all clear as to how TNF- α can act as a growth stimulating agent in cell lines as in WI-38 or U-373 and be at the same time cytotoxic to some others like L-929, WEHI-164 and MCF-7 (TABLE 1) although it has been suggested that a 138 K polypeptide is involved at the level of TNF- α binding to its receptor on the cell surface, in mediating its cytotoxicity. Recent investigations have determined that cytotoxicity of TNF- α could be mediated by production of free radical species. Incubation of TNF- α with TNF- α sensitive mouse tumorigenic fibroblasts L-M cells were shown to produce hydroxyl radicals (\cdot OH) as detected by evolution of methane gas upon addition of dimethyl sulfoxide (Yamauchi et al, 1989). The production of the \cdot OH under TNF- α cytotoxicity was responsive to dose of TNF- α and was shown by the above investigators to be inhibited by iron chelators like 2,2-bipyridine. The addition of iron chelator inhibits iron-catalyzed Fenton reaction thus suppressing the production of \cdot OH. The production of free radical species have been indirectly inferred by many other studies. For example TNF- α induces mitochondria to swell with an accompanied reduction in the

number of cristae (Matthews et al, 1987). This is indicative of free radical involvement. Similarly it has been shown that TNF- α cytotoxicity is suppressed by mitochondrial electron transport chain inhibitors (Kull et al. 1981, Watanabe, et al 1988) and inhibitors of arachidonic acid metabolism (Matthews, et al 1987). Both the mitochondrial electron transport chain (Cadenas et al, 1980) and the arachidonic acid cascade (Kuehl, et al 1980) are known to involve generation of $\cdot\text{OH}$. Thus it is possible that TNF- α induced production of $\cdot\text{OH}$ radicals could be via activation of the above mentioned metabolic pathways. Peroxidation of membrane lipids (Suffys et al, 1987) release of lysosomal enzymes (Watanabe et al, 1988) and fragmentation of DNA (Dealtoy, et al. 1987), observed under TNF- α toxicity could all be mediated via the production of $\text{OH}\cdot$ radical. Cytotoxicity of TNF- α has also been known to be partially mediated by other reactive species of oxygen like superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) as determined in a system of macrophage activated cell killing (Grace, et al 1988). The role of oxidant injury in tumor cells susceptible to TNF- α was investigated by measuring the levels of intracellular glutathione in these cells (Zimmerman, et al 1989). Production of these radical species, in cells susceptible to TNF- α , results in a high oxidative stress. If the cellular machinery cannot gear itself to handle this oxidant attack cell death ensues. It was shown that there is an inverse correlation between glutathione levels and cellular susceptibility to TNF- α . Thus if intracellular metabolism of TNF- α leads to the production of free radical species, the susceptibility of a cell to killing by TNF- α could be influenced by its content of enzymatic antioxidants such as Cu/Zn-superoxide dismutase (Cu/Zn-SOD), Mn-superoxide dismutase (Mn-SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rx), glucose-6-phosphate dehydrogenase (G-6-PDH), cytochrome P-450 reductase and non enzymic antioxidants like total glutathione content, vitamin E, and Vitamin A. For example, it has been illustrated that incubation of cells with TNF- α leads to a specific induction in the levels of mRNA as well as protein for Mn-SOD (Grace et al, 1988) *in vitro* as well as *in vivo*. The induction of Mn-SOD mRNA was independent of cell type. It was proposed by the above authors that Mn-SOD could be one of the defense enzymes involved in protecting the cells from the cytotoxic effects of TNF- α . This further supports the free radical pathway of cell killing of

TNF- α . TNF- α cytotoxic pathway could be operative by generating oxygen radicals like O_2^- at a mitochondrial site (Matthews, 1983) and induction of mRNA and enzyme levels of Mn-SOD could then protect cells from the action of TNF- α by dismutation of O_2^- .

Cellular Interaction of Reactive Oxygen Species and Tissue Damage:

Many of the toxic effects associated with oxygen are due to the formation of oxygen radicals (Gerschman, 1981). This idea was developed by Fridovich into the "superoxide theory of oxygen toxicity" (Fridovich, 1974, 1975, 1978). The susceptible targets for action of oxygen radicals are the polyunsaturated fatty acid (PUFA) side chain of cell and organelle membranes (specifically that of the mitochondria), cellular DNA, and cellular proteins. The superoxide dismutases accelerate the dismutation reaction removing O_2^- at the expense of forming hydrogen peroxide. The hydrogen peroxide, which is also a very reactive species, is removed by the peroxisomal enzyme, catalase. Hydrogen peroxide produced by the dismutation of O_2^- by SOD can contribute to $\cdot OH$ formation via iron-catalyzed reactions. The $\cdot OH$ radical so produced can initiate peroxidation of membrane lipids as well as cause damage to cellular DNA.

PUFA contain two or more carbon-carbon double bonds which makes them a good electron sink and therefore make them susceptible to damage by free radical attack. Abstraction of a hydrogen atom from the carbon chain of PUFA results in the formation of a carbon-centered radical ($C\cdot$). This triggers the peroxidative chain of events as shown (Figure 2). Abstraction of a proton followed by oxygen uptake by the conjugated diene gives rise to the formation of peroxy radical starting a chain of events to form lipid hydroperoxides, cyclic peroxides and cyclic endoperoxides. $\cdot OH$, peroxy and alkoxy radicals have sufficient energy to initiate the process of hydrogen abstraction leading to lipid peroxidation cascade. O_2^- and H_2O_2 are not good candidates for this event. The biological effect of such a lipid peroxidation event can be disastrous, resulting in structural damage to lipid bilayer making them more prone to oxidative stress (Gutteridge, 1978). The membrane damage can result in loss of semipermeability, release of hydrolytic enzymes, release of iron and copper complexes all aiding in the toxicity events stemming from the peroxidation process.

The Antioxidant Defense System:

In face of the potential adverse effects of oxidative stress (ie oxygen and it's reactive intermediates), the cellular machinery must evolve an efficient detoxification system. A number of enzymes and compounds are present in the cell which can protect the cellular components from the deleterious effects of activated oxygen species. The enzymatic system consists of three basic enzymes: the superoxide dismutase (SOD), catalase (CAT), and peroxidases, of which glutathione peroxidase (GSH-Px) is the most common form in mammalian cells. Different organelles are assigned for different enzymes. For example Cu/Zn-SOD is located in the cytosol whereas Mn-SOD is found in mitochondria. Glutathione peroxidase has two distinct isozymes: one found in the cytosol and one in mitochondria. Catalase is predominantly found in the peroxisomes. The network of these defense enzymes and their detoxification action are shown in Figure 4 a, b, and c. Removal of O_2^- and H_2O_2 from the biological system prevents the iron-catalyzed Haber-Weiss reaction from occurring. Among O_2^- , H_2O_2 and $\cdot OH$, the $\cdot OH$ is by far the most reactive of the three species. Thus the antioxidant enzyme system has a major function in preventing the $\cdot OH$ formation. Equally important is the nonenzymatic antioxidant defense system which largely consists of small molecular weight compounds. They include β -carotene (scavenger of 1O_2), vitamin E (nonspecific inhibitor of lipid peroxidation), mannitol (inhibitor of $\cdot OH$), iron chelators (which bind to iron and prevent $\cdot OH$ formation), glutathione and other thiols (inhibitor of $\cdot OH$ and cofactor of GSH-Px), vitamin C (scavenger O_2^- , $\cdot OH$ and 1O_2) and uric acid (scavenger of 1O_2 and $\cdot OH$). The lipid soluble antioxidants are vitamin E, vitamin A where are water soluble antioxidants are uric acid and vitamin C. Vitamin C probably acts as an antioxidant for vitamin E at the surface of lipid bilayer by scavenging the vitamin E radical and reducing it back to the original form (Barton et al; 1983). It should be emphasized that the cell responds to a given oxidative stress by orchestrating an interactive enzymatic as well as nonenzymatic antioxidant defense.

Antioxidant Defense of Tumor Cells:

A large number of studies have been performed on antioxidants in tumor cells. Tumor cells have abnormal activities of antioxidant enzymes as compared to control cells. Tumor cells have been shown to be nearly always low in Mn-SOD activity, usually low in Cu-Zn SOD activity, and almost always low in catalase activity (Oberley et al 1979). GSH-Px activity is highly variable, from very low to very high. Mn-SOD activity has been shown to be dramatically reduced in over 80 different types of human and murine neoplastic cells, whether spontaneous or oncogenically induced *in vivo* or *in vitro* by chemicals, viruses, or transplantation (Oberley et al 1979, 1982). The enzyme levels may be low in cells due to a low substrate (free radicals) levels. However, several investigations have shown that neoplastic cells have the capability to produce superoxide radical, the substrate for SOD (Oberley, 1979, 1982). Thus it appears that SOD, an inducible enzyme, is not low in the tumor cells because O_2^- is low. This suggests that diminished amounts of Mn-SOD activity coupled with superoxide production in the cancer cells may be a general characteristic of tumor cells. It has been hypothesized that low SOD activity in tumors is a result of lowered oxygen concentration (Halliwell, 1985) leading to an anoxic condition specially at their centers. There is however considerable evidence that O_2 or O_2^- are not the reason for low SOD activity. O_2^- causes increase in SOD activity in normal cells but not in fibrosarcoma cells in culture (Simon et al, 1981).

Tumor cells in general have a high level of GSH, although exceptions have been known (Meister and Griffith, 1979). The growth rate of human skin tumors is proportional to GSH concentration (Engin, 1976). Even though the levels of GSH are usually elevated in tumors, the activity of GSH-Px and GSH-Rx are variables. Enzymes such as SOD, CAT, GSH-Px and GSH-Rx have been measured in a wide variety of mouse tumors as compared with normal tissue levels (Misdale et al, 1983). The above investigators found that the activities of SOD, CAT and GSH-Rx were in general lower in the tumor than in normal tissue while that of GSH-Px were comparable. These data are consistent with the idea that tumors generally have low SOD and CAT levels but have variable amounts of GSH-Px.

Tumors of viral origin have been shown to incorporate viral

insertion sequences which have the ability to turn off and turn on genes (Tewin, 1980). The viral proteins have the potential to moderate gene expression. Viruses have been known to turn off Mn-SOD gene and turn on the cell proliferation gene. Alternatively, viruses may contain their own gene for cell proliferation and for alterations in the host defense enzyme status.

Recently we have determined that some Abelson-murine leukemia virus (A-MuLV) transformed clones of mouse embryonic fibroblasts (normal cells) are differentially susceptible to TNF- α as compared with the control cell lines (Chapter 1). It has been known that susceptibility of a cell to killing by TNF- α might be influenced by its content of cellular antioxidants such as Cu/Zn-SOD, Mn-SOD, GSH-Px, GSH-Rx and total glutathione (GSH) (Grace et al, 1988). As viral transformation has the potential to alter the antioxidant status of the cell, it is possible that differential susceptibility to TNF- α by the normal cells and the A-MuLV transformed cells could be due to an altered level of antioxidant enzymes. Thus differences in the antioxidant enzymes of the above cells could make them differentially capable of handling the reactive species of oxygen implicated to be produced as a consequence of intracellular metabolism of TNF- α . The present study is aimed at determining not only the defense status of CL.7, 3R.1 and 6R.1 clones but also to determine the alteration in their antioxidant levels in response to the action of TNF- α as a function of time.

MATERIALS AND METHODS

CELLS:

CL.7, 3R.1 and 6R.1 clones were cultured in Dulbeccos modified Eagles medium (DMEM), with 10 % fetal calf serum (FCS), with 10 units/ml penicillin and 10 μ g/ml streptomycin at pH 7.4 in a 37°C incubator with 5% CO₂. The cells were purchased from American Type Culture Collection (ATCC).

TNF- α :

TNF- α was immunopurified from lipopolysaccharide (LPS)

activated RAW 264.7 macrophage supernatant on an anti-TNF- α Ab column as described (Chapter 1). The specific activity of TNF- α as determined on L929 cytotoxicity bioassay was 6×10^5 units/mg protein.

Induction study:

Cells in 75 cm² flasks, in duplicate, were incubated with and without 100 units/ml TNF- α , 12 ml per flask, for 0, 2, 4, 8 and 16 hr in complete medium without actinomycin D. After the incubation time cells were scraped and spun down, in complete media, at 1500 rpm for 15 minutes. The cells were further washed with 10 ml of Hanks balanced salt solution (HBSS) without phenol red. The cell pellet was resuspended in 600 μ l of HBSS without phenol red and were frozen in six 100 μ l fractions at -70° C till assayed.

Determination of Proteins:

Protein levels were determined by the dye binding method of Bradford (Bradford 1976). The samples were freeze thawed three times and were diluted 1:4 in dH₂O. 20 μ l of 1:4 dilution of sample was mixed with 50 μ l water in six replicates. To each of these replicates were added 5 ml of Bradford reagent and incubated for 5 minutes at room temperature. The samples were read at 595 nm in disposable 1 ml cuvettes in a Shimadzu UV-160 spectrophotometer against a bovine serum albumin (BSA) standard curve. The protein levels of the replicate samples were expressed as mean \pm S.D.

Assay for Cu/Zn-SOD and Mn-SOD:

Superoxide dismutases (SOD) catalyze the dismutation of the superoxide radical:



The assay was performed in a 96 well microtiter plate as a modification of the cytochrome-c reduction by O₂⁻, generated enzymatically by a xanthine-xanthine oxidase system, (McCord et al, 1969) as described (APPENDIX-4). The total reaction volume of the assay system was 100 μ l. The total SOD content of the samples was determined using the buffer 0.05 M potassium phosphate buffer

(KPi), pH7.8, 10^{-4} M EDTA. The Mn-SOD content of the samples was determined using the above buffer in the presence of 2mM KCN (CN⁻ inhibits Cu/Zn-SOD). The conversion of xanthine to uric acid by xanthine oxidase produced the superoxide radical. The amount of superoxide present could be assayed by the reduction of cytochrome C monitored at 550 nm. The rate of cytochrome C reduction reflected the rate of superoxide production. In presence of SOD, the superoxide, produced via the X.O pathway, was scavenged at an unknown rate. The extent to which the enzyme decreased the rate of cytochrome C reduction was used to determine the activity of SOD in a given sample. This determination in absence of CN⁻ gave total SOD and in presence of CN⁻ give Mn-SOD. The Cu/Zn-SOD content derived by subtracting Mn-SOD values from total SOD content. The rates were determined as m OD/minute. We calculated the percent inhibition of cytochrome C reduction for each sample. Defining 50 percent inhibition of cytochrome C reduction to be 0.1 U

$$\% \text{ inhibition} / 50 \% = 10 \times \# \text{ Units}$$

knowing units/ μ l in undiluted sample and mg/ μ l in undiluted sample we calculated the units SOD/mg protein. Thus the specific activity of both Cu/Zn-SOD and Mn-SOD could be calculated as above. The details of the microtiter assay for both Cu/Zn-SOD and Mn-SOD was described in APPENDIX-4.

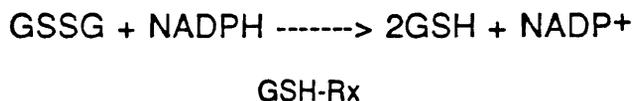
Assay for Total Glutathione:

Glutathione (GSH) is a tripeptide of cysteine, glycine and glutamic acid and is the principal nonprotein sulfhydryl compound present in the tissues and is a measure of cellular oxidative stress. Control and TNF- α (100 units/ml) treated BALB/c CL.7, A-MuLV 3R.1 and A-MuLV 6R.1 cell samples were analyzed for total glutathione content by a microtiter plate assay, adapted from the kinetic assay presented by Tietze (1969), as described (APPENDIX-5). A plot of total glutathione in μ g per mg protein was obtained for the above three cell lines as a function of TNF- α incubation time.

Assay for Glutathione Reductase (GSH-Rx):

GSH-Rx reduces oxidized glutathione (GSSG) to restore intracellular concentration of GSH, using NADPH as a cofactor. The

enzyme is assayed spectrophotometrically at 340 nm by monitoring the decrease in NADPH concentration.



BALB/c CL.7, A-MuLV 3R.1 and A.MuLV 6R.1 clones treated with TNF- α (100 units/ml) for 0,2,4,8 and 16 h were assayed for GSH-Rx by a microtiter plate assay adapted from the method presented by Sies, et al (1984). The assay was performed as described in APPENDIX-6. GSH-Rx levels in units per μg protein, were plotted for the cell lines as a function of the TNF- α incubation time.

Assay for Glutathione Peroxidase:

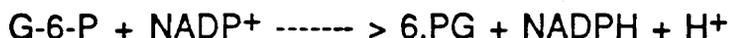
Glutathione peroxidase (GSH-Px) catalyzes the reduction of hydroperoxides with glutathione as a reductant.



It is a major antioxidant defense enzyme in the cytosol of cells which protects cells from the oxidative damage due to hydrogen peroxide as well as lipid hydroperoxides. The glutathione peroxidase levels in cells incubated with 100 units/ml of TNF- α for various time intervals were determined by a microtiter modification of the assay (Flohl et al 1984) as was described in APPENDIX-7. The GSH-Px levels, in units/ μg protein, were plotted for the cell lines as a function of TNF- α incubation time.

Assay for Glucose-6 Phosphate Dehydrogenase (G-6-PDH):

G-6-PDH is responsible for catalyzing the first reaction in the pentose phosphate shunt. Glucose-6-Phosphate (G-6-P) is oxidized to 6-phosphogluconate (6-PG) with a concomitant reduction of NADP to NADPH. The enzyme catalyzes the following reaction:

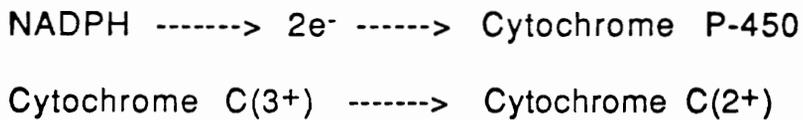


The G-6-PDH assay was performed using a modified version of the Sigma diagnostic kit (Sigma Chemical Co, MO, Procedure #345-UV). The assay was performed in a microtiter plate as described

(APPENDIX-8). G-6-PDH levels in normal and A-MuLV transformed cells incubated with 100 units/ml TNF- α for 0,2,4,8 and 16 hr were determined and plotted as units/ μ g protein for the three cell lines as a function of TNF- α incubation time.

Assay for Cytochrome P-450 Reductase:

Cytochrome P-450 reductase is a NADPH dependent flavoprotein necessary for transfer of electrons from NADPH to the monooxygenase drug metabolizing system. A reaction scheme used to assay the enzyme activity is as follows:



The cytochrome P-450 assay was performed in a microtiter plate system adapted from Dallner (1963) as described (APPENDIX-9). Cytochrome P-450 was quantitated, in normal and A-MuLV transformed cells incubated with 100 units/ml TNF- α for 0,2,4,8 and 16hr, as units/ μ g protein.

Statistical Analysis : The statistical analysis was performed by a paired t test comparison. The probability of $p \leq 0.05$ was taken to be significant. The results were expressed as mean \pm S.D of six replicate readings.

RESULTS

Cu/Zn-SOD, Mn-SOD, cytochrome P-450 reductase, total glutathione, GSH-Rx, GSH-Px and G-6-PDH levels were determined as described in the material and methods section. The antioxidant status under A-MuLV transformation was depicted in Figure 5. Cu/Zn-SOD as well as Mn-SOD for the 3R.1 clone expressed as units/mg protein were higher than the CL.7 and the 6R.1 clones ($p < 0.05$, Figure 6). These enzymes were measured using the microtiter assay system as described in Appendix 4. 3R.1 and 6R.1 clones possessed more Cu/Zn-SOD as compared to the CL.7 clone (Figure 6). Contrary to the observations (Oberley et al 1979, 1982) that neoplastic cells have reduced levels of Mn-SOD, the 3R.1 clone had a significantly higher Mn-SOD content than the CL.7 and the 6R.1.

clones. Similarly neoplastic cells in general do have a reduced level of cytochrome P-450 but our finding suggests that both the A-MuLV transformed clones had a significantly higher levels of cytochrome P-450 than the untransformed CL.7 clone ($p < 0.05$, Figure 7). Cytochrome P450 was quantitated using the microtiter assay system as described (Appendix 9). GSH-Rx, GSH-Px and total glutathione values were all significantly higher in 3R.1 clone than in the CL.7 clone ($p < 0.05$). 6R.1 clone had only G-6-PDH levels higher than CL.7 (Figure 8). Similarly G-6-PDH was higher in 3R.1 than in CL.7 but was not significantly different in 6R.1 as compared to CL.7 (Figure 9). From the analysis of the constitutive levels of the antioxidant enzymes in the three clones tested it was seen that CL.7 clone had low levels of Cu/Zn-SOD, Mn-SOD, Cytochrome-P450, GSH-Px, GSH-Rx, and G-6-PDH. The 3R.1 clone had a significantly higher level of Cu/Zn-SOD, Mn-SOD, Cytochrome-P450, total glutathione, GSH-Rx, GSH-Px, as well as G-6-PDH as compared to the CL.7 clone ($p < 0.05$). The 6R.1 clone however had only cytochrome P450 and GSH-Rx significantly higher than the CL.7 clone. Thus most of the antioxidant enzymes were expressed at a lower level for CL.7 and 6R.1 clones than for the 3R.1 clone. These results would suggest that among the three clones studied 3R.1 clone would be best prepared to cope with the TNF- α induced oxidative stress. These results although explain why 3R.1 would be a TNF- α insensitive clone, the reason why the normal (CL.7) clone would also be TNF- α insensitive was not clear from the constitutive defense enzyme status.

The effect of time incubation with 100 units/ml of TNF- α on the defense enzyme levels was investigated for the normal and the A-MuLV transformed clones. TNF- α induced the Cu/Zn-SOD levels only in the TNF- α insensitive (CL.7) cell line (Figure 10). Similarly TNF- α induced Mn-SOD levels only in CL.7 clone (Figure 11). TNF- α however suppressed the Mn-SOD levels in 3R.1 progressively with incubation time (Figure 11). Cytochrome P-450 levels were inducible in TNF- α insensitive normal (CL.7) and transformed clone (3R.1) (Figure 12). The induction of the enzyme however was transient in the case of CL.7 (maximum at 4h: TNF- α) whereas the induced cytochrome P-450 levels were stable for the case of 3R.1 clone (Figure 12). Surprisingly, the total glutathione levels (GSH +

GSSG) were enhanced for the two transformed clones 3R.1 and 6R.1 (Figure 13). GSH-Rx remained induced in 3R.1 and 6R.1 clones with TNF- α incubation time whereas TNF- α transiently induced the GSH-Rx levels in the normal insensitive clone CL.7 (Figure 14). Similarly GSH-Px levels were inducible in CL.7 with TNF- α incubation time although there was a noticeable suppression of GSH-Px in 3R.1 (Figure 15) with time incubation with TNF- α . Similarly G-6-PDH levels were inducible in CL.7 while they remained non-induced for 3R.1 and 6R.1 clones (Figure 16). From the defense enzyme induction data we concluded that for the CL.7 clone Cu/Zn-SOD activity was inducible around 4-8 hr (Figure 10), Mn-SOD activity was inducible throughout the 16 hr incubation time (Figure 11), cytochrome P450 level was inducible between 2 to 6 hr (Figure 12), glutathione reductase was inducible around 2 hr, GSH-Px (Figure 15) and G-6-PDH (Figure 16) levels were induced around 4 to 8 hr of TNF- α incubation. Thus although clonal constitutive levels of the defense enzymes were found to be low for CL.7, most of the key defense enzyme activities were inducible when CL.7 was incubated with TNF- α . In contrast most of the defense enzymes for 3R.1 and the 6R.1 clones remained non-induced.

DISCUSSION

TNF- α has been implicated to elicit its cytotoxic effect via an oxidative process. The reactive oxidants could manifest at the organelle level by inducing mitochondrial toxicity, fragmentation of cellular DNA, release of lysosomal enzymes and peroxidation of membrane lipids. The cellular machinery evolved an array of defense enzymes (Figure 4b) to protect itself from this oxidative stress. We have investigated the antioxidant defenses in normal (CL.7) and A-MuLV transformed clones (3R.1 and 6R.1) under the action of TNF- α . The cellular antioxidants such as Cu/Zn-SOD, Mn-SOD, GSH-Px, GSH-Rx, total glutathione (GSH), G-6-PDH, and Cytochrome P450 were monitored. The alterations in their antioxidant levels in response to the action of TNF- α as a function of time was also determined. We have developed sensitive microtiter assays for several of these enzymes to measure their levels in a smaller reaction volume. The 3R.1 clone had a significantly higher TNF- α noninducible Cu/Zn-SOD

and Mn-SOD levels than the CL.7 and the 6R.1 clones ($p < 0.05$). The Cu/Zn-SOD and Mn-SOD in CL.7 clone were low but inducible with TNF- α incubation time. The constitutive cytochrome P450 levels were found to be higher in 3R.1 clone than in CL.7 or 6R.1 clones. The cytochrome P450 in 3R.1 and CL.7 clones unlike in 6R.1 were inducible with TNF- α incubation time. The A-MuLV transformed clones, unlike CL.7, did not induce their glutathione reductase levels with TNF- α incubation time. GSH-Px was found to be significantly higher in 3R.1 than in CL.7 or 6R.1 clones. The GSH-Px was found to be inducible with TNF- α incubation time in CL.7 as well as 3R.1 cell lines.

From the above results we concluded that CL.7 protected itself from TNF- α cytotoxicity by inducing its otherwise low levels of defense enzymes with TNF- α incubation time. CL.7 is thus capable of handling the TNF- α induced oxidative stress by inducing its defense enzyme activities. The 3R.1 clone although does not induce its defense enzymes upon TNF- α action was able to protect itself from a TNF- α mediated oxidative damage due to a high level of constitutive defense enzyme status. The 6R.1 clone on the other hand neither had high levels of antioxidant enzymes nor were its enzymes inducible with TNF- α incubation. Thus 6R.1 clone was not prepared to cope with the oxidative stress induced by TNF- α action. It was concluded that the susceptibility of a clone to TNF- α could in part depend on the constitutive defense enzyme levels or on the inability of the clone to induce its defense enzymes when exposed to the action of TNF- α .

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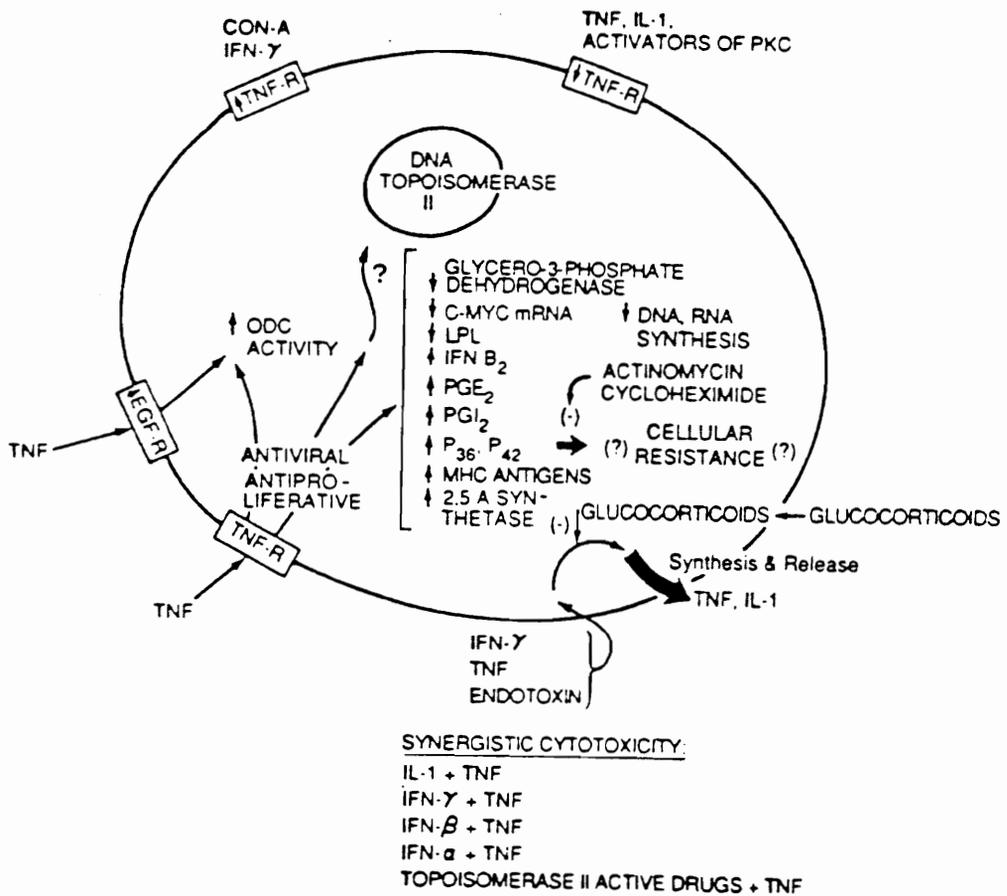


Figure 1 (a): An Overview Of The Effect Of TNF- α At The Cellular Level. Produced from : Rosenblum et al. (1989) vol.9, pp25.

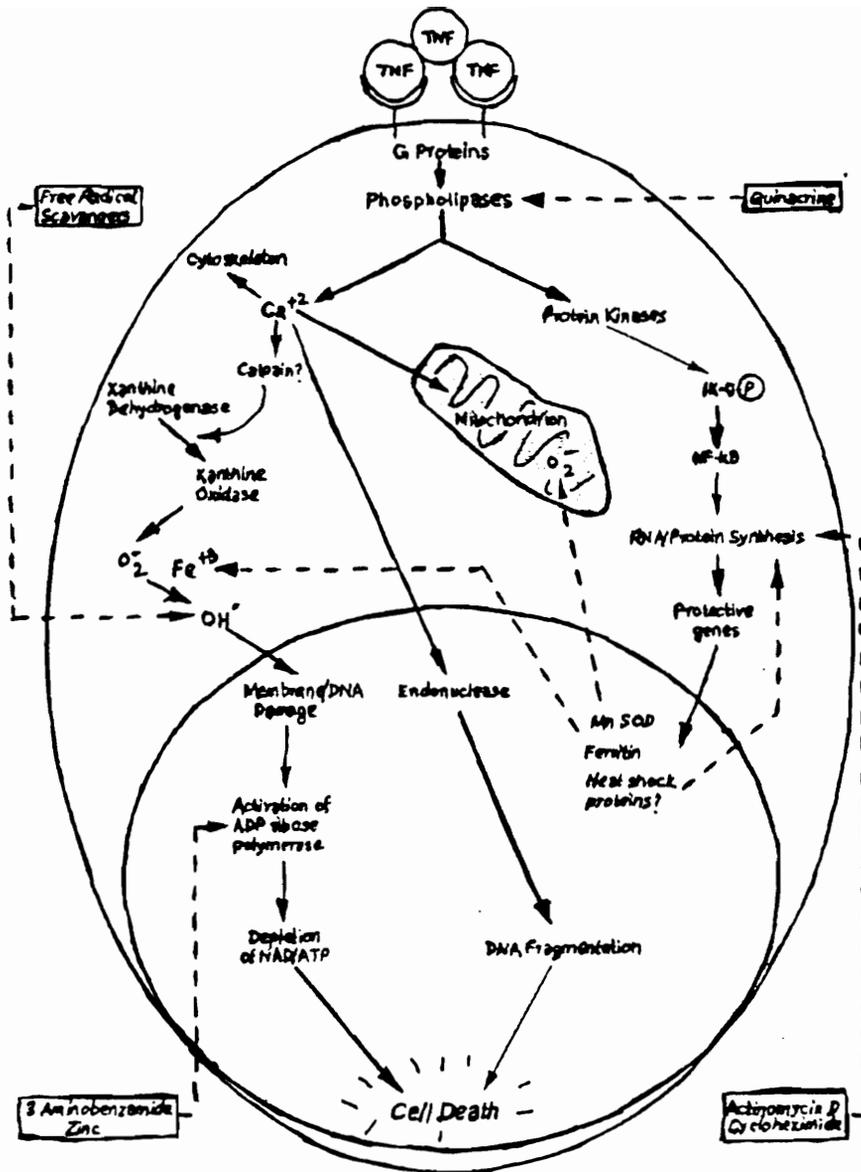


Figure 1 (b) : TNF- α Cytotoxic Pathway, Involvement of Free radicals And Defence Enzymes.

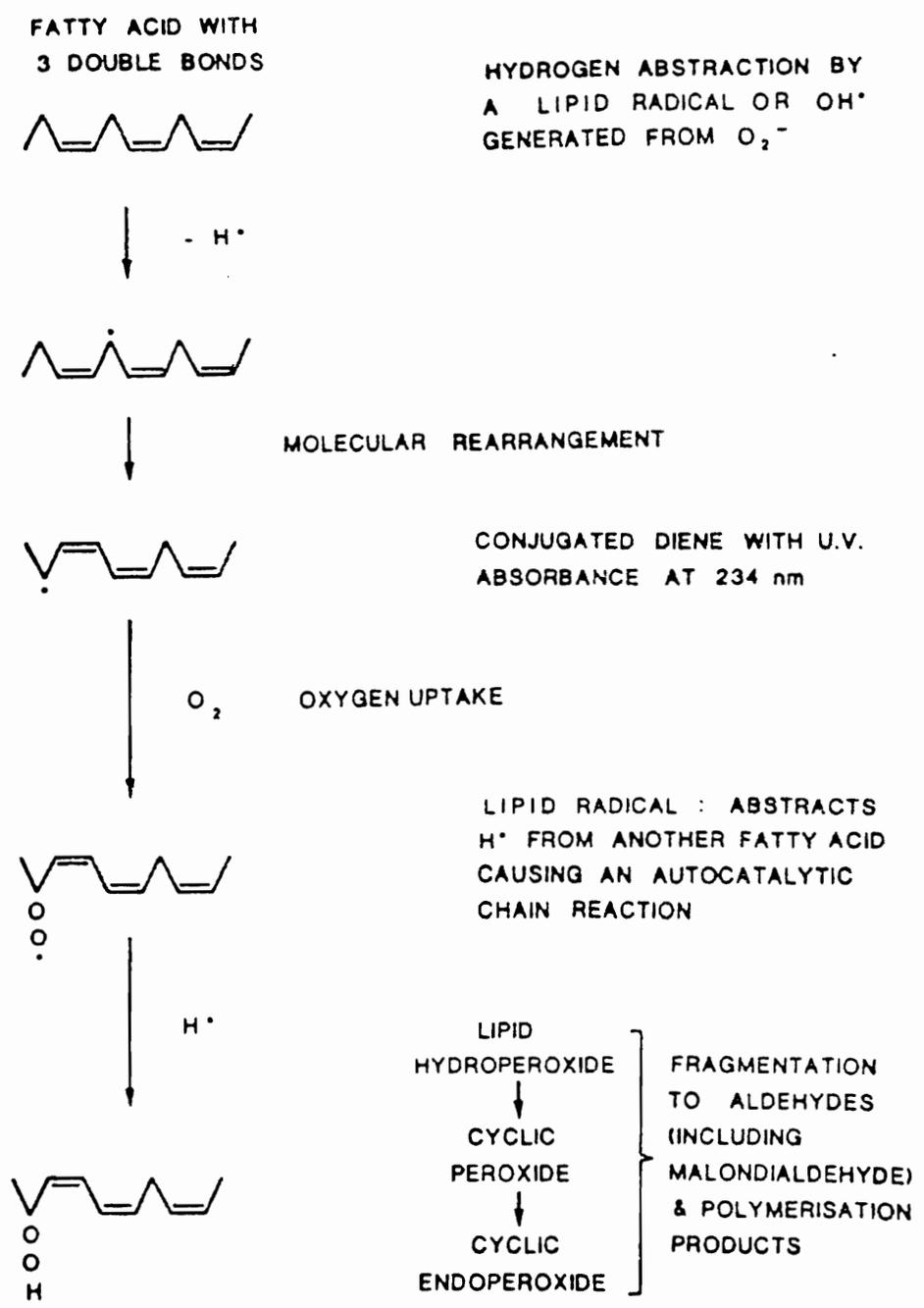


Figure 2 : A Proposed Mechanism of Lipid Peroxidation Events. Produced from : Oberley, et al (1986).

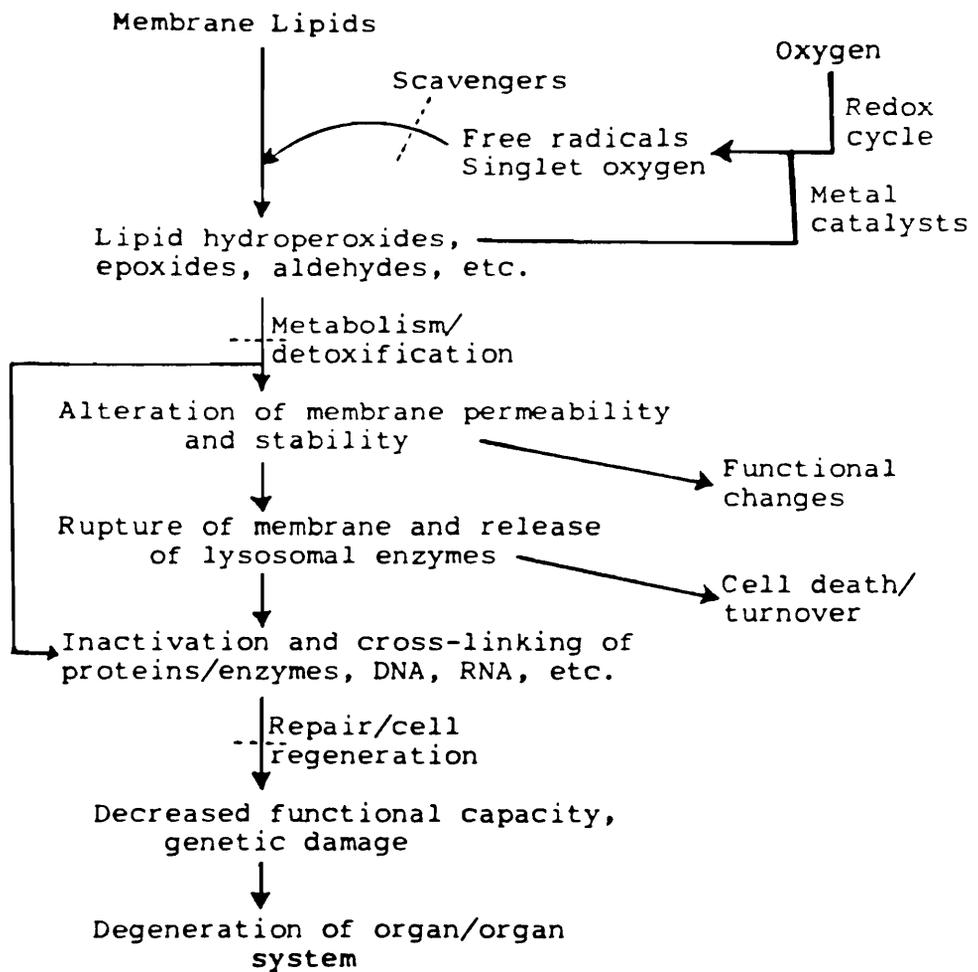


Figure 3 : Mechanism of Free radical Mediated Tissue Injury. Produced from : Oberley, et al (1986).

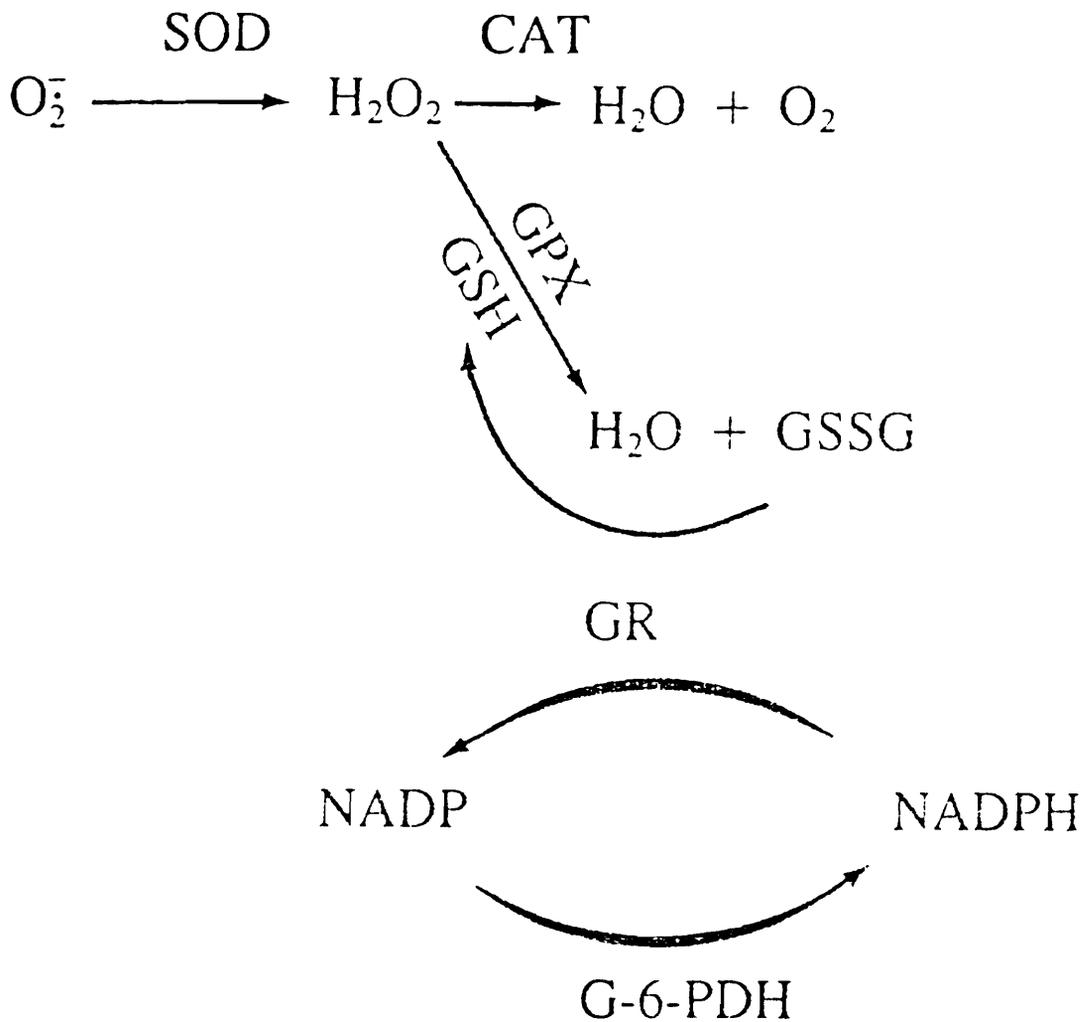


Figure 4 (a) : Crucial Defence Enzymes Involved In Mediating Protection Against Free Radical Attack: (Gerschman, 1981).

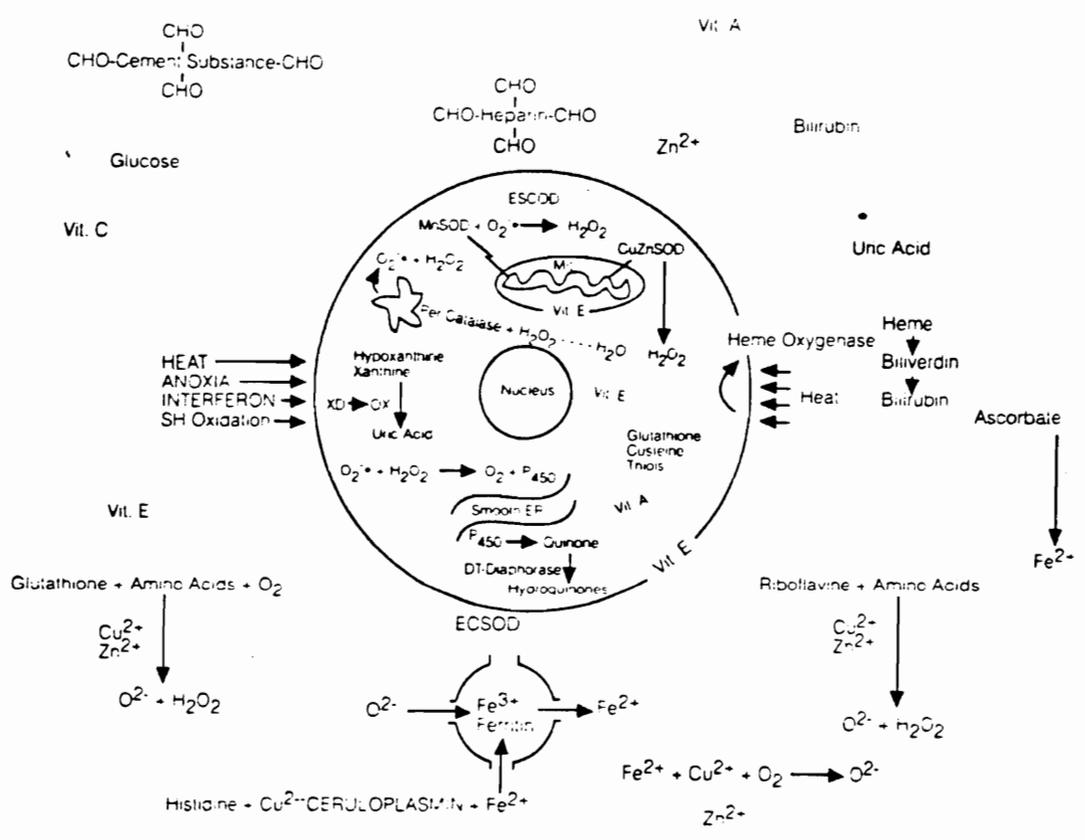


Figure 4 (b) : Array of Defense Enzymes With Their Cellular Location. From: Sp bulletin (1991), J. of the Lab Technology for bio-research.

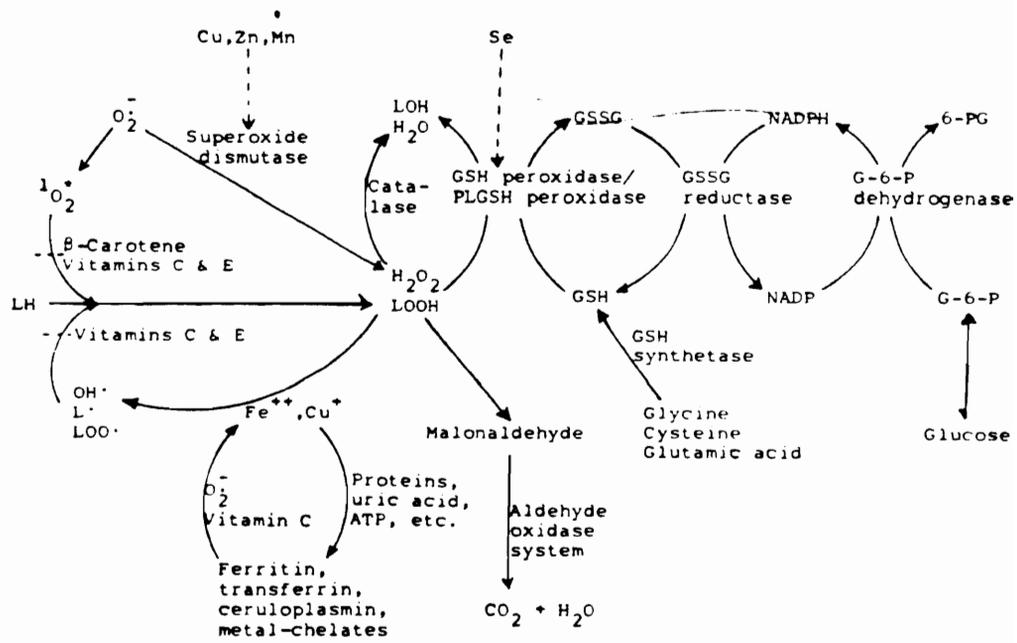


Figure 4 (c) : Major Cellular Antioxidant Defence Systems

ANTIOXIDANT STATUS UNDER AbLV TRANSFORMATION

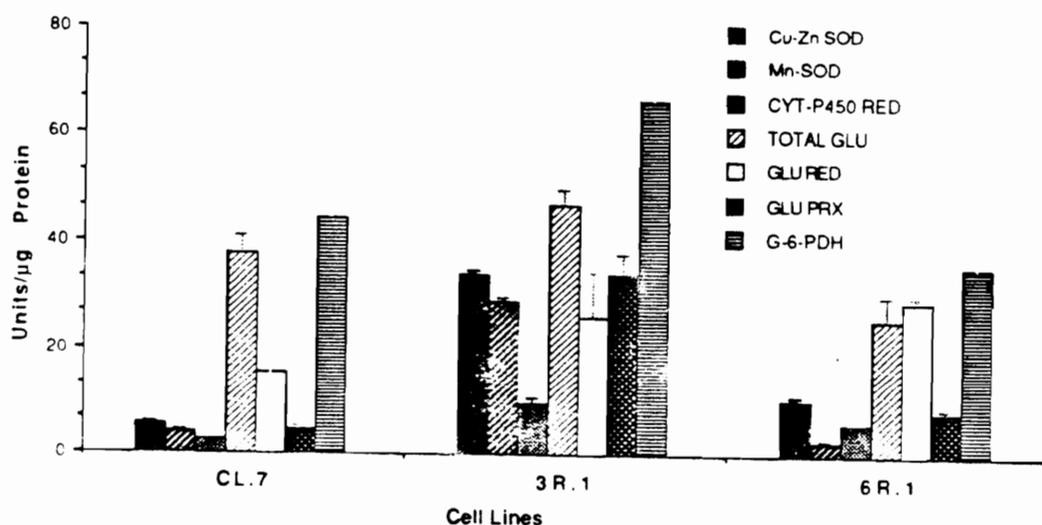


Figure 5 : An Overview of The Antioxidant Status Under A-MuLV Transformation. The values of units enzyme/mg protein as well as total glutathione in $\mu\text{g}/\text{mg}$ protein were determined for CL.7, 3R.1, as well as 6R.1 cells using microtiter assay systems developed as described in the materials and methods section.

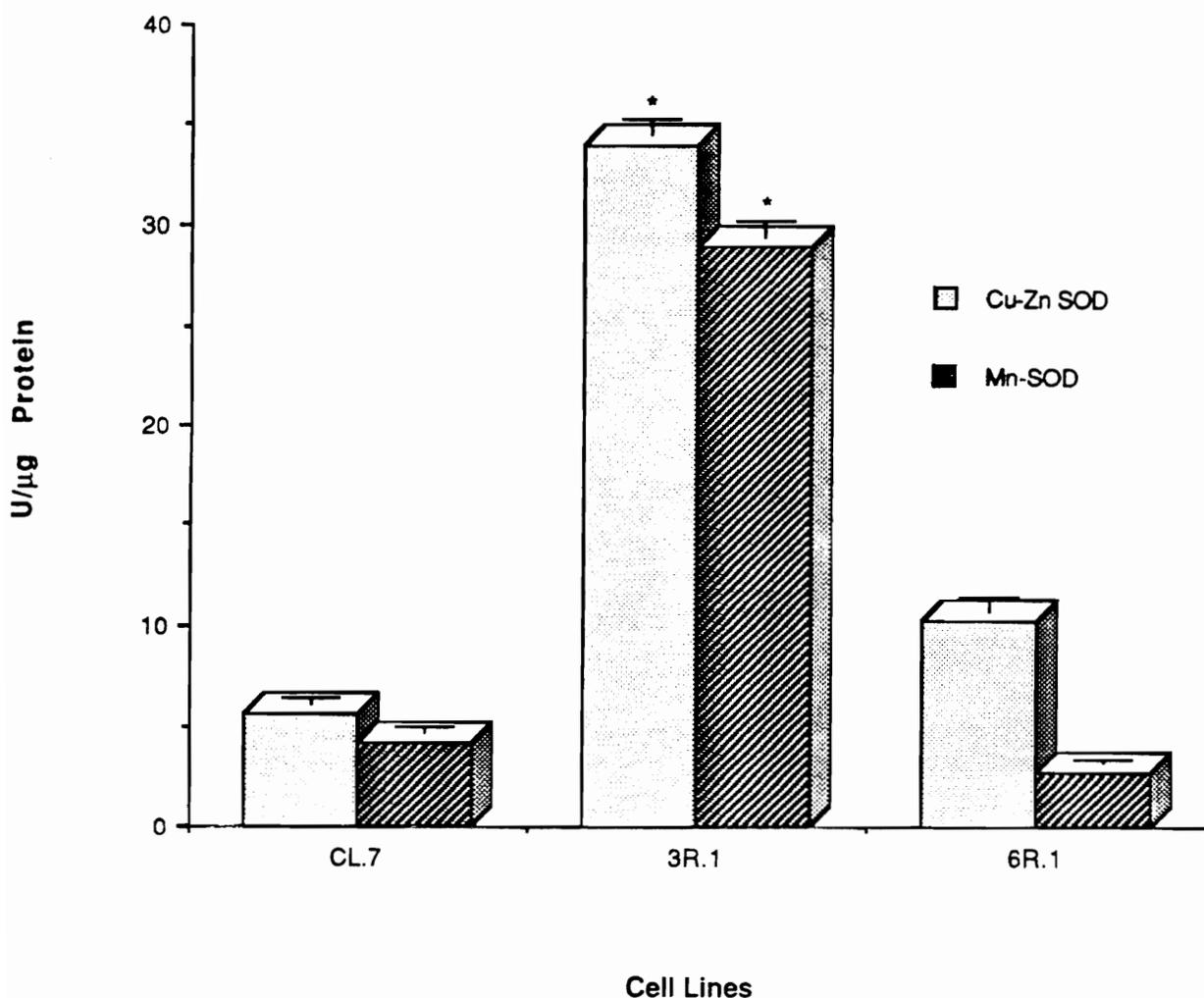


Figure 6 : The Cu/Zn - SOD and Mn-SOD Under A-MuLV Transformation. The quantitation of Cu/Zn- SOD and Mn-SOD levels CL.7, 3R.1, and 6R.1 were done in six replicates by a cytochrome C reduction microtiter assay as described (appendix-4). The 3R.1 (TNF- α insensitive) cell line has a significantly higher ($p < 0.05$) level of both Cu/Zn- SOD and Mn-SOD than the CL.7 and 6R.1 clones.

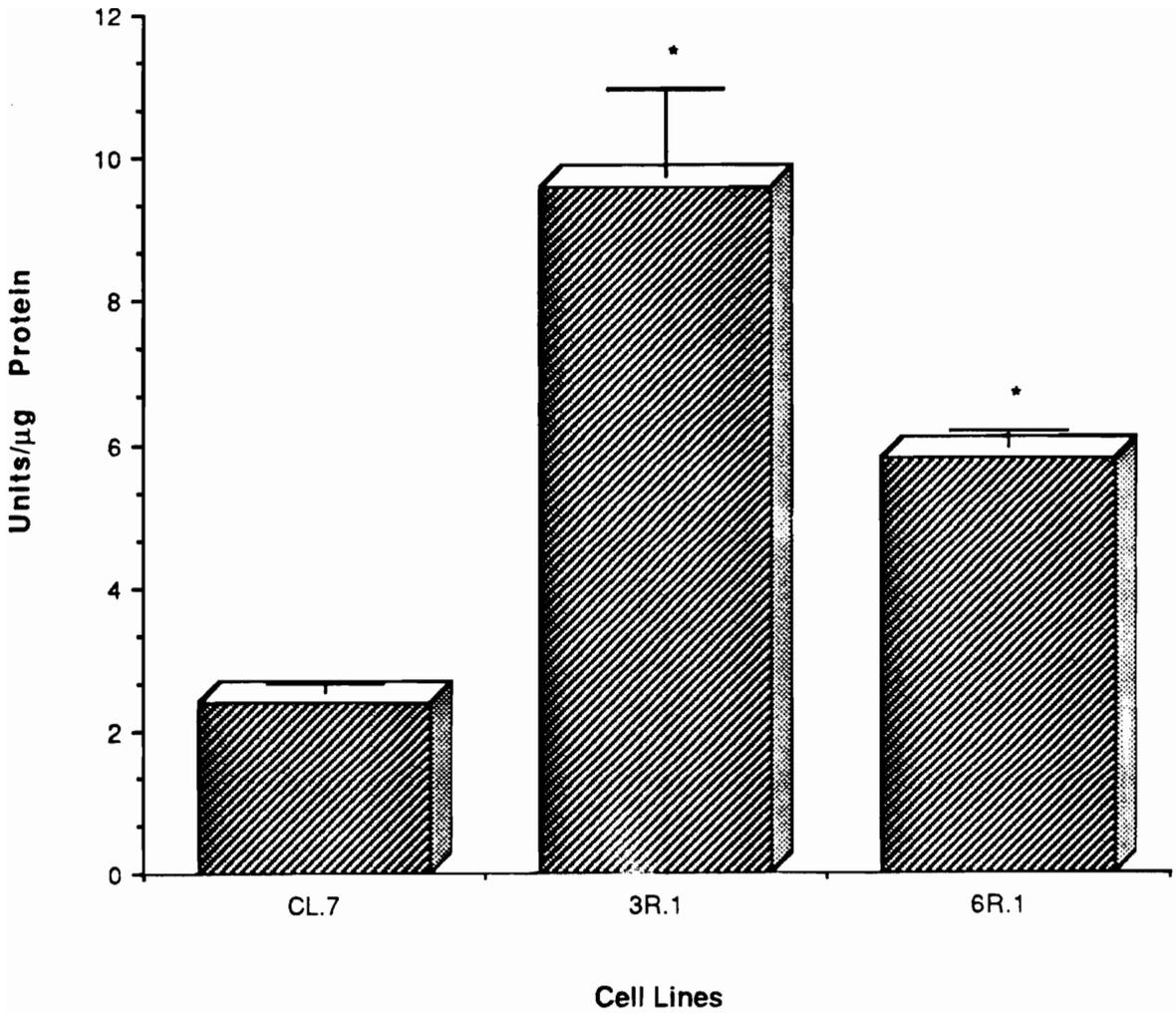


Figure 7 : The Determination of Cytochrome P450 . Cytochrome P 450 levels of CL.7, 3R.1, and 6R.1 clones were determined as units/mg protein in six replicates, presented as mean \pm S.D , by a microtiter assay system (appendix-9).

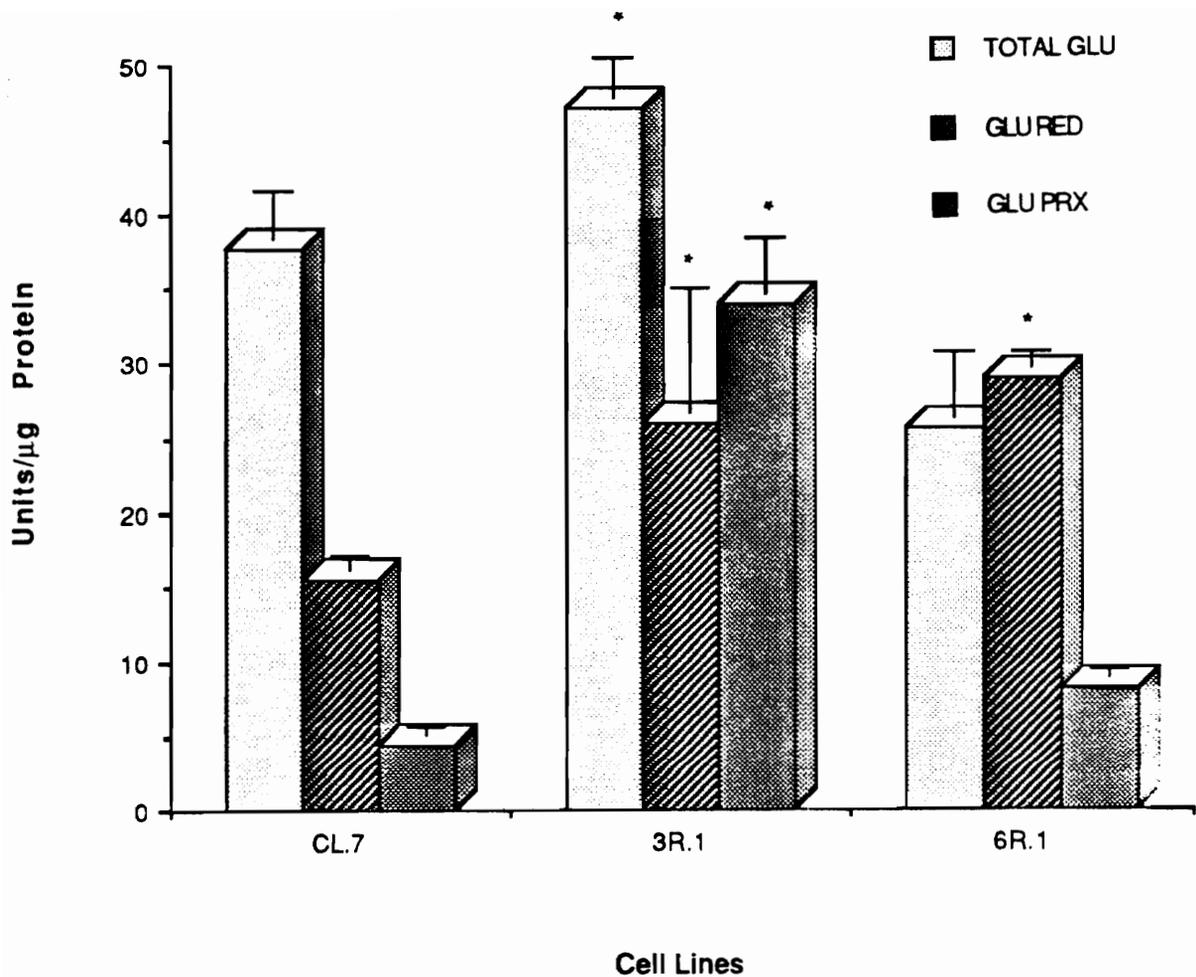


Figure 8 : Determination of total glutathione (in $\mu\text{g}/\text{mg}$ protein). Glutathione reductase (units/mg protein), and glutathione peroxidase (units/mg protein) were carried out for CL.7, 3R.1, and 6R.1 clones using microtiter assay systems described in appendices 5, 6, and 7. The values are represented as mean \pm S.D.

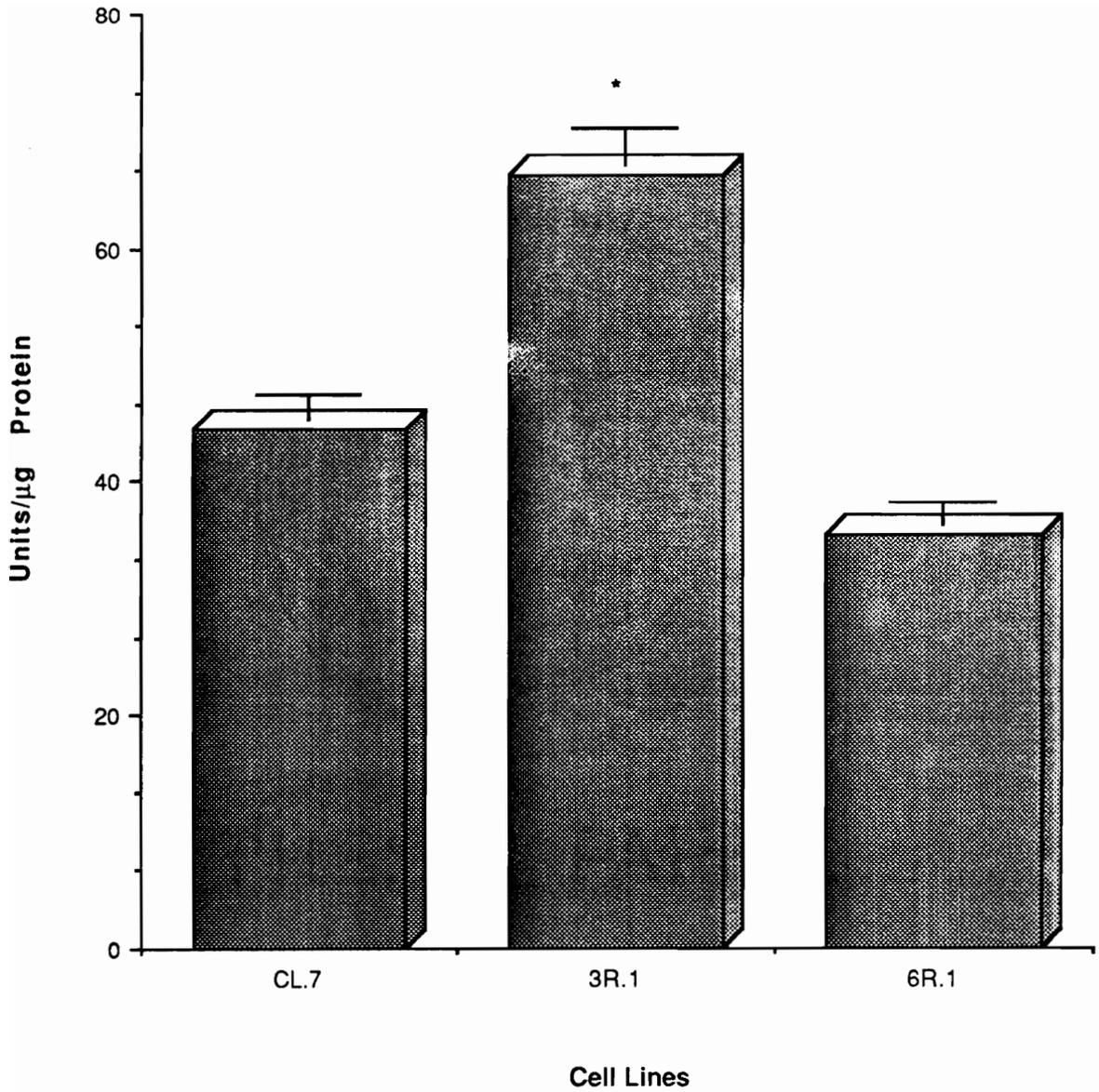


Figure 9 : G-6-PDH Levels Under A-MuLV Transformation. The values are determined as mean \pm S.D of six replicate wells in a microtiter assay system as described (appendix-8).

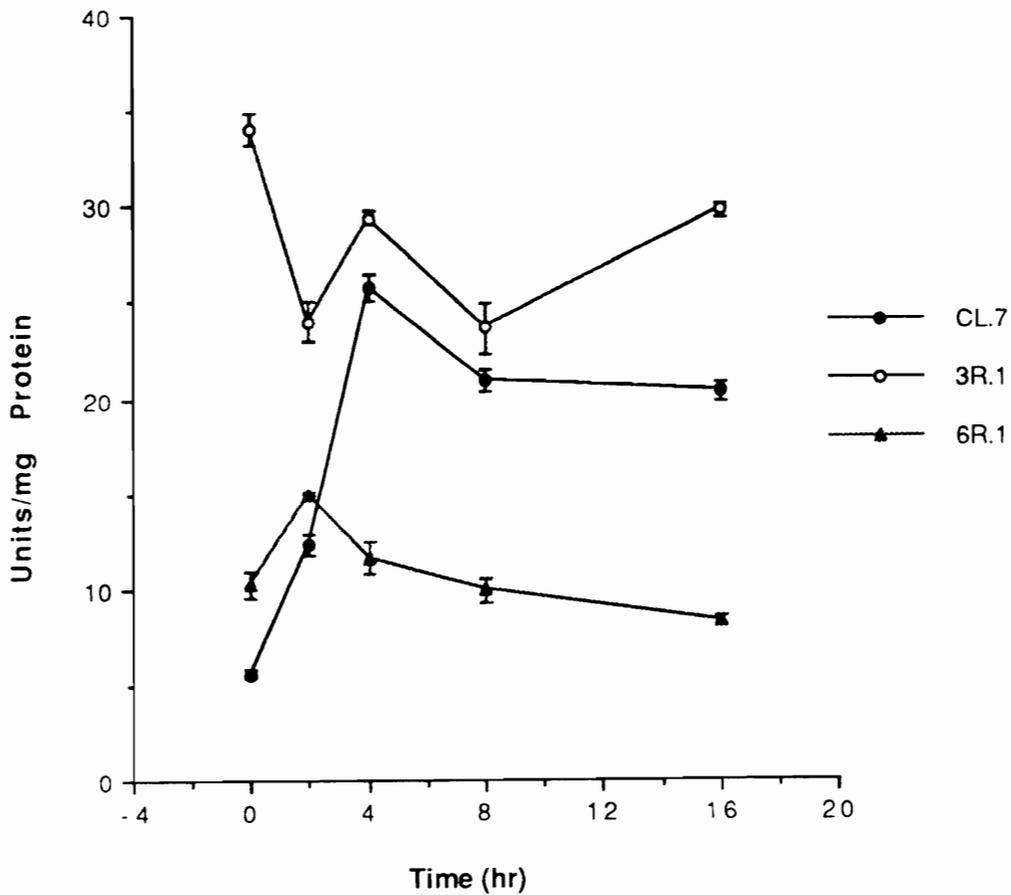


Figure 10 : Effect of TNF- α on Cu/Zn-SOD . CL.7, 3R.1, and 6R.1 cells incubated with TNF- α (100 units/ml) for different times were assayed for Cu/Zn-SOD using the microtiter assay system as described (appendix-4). The values of six replicates are presented as mean \pm S.D in units/mg protein. The CL.7 clone induces the Cu/Zn-SOD levels when exposed to TNF- α .

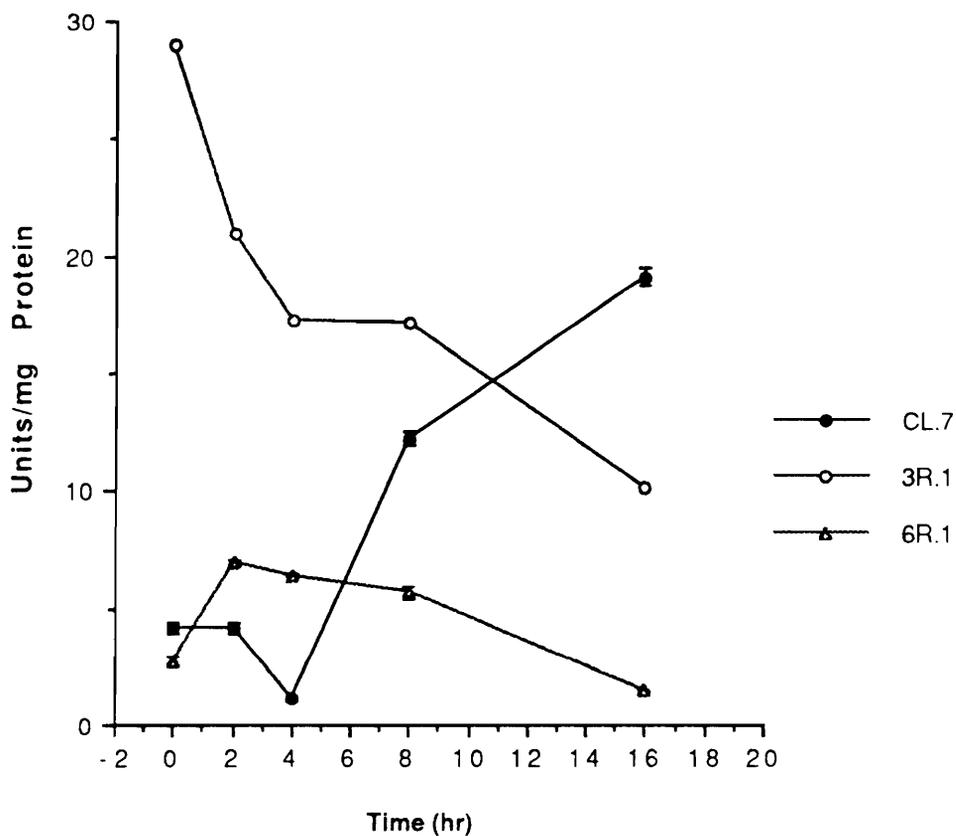


Figure 11 : Effect of TNF- α on Mn-SOD. Mn-SOD were quantitated by cyanide inhibited levels of total SOD as described (appendix - 4) in the microtiter assay system. The data presented shows that the TNF- α insensitive CL.7 clone induces its Mn-SOD with TNF- α (100 units/ml) incubation time. The 3R.1 has a significantly ($p < 0.05$) higher level of Mn-SOD than that for 6R.1 clone.

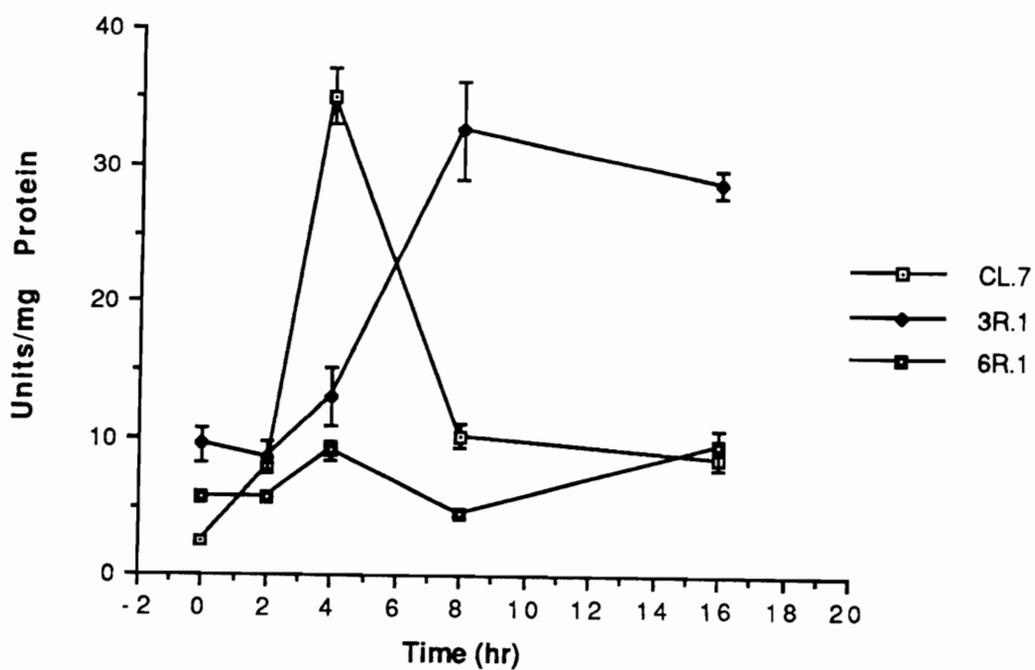


Figure 12 : Effect of TNF- α (100 units/ml) on cytochrome P 450 reductase levels of CL.7, 3R.1, and 6R.1 clones as a function of incubation time. The enzyme assay was performed in six replicates as described (appendix-9). The data is presented as mean \pm S.D of units/mg protein.

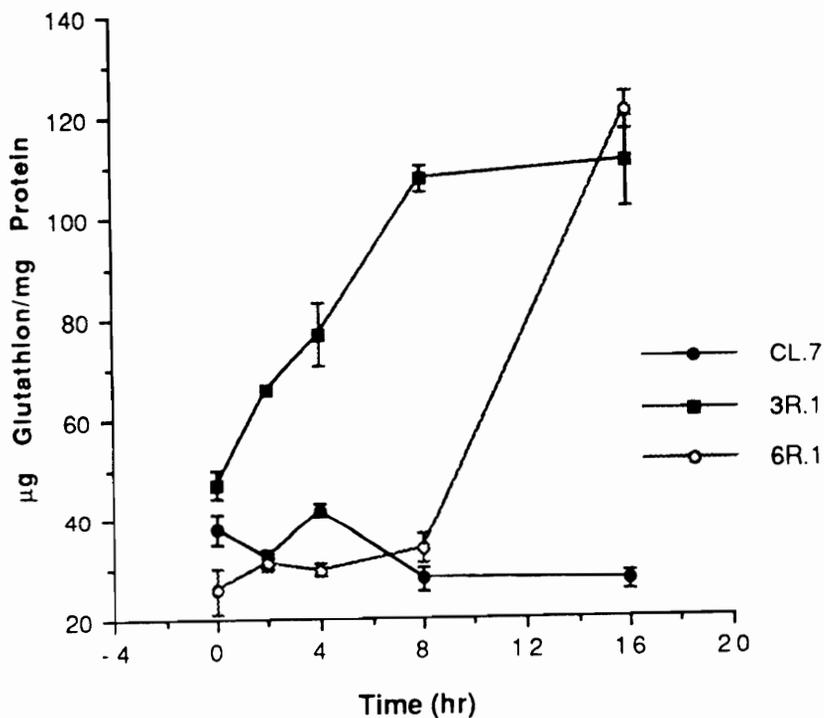


Figure 13 : Effect of TNF- α (100 units/ml) on total glutathione levels of CL.7, 3R.1, and 6R.1 clones as a function of incubation time. The assay was performed in six replicates as described (appendix-5). The data is presented as mean \pm S.D of μg glutathione/mg protein.

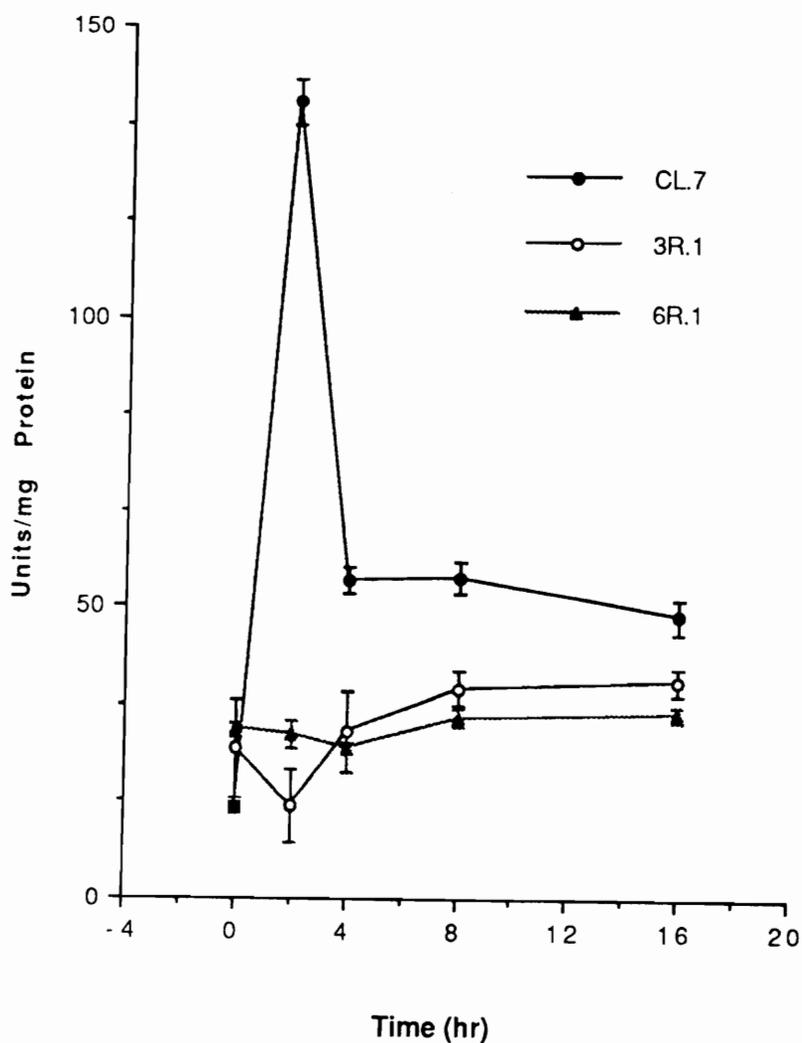


Figure 14 : Effect of TNF- α (100 units/ml) on glutathione reductase levels of CL.7, 3R.1, and 6R.1 clones as a function of incubation time. The enzyme assay was performed in six replicates as described (appendix-6). The data is presented as mean \pm S.D of units/mg protein.

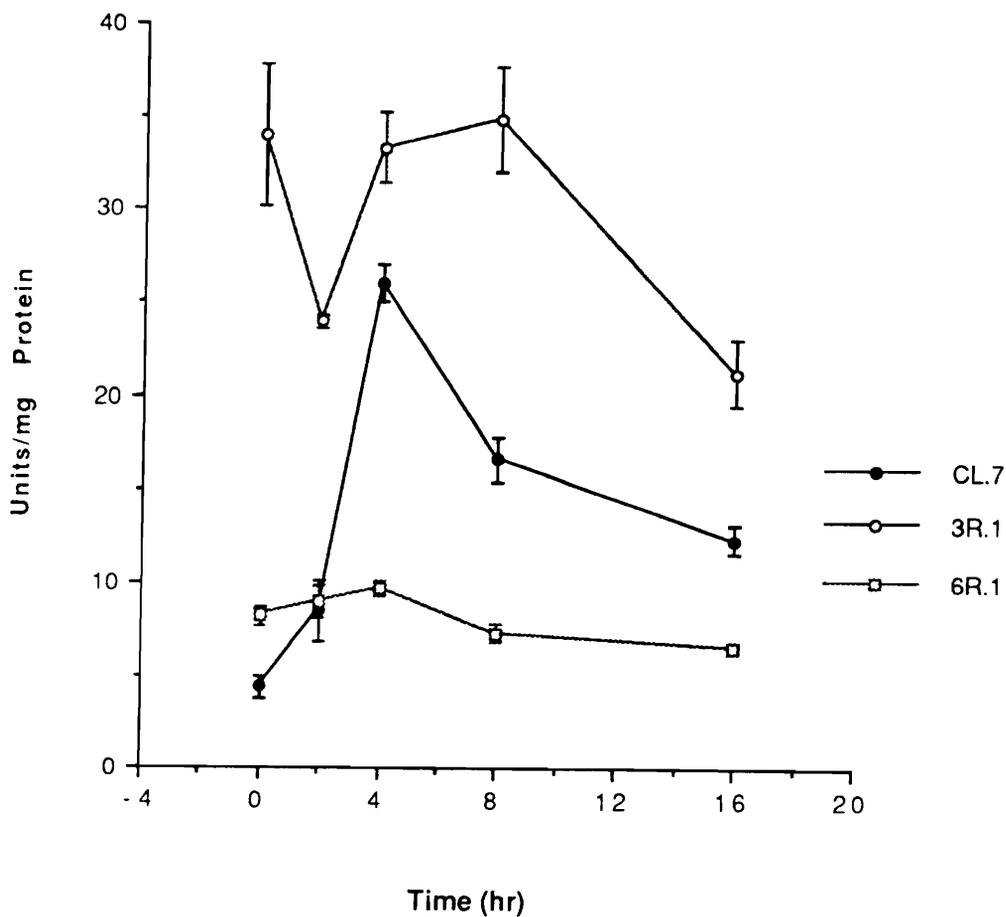


Figure 15 : Effect of TNF- α (100 units/ml) on glutathione peroxidase levels of CL.7, 3R.1, and 6R.1 clones as a function of incubation time. The enzyme assay was performed in six replicates as described (appendix-7). The data is presented as mean \pm S.D of units/mg protein.

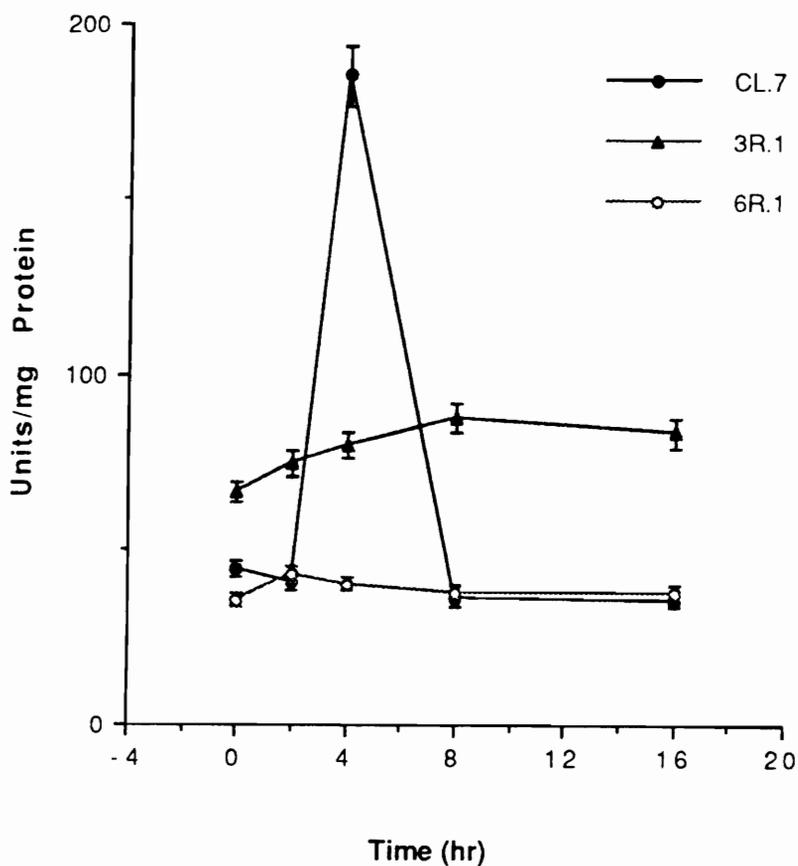


Figure 16 : Effect of TNF- α (100 units/ml) on G-6-PDH levels of CL.7, 3R.1, and 6R.1 clones as a function of incubation time. The enzyme assay was performed in six replicates as described (appendix-8). The data is presented as mean \pm S.D of units/mg protein.

TABLE - 1

Cytotoxic, Cytostatic, Null, And Growth Enhancing Effects Of TNF- α On Various Cell Lines.

Human Cells

Growth Enhancement

| | |
|---------------------------------|---------------------------|
| CCD-18Co (normal colon) | LL-24 (normal lung) |
| Detroit 551 (normal fetal skin) | WI-38 (normal fetal lung) |
| FS-4 (foreskin fibroblasts) | WI-1003 (normal lung) |
| HS-27F (foreskin fibroblasts) | U-373 (astrocytoma) |

Resistant^b

| | |
|---|---------------------------------------|
| A-427 (lung carcinoma) | LS-174T (colon carcinoma) |
| A-549 (lung carcinoma) | Panc (pancreatic carcinoma) |
| Calu-3 (lung carcinoma) | RD (rhabdlosarcoma) |
| FHS-74 Int (fetal interstitial epithelia) | Saos 2 (osteogenic carcinoma) |
| G-361 (melanoma) | SK-CO-1 (colon carcinoma) |
| GM-2504 (trisomic foreskin fibroblasts) | SK-LU-1 (lung carcinoma) |
| HeLa (cervical carcinoma) | SK-OV-3 (ovarian carcinoma) |
| HS-294T (melanoma) | SK-UT-1 (uterine carcinoma) |
| | T-24 (bladder carcinoma) |
| | WI-38 VA-13 (SV 40-transformed WI-38) |
| HS-939SK (skin fibroblasts) | 184 (primary breast epithelial cells) |
| HT-1080 (fibrosarcoma) | 5637 (bladder carcinoma) |
| K-562 (erythroblastic leukemia) | |
| KB (oral epidermoid carcinoma) | |

Cytotoxic or Cytostatic Effect^c

| | |
|-----------------------------|-----------------------------|
| ACHN (renal carcinoma) | SK BR-3 (breast carcinoma) |
| BT-20 (breast carcinoma) | SK CO-1 (colon carcinoma) |
| BT-475 (breast carcinoma) | SK LU-1 (lung carcinoma) |
| HS-939T (melanoma) | SK MEL-109 (melanoma) |
| HT-29 (colon carcinoma) | SK OV-4 (ovarian carcinoma) |
| HT-1376 (bladder carcinoma) | WiDr (colon carcinoma) |
| MCF-7 (breast carcinoma) | ZR-75-1 (breast carcinoma) |
| ME-180 (cervical carcinoma) | ZR-75-30 (breast carcinoma) |

Murine Cells

Resistant^b

| | |
|---------------------------|------------------|
| B16F10 (melanoma) | S-49 (lymphoma) |
| CMT-93 (rectal carcinoma) | U3 (fibroblasts) |

Cytotoxic or Cytostatic Effect^c

| | |
|--------------------|-------------------------------|
| B6MS2 (sarcoma) | Meth A (breast sarcoma) |
| B6MS5 (sarcoma) | MMT (breast carcinoma) |
| CMS4 (sarcoma) | SAC (Moloney-transformed 3T3) |
| CMS16 (sarcoma) | WEHI-164 (sarcoma) |
| L-929 (fibroblast) | |

Produced from: Rosenblum, et al (1989). Critical Rev. in Immunology Vol 9. pp27.

APPENDIX - 1**ROLE OF SUPEROXIDE RADICAL IN CYTOTOXIC
RESPONSE OF TNF- α : AN EPR STUDY****ABSTRACT**

Recent investigations have demonstrated the generation of reactive species of oxygen, in particular hydroxyl radicals, under the action of TNF- α on TNF- α -sensitive cell lines as determined indirectly by the production of methane in presence of dimethylsulfoxide (DMSO) (Yamauchi, et al 1989). These authors however have failed to demonstrate the primary reactive species in this reaction. We have directly quantitated the radical species produced under the action of TNF- α by using spin trapping in conjunction with electron paramagnetic resonance (EPR) spectrometry. L929 fibrosarcoma cells were incubated for a fixed time with and without 100 units/ml TNF- α . PBN, a spin trapping agent, was added at a final concentration of 14 mM, 1/2 hour prior to the incubation period. Incubation with TNF- α produced a three line spectrum with hyperfine splitting constants of $a_N = 14.75\text{G}$ and $a_{H^B} = 2.75\text{ G}$ characteristic of PBN-OOH adduct. Cytotoxicity of TNF- α to L929 cells have been known to be inhibited significantly in the presence of 70 μM desferrioxamine-MnO₂ complex (known superoxide dismutase mimic). In our system incubation of cells with 10 μM desferrioxamine-MnO₂ complex inhibited the formation of PBN-OOH adduct by 30% indicating that superoxide (O₂⁻) radicals are produced during TNF- α action. Thus the involvement of O₂⁻ production in L929 could be the causative factor of cell killing by immunotherapeutic agent TNF- α .

INTRODUCTION

Mechanism of TNF- α Action:

Tumor necrosis factor- α (TNF- α) is a polypeptide hormone of molecular weight 17 K known for its antitumor activity *in vitro* and *in vivo* (Old, 1985, Beutler, et al., 1986). This macrophage/monocyte derived anticancer cytokine has been recognized for its pleiotropic biological capacities. Besides its cytostatic and cytotoxic action, it influences the growth and differentiation of many normal and transformed cell lines (Nathan, 1987, Le, et al, 1987). Excellent reviews are available for the myriad biological properties of TNF- α (Fong, et al, 1989, Beutler, et al, 1989). The mechanisms by which TNF- α induces its diverse cellular responses are not fully understood. Like other cytokines, TNF- α elicits its cellular response via binding to a cell surface receptor (Baglioni, et al, 1985, Aggarwal, et al, 1988). After TNF- α binds its receptor, it is internalized via the classical receptor-mediated endocytic pathway i.e., via coated pits and vesicles and finally accumulates in the lysosomes (Mosselmans, et al, 1988). Agents such as colchicine and chloroquine which block the events of internalization and degradation of receptor bound TNF- α are known to effectively inhibit TNF- α cytotoxicity (Ruff, et al., 1981). The receptor binding is necessary, but is not the sole condition for eliciting of TNF- α cytotoxicity. The reason being that TNF-R are expressed to equal degree in both normal and malignantly transformed cells. (Scheurick, et al, 1986; Aggarwal, et al, 1985; Kull, et al, 1985; Tsujimoto, et al, 1985). Thus TNF- α responsiveness is not related to the number of TNF- α high affinity binding sites. To date, little is known about the molecular basis of TNF- α cytotoxicity. Post receptor binding events include increase in membrane fluidity and permeability (Anghileri, et al, 1987), activation of arachidonic acid cascade via an activated phospholipase A₂ (Godfrey, et al, 1987), a concomitant increase in prostaglandin E₂ production (Dayer, et al, 1985) and activation of protein kinase coupled pathways (Hinsel, et al, 1987). In rat mesangial cells, TNF- α was found to stimulate the

synthesis of PGE₂, PGF_{2a}, and 6-keto-PGF_{1a} as well as adenosine 3'-5' cyclic monophosphate (cAMP) levels in a dose dependent process (Baud, et al, 1988). In studying the cytolysis of L929 cells by TNF- α , it was found that mitochondria were the first site to be affected. The mitochondria were swollen and had reduced number of cristae leading to a belief that free radicals could be involved in the cytolytic action of TNF- α (Mathews, et al, 1987). TNF- α has been shown to induce mRNA levels of mitochondrial Mn-Superoxide Dismutase (Mn-SOD) (Grace, et al, 1988) which is a known scavenger of potentially toxic superoxide radical. The biological significance of oxygen derived free radical species have been well established. Oxygen free radicals like hydroxyl radical (\cdot OH), superoxide radical (O_2^-) and related derived reactive species of oxygen like hydrogen peroxide (H_2O_2) initiate peroxidative events resulting in damage to cellular phospholipids, proteins, DNA and RNA (Bus, et al, 1979; Slater, et al, 1984; Moody, et al, 1982; Lesko, et al, 1980; Cerutti, et al, 1983). A protection against such oxidative attack is conferred by various water soluble and lipid soluble antioxidants as well as by enzymes such as Cu/Zn-SOD, Mn-SOD, catalase, glutathione peroxidase etc (DiGuseppi, et al, 1984; Bus, et al, 1979; Slater, et al, 1984; McCord, 1979; Fridovich, 1978; Meister, 1983). Further, glutathione and other sulfhydryl compounds aid in the detoxification of reactive electrophilic metabolites of oxygen and lipid radical species thereby serving as an indicator of oxidative damage to a cell. Higher levels of intracellular glutathione have been shown to be correlated with *in vivo* tumor resistance to rhTNF- α (Zimmerman, et al, 1989) thereby proving that tumor cell sensitivity to TNF- α is somehow dependent on it's capability to withstand oxidative stress. The authors claim that *in vivo* action of TNF- α resembles that of damage due to ionizing radiation in that there is DNA fragmentation (Schmid, et al, 1986) and indirect evidence of involvement of reactive oxygen species and free radical production leading to membrane lipid peroxidation and subsequent lysosomal enzyme release (Wills, et al, 1966). Inhibition of TNF- α cytotoxicity with DMSO (\cdot OH radical scavenger) with concomitant release of gaseous methane has been taken as major evidence of intracellular hydroxyl radical production under cytotoxicity of rhTNF- α in TNF- α sensitive cell lines (Yamauchi, et al, 1989). However production of methane does not make \cdot OH radical the sole free radical produced under TNF- α

cytotoxicity.

To determine the nature of free radicals involved in TNF- α cytotoxic pathway we attempted to directly ascertain the kind of free radical(s) produced by using spin trapping in combination with electron paramagnetic resonance (EPR) technique. The principle of spin trapping involves the conversion of highly reactive short lived free radicals to relatively long lived inert radicals which can then be detected by electron paramagnetic resonance spectroscopy. The present investigation aimed at trapping and detection of any free radical produced in the cytotoxic pathway of TNF- α on L929 cells. The spin trap used in this study was PBN.

MATERIALS AND METHODS

Cells: L929 fibrosarcoma cells were grown in DMEM 90%, FCS 10 %, 10 units/ml penicillin, 10 $\mu\text{g/ml}$ streptomycin, 3.7 g/l sodium bicarbonate and 25 mM HEPES at 37°C in 5% CO₂ in a humidified atmosphere.

Cytokine: TNF- α was obtained by an immunoaffinity purification of mouse TNF- α from *E. coli* LPS induced, A-MuLV immortalized, RAW 264.7 murine macrophage supernatant on an anti-TNF- α antibody agarose column and its specific activity was determined by a bioactivity assay (Larrick et al, 1989) to be 2.5×10^5 units/mg protein.

Spin Trapping with PBN

Duplicate cultures of L929 fibrosarcoma cells were incubated for 1/2 hr, 3 hr, 5 hr, 10 hr and 15 hr with and without 100 units/ml TNF- α , 10 ml per flask, in DMEM without FCS in 25 mM HEPES, 10 units/ml penicillin, 10 $\mu\text{g/ml}$ streptomycin, 1 $\mu\text{g/ml}$ actinomycin D, pH 7.4 with 5% CO₂ at 37°C. One half hour prior to the end of the incubation period, 1 ml of 154 mM PBN in DMEM (without FCS) was added to obtain a final concentration of 14 mM PBN. At the end of the incubation period, cells were scraped with a cell scraper in the 11 ml of incubation media. The cell suspension was quickly removed to a 100 ml erlenmeyer flask. To a total of 22 ml of cell suspension

(2 confluent culture) we added 22 ml of chloroform, 44 ml methanol and 11 ml of 0.9% NaCl. This mixture was shaken vigorously at room temperature for 10 minutes and then spun down at 5000 g for 15 minutes at 4°C. The bottom chloroform layer was aspirated and vacuum evaporated to dryness and resuspended in 200 µl of chloroform. The sample was taken up in an ESR capillary tube and placed in the sample cavity of a Bruker D-200 X-band spectrometer.

The EPR Setting

The samples were run on the EPR spectrometer using a modulation frequency of 100 KHz at a microwave power of 10 mwatts with a 5G modulation amplitude and 0.64s as time constant. The scan rate was set for 500 sec at a receiver gain of 6.3×10^5 . The sample was scanned at a center field (CF) of 3483 G and sweep width (SW) of 100 G at a microwave frequency of 9.78 GHz.

RESULTS

The spectra obtained for L929 cells incubated for one half hour with PBN without and with 1 µg/ml actinomycin D was shown in Figure 2. Figure 3 showed the scan of 3 hr, 5 hr, 7 hr, 8 hr, 9 hr, 10 hr and 15 hr of TNF-α incubation in presence of 1 µg/ml actinomycin D. In a separate experiment, we determined the signal of 8 hr TNF-α incubated cells with and without actinomycin D (Figure 4). As seen in Figure 2, actinomycin D incubated cells for one half hr, produced a carbon centered radical as spin trapped with PBN. The carbon centered radical could be produced due to metabolism of actinomycin D by the drug metabolizing enzymes like cytochrome P450 on endoplasmic reticulum. The cell samples incubated with TNF-α produced a spectrum with a peak at 9 hr (Figure 3). Analysis of the 9 hr spectrum gave values of $a_N = 14.75\text{G}$ and $a_{HB} = 2.75\text{ G}$. It was concluded that the spectrum could be that of PBN-OOH adduct (Buettner, 1987). Incubation of cells with 10µM solution of filter sterilized desferrioxamine and MnO₂ complex (known to be a superoxide mimic, Darr et al, 1987) inhibited the formation of PBN-OOH adduct by 30%. This showed that TNF-α cytotoxicity was via a superoxide (O₂⁻) production pathway (Figure 5). Thus TNF-α known

to be cytotoxic to L929 fibrosarcoma cells could be eliciting its cytotoxicity via production of superoxide radicals. Interestingly, desferrioxamine alone is known to inhibit toxicity of TNF- α by 90% at a concentration of 70 μ M as determined by a 3-day assay on L929 cells (Matthews, et al, 1988). Figure 3 shows that the radical production starts as early as 3 h and peaks at 9 h and decays by around 15 hr.

DISCUSSION

Our finding that O_2^- radicals are involved in TNF- α cytotoxicity is in concordance with two separate investigations. Firstly, according to our finding, superoxide radicals produced around 3 hr to 10 hr could be responsible for peroxidation of membrane lipids found only around 10 to 20 hr (Matthews et al, 1988). Secondly, O_2^- radicals produced could be converted to hydroxyl radicals ($\cdot OH$) which in turn could be the major radical responsible for DNA fragmentation, lipid peroxidation and cell death. Production of $\cdot OH$ radicals in the pathway of TNF- α action was measured indirectly by production of methane when cells were incubated in presence of dimethyl sulphoxide (DMSO) at a concentration of 100 mM as determined by Yamauchi et al, 1989. $\cdot OH$ production could find its origin in the O_2^- radicals as the original radical produced under TNF- α cytotoxic action. A model of TNF- α cytotoxicity thus appears to be the following: binding of TNF- α to its specific cell surface receptor, internalization of ligand-receptor complex, toxicity to mitochondria, production of O_2^- , production of $\cdot OH$, fragmentation of DNA, peroxidation of membrane lipids, release of lysosomal enzymes and cell death. Our model does not explain how TNF- α acts as a growth factor for normal human fibroblasts like FS-4. Such a two-pronged effect of TNF- α required further investigation at the molecular level. Nonetheless, involvement of O_2^- production as a cause of ultimate cell death in cancerous cells like L929 by TNF- α could be significant in that the cell killing by chemotherapeutic drugs like adriamycin is comparable to that of immunotherapeutic agent like TNF- α i.e., both effect cell death via production of free radicals.

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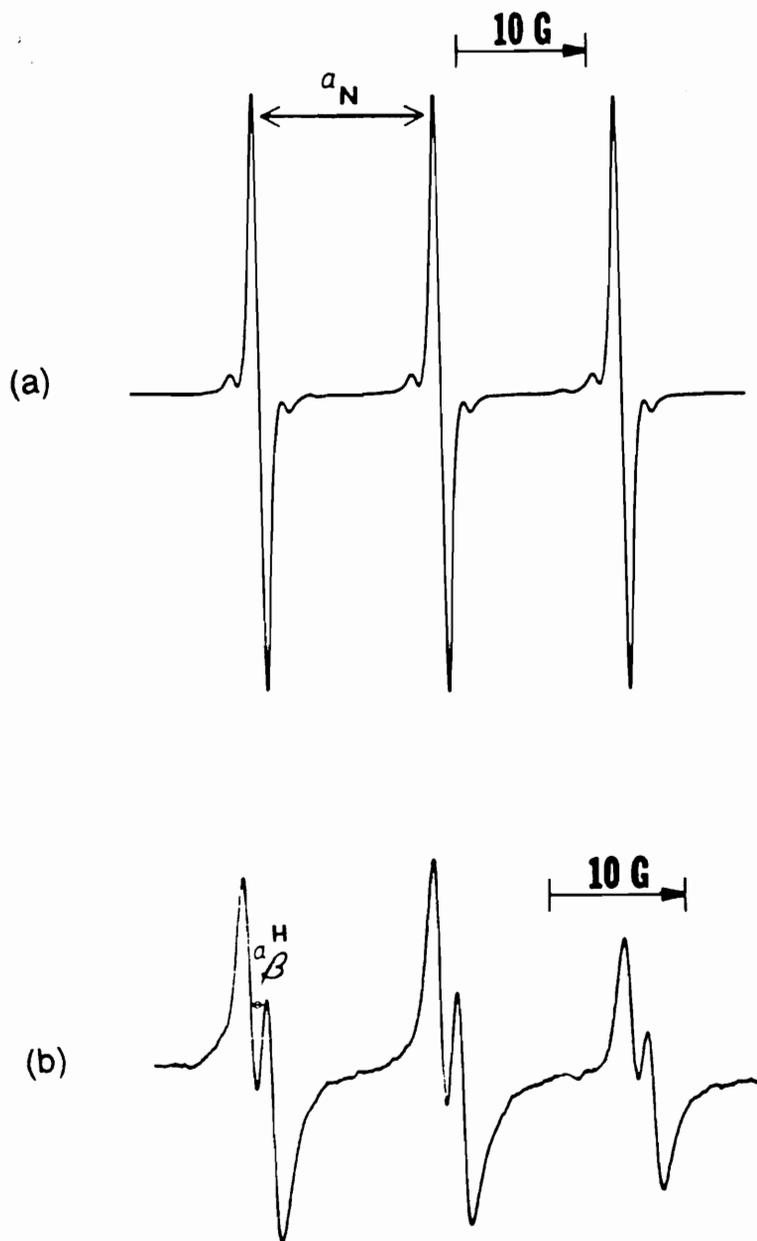


Figure 1 : EPR spectral coupling constants. (a) A typical EPR spectrum of a nitroxyl radical showing the coupling constant a_N (G value) associated with it. (b) Fine structure splitting of the three nitrogen lines by β hydrogen.

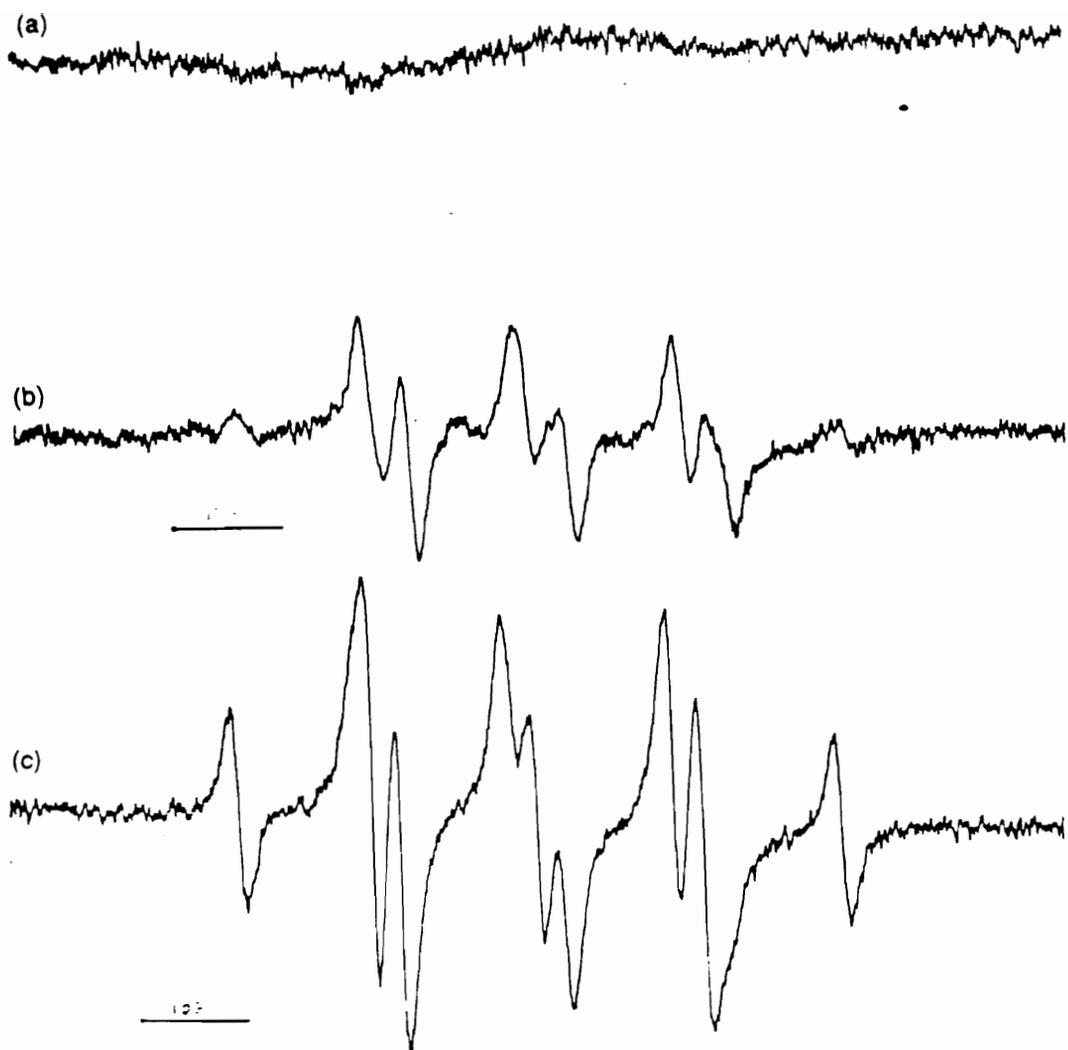


Figure 2 : EPR spectrum of PBN adduct formed under TNF- α cytotoxicity. EPR spectrum of L929 chloroform extract for cells incubated (a) without PBN, (b) with PBN + 1 $\mu\text{g/ml}$ actinomycin D, and (c) with PBN + 1 $\mu\text{g/ml}$ actinomycin D + 100 units/ml TNF- α for 15 hrs.

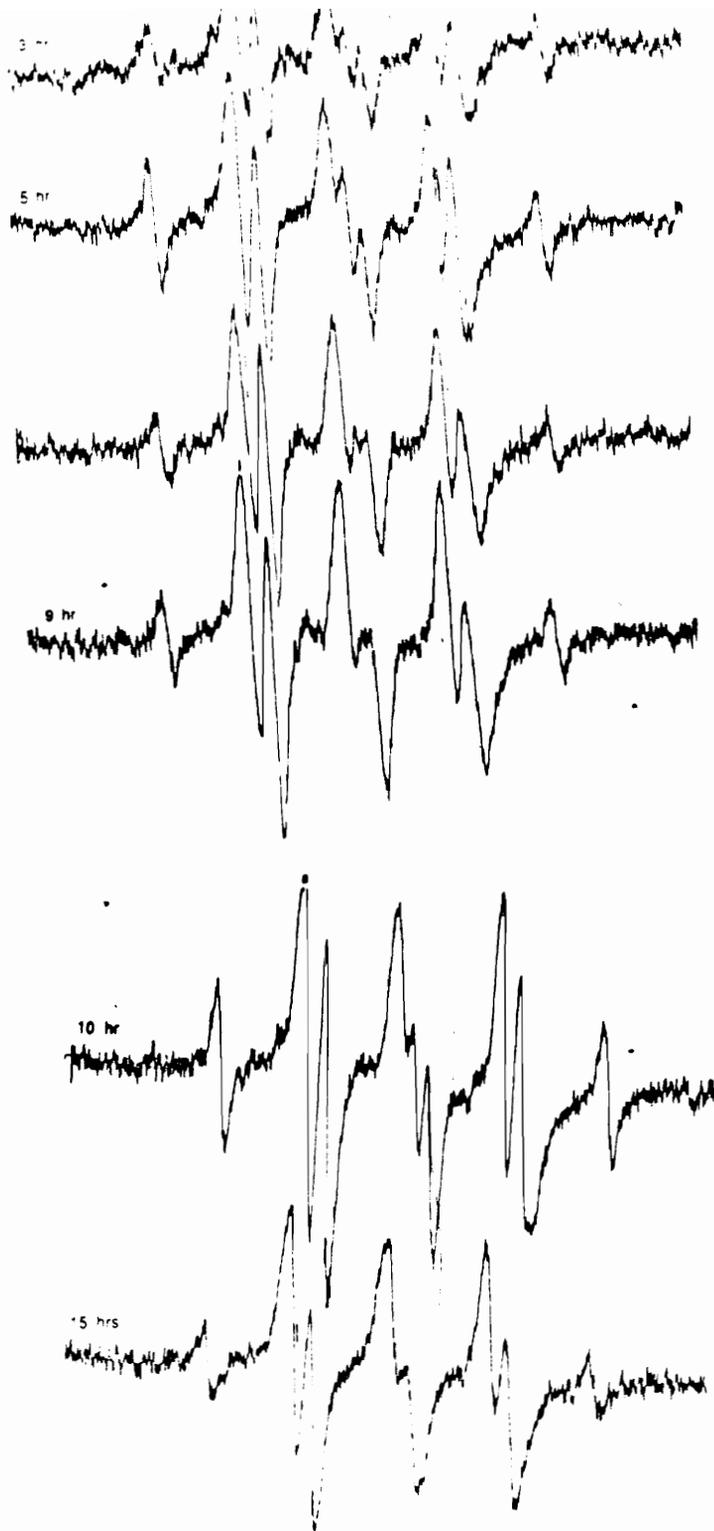


Figure 3 : Spectra of PBN adduct with time incubation of TNF- α . PBN adduct spectrum of chloroform extract from L929 cells incubated with 100 units/ml TNF- α for 3, 5, 7, 8, 9, 10, and 15 hrs. The experimental protocol and the EPR settings were as described in the methods section.



Figure 4 : Effect of time incubation and TNF- α sensitizer, actinomycin D on PBN adduct spectra. (a) The stability of the PBN-adduct EPR signal of the 8 hr TNF- α incubated L929 chloroform extract. The trace is at the maximum of the second peak for 3 hrs. (b) The 8 hr PBN adduct spectrum of TNF- α incubated chloroform extract of L929 cells without actinomycin D. (c) The 8 hr PBN adduct spectrum of TNF- α incubated chloroform extract of L929 cells with actinomycin D.

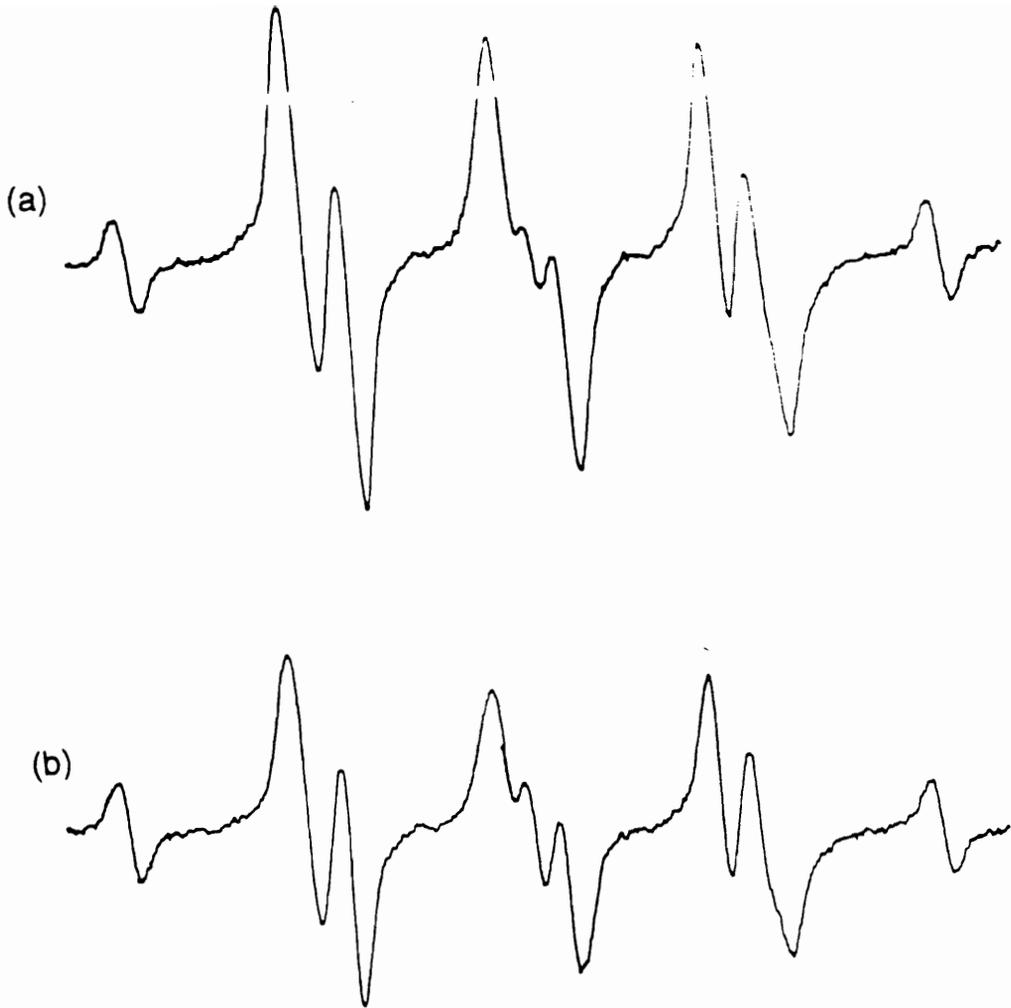


Figure 5 : The inhibition of PBN adduct spectrum with desferoxamine-MnO₂ complex. The 9 hr PBN adduct spectrum of L929 cells with 100 units/ml TNF- α incubated in absence and presence of 10 μ M desferoxamine-MnO₂ complex as described. A 30 % reduction in the PBN-adduct signal further confirms that O₂⁻ is implicated in TNF- α cytotoxicity.

APPENDIX - 2**HYDROGEN PEROXIDE PRODUCTION UNDER TNF- α
CYTOTOXICITY: A FLOW CYTOMETRIC APPROACH****ABSTRACT**

The objective of the present study was to test the hypothesis that TNF- α induced cellular toxicity is mediated in part by the production of intracellular H₂O₂ in TNF- α susceptible cell lines. Use of 2',7'- dichlorofluorescein (DCFH) is one of the most convenient ways by which intracellular H₂O₂ can be measured. The green fluorescence of DCF measured at emission wave length of 520 nm in conjunction with flow cytometry allowed for the quantitation of intracellular H₂O₂ on a cell by cell basis. TNF- α sensitive L 929 cell samples were analyzed by a two parameter plot of forward angle light scatter (linear scale) and 90° light scatter (log scale) using a flow cytometer. The lin-log plot showed two distinct subpopulations namely small cells (MAP 1) and the larger cells (MAP 2) which were analyzed separately and jointly for green fluorescence of DCF. MAP 2 cells were found to be more viable subpopulation of L929 cells and MAP 1 cells were found to be the more non-viable subpopulation as determined by the viability of the two cell subpopulations under TNF- α action. The H₂O₂ production in MAP 2 cells increased sharply with concentration as well as time incubation of TNF- α . The total cell population (MAP 3 cells) however did not show any significant change in H₂O₂ production. This was hypothesized to be due to the compensating loss of H₂O₂ production by MAP 1 cells accompanying the enhanced production of H₂O₂ by the MAP 2 cells. We concluded therefore that H₂O₂ was one of the reactive species of oxygen produced when L929 cells were incubated with TNF- α . Antioxidants like dimethyl sulfoxide (DMSO) and vitamin E were known inhibitors of cytotoxicity by TNF- α . The effect of these antioxidants in blocking the H₂O₂ production by TNF- α in MAP 1, MAP 2, and MAP 3 cells was investigated. We determined the effect of various lipid

soluble antioxidants on H_2O_2 production. DMSO, at 500 mM, increased the H_2O_2 production in all the cell subpopulations. Vitamin E at both 0.1 and 1.0 mM concentrations decreased the production of TNF- α induced H_2O_2 production in MAP 2 and MAP 3 cells. Thus the status of vitamin E in inhibition of peroxidative chain reaction could not only be at the nonspecific termination of lipid peroxidation but also as an inhibitor of H_2O_2 production. Vitamin A at 0.1 mM had little effect on H_2O_2 levels but at 1.0 mM, was synergistic with TNF- α , in increasing the H_2O_2 production (which could explain the cellular toxicity of vitamin A at a high enough concentration). Thus the hypothesis that the TNF- α cytotoxicity was mediated in part by intracellular production of H_2O_2 was confirmed.

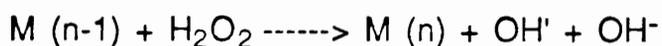
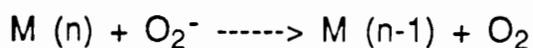
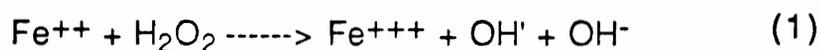
INTRODUCTION

Tumor necrosis factor- α (TNF- α) is a polypeptide hormone of 17 K molecular weight known for its antitumorogenic activities *in vitro* and *in vivo* (Old, 1985; Beutler, et al. 1986). This macrophage/monocyte derived anticancer cytokine has been recognized for its pleiotropic biological capacities. Besides its cytostatic and cytotoxic action, it influences the growth and differentiation of many normal and transformed cell lines (Nathan, 1987; Le et al. 1987). Excellent reviews are available for the myriad biological properties of TNF- α (Fong et al. 1989, Beutler et al. 1989). The mechanisms by which TNF- α induces its diverse cellular responses are not fully understood. Like other cytokines TNF- α elicits its cellular responses via binding to a cell surface receptor (Baglioni, et al. 1985; Aggarawal, et al. 1985, Tsujimoto, et al. 1986, Mosselmans, et al. 1988). After TNF- α binds its receptor, it is internalized via the classical receptor mediated endocytic pathway ie. via coated pits and vesicles and finally accumulates in the lysosomes (Mosselmans, et al. 1988). It has been shown that agents such as colchicine and chloroquine which would block the events of internalization and degradation of receptor bound TNF- α is known to effectively inhibit TNF- α cytotoxicity (Ruff, et al., 1981). The receptor binding is although necessary is however not sufficient

condition for the eliciting of TNF- α response. This is so because normal and malignantly transformed cells express TNF-receptors (TNF-R) to an equal degree (Scheurich, et al. 1986; Aggarawal, et al. 1985; Kull, et al. 1985; Tsujimoto et al. 1985). To date however little is known about the molecular basis of TNF- α action. Post receptor binding membrane events include increase in membrane fluidity and permeability (Anghileri et al. 1987), activation of arachidonic acid cascade via an activated phospholipase A₂ (Godfrey, et al. 1987) a concomitant increase in prostaglandin E₂ production (Dayer et al. 1985) and activation of protein kinase coupled pathway (Hensel, et al. 1987). In rat mesangial cells TNF- α was found to stimulate the synthesis of PGE₂, PGF_{2 α} , and 6-keto- PGF_{1 α} as well as adenosine 3'-5' cyclic monophosphate (cAMP) in a dose dependent manner(Baud et al. 1988). At an intracellular site mitochondria were the first organelles to be affected, were swollen and had reduced number of cristae. These observations implicate the involvement of free radicals in the cytolytic path way of TNF- α (Mathewes et al. 1987). At a molecular level TNF- α has been shown to induce mitochondrial Mn-superoxide dismutase (Mn-SOD) as well as Mn-SOD mRNA (Grace, et al. 1988). Mn-SOD is a known scavenger of potentially toxic superoxide radical which, upon induction, has the ability to protect cells from the TNF- α mediated cellular toxicity.

The biological significance of oxygen derived free radical species have been well established. Oxygen free radicals like hydroxyl radical (OH \cdot), superoxide radical (O₂ \cdot^-) and related derived reactive species of oxygen like hydrogen peroxide (H₂O₂) initiate the peroxidative events resulting in damage to cellular phospholipids, proteins, DNA and RNA (Bus et al. 1979; Slater et al. 1984; Moody et al. 1982; Lesko et al 1980; Cerutti et al. 1983). A protection against such oxidative attack is conferred by various water soluble and lipid soluble antioxidants as well as by enzymes such as Cu/Zn-SOD, Mn-SOD, catalase, glutathione peroxidase etc (DiGuseppi et al 1984, Bus et al. 1979, Slater et al.,1984; McCord, 1979; Fridovich, 1978, Meister 1983). Further, glutathione and other sulfhydryl compounds aid in the detoxification of reactive electrophilic metabolites of oxygen and lipid radical species thereby serving as an indicator of oxidative damage to a cell. A higher levels of intracellular

glutathione have been shown to be correlated with *in vivo* tumor resistance to rh TNF- α (Zimmerman et al.1989) thereby proving that tumor cell sensitivity to TNF- α is somehow dependent on its capacity to withstand oxidative stress. The authors claim that *in vivo* action of TNF- α resembles that of damage due to ionizing radiation in that there is DNA fragmentation (Schmid et al. 1986) and indirect evidence of involvement of reactive oxygen species (Yamauchi et al. 1989) and subsequent lysosomal enzyme release (Wills, et al. 1966). Inhibition of TNF- α cytotoxicity with DMSO (OH' radical scavenger) with concomitant release of gaseous methane has been taken as a major evidence of intracellular hydroxyl radical production under cytotoxicity of rh TNF- α in TNF- α sensitive cell lines (Yamauchi et al. 1989). However, the production of 'OH could be a result of interaction of H₂O₂, hypothesized to be formed earlier in the pathway. H₂O₂, although is the least reactive of oxygen metabolites is however the central species around which other oxygen species interact. This further could explain the production of OH' radical either via the Fenton reaction (equation 1), the Haber-Weiss reaction (equation 2), or the metal catalyzed Haber-Weiss reaction (equation 3).





To test the hypothesis that H₂O₂ is produced in the TNF- α cytotoxicity pathway it is imperative to measure the intracellular hydrogen peroxide levels. Many methods have been published to quantitate H₂O₂ production in biological systems. They include assaying the release of ¹⁴C O₂ from carboxylated compounds

(Klebanoff, et al. 1971), oxidation of scopoletin (Root, et al. 1975), use of catalase (Zatti et al., 1968), chemiluminescent detection (Allen, et al. 1972) and use of 2',7'- dichlorofluorescein (DCFH) (Bass et al. 1983; Burow et al., 1987; Rothe et al. 1990). Use of DCFH is one of the most convenient way by which intracellular H_2O_2 can be measured. Non-fluorescent DCFH is first loaded into the vital cells by enzymatic cleavage of the membrane-permeable 2',7'-dichlorofluorescein-diacetate (DCF-DA). Once it enters the cell, DCF-DA is cleaved by an intracellular nonspecific esterase to form an intracellular DCFH. Intracellular DCFH is oxydised to fluorescent 2',7'- dichlorofluorescein (DCF) upon reaction with intracellular H_2O_2 . The green fluorescence of DCF can be measured at emission around 520 nm. The usefulness of the assay is evident when it is applied in conjunction with flow cytometry. This allows for measurement of intracellular H_2O_2 on a cell by cell basis. Such flow cytometric methods have recently been used for the rapid measurement of intracellular H_2O_2 in human neutrophils (Rothe et al, 1990).

The objective of the present study was to test the hypothesis that TNF- α induced cellular toxicity is mediated in part by the production of intracellular H_2O_2 in TNF- α susceptible cell lines.

MATERIALS AND METHODS

Cell Culture : L929 fibrosarcoma cells, known to be very sensitive to TNF- α , were grown in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal calf serum (FCS) with 10 units/ml penicillin and 10 μ g/ml streptomycin and 25 mM HEPES, pH 7.4 at 37^o C in a humidified incubator with 5% CO₂.

TNF- α : Murine TNF- α was from *E Coli* lipopolysaccharide (LPS) activated, leukemia virus immortalized, macrophage cell line RAW 264.7 supernatant. The TNF- α was immunopurified on an anti- TNF- α antibody column (chapter 1) and was determined by a TNF- α cytotoxicity bioassay (Larrik et al. 1989) to have specific activity of 6 x 10⁵ units/ mg protein.

Cell Staining & TNF- α Cytotoxicity Assay : L 929 cells were grown to confluency in 24 well plates (2 x 10⁵ cells/well). Confluent cells were washed 2 x with media without fetal calf serum. DCF-DA

(Molecular Probes, OR), weighing 0.974 mg, was desolved in 100 μ l of 100 % ethanol. A 20 μ M stock solution of DCF-DA was prepared in the culture medium. Cells were incubated with 500 μ l of 10 μ M DCF-DA for 30 minutes in dark. After the DCF-DA loading, the media was removed and control and DCF-DA treated cells were split into two subgroups. The control cells were incubated with and without TNF- α (100 units/ml) in 1 ml of media supplemented with 10 % FCS and 1 μ g/ml of actinomycin D. Similarly the DCF-DA treated cells were incubated with and without 100 units/ml TNF- α in media suplimented with 10 % FCS, 10 μ g/ml actinomycin-D, and 10 μ M DCF-DA. After the predetermined incubation times (0, 5, 10 & 15 hr) the samples were scraped from the culture dish and immediately analyzed by flow cytometry. A similar experiment was performed for different concentrations of TNF- α at a fixed (15 hr) incubation time and the samples were analyzed by flow cytometry.

Flow Cytometry: Hydrogen peroxide generated during oxidative metabolism converts the trapped, intracellular, nonfluorescent probe, DCFH, to the highly fluorescent form, DCF. After incubation with and without TNF- α the samples were analyzed for DCF fluorescence by flow cytometry. The amount of fluorescent DCF generated per cell, which is a measure of oxidative product formation, was indicated by the mean channel number of the generated histograms. Prior to running the cells on the flow cytometer, the cells were filtered through a 37 μ m nylon mesh. Three values per cell were collected : a) forward angle light scatter, b) 90 $^{\circ}$ light scatter and c) green fluorescence. Filters were used as follows : a) 488 nm long-pass dichroic for 90 $^{\circ}$ light scatter, b) 457 to 502 nm laser blocker in conjunction with a 515 nm long-pass absorbance filter and c) 550 nm long-pass dichroic and 525 nm band-pass filters for green fluorescence measurement. Forward angle light scatter and green fluorescence were collected by linear integral mode, and 90 $^{\circ}$ light scatter was collected by log integral mode. Cell numbers per channel, as a function of fluorescence intensity, were collected on single parameter histograms with 256 channels and were gated on forward angle light scatter and 90 $^{\circ}$ light scatter on dual parameter histogram (64 x 64 channels resolutions) defining the cell population of interest. The total number of counts for each histogram was 1 x 10 5 cells. Standard beads (Coulter

Cytomerty, Hialeah Florida) were used to standardize the instrument prior to each day's testing.

Effect Of Lipid Soluble Antioxidants On TNF- α -Induced DCF green fluorescence: Lipid soluble antioxidants like dimethyl sulfoxide (DMSO), β -carotene (vitamin A), and α -tocopherol (vitamin E) have been implicated in inhibition of TNF- α cytotoxicity. We examined the effect of DMSO (50, 100, and 500 mM), vitamin A (0.1 and 1.0 mM) and vitamin E (0.1 and 1.0 mM) on TNF- α (100 units/ml) induced H_2O_2 production as monitored by green fluorescence of DCF. The protocol for the experiment was exactly as previously described except that the TNF- α incubation time was fixed at 15 hr.

Statistical Analysis : The statistical analysis was performed by a paired t test comparison. The probability of $p \leq 0.05$ was taken to be significant. Unless otherwise mentioned the results were expressed as mean \pm S.D of six replicate readings.

RESULTS

L 929 cell samples were analyzed by a two parameter plot of forward angle light scatter (linear scale) and 90° light scatter (log scale) in a flow cytometer. The lin-log plots showed two distinct subpopulations (Figure 1). The small cells (MAP 1) and the large cells (MAP 2) were analyzed separately and jointly for green fluorescence of DCF. The green fluorescence of representative samples of L929 MAP 1 cells, MAP 2 cells and MAP 1 and MAP 2 cells taken together (MAP 3 cells) along with the two dimensional lin-log four-quadrant plot at different time incubations with TNF- α (100 units/ml) were shown in Figures 1 (a) thru 1 (i). The treatment groups were given the general designation : DCF/TNF (t). For example +/- (15) represented samples pretreated with DCF-DA and incubated without TNF- α for 15 hr and +/- (10) represented samples pretreated with DCF-DA and incubated with TNF- α for 10 hr.

A major difference between use of DCF-DA to quantitate H_2O_2 production for example in activated neutrophils and to use it to monitor H_2O_2 production under TNF- α cytotoxicity was the incubation time. The demand made on DCF-DA to be a H_2O_2

production reporter was definitely very different. DCF-DA after being taken up by cells would not only have to remain an efficient indicator of H_2O_2 production in the span of 15 to 18 hrs but also it should not by itself change the viability of the cells. The $-/-$ (0) plot (Figure 1. a) showed that DCF-DA untreated cells had no fluorescence. The $+/-$ (0) sample showed a good loading of cells with DCF-DA. $+/-$ (0), $+/-$ (5), $+/-$ (10), and $+/-$ (15) represented the green fluorescence of DCF-DA loaded control (no $TNF-\alpha$) cells at 0, 5, 10, and 15 hr. The green fluorescence mean channel number drastically decreased by 5 hr incubation with DCF-DA and remained steady between 5 hr to 15 hr incubation time (Figures 1c thru 1e). This steady state fluorescence of DCF between 5 hr and 15 hr in control cells showed that any significant increase in DCF fluorescence over this base line in treated cells would be an indicator of production of intracellular H_2O_2 in this time interval. Effect of time incubation with $TNF-\alpha$ (100 units/ml) for 5, 10, and 15 hr on lin-log population distribution and green fluorescence of MAP 1 (small cells), MAP 2 (large cells) and MAP 3 (total cell population) was plotted as shown in Figures 1 f thru 1 h. As shown in figures 1 f thru 1 h the MAP 2 cell population density decreased with $TNF-\alpha$ incubation time. The population density of MAP2 cells decreased ($p < 0.05$) with $TNF-\alpha$ dilution. Contrastingly the population density of MAP 1 cells, however, increased significantly with concentration of $TNF-\alpha$ (Figure 2). The total number of cells counted per run being a constant (10,000 cells), along with the fact that the increase in MAP 1 cells being equal to the decrease in MAP 2 cells (Figure 2) we concluded that the larger MAP 2 cells under $TNF-\alpha$ cytotoxicity go to make the smaller MAP 1 cells. Moreover percent viability curve with time incubation of $TNF-\alpha$, was equivalent to the loss of MAP 2 cells, (Figure 3), and percent viability curve, with $TNF-\alpha$ incubation time, was inversely equivalent to the growth of MAP 1 cells (Figure 4). Thus MAP 2 cells were the more viable subpopulation of L929 cells and MAP 1 cells were the more non viable subpopulation of L929 cells.

The green fluorescence of MAP 1 cells with incubation time of $TNF-\alpha$ was shown in Figure 5. The figure showed that the non-viable cells actually produced less H_2O_2 (as measured by DCF fluorescence) with $TNF-\alpha$ incubation time than the normal cells. The H_2O_2

production in MAP 2 cells were shown in Figure 6. Clearly H_2O_2 produced by MAP 2 cells increased significantly with time incubation of $TNF-\alpha$ ($p < 0.05$). The total cell population however did not show any significant increase in H_2O_2 production (Figure 7). It was possible that the enhanced production of H_2O_2 by the MAP 2 cells could compensate for the loss of H_2O_2 production by MAP 1 cells. We concluded therefore that H_2O_2 was one of the reactive species of oxygen produced in viable L 929 cells when these cells were incubated with $TNF-\alpha$ as a function of $TNF-\alpha$ incubation time.

Starting with a stock solution of $TNF-\alpha$ (2×10^4 units/ml) we performed dilutions of 1 : 100, 1 : 200, 1 : 400, 1 : 800, and 1 : 1600. Both the MAP 2 and MAP 3 subpopulations had enhanced H_2O_2 production under exposure for 15 hr with $TNF-\alpha$ concentrations ranging from 10 to 200 units/ml in a 1 ml incubation volume (Figure 8). The production of H_2O_2 for MAP 1 (Figure 9), MAP 2 (Figure 10) and MAP 3 (Figure 11) cells were shown as mean \pm S.D. As seen from figure 9 the MAP 1 cell H_2O_2 production was not sensitive to $TNF-\alpha$ dilution. Figure 10 showed that MAP2 cells H_2O_2 production was sensitive to $TNF-\alpha$ concentration although the increase in H_2O_2 production might not be significant in the range of $TNF-\alpha$ concentration tested. The MAP3 cells showed an increase in H_2O_2 production with $TNF-\alpha$ dilution. This increase was however not statistically significant.

That the production of H_2O_2 under $TNF-\alpha$ cytotoxicity was mainly intracellular was apparent from the cell trapped DCF fluorescence data as well as from the percent viability data obtained under $TNF-\alpha$ cytotoxicity in the presence of various antioxidants like Cu/Zn-superoxide dismutase (SOD), catalase, manitol and DMSO. Firstly as DCF was trapped inside the cell the DCF fluorescence was due to intracellular H_2O_2 levels. As seen in Figure 12 the extracellular SOD, catalase, and manitol had little effect in protecting the L929 cells from $TNF-\alpha$ cytotoxicity. Catalase however offered a slight protection. Due to an insignificant effect of extracellular catalase on cell viability as well as DCF fluorescence it was concluded that H_2O_2 was produced primarily at an intracellular site.

We investigated the effect of various lipid soluble antioxidants on the H_2O_2 production by MAP 1, MAP 2 and MAP 3 cells under TNF- α (100 units/ml) cytotoxicity. DMSO, known to inhibit TNF- α cytotoxicity in the range of concentrations investigated, had little effect in inhibiting H_2O_2 production in MAP1(Figure13), MAP2 (Figure 14) and MAP 3 (Figure15) cells at lower concentrations. DMSO, at 500 mM, did however increase H_2O_2 production in all the cell subpopulations. Vitamin E (0.1 and 1.0 mM) decreased the TNF- α induced H_2O_2 production in MAP 2 (Figure 14) and MAP 3 (Figure 15) cells. Thus the status of vitamin E in inhibition of peroxidative chain reaction could not only be in nonspecific termination of lipid peroxidation but also an inhibition of H_2O_2 production. Vitamin A (0.1 mM) had no effect on H_2O_2 production but at 1.0 mM, was synergistic with TNF- α , in increasing the H_2O_2 production ($p < 0.05$). That TNF- α cytotoxicity appeared to be mediated in part by intracellular production of H_2O_2 was suggestive from the flow cytometric data.

DISCUSSION

A major difference between use of DCF-DA to quantitate H_2O_2 production in activated neutrophils and to use it to monitor H_2O_2 production under TNF- α cytotoxicity is the incubation time. Loading of DCF-DA in cells at 37 $^{\circ}$ C typically takes 15 minutes (Burow et al. 1987). Activation of DCF-DA loaded neutrophils with phorbol 12-myristate 13-acetate (PMA) produces hydrogen peroxide for a maximum period of one hour (Rothe, et al., 1990). In comparison to this system the TNF- α cytotoxicity bioassay is typically a 15 hr to 18 hr assay. Thus the demand made on DCF-DA to be a H_2O_2 production reporter is definitely very different. DCF-DA after being taken up by cells should not only remain an efficient indicator of H_2O_2 production in the span of 15 to 18 hrs but also it should not by itself change the viability of the cells.

TNF- α cytotoxicity known to be mediated via an oxidative reaction was shown, using flow cytometry, to produce intracellular H_2O_2 . This H_2O_2 production was sensitive to, both concentration as well as incubation time of, TNF- α . The L929 cells were analyzed by

flow cytometry as two subpopulations namely MAP1 and MAP2 cells. The MAP1 cells were smaller and the less viable subpopulation than the MAP2 cells as was determined by the viability study under the action of TNF- α (Figures 3 and 4). DCFDA was established to be an indicator of intracellular H₂O₂ production between 5 hr and 15 hr during which time the control cells expressed a steady state DCF fluorescence. The MAP 2 cells green fluorescence indicated that there was a significant increase in the kinetics of H₂O₂ production when these cells were subjected to TNF- α in the range of incubation times between 5 hr and 15 hr (Figure 6). H₂O₂ production in MAP2 cells was also sensitive to TNF- α concentration (Figure 8). Interestingly, maximal TNF- α binding and internalization was known to be completed around 4 hr of TNF- α incubation in cell culture systems. That the temporal production of H₂O₂ coincided with the time by which maximal TNF- α internalization (Larrik et al. 1990) occurred, fitted well with the hypothesis of TNF- α induced oxidative killing. The production of H₂O₂ could possibly take place as presented in SCHEME-1. DMSO, a known OH' scavenger, and a known inhibitor of TNF- α , at lower concentrations had little effect and at higher concentrations (500 mM) had an enhancing effect for H₂O₂ production in MAP1, MAP2, and MAP3 cells (Figures 13, 14, 15). Increased H₂O₂ production at a higher DMSO concentration could possibly be due to the fact that DMSO by removing the OH' radical from the product side of Fenton or Haber-Weiss reaction could tilt the equilibrium to the right. Because the presence of catalase, a known H₂O₂ scavenger, in the extracellular environment had little effect in inhibiting cytotoxicity due to TNF- α (Figure 12) most of the H₂O₂ was probably produced at an intracellular site. The production of H₂O₂, as monitored by flow cytometry, fitted rather nicely into the model of involvement of free radical species of oxygen under TNF- α cytotoxicity. A possible mechanism of TNF- α cytotoxicity is given (SCHEME-1) which shows that the H₂O₂ production as an important intermediate step.

SCHEME -1

TNF- α ---> TNF-Receptor Complex ---> Internalization ---> Mitochondrial Toxicity ---> Generation Of O_2^- ---> Dismutation by Mn-SOD ---> **H₂O₂ Production** ---> OH' Production (via Fenton/Haber-Weiss Chemistry) ---> Lipid Peroxidation ---> Cell Death.

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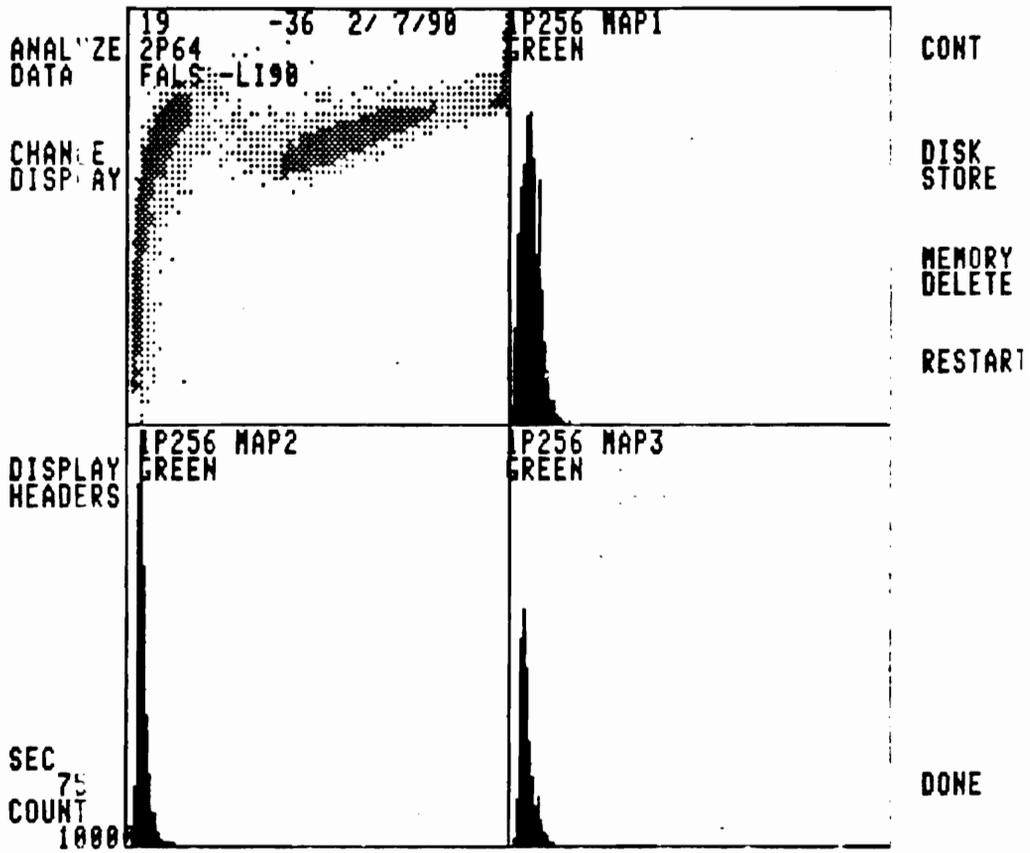
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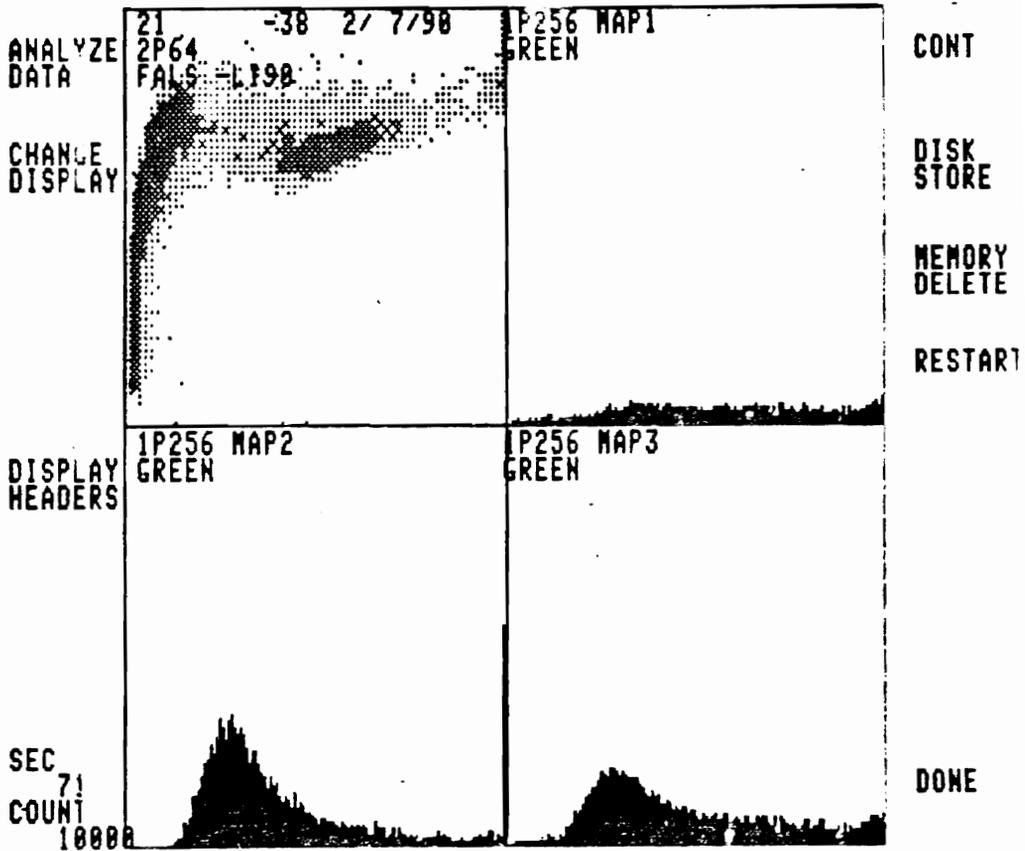
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Figure 1 : Four quadrant representative linear vs. logarithmic plot of L929 cells for DCF florescence (indicator of H_2O_2 production). The treatment groups were designated as DCF/TNF- α (t) as mentioned in the results section. The representative groups were designated as (a) (-/-) 0 hr; (b) (+/-) 0 hr; (c) (+/-) 5hrs; (d) (+/-) 10 hrs; (e) (+/-) 15 hrs; (f) (+/+) 5 hrs; (g) (+/+) 10 hrs; and (h) (+/+) 15 hrs.

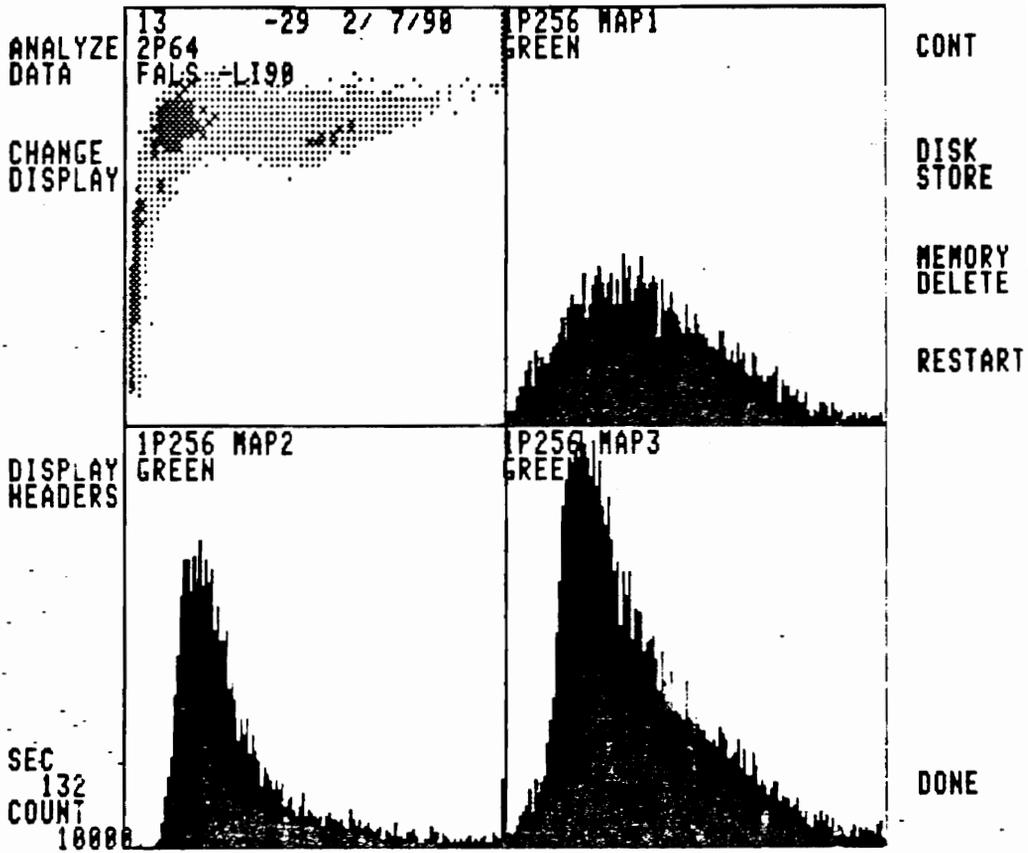
(a) (-/-) 0 hr;



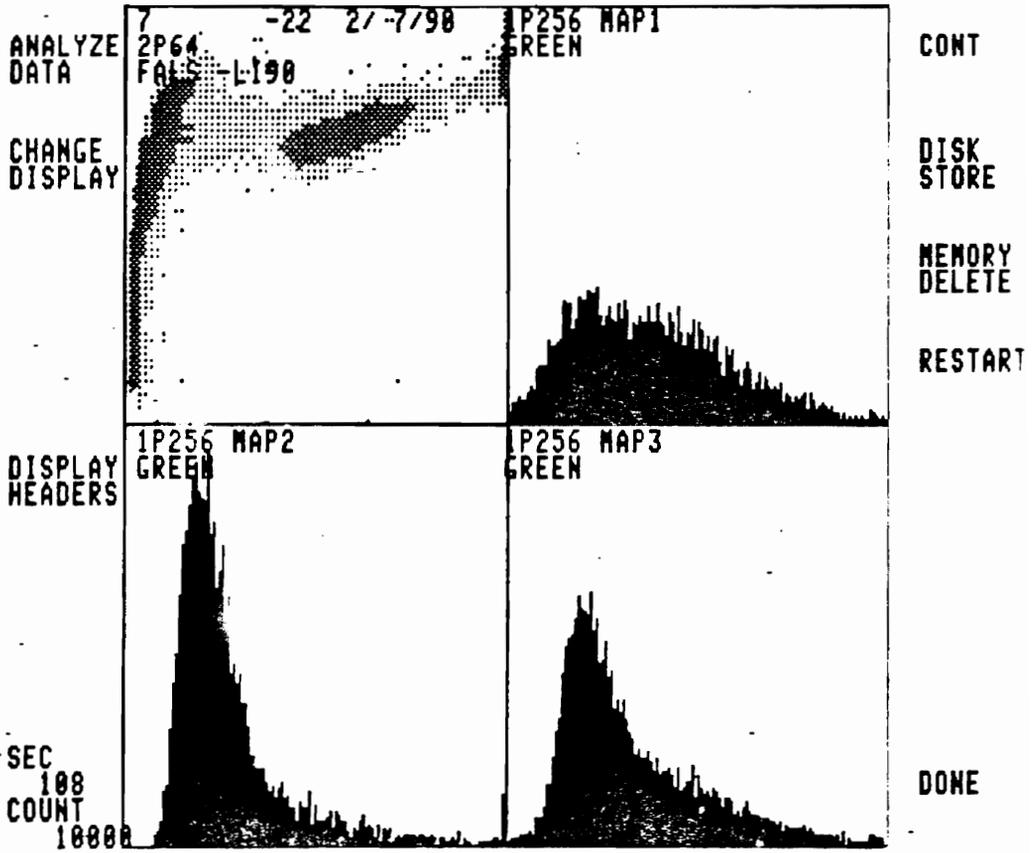
(b) (+/-) 0 hr



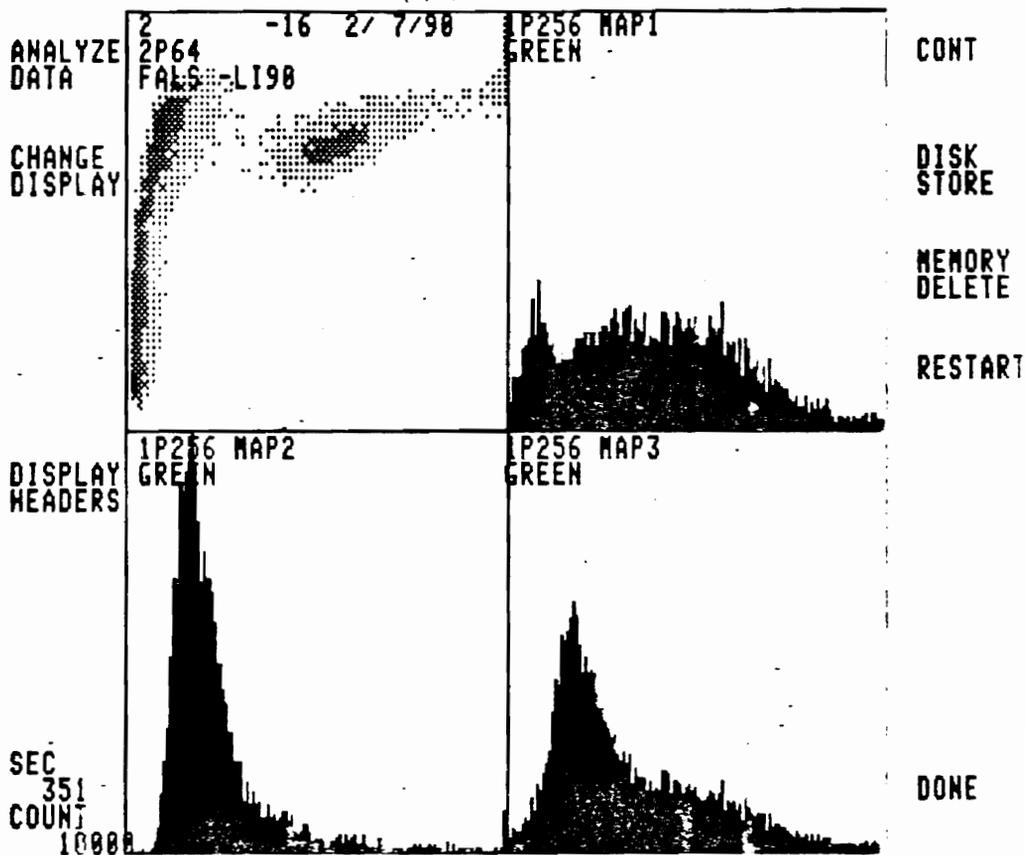
(c) (+/-) 5hrs



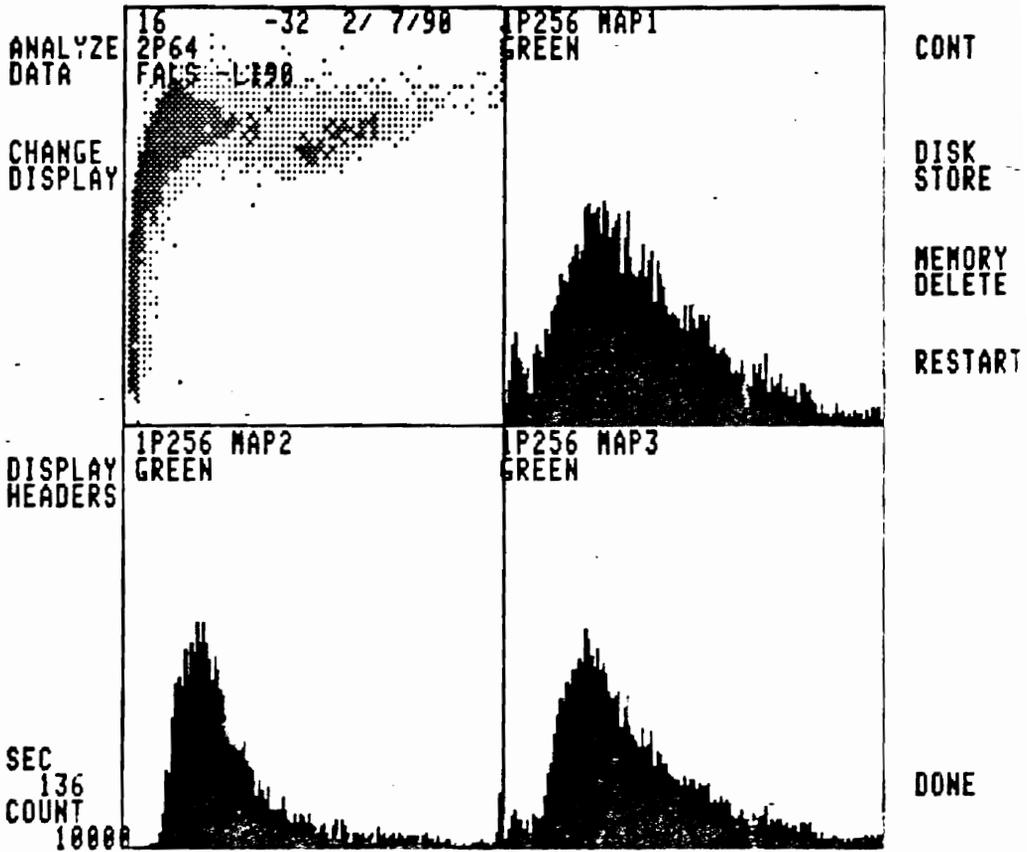
(d) (+/-) 10 hrs



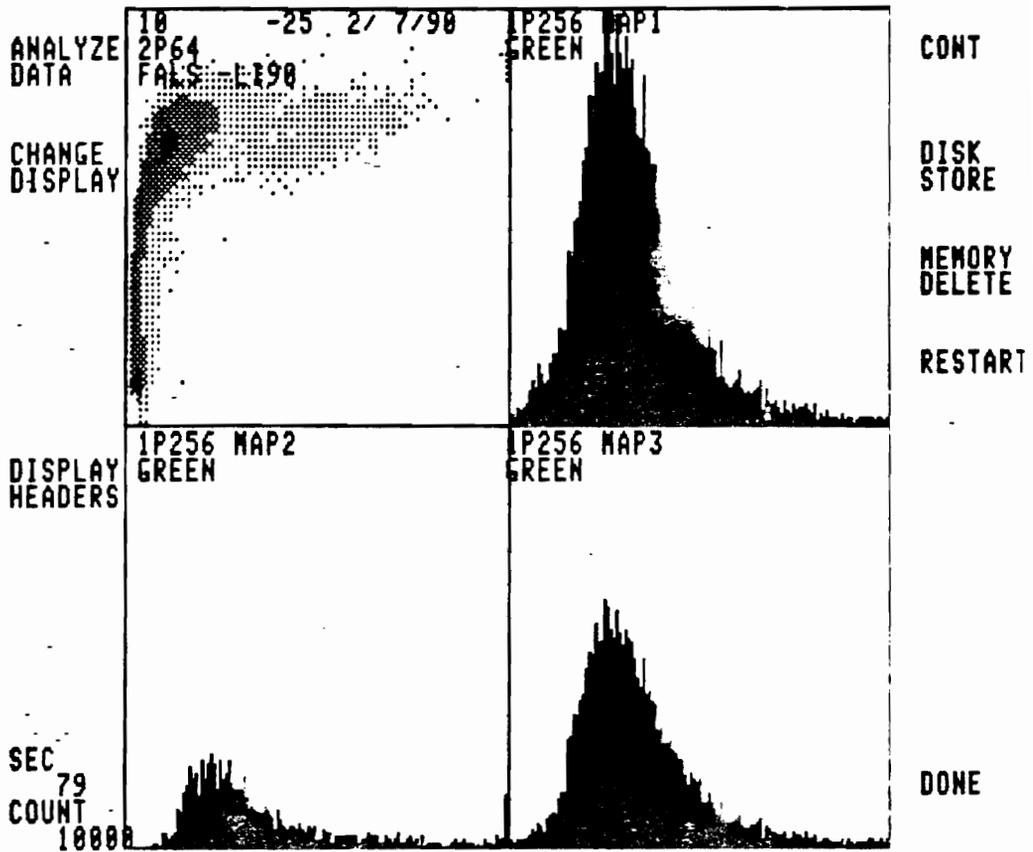
(e) (+/-) 15 hrs



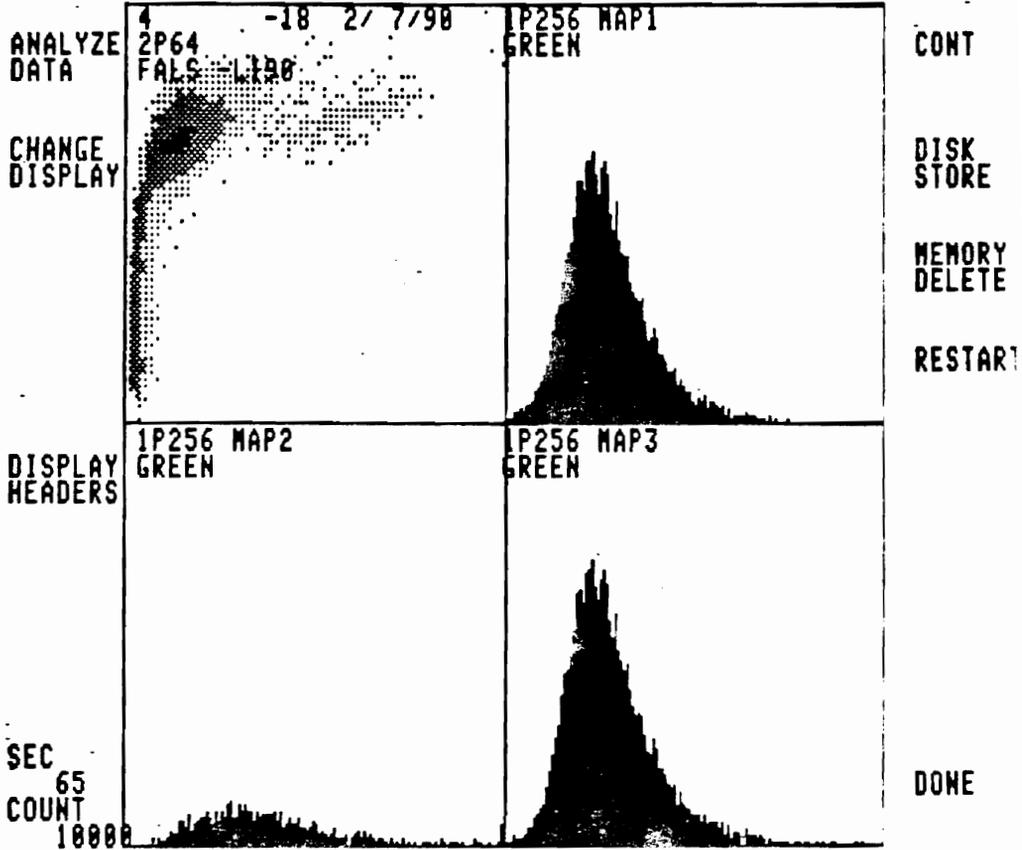
(f) (+/+) 5 hrs



(g) (+/+) 10 hrs



(h) (+/+) 15 hrs.



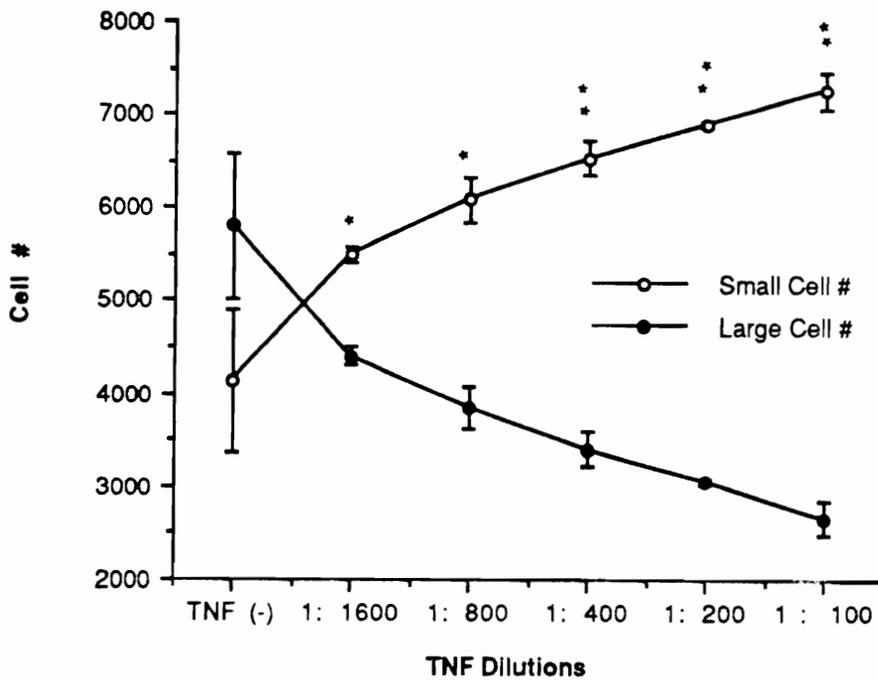


Figure 2 : Effect of TNF- α concentration on The Population Of MAP 1 and MAP 2 cells. MAP 1 (small cells), and MAP 2 (large cells) are the two subpopulations of L929 cells as described in the results section. Confluent L929 cells were incubated in triplicate without TNF- α as well as 1 : 100, 1 : 200, 1 : 400, 1 : 800, 1 : 1600 dilution of a 10,000 units/ml TNF- α stock for 15 hrs. Of a total of 10,000 cells counted per run, the number of MAP 1 and MAP 2 cells

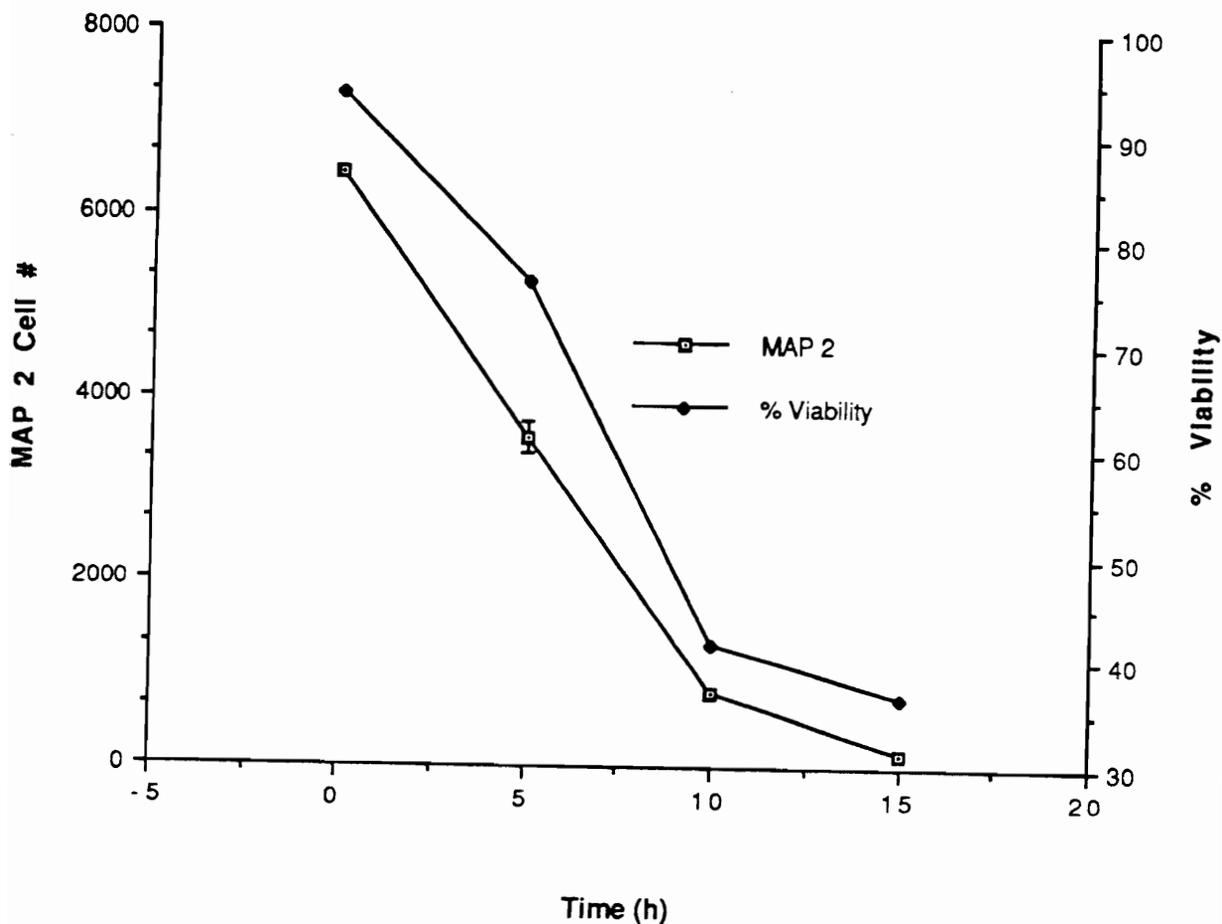


Figure 3 : MAP 2 Cell Number vs. Viability Under TNF- α Cytotoxicity. L929 cells in 24 well plates were incubated with 100 units/ml of TNF- α for 0, 5, 10, and 15 hrs in 1 ml of media containing 1 μ g/ml actinomycin D. Cells were simultaneously analyzed for viability (tripan blue exclusion) and number of MAP 2 cells in 10,000 cells counted by flow cytometry. The viability was plotted with MAP 2 cell number as a function of TNF- α incubation time.

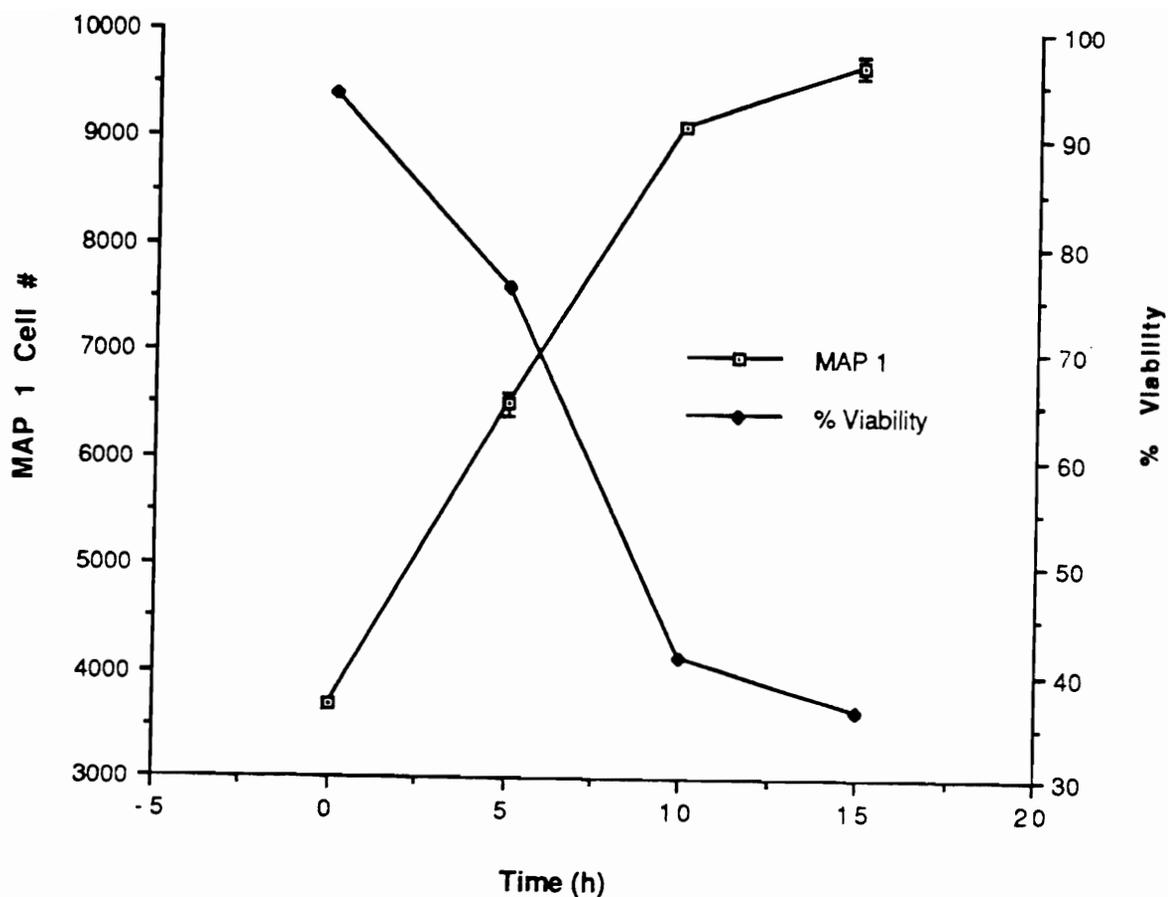


Figure 4 : MAP 1 Cell Number Vs. Viability Under TNF- α Cytotoxicity. L929 cells in 24 well plates were incubated with 100 units/ml of TNF- α for 0, 5, 10, and 15 hrs in 1 ml of media containing 1 μ g/ml actinomycin D. Cells were simultaneously analyzed for viability (tripan blue exclusion) and number of MAP 1 cells in 10,000 cells counted by flow cytometry. The viability was plotted with MAP 1 cell number as a function of TNF- α incubation time as shown.

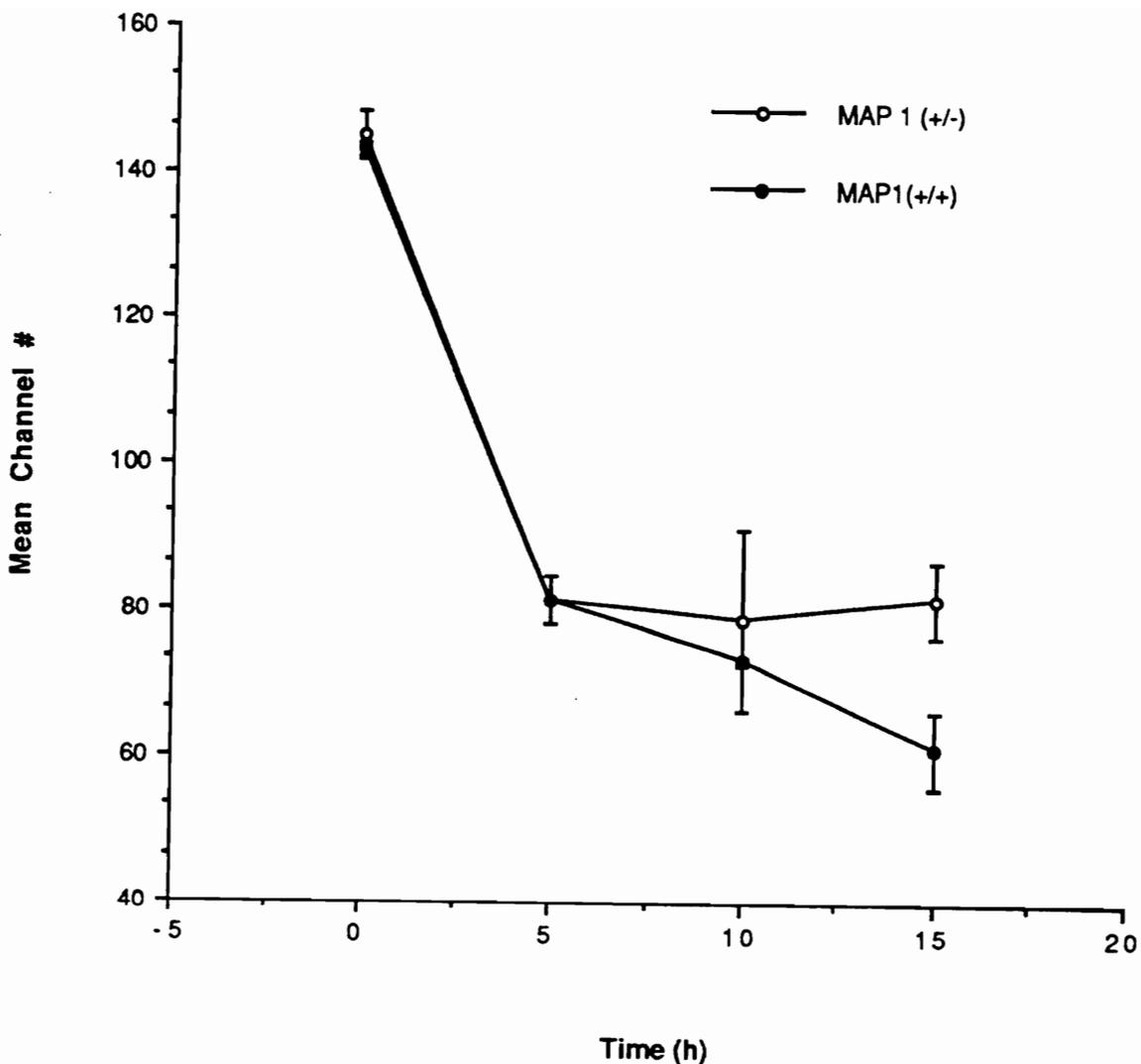


Figure 5 : Green Florescence of MAP 1 Cells With TNF- α Cytotoxicity. Triplicate confluent L929 cultures in 24 well plates were preincubated with DCFDA as described in the methods section and treated with 100 units/ml TNF- α for 0, 5, 10, and 15 hrs and analyzed for green florescence. The mean channel numbers of triplicate culture are represented as mean \pm S.D for the MAP 1 cells.

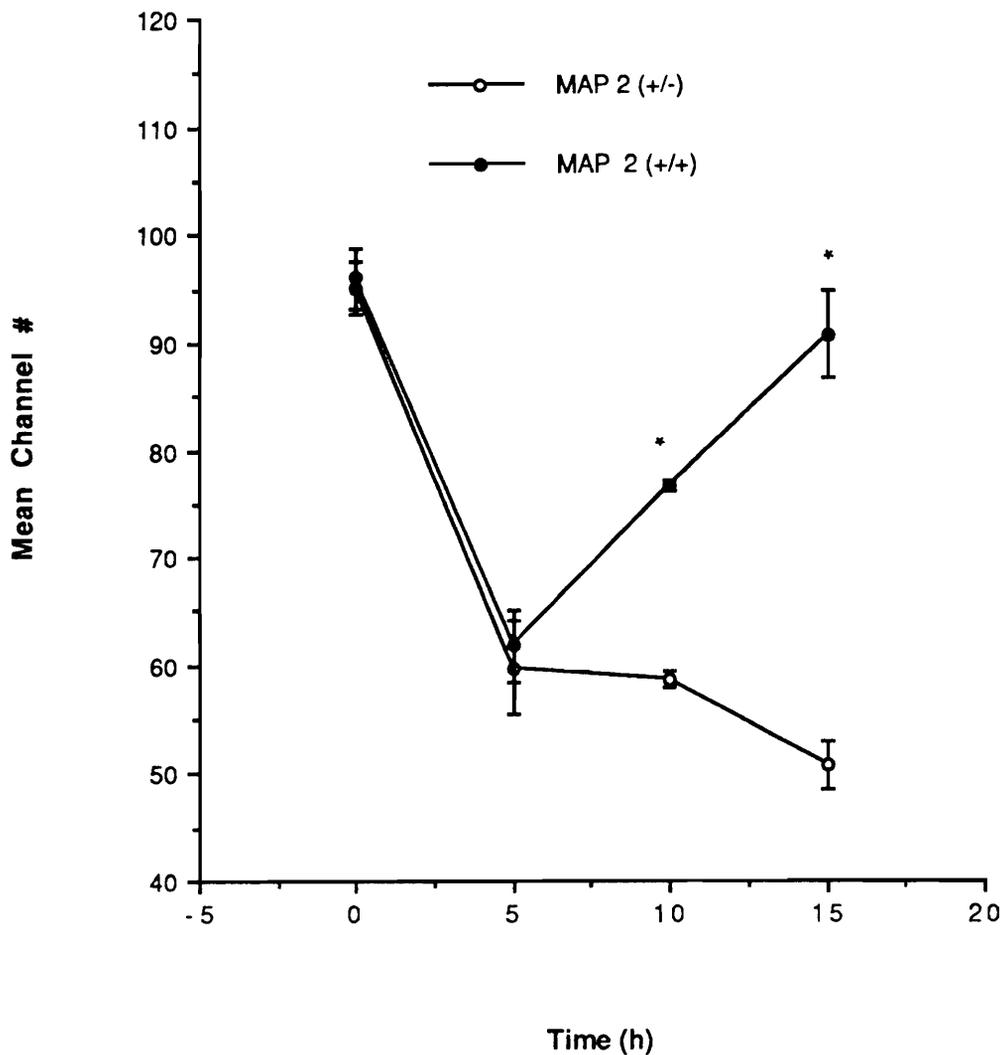


Figure 6 : Green Florescence of MAP 2 Cells With TNF- α Cytotoxicity. Triplicate confluent L929 cultures in 24 well plates were preincubated with DCFDA as described in the methods section and treated with 100 units/ml TNF- α for 0, 5, 10, and 15 hrs and analyzed for green florescence. The mean channel numbers of triplicate culture are represented as mean \pm S.D for the MAP 2 cells.

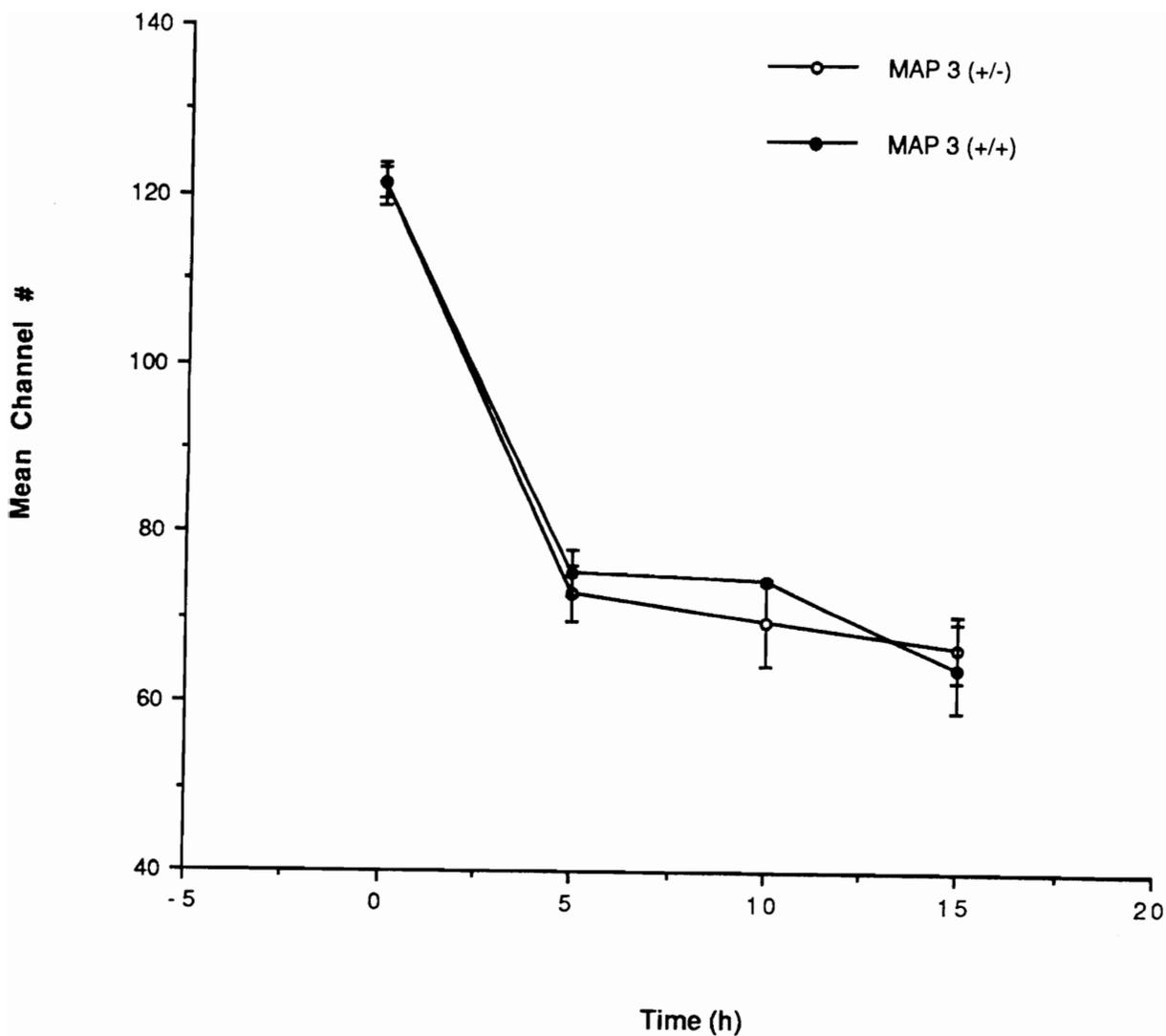


Figure 7 : Green Florescence of MAP 3 Cells With TNF- α Cytotoxicity. Triplicate confluent L929 cultures in 24 well plates were preincubated with DCFDA as described in the methods section and treated with 100 units/ml TNF- α for 0, 5, 10, and 15 hrs and analyzed for green florescence. The mean channel numbers of triplicate culture are represented as mean \pm S.D for the MAP 3 cells.

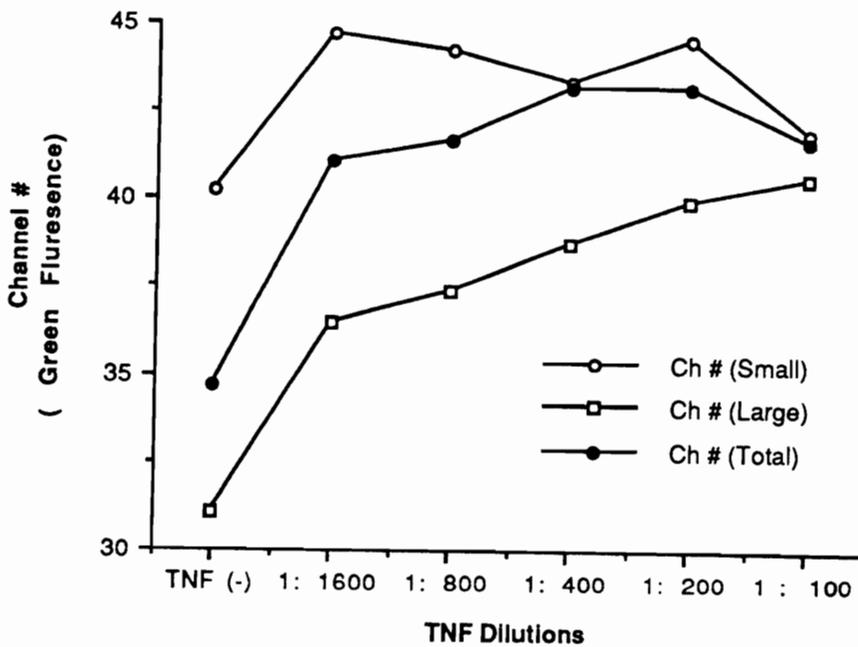


Figure 8 : Hydrogen Peroxide Production With TNF- α dilution. Confluent L929 cells in 24 well plates preincubated with DCFDA were cultured in presence of TNF- α at different concentrations as shown. The stock TNF- α concentration was 10,000 units/ml. The green florescence of MAP 1 (small), MAP 2 (large), and MAP 3 (total) cells are plotted (without statistics) with TNF- α dilution as shown. The MAP 2 (viable cells) as well as MAP 3 cells show an induction of H₂O₂ production with TNF- α dilution.

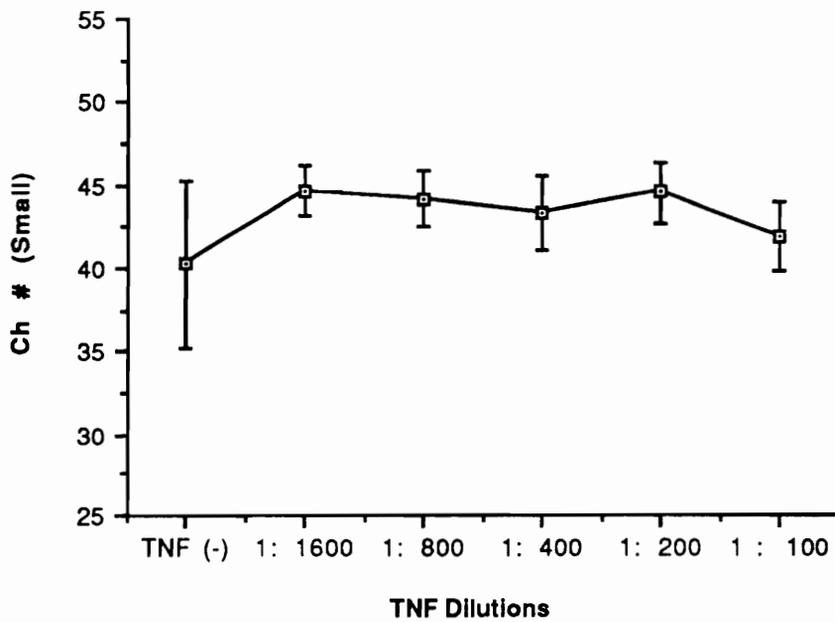


Figure 9 : The plot of MAP 1 cell H₂O₂ production (with statistics) with TNF- α dilution. The conditions were exactly same as in the Figure 8.

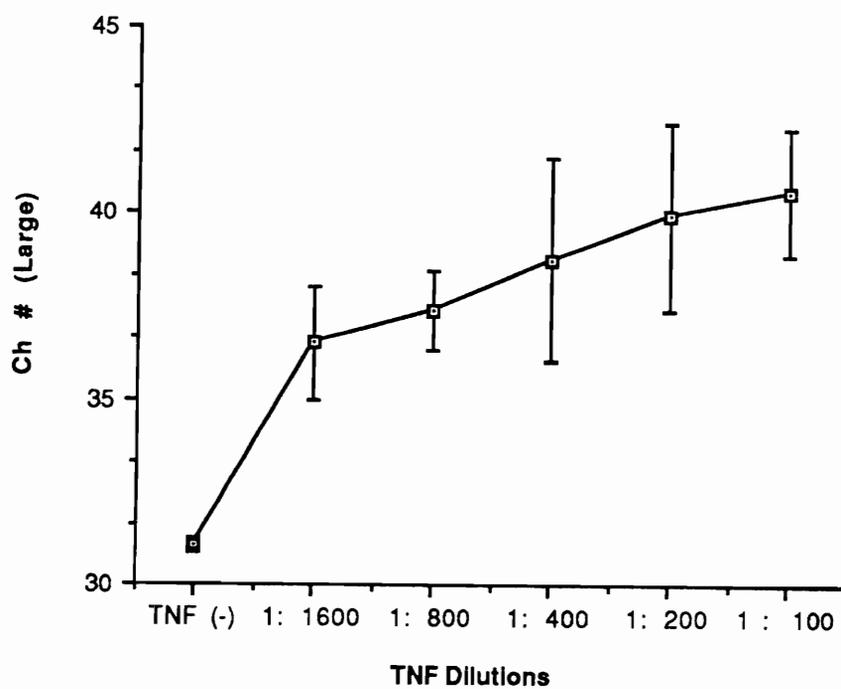


Figure 10 : The plot of MAP 2 cell H_2O_2 production (with statistics) with TNF- α dilution. The conditions were exactly same as in the Figure 8.

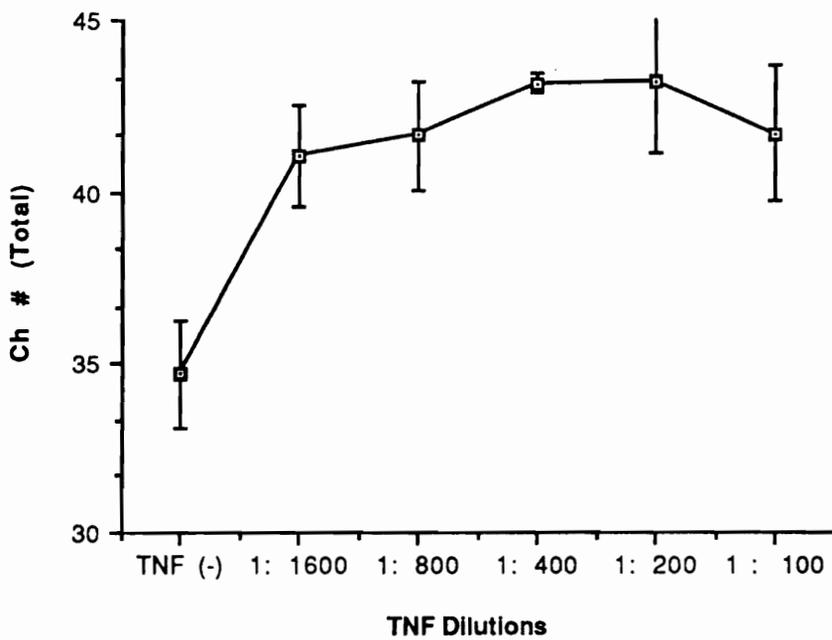


Figure 11 : The plot of MAP 3 cell H_2O_2 production (with statistics) with $TNF-\alpha$ dilution. The conditions were exactly same as in the Figure 8.

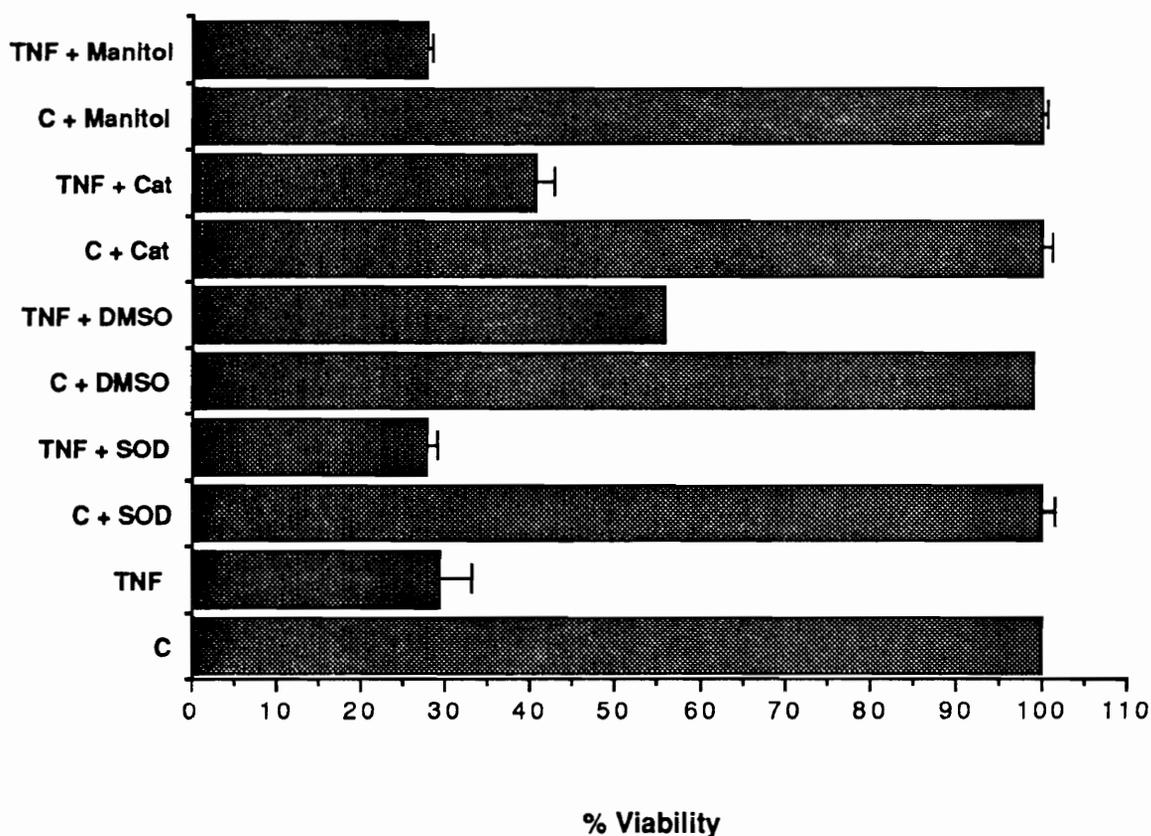


Figure 12 : Protection against cytotoxicity of TNF- α by antioxidants. The incubation time was 15 hrs with/without 100 units/ml TNF- α , with/without SOD (50 μ g/ml), with/without catalase (50 μ g/ml), with/without manitol (6 mM), and with/without DMSO (100 mM). % viability was determined using a tripan blue exclusion method. DMSO at 100 mM protects against TNF- α .

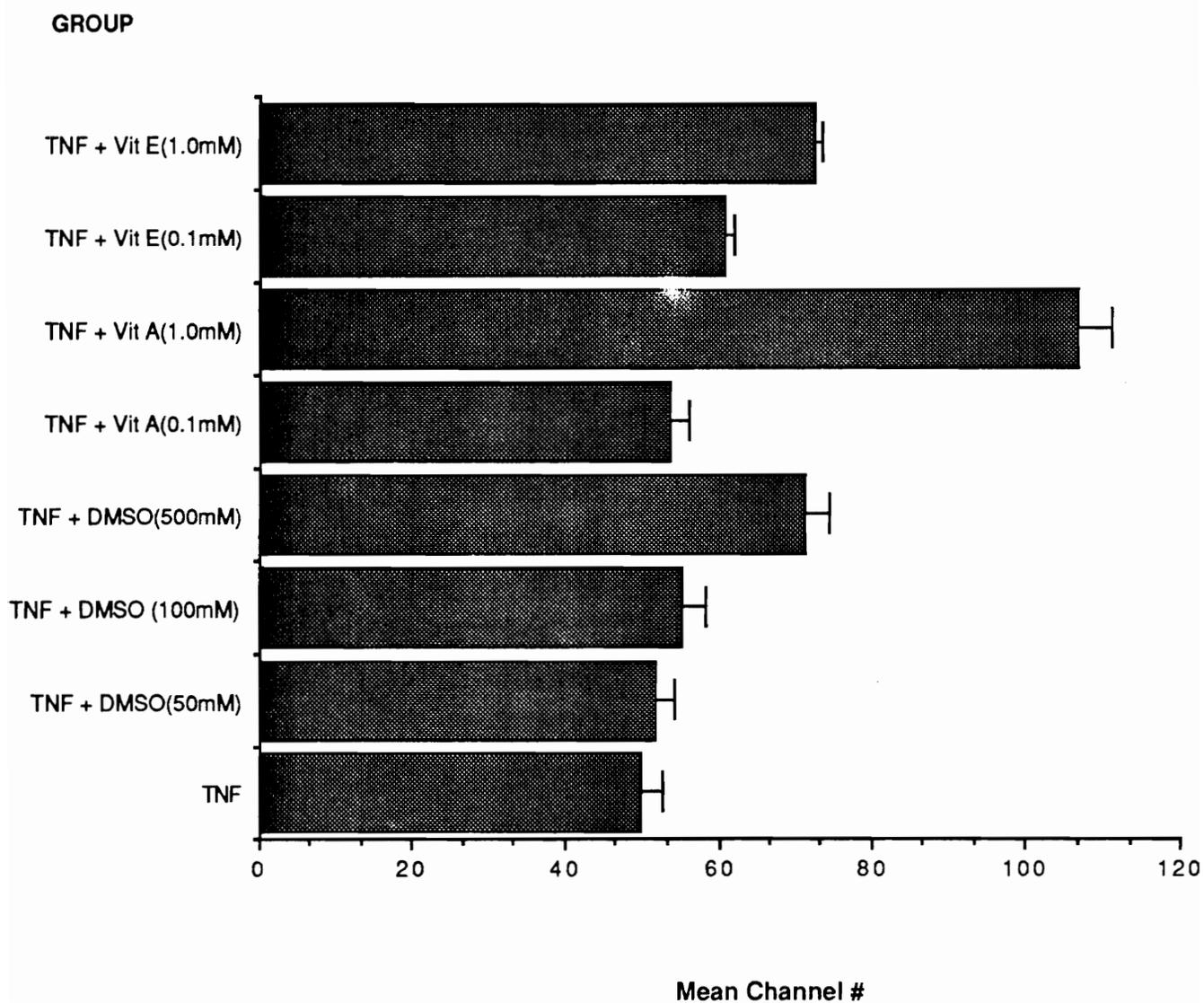


Figure 13 : Modulation In The Production of TNF- α Induced H₂O₂ By Lipid soluble antioxidants in MAP 1 cells. The experimental details are as in the methods section.

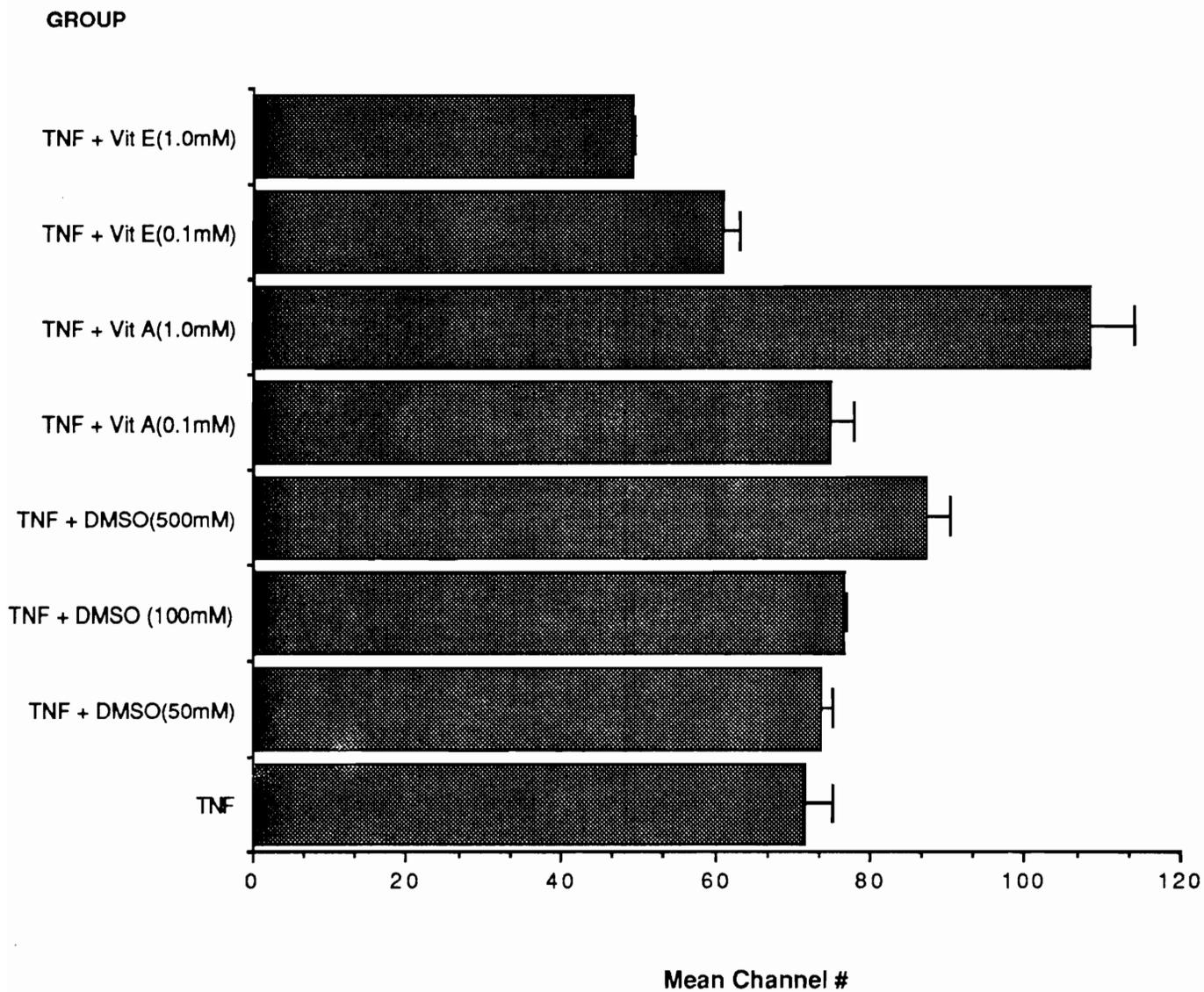


Figure 14 : Modulation In The Production of TNF- α Induced H₂O₂ By Lipid soluble antioxidants in MAP 2 cells. The experimental details are as in the methods section.

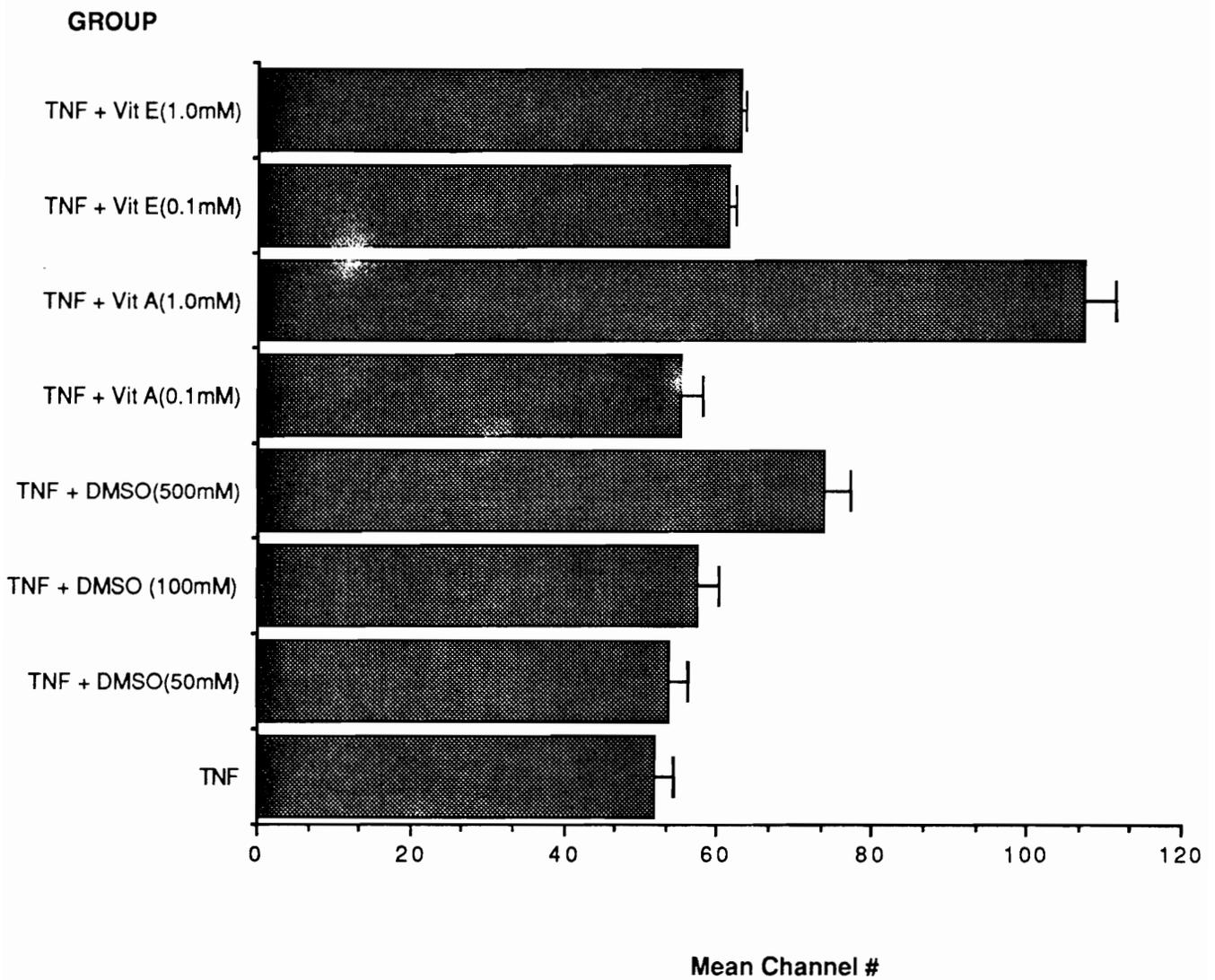


Figure 15 : Modulation In The Production of TNF- α Induced H₂O₂ By Lipid soluble antioxidants in MAP 3 cells. The experimental details are as in the methods section.

APPENDIX - 3**USE OF TEMPO IN MONITORING TNF- α CYTOTOXICITY
IN L929 CELLS BY EPR****ABSTRACT**

TNF- α cytotoxicity is known to act via an oxidative process. The oxidative damage to the cells result in peroxidation of membrane lipids. Although (malondialdehyde) MDA production happens to be a late event in the oxidative cascade, it is, however, a poor indicator of TNF- α cytotoxicity. In this investigation, it was demonstrated that the TNF- α -induced peroxidative event could be monitored by measuring the differential quenching of the spin label 2, 2, 6, 6- tetramethyl-1-piperidine-n-oxyl (TEMPO) using EPR spectroscopy. The rate of quenching of TEMPO by the TNF- α sensitive L929 cells was hypothesized to be an indicator of TNF- α cytotoxicity. The cell-induced rate of loss of EPR signal of nitroxide spin label, TEMPO was shown to depend on TNF- α concentration, time of incubation of TNF- α and time of incubation of TEMPO with cells. Decrease in the rate of reduction of TEMPO with time of incubation of TNF- α correlated with time kinetics and concentration of TNF- α cytotoxicity respectively. The loss of TEMPO EPR signal under the action of TNF- α was determined to be at an extracellular domain of the lipid bilayer as elucidated by the quenching and broadening of the TEMPO signal in presence of NiCl₂. From the above data, we concluded that the reduction rates of a spin label was indicative of the peroxidative stress on a cell membrane. Spin labels inhibit lipid peroxidation and in the process get reduced to a non-paramagnetic form. Thus reduction of a lipid soluble spin label like TEMPO as monitored by EPR could be an useful reporter to study TNF- α cytotoxicity.

INTRODUCTION

Electron paramagnetic resonance (EPR) of nitroxide spin labels are used to monitor many biochemical and biophysical properties of cells (Cafiso and Hubbell, 1981; Symons, 1982; Gaffney, et al, 1977; Marsh and Watts, 1982). However, a major set back in the use of nitroxide spin labels in biological systems is their metabolism to non-paramagnetic products, specifically to hydroxylamines (Swartz, et al, 1985; Aracaia, et al, 1983; Gaffney, 1976; PaLeos, et al, 1977). The cell-induced loss of EPR signal of nitroxide spin labels depends on the presence of charged groups on the nitroxide that influence it's entry into the cells. The reduction of nitroxide has been conjectured to be an intracellular process (Harold, et al, 1986). The spin labels and their corresponding hydroxylamines have the ability to inhibit NADPH-dependent, Fe^{2+} catalyzed, lipid peroxidation in hepatic microsomes (Ulf, et al, 1989). We hypothesize that if a nitroxide spin label can inhibit cellular lipid peroxidation with it's concomitant reduction, the rate of reduction of the nitroxide label could be different for the cells that are normal and the cells that are under oxidative stress. Thus, for a given cell line, cells that are undergoing rapid peroxidation of its cellular lipids would quench nitroxide spin labels at a slower rate than the normal cells.

Tumor necrosis factor- α (TNF- α) is a multifunctional protein that is secreted by activated macrophages and elicits hemorrhagic necrosis of certain tumors *in vivo* as well as cytotoxicity to several murine and human cell lines *in vitro* (Carswell, 1975; Haranaka, et al, 1981; Williamson, et al, 1983). Apart from its cytostatic, cytotoxic and null effect on several human tumor cell lines, TNF- α has also been shown to be growth promoting in normal human fibroblasts (Sugarman, et al, 1985; Vilcek, et al, 1986).

Several lines of evidence implicate oxygen radicals as mediators of TNF- α -induced cytotoxicity. It has been observed that TNF- α treated cells produce malonyldialdehyde (MDA) (a measure of free radical damage) detectable around 8 hr of TNF- α incubation and maximally produced around 40 hr (Matthews, et al, 1987). Hydroxyl radical formation (inhibited by dimethyl sulfoxide) was detected at

around 18 hr of incubation with TNF- α in tumorigenic mouse fibroblast L-M cells (Yamavehi, et al, 1989) and that cytotoxicity of TNF- α is more in cells which are incapable to scavenge the radicals (Zimmerman, et al, 1989; Wong, et al, 1988). Thus, TNF- α cytotoxicity is envisioned to be via an oxidative process. The oxidative damage to the cells result in peroxidation of membrane lipids. Although MDA production happens to be a late event in the oxidative cascade, it is, however, a poor indicator of TNF- α cytotoxicity.

In this investigation, we demonstrated the TNF- α -induced peroxidative event, by measuring the differential quenching of the spin label 2, 2, 6, 6- tetramethyl-1-piperidine-n-oxyl (TEMPO) (Sigma Chemical Co., MO) signal in L929 cells treated with and without TNF- α using EPR spectroscopy. The rate of quenching of TEMPO by the cells was hypothesized to be an indicator of TNF- α cytotoxicity.

MATERIALS AND METHODS

Cell culture:

L929 (mouse fibrosarcoma) cells and RAW 264.7 (Abelson murine leukemia virus transformed and immortalized BALB/c mouse macrophage) cells were obtained from the American Type Culture Collection (ATCC), Rockville, MD. The cells were cultured in 90% Dulbecco's modified Eagle's medium, 10% heat inactivated, filter sterilized, fetal calf serum (FCS) (< 0.1 ng/ml endotoxin), 10 units/ml penicillin, 10 μ g/ml streptomycin and 3.7 g/l sodium bicarbonate pH 7.4 at 37 $^{\circ}$ C in a 5% CO $_2$ incubator.

Murine TNF- α :

The cytokine TNF- α was produced and immunopurified from the LPS treated RAW 264.7 macrophage supernatant. The immunopurification was performed on an anti-TNF- α antibody column as described (Chapter 1). The specific activity of TNF- α was determined to be 6 x 10 5 units/mg proteins as determined by the

reduction of a tetrazolium dye by a L929 cytotoxicity bioassay (Larrik, et al, 1989).

EPR Assay for TEMPO Quenching:

Confluent cultures of L929 cells in 25 cm² flasks were incubated with different concentrations (0 to 400 units/ml) of TNF- α for 0, 5, 10 and 15 hr in 6 ml of media supplemented with 1 μ g/ml actinomycin D. At the end of the incubation time, the cells were scraped (10^7 cells/25 cc flask) and washed by centrifugation, (600 rpm for 10 minutes), with 6 ml of Hanks balanced salt solution (HBSS) (Sigma Chemical Co., MO). The cell pellets were resuspended in 150 μ l of HBSS. 90 μ l of the cell suspension was incubated with 10 μ l (0.1 mM) TEMPO for 0 to 30 minutes. The cell suspension was taken up in a capillary tube and placed in the EPR cavity to monitor the TEMPO signal.

Monitoring Intracellular Decay of TEMPO:

Cells incubated with and without TNF- α (100 units/ml) were incubated in presence of 0.01 mM TEMPO for 15 minutes, with and without 50 mM (final concentration) NiCl₂. NiCl₂ is known to be a cell non-permeable paramagnetic ion that broadens the EPR spectrum of extracellular nitroxides (Harold, et al, 1986). This facilitates in the measurement of intracellular TEMPO. This study was performed to assay whether cytotoxicity of cells to TNF- α altered the intracellular quenching of TEMPO.

EPR Settings:

The triplet (1:1:1) TEMPO signal was monitored on an ER 200-D SRC EPR spectrometer (Bruker Instruments, Inc). The EPR parameters were: modulation amplitude, 1G (at 100 KHz), receiver gain, 4×10^5 (at signal channel time constant of 640 ms), the center field 3480 G was scanned with a sweep width of 50 G at 13 mwatts microwave power (9.78 GHz).

RESULTS

The EPR spectrum of 0.01 mM TEMPO in HBSS is shown as a 1:1:1 spectrum in Figure 1(a). Figure 1(b) shows the peak heights of the third peak of the TEMPO signal (at 0.01 mM final concentration) when TEMPO was incubated in the presence of cells without TNF- α (top) or cells treated with 100 units/ml TNF- α in 6 ml of media (bottom), for 15 h. The peak heights were recorded at the indicated time of incubation of the cells (control and TNF- α treated) with TEMPO. As is seen in Figure 1(b) viable, control, L929 cells rapidly quench the TEMPO signal (top) where as an equal number of cells treated with TNF- α quenched the TEMPO signal at a very slow rate (bottom). The reduction rate of the triplet signal with time of incubation with TEMPO for cells treated with or without 100 units/ml TNF- α is shown in Figure 2. This large difference in the rate of quenching of TEMPO signal forms the basis of a new, simple, sensitive and highly reproducible TNF- α cytotoxicity assay system using EPR spectrometry.

L929 cells, in (25 cm²) flasks, treated with 0 to 400 units/ml of TNF- α for 15 h were handled in a similar manner and the peak height at 15 minutes incubation with TEMPO were plotted as a function of TNF- α concentration (Figure 3, TABLE 2). The Figure 3 showed that the inability of cells (exposed to TNF- α), to quench TEMPO signal increased with TNF- α concentration (at 15 h incubation). Thus TNF- α induced alteration in the ability of L929 cell membrane to quench TEMPO was responsive to TNF- α concentration.

The kinetic of reduction of TEMPO signal with time incubation of cells with 100 units/ml TNF- α for 0, 5, 10 and 15 hr was shown in Figure 4. With increasing incubation time of the L929 cells with TNF- α , the cells lost the ability to quench the TEMPO signal. The reduction rate of TEMPO quenching decreased with increasing incubation time with TNF- α . When this negative reduction rate was plotted with TNF- α incubation time, the curve so obtained, resembled the tetrazolium dye reduction (% cytotoxicity) curve in the TNF- α cytotoxicity bioassay as performed in a 96 well plate

(Figure 5). Thus quenching of TEMPO by cells treated with TNF- α was an efficient measure of TNF- α cytotoxicity.

The quenching of TEMPO signal with control and TNF- α treated cells in absence and presence of 50 mM NiCl₂ is shown in Figure 6 (a thru d). Figure 6(a) shows the signal height of TEMPO with control cells after 15 minutes of incubation without (-) and with (+) 50 mM NiCl₂ at 0.01 mM TEMPO concentration. The unquenched 0.01 mM TEMPO peak is as in Figure 1(a). Figure 6(a) shows that most of the quenching of TEMPO signal is at the membrane site of the extracellular environment. Figures 6(b) thru 6(d) depict total TEMPO quenching (left) and intracellular TEMPO quenching (right) for cells incubated for 5, 10 and 15 h with 100 units/ml TNF- α . It is obvious from the data that TEMPO signal is quenched by the cell membrane specifically at an extracellular environment as NiCl₂ broadened the extracellular TEMPO signal, which resulted in a little (intracellular) signal for cells treated with and without TNF- α .

TEMPO quenching by TNF- α treated cells was monitored in presence of various antioxidants. Superoxide dismutase (100 units/ml) and catalase (1000 units/ml) had little effect in inhibiting TNF- α cytotoxicity as monitored by TEMPO quenching (Figure 7). Cells treated with 1 mM and 10 mM α -tocopherol (vitamin E) and 0.1 mM β -carotene (Figure 8) enabled the cells to retain their TEMPO quenching ability. These lipid soluble antioxidants are known to inhibit TNF- α cytotoxicity. Dimethyl sulfoxide (DMSO) is another lipid soluble antioxidant (hydroxyl radical scavenger) known to inhibit the TNF- α cytotoxicity (Larrik, et al, 1990) in L929 cells. The ability of TNF- α treated L929 cells in absence and presence of DMSO (0 to 500 mM) to quench TEMPO was tested. As shown in Figure 9, inhibition of TNF- α cytotoxicity by DMSO can very easily be shown by TEMPO quenching using EPR spectroscopy. The cytoprotectiveness of DMSO was illustrated to be responsive to DMSO concentration. TNF- α cytotoxicity and its inhibition by antioxidants could therefore be demonstrated by the quenching of TEMPO signal using EPR technique. Thus TEMPO previously known to inhibit lipid peroxidation was demonstrated to be an efficient reporter of the TNF- α induced cytotoxic event.

DISCUSSION

The cell induced reduction rates of spin label TEMPO incubated with L929 cells with and without TNF- α was distinctly different (TABLE 1, Figure 2). This cell-induced loss of EPR signal of TEMPO, was shown in the TNF- α sensitive cell line L929 to depend on TNF- α concentration (Figure 3). In the TNF- α concentration range tested the L929 cells progressively lost their ability to quench TEMPO signal. Higher the concentration of TNF- α , higher was the TEMPO peak resulting from a decreased ability of the L929 cells to quench TEMPO. Time incubation of TNF- α showed that L929 cells expressed decreasing rates of TEMPO quenching (Figure 4). Decrease in the rate of reduction of TEMPO with time of incubation of TNF- α correlated with time kinetics and concentration of TNF- α cytotoxicity respectively. The negative rate of TEMPO decay compared well with the cytotoxicity curve as presented in Figure 5. This demonstrated that TEMPO quenching, measured by EPR spectrometer, was a sensitive technique to monitor TNF- α cytotoxicity. The cellular site for TEMPO quenching was determined to be on the extracellular domain. This was concluded by use of membrane impermeant NiCl₂ (a known broadner of TEMPO signal) in conjunction with TEMPO quenching experiments. Thus any TEMPO signal that was measured in presence of NiCl₂ would originate at an intracellular site. It was observed in Figure 6 that the intracellular TEMPO signal was not responsive to TNF- α incubation with L929 cells. This suggested that the TEMPO quenching was at an extracellular site.

Quenching of TEMPO, taken as a measure of TNF- α cytotoxicity, was monitored in presence of various water soluble and lipid soluble antioxidants. This was done to test the validity of TEMPO quenching technique in being a faithful reporter of TNF- α cytotoxicity. L929 cells incubated for 15 hr in presence of SOD, catalase, vitamin E, vitamin A, and DMSO were monitored for TEMPO quenching (TABLE \$ a, b). As was expected, increasing concentration of DMSO (0 to 500 mM), increased the ability of cells to quench TEMPO (Figure 9) showing thereby that protective effects of DMSO against TNF- α cytotoxicity was demonstrated by TEMPO quenching. Similarly, vitamin A (0.1 mM) and vitamin E (1.0 mM) in incubation media

enhanced the ability of TNF- α treated cells to quench TEMPO. This again demonstrated the ability of TEMPO quenching technique to be a faithful reporter of TNF- α cytotoxicity.

From the above data, we concluded that the reduction rates of a spin label was indicative of the peroxidative stress on a cell. Not only did spin labels inhibit lipid peroxidation (Ulf, et al, 1989), but also, in comparison to normal cells, got reduced differentially by cells under peroxidative chain reactions. This was elucidated to be the case for L929 cells under TNF- α cytotoxicity. TNF- α incubation lead a cell through a peroxidative process (Larrik, et al, 1990). This fact coupled with observation that a TNF- α sensitized cell reduced nitroxide spin label, like TEMPO, at a much slower rate than non-sensitized cells, thus demonstrated that reduction of a lipid soluble spin label like TEMPO could be an useful reporter to monitor TNF- α cytotoxicity.

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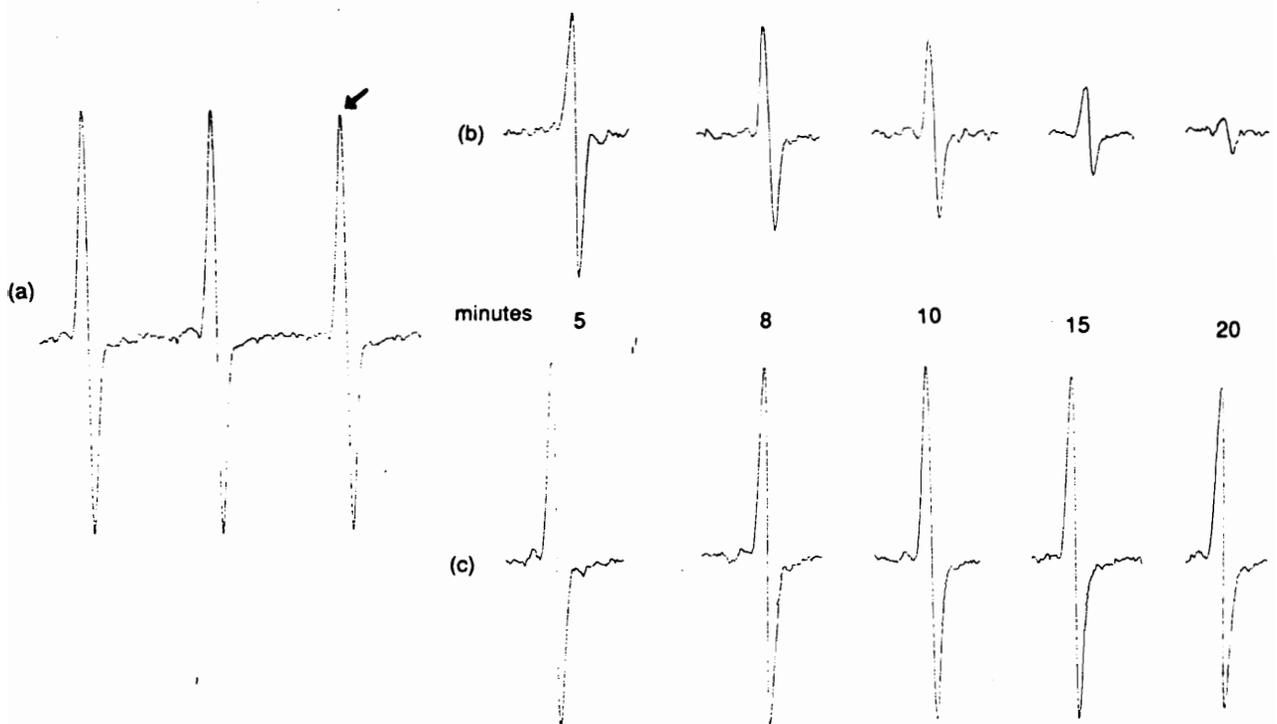


Figure 1 : EPR spectrum of TEMPO signal. (a) TEMPO at 0.01 mM in HBSS. (b) Quenching of 0.01 mM TEMPO signal with viable L929 (6×10^6) cells in a $100 \mu\text{l}$ reaction volume. The quenching of the TEMPO peak (arrow) is rapid. (c) Quenching of 0.01 mM TEMPO signal with TNF- α (100 units/ml, 15 hrs) treated cells is much slower. The repeat spectra are that of the third peak of the TEMPO signal at 5, 8, 10, 15, and 20 minutes of TEMPO incubation.

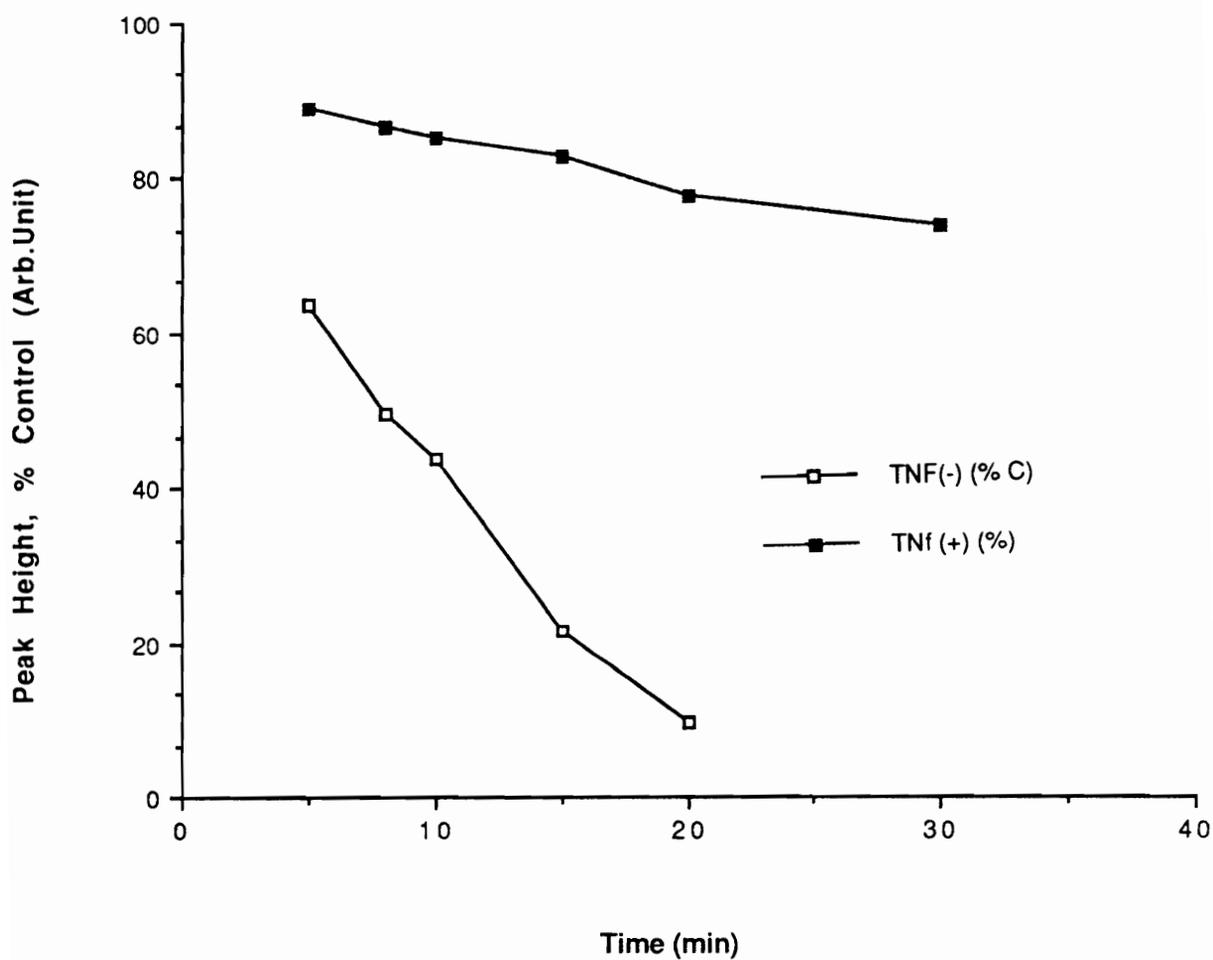


Figure 2 : The TEMPO reduction rate (monitored by EPR) with L929 cells incubated in absence or presence of 100 units/ml TNF- α for 15 hr.

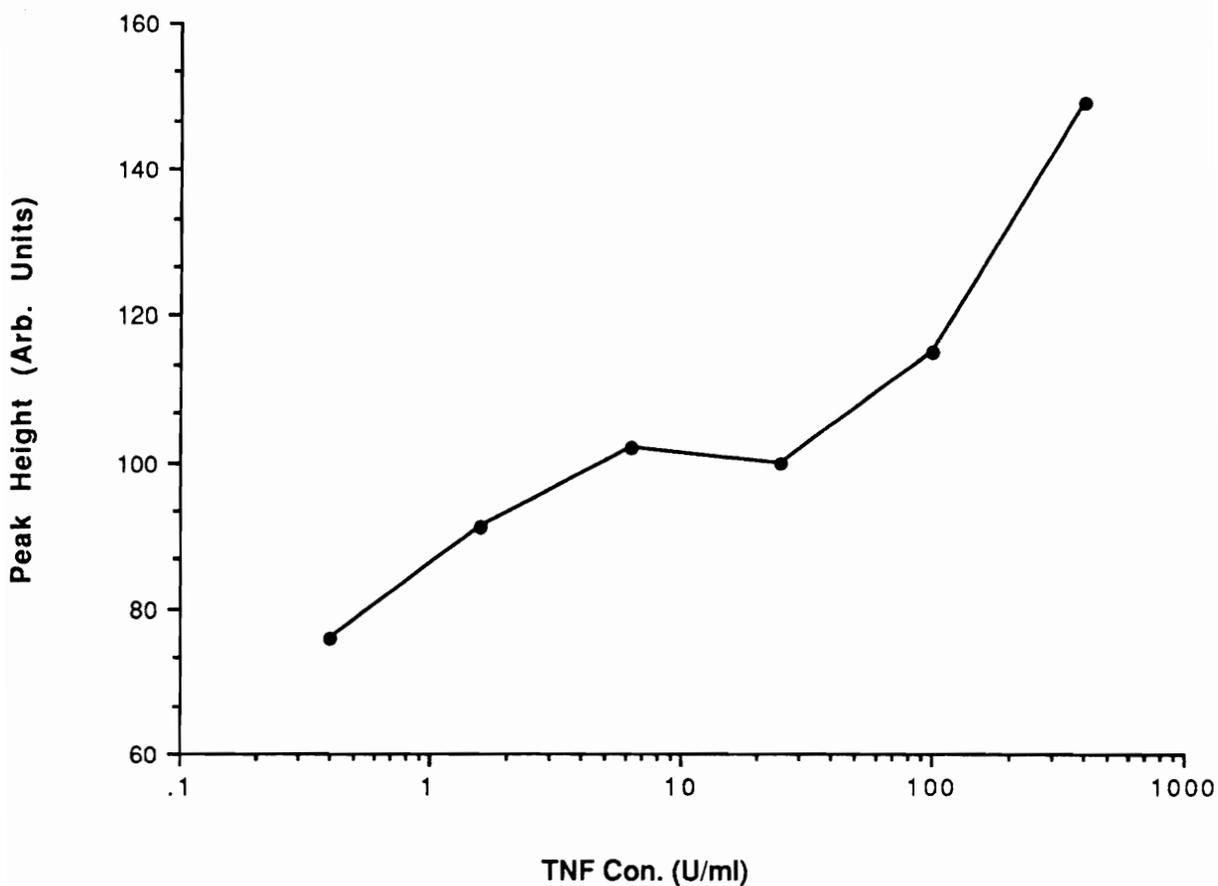


Figure 3 : Effect of TNF- α concentration in units/ml on quenching of TEMPO signal. L929 cells were incubated with different concentrations of TNF- α for 15 hrs. The peak heights of TEMPO was monitored at 15 minutes of TEMPO incubation.

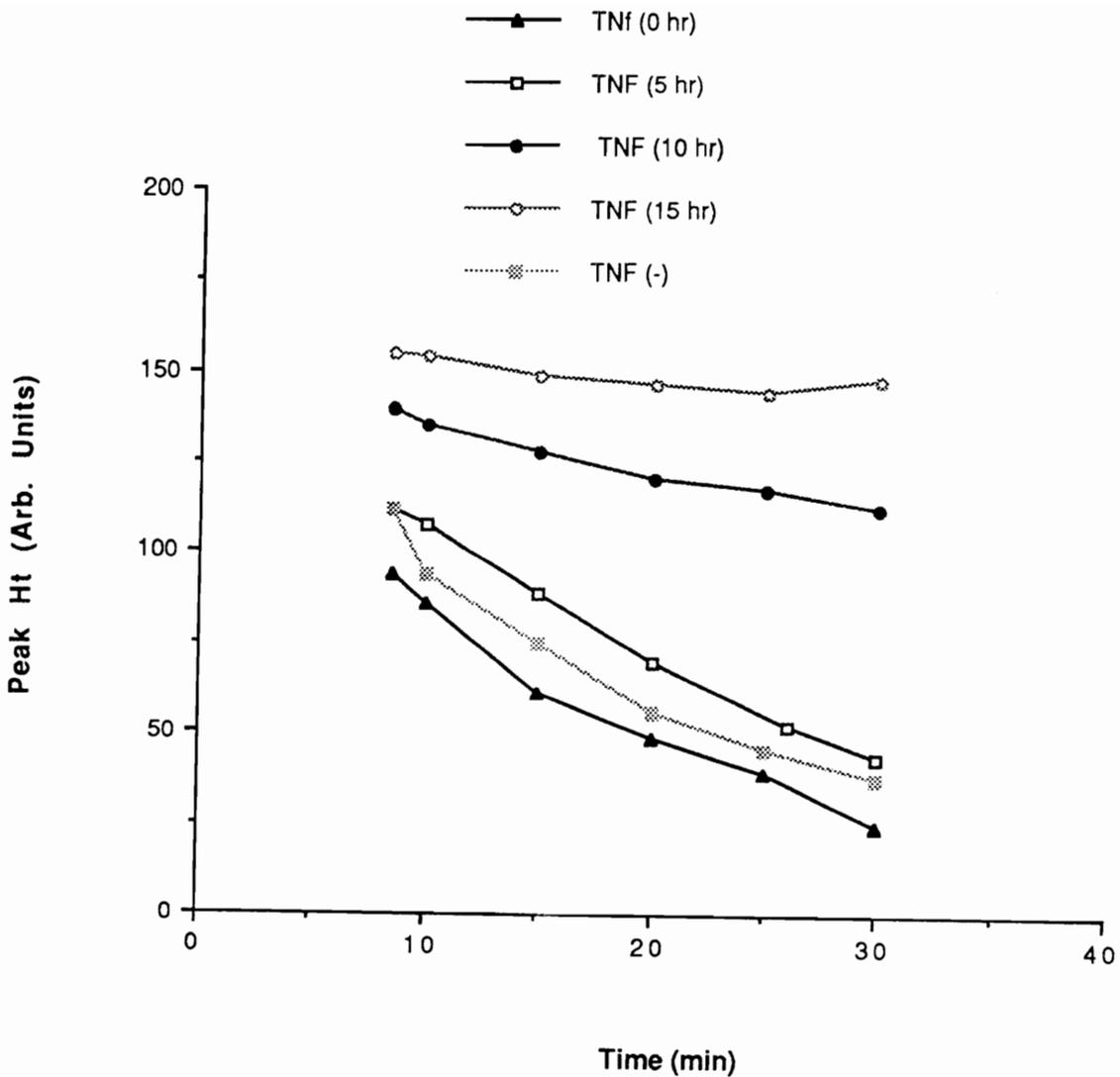


Figure 4: Effect of TNF- α incubation time on the rate of TEMPO quenching. Cells incubated for 0, 5, 10, and 15 hrs with TNF- α at 100 units/ml were washed and pelleted. To 6×10^6 cells in 90 μ l HBSS was added 10 μ l of 0.1 mM TEMPO. The TEMPO quenching was monitored as the decrease of the third peak in 30 minutes. It is seen that the inhibition of TEMPO quenching increases with TNF- α incubation time.

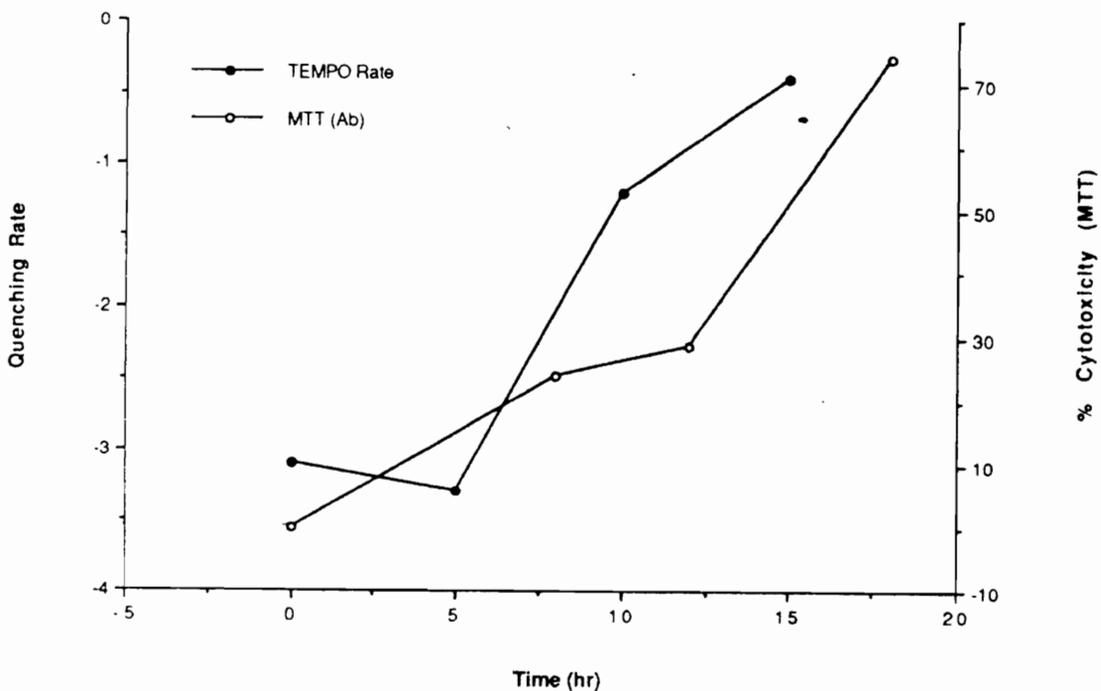


Figure 5 : Comparison of MTT Assay With TEMPO Quenching Rate. The negative slopes from Figure 4 depicts the rate of quenching of TEMPO by L929 cells treated with TNF- α for different incubation times. The quenching rate of TEMPO is plotted with % cytotoxicity indices as determined by the tetrazolium dye (MTT) reduction assay for different TNF- α incubation times. As depicted, the rate of TEMPO quenching can be used as an index of measurement of TNF- α cytotoxicity.

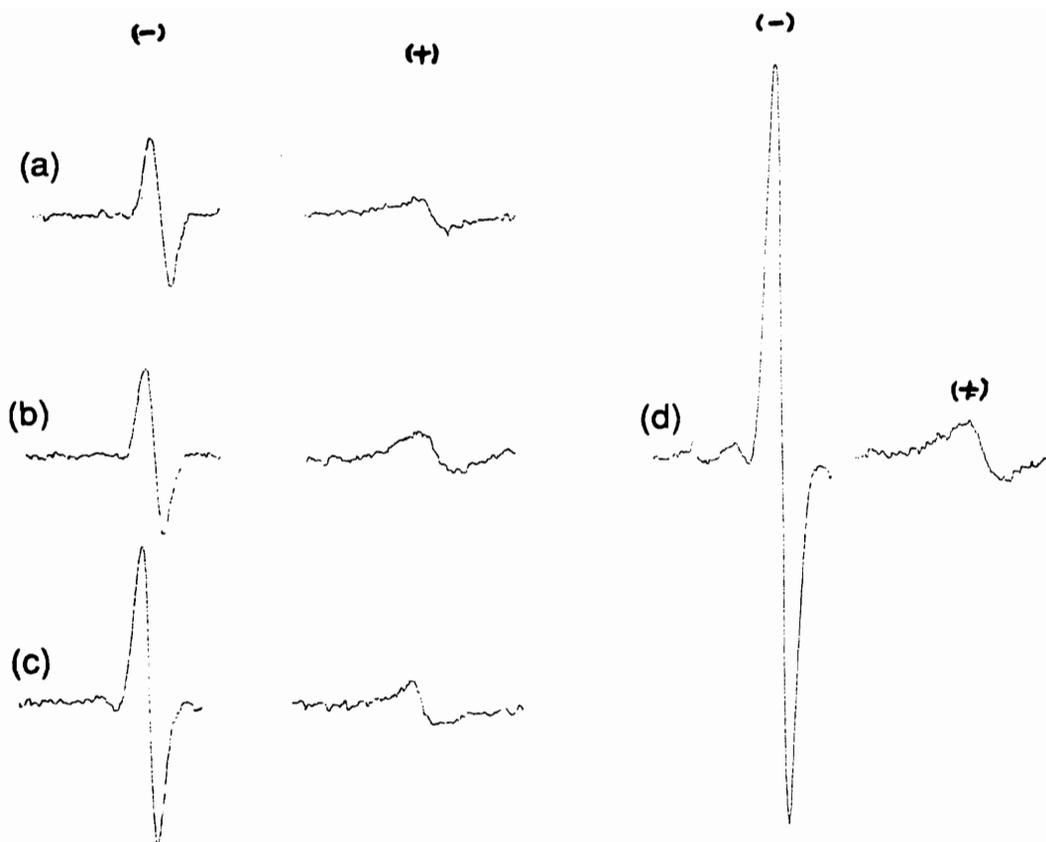


Figure 6: TEMPO Quenching Under TNF- α Cytotoxicity is a Membrane Event on The Extracellular Domain. L 929 cells treated for (a) 0 hr, (b) 5 hrs, (c) 10 hrs, (d) 15 hrs with 100 units/ml TNF- α , were incubated in absence (-) and presence (+) of 50 mM NiCl₂ (a cell nonpermeant that broadens the extracellular TEMPO signal) with 0.01 mM TEMPO for 15 minutes. The peak heights measured were as shown. The result shows that the intracellular quenching of TEMPO (NiCl₂ treated cell quenching of TEMPO peak heights) is independent of TNF- α incubation time.

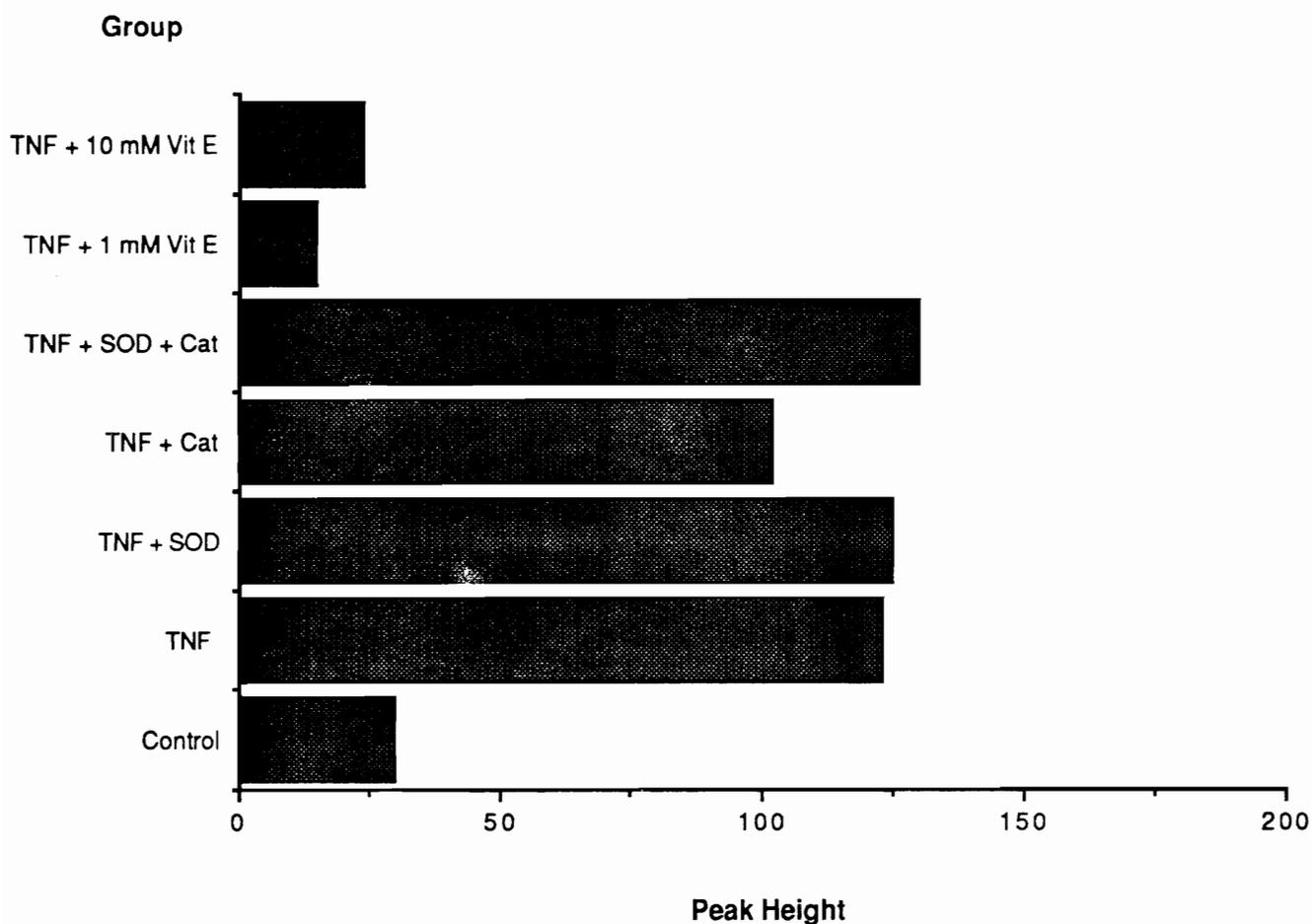


Figure 7 : Effect of Antioxidants on TNF- α Cytotoxicity As Monitored By TEMPO Quenching. As quenching of TEMPO signal is seen to be a measure of TNF- α cytotoxicity (which proceeds via an oxidative pathway) we attempt to determine the effect of antioxidants on TNF- α cytotoxicity by monitoring TEMPO quenching. The TNF- α incubation was at 100 units/ml for 15 hrs. The TEMPO was at 0.01 mM final concentration in 100 μ l containing 6×10^6 cells. The TNF- α cytotoxicity was performed in absence and presence of SOD (100 units/ml), catalase (1000 units/ml), and vitamin E (1.0 and 10.0 mM).

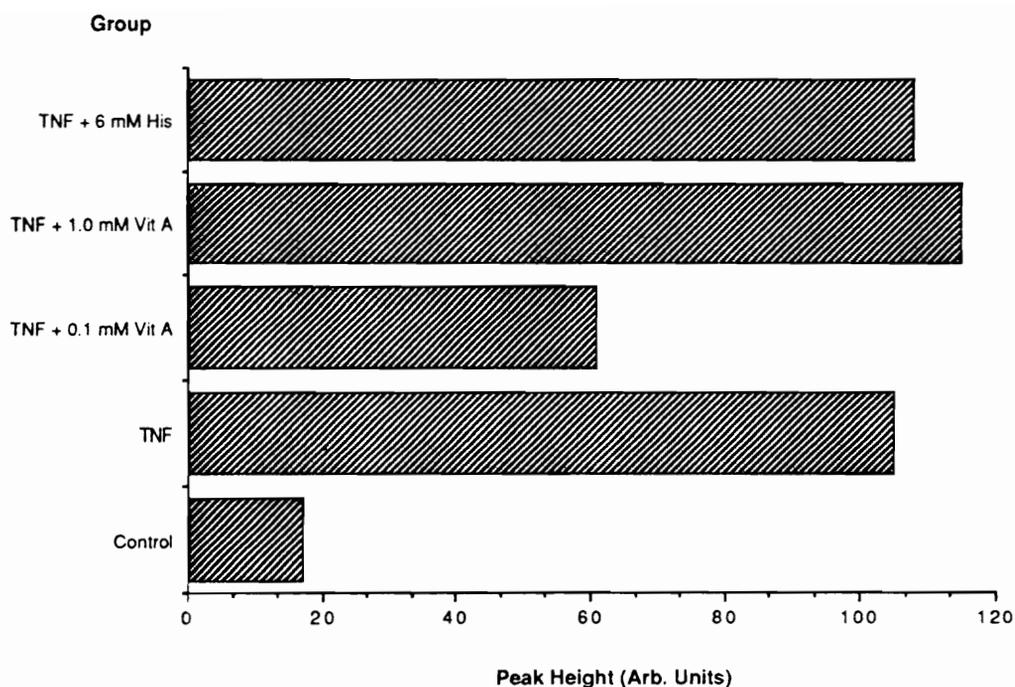


Figure 8 : Effect of Antioxidants on TNF- α Cytotoxicity as Monitored by TEMPO Quenching. The TNF- α incubation was at 100 units/ml for 15 hrs. The TEMPO was at 0.01 mM final concentration in 100 μ l containing 6×10^6 cells. The TNF- α cytotoxicity was performed in absence and presence of vitamin A (0.1 and 1.0 mM) and histidine at 6 mM.

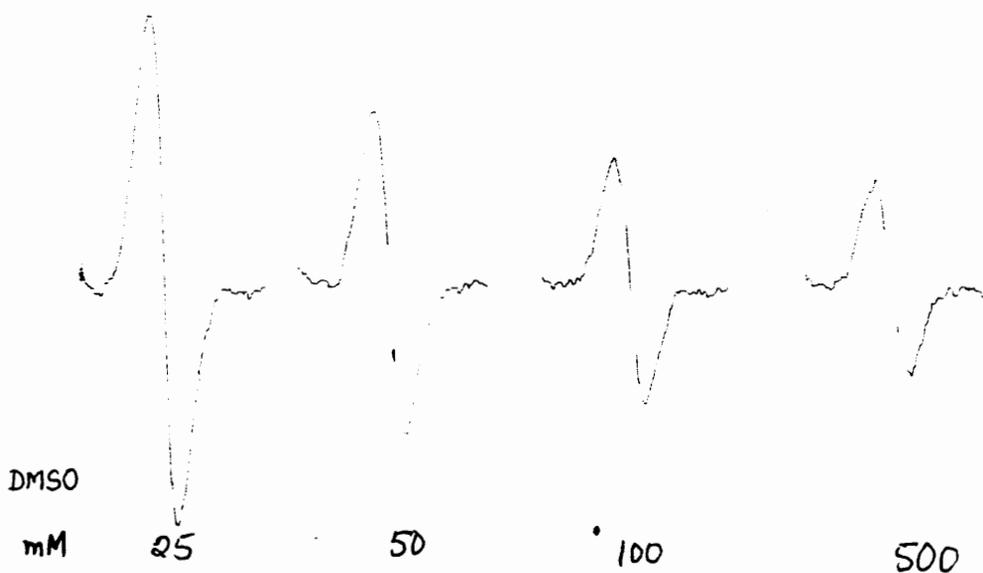


Figure 9 : Protection of TNF- α cytotoxicity by DMSO (0 - 500 mM) as Monitored Using TEMPO Quenching. The measurement of TEMPO peak height at 15 minutes of TEMPO incubation were performed immediately after a 15 hrs incubation of control and DMSO treated cells with 100 units/ml TNF- α . The ability of TNF- α treated cells to quench TEMPO signal at a higher rate with increasing concentration of DMSO proves that DMSO provides its protective role against TNF- α cytotoxicity and that this toxicity can be monitored by EPR using TEMPO quenching.

TABLE 1**EFFECT OF TNF- α ON QUENCHING OF TEMPO SIGNAL BY EPR**

| Time (Min) | TNF(-) | TNF (+) | *TNF(-) (% C) | *TNF(+) (% C) |
|------------|--------|---------|------------------|------------------|
| 5 | 86 | 120 | 63.7 | 88.9 |
| 8 | 67 | 117 | 49.6 | 86.7 |
| 10 | 59 | 115 | 43.7 | 85.2 |
| 15 | 29 | 112 | 21.5 | 83.0 |
| 20 | 13 | 105 | 9.6 | 77.8 |
| 30 | | 100 | | 74.1 |

Control (C) height of TEMPO was 135 as measured by EPR, of 0.01 mM TEMPO in HBSS (figure 1(a)). The concentration of TNF- α used was 100 U/ml in 6 ml media per culture dish, as mentioned in the methods section.

TABLE - 2
EFFECT OF TNF- α CONCENTRATION ON QUENCHING OF
TEMPO SIGNAL

| TNF- α (Units/ml) | Peak Height (mm) | Peak Height(%Control) (mm) |
|--------------------------|---------------------|-------------------------------|
| 400 | 149 | 100 |
| 100 | 115 | 85.2 |
| 25 | 100 | 74.1 |
| 6.25 | 102 | 75.5 |
| 1.6 | 91 | 67.4 |
| 0.4 | 76 | 56.3 |

Control value was for 0.01 mM TEMPO in HBSS equalled 135 mm for the third peak. All peak heights were monitored at 15 minutes of TEMPO incubation.

TABLE 3**EFFECT OF TNF- α INCUBATION TIME ON THE RATE OF QUENCHING OF TEMPO SIGNAL**

| Time (min) | Peak Heights (mm) | | | | |
|---------------------|-------------------|-------|------|------|-------|
| (TEMPO Incubation.) | TNF (-) | 0 hr | 5 hr | 10hr | 15 hr |
| 8.5 | 112 | 94 | 112 | 140 | 155 |
| 10 | 94 | 86 | 108 | 135 | 154 |
| 15 | 75 | 61 | 89 | 128 | 149 |
| 20 | 56 | 49 | 70 | 121 | 147 |
| 25 | 46 | 39 | | 118 | 145 |
| 26 | | | 52 | | |
| 30 | 38 | 25 | 44 | 113 | 148 |
| Quenching Rate | - 3.3 | - 3.1 | -3.3 | -1.2 | -0.4 |

TABLE 4(a)
EFFECT OF ANTIOXIDANTS ON TNF- α CYTOTOXICITY
AS MONITORED BY TEMPO QUENCHING USING EPR

| Group | Peak Height (mm) |
|--------------------------|------------------|
| TNF(-) | 30 |
| TNF(+) | 123 |
| TNF(+) + SOD | 125 |
| TNF(+) + CATALASE | 102 |
| TNF(+) + SOD + CATALASE | 130 |
| TNF (+) + 1 mM VITAMIN E | 15 |
| TNF(+) + 10 mM VITAMIN E | 24 |

TABLE 4(b)
EFFECT OF ANTIOXIDANTS ON TNF- α CYTOTOXICITY AS
MONITORED BY TEMPO QUENCHING USING EPR

| Group | Peak Height (mm) |
|---------------------------|------------------|
| TNF(-) | 17 |
| TNF(+) | 105 |
| TNF(+) + 0.1 mM Vitamin A | 61 |
| TNF(+) + 1.0 mM Vitamin A | 115 |
| TNF(+) + HISTIDINE | 108 |

TABLE 5
COMPARISON OF CYTOTOXICITY CURVE OF TNF- α AS MONITORED BY EPR AND BY MTT DYE BINDING ASSAY

| Time (h) | Rate of TEMPO Quenching EPR | % Cytotoxicity by MTT* |
|----------|-----------------------------|------------------------|
| 0 | -3.1 | 0 |
| 5 | -3.3 | |
| 8 | | 23.9 |
| 10 | -1.2 | |
| 12 | | 28.7 |
| 15 | -0.4 | |
| 18 | | 74.1 |

* Data for % cytotoxicity using MTT dye is from Chapter 1. Units of TNF- α used in both assays were around 60 units per million cells.

In EPR Assay:

Incubation was performed in a 25 cm² flask in 6 ml of TNF- α (100 U/ml) 600 units of TNF- α was used. The amount of cells in 25 cc flask was $\sim 10^7$ cells, therefore units of TNF- α used was 60 units/ml.

In MTT Assay:

Incubation was performed in 96 well plate in 100 μ l of TNF- α (100 U/ml). Units of TNF- α was 66 units/million cells.

APPENDIX - 4

A MICROTITER ASSAY FOR Cu/Zn-SOD & Mn-SOD

ABSTRACT

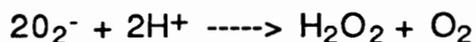
We present a microtiter assay system for monitoring the Cu/Zn-superoxide dismutase (SOD) and Mn-SOD levels as a micro version of cytochrome C reduction assay for SOD. The assay presented here retains the sensitivity of the cytochrome C reduction assay and facilitates the handling of small sample volume. The assay is simple, fast and reproducible and allows for higher number of replicate reactions giving a better statistical average.

INTRODUCTION

Many direct and indirect assays for superoxide dismutase (SOD) have been published (Reviewed by: Flohle et al. 1984) but none of the assays are sensitive for measuring the SOD content in cell extracts from a relatively small number of cells. The current assay is a micro version of the cytochrome-C reduction assay for SOD as published by McCord & Fridovich, J. Biol. Chemistry 244, 6049 (1969). The original assay has been known to be sensitive and applicable in both pure systems and crude extracts. However, the assay is not sensitive for extracts of relatively small number (10^4) of cells grown in tissue culture. This is because, the spectrophotometric assay requires at least 1 ml of reaction volume and the small amount of cell extract in 1 ml reaction volume makes final sample SOD concentration too low to significantly affect the rate of cytochrome-C reduction and further, not much sample is available for repeating the assays for a meaningful statistical interpretation. The reduction of the volume to 100 μ l in a microtiter plate assay makes the colorimetric reaction ideally suited to serve as a fast, sensitive, and reproducible test for monitoring SOD levels from cells in culture. It should be noted that Fridovich et al (1988) did publish a picomolar assay system to monitor SOD at the cellular level but the assay system is not reproducible.

PRINCIPLE

SOD catalyzes the dismutation of the superoxide radical



The extent to which the enzyme decreases the rate of a measurable O_2^- -dependent cytochrome C reduction is used to determine the activity of SOD in a given sample. The conversion of xanthine to uric acid by xanthine oxidase produces the O_2^- radical. The amount of O_2^- present can be directly assessed by monitoring the reduction of ferricytochrome C at 550 nm. At 25°C this reaction is linear for several minutes with no lag time. One unit of SOD activity is defined as the quantity of SOD required to produce 50% inhibition in the rate of cytochrome C reduction, under specified conditions.

The light path for normal spectrophotometric measurement is 1 cm whereas light path for a 100 μ l volume in a 96 well plate is 0.3 cm. Therefore to calculate unknown concentration terms from a 96 well plate reader absorbance readings the extinction coefficient is multiplied by a factor of 3.3 (Pick E et al., 1981).

MATERIALS

Superoxide dismutase (SOD) (type I, 3000 units/mg protein), ferricytochrome c (type III), xanthine and xanthine oxidase were purchased from Sigma Chemical Co, MD. The total SOD content was measured in 0.05 M potassium phosphate, pH 7.8, containing 10^{-4} M EDTA (buffer-A). The Mn-SOD levels were measured in buffer A supplemented with 2mM KCN (buffer B). The stock solution of xanthine and cytochrome c were made 1 mM each in buffer A.

PROCEDURE

Buffers A and B were placed in water bath at 25°C. SOD, xanthine oxidase, cytochrome c and tissue samples were kept on ice. As some cytochrome c would already be in reduced form, the concentration of ferri-cytochrome c was routinely monitored by a dithionite reduction of ferri-cytochrome c. A reaction mixture consisted of 940 μ l buffer A, 50 μ l xanthine and 10 μ l cytochrome c.

100 μ l/well of above mix was dispensed into six wells and absorbance measured using the 550nm filter in the 96 well plate reader (Molecular Devices Inc.).

The microtiter plate was read using SOFTmax software for MAX line microplate reader.

After the proper amount of cytochrome C has been determined, the plate reader was set with the following parameters:

| | | | |
|---------------|------------------------------|--------------|--------------|
| Mode: | Kinetic (single wave length) | Calibration: | On |
| wavelength: | 550 nm filter | Automix: | On |
| Data display: | Raw mOD/min. (Vmax) | Autoprint: | On |
| OD limit: | 0.2 | Data File: | SODassay |
| Lag time: | 0:00 min. | Run time: | 2:30 minutes |
| Description: | Xanthine Oxidase Test | | |

Using previously determined reaction composition an adjustable amount of xanthine oxidase (1:4 dilution of stock) was added with buffer compensation till the rate of cytochrome c reduction reached 7.5 mOD/min. After determining the amount of cytochrome c and xanthine oxidase to be used, SOD standards were prepared in buffer A having 0, 0.1, 0.5, 1, 2 and 3 units of SOD/100 μ l of reaction mixture (in six replicates). One units of SOD was defined as the amount of SOD that gives 50% inhibition of cytochrome c reduction plot. The kinetic plot display (in six replicates) of control cytochrome c reduction and reduction of cytochrome c in the presence of 0.1, 0.5, 1, 2 and 3 units of Cu/Zn-SOD (Sigma Chemical Company) was shown in Figure 1. The percent inhibition of cytochrome c by various levels of SOD was presented in TABLE 1. Cu/Zn-SOD is known to be inhibited by KCN. TABLE 2 showed the effect of CN⁻ on Cu/Zn-SOD activity. As shown in TABLES 1 & 2, it was demonstrated using the microtiter plate assay that the Cu/Zn-SOD catalyzed the inhibition of cytochrome c reduction in a dose dependent manner and KCN at 0.2 and 2 mM releaved the inhibition dose dependently. Using buffer A one could get total SOD activity and using buffer B one could obtain the Mn-SOD activity, as Mn-SOD

was not inhibited by 2mM CN⁻. Using this procedure the Cu/Zn- and Mn- SOD contents in three different cell lines were measured. Figure 2 showed the specific activities of Cu/Zn-SOD and Mn-SOD in normal (CL.7) and transformed cells (3R.1 & 6R.1). As was true in most transformed cells, the Cu/Zn-SOD and Mn-SOD specific activities of 6R.1 were significantly ($p < 0.05$) lower than that for the normal CL.7 clone.

COMMENTS

The assay system retains the sensitivity of the conventional cytochrome c reduction assay and facilitates the handling of small sample volume. The assay is simple, fast and reproducible and allows for monitoring higher number of replicate reactions simultaneously.

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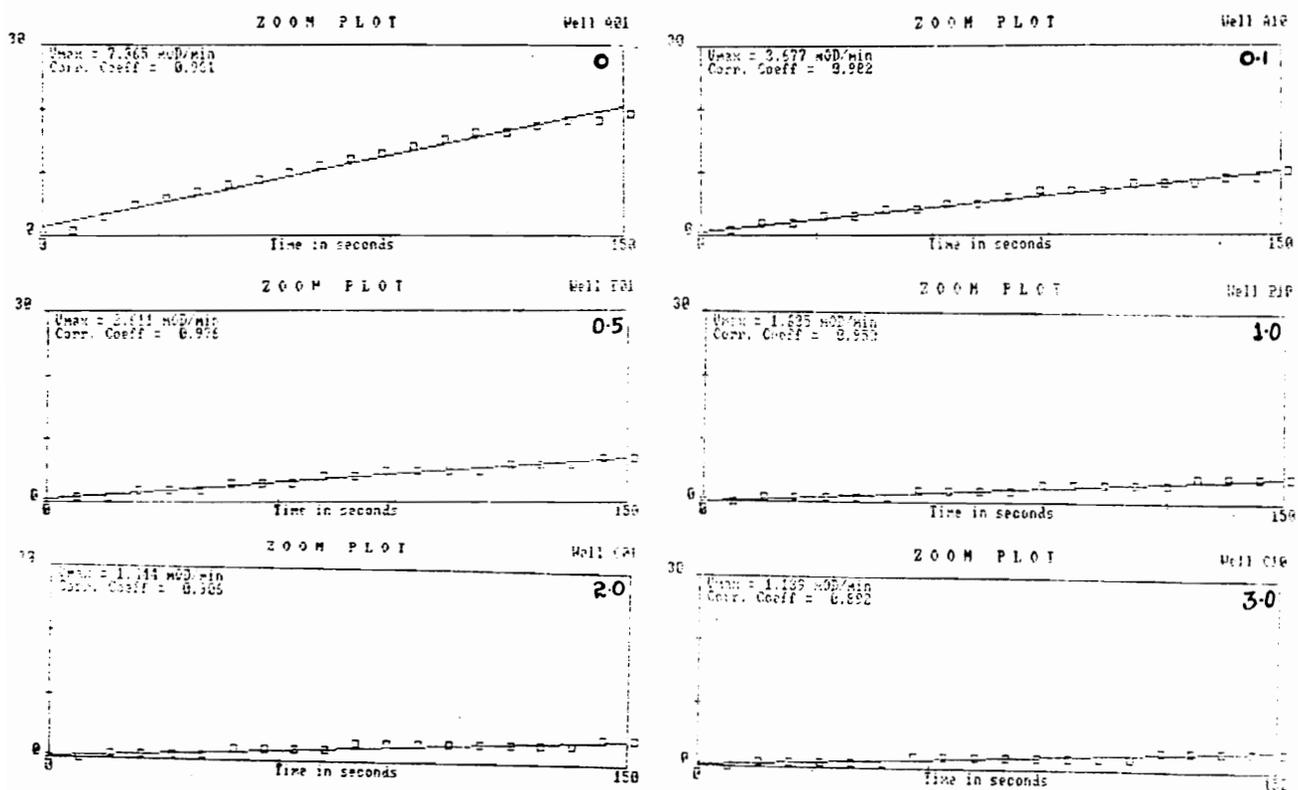


Figure 1 : Cytochrome c reduction in presence of 0 to 3 units of SOD as monitored by microtiter plate assay.

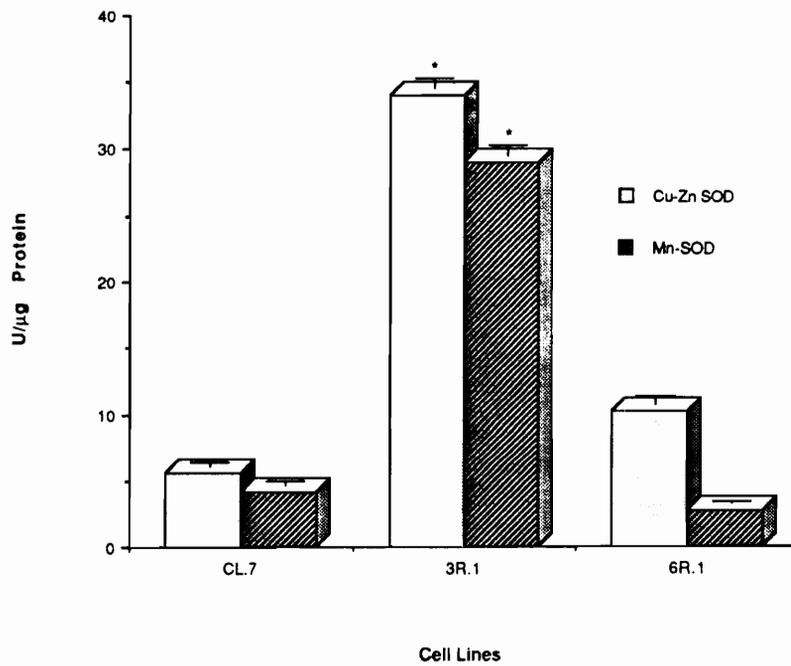


Figure 2 : Specific activities of Cu/Zn-SOD & Mn-SOD in normal (CL.7) and leukemic (3R.1 & 6R.1) clones as determined by a microtiter assay.

TABLE 1
SOD Inhibitable Cytochrome c Reduction As Monitored By
A Microtiter Assay System

| Units SOD * | mO.D/ml | % Inhibition |
|-------------|-------------|--------------|
| Control | 7.26 ± 0.08 | 0 |
| 0.1 U SOD | 3.75 ± 0.39 | 48.3 |
| 0.5 U SOD | 2.41 ± 0.11 | 66.8 |
| 1.0 U SOD | 1.62 ± 0.24 | 77.7 |
| 2.0 U SOD | 1.37 ± 0.12 | 81.1 |
| 3.0 U SOD | 1.08 ± 0.11 | 85.1 |

* SOD was quantitated using the method of McCord and Fridovich (1969).

TABLE 2
KCN Inhibitable Cu/Zn-SOD Activity

| Group | mO.D /ml | % Inhibition |
|--|-------------|--------------|
| Cyt c | 7.91 ± 0.04 | 0 |
| Cyt c + 0.1 U SOD | 4.25 ± 0.11 | 46.3 |
| Cyt c + 0.1 U SOD + 0.2 mM CN ⁻ | 5.05 ± 0.30 | 36.2 |
| Cyt c + 0.1 U SOD + 0.2 mM CN ⁻ | 7.94 ± 0.28 | ~ 0 |

The inhibition of SOD activity was determined in three replicates and is presented as mean ± S.D.

APPENDIX - 5

A MICROTITER ASSAY FOR TOTAL GLUTATHIONE

ABSTRACT

There are many assays that have been developed to quantitate the total GSH levels in tissues. The assay system presented here is a micro-version of the enzymatic assay presented by Tietze, 1969 and can detect total glutathione levels, in a 100 μ l reaction volume at about 0.01 nmol glutathione. This assay is very simple, quick, and sensitive way to determine the total glutathione levels in tissue culture samples precisely with meaningful statistical interpretation.

INTRODUCTION

Glutathione (GSH) is a tripeptide of cysteine, glycine, and glutamic acid and is the principal non-protein sulfhydryl compound present in the tissues. It plays a major role in the process of detoxification of reactive metabolites (Reed, 1985; Chance et al., 1979). A decrease in the level of glutathione makes the biological system susceptible to reactive electrophilic and oxidative stress. GSH is involved in several lines of defence against peroxidative damage. It is a known scavenger of hydroxyl radical (\cdot OH). Apart from its free radical scavenging properties and its ability to inhibit the process of lipid peroxidation by forming conjugation products with several electrophilic intermediates (Jacoby, 1978), GSH is a physiological substrate of GSH-peroxidases which are known to reduce lipid hydroperoxides (Flohl, 1982). Cells depleted of their GSH levels have an impaired ability to cope with peroxidative injury and thus form an important component of cellular antioxidant defence. For example, it has been shown that tumor cells which are susceptible to killing by tumor necrosis factor- α (TNF- α) via a peroxidative process have reduced levels of intracellular GSH (Zimmerman et al., 1989).

There are many assays that have been developed to quantitate the total GSH levels in tissues (reviewed by Sies et al., 1984). The kinetic assay (Tietze, 1969) determines the sum of GSH and GSSG in a 1 ml reaction system where the sample of 100 μ l typically contains 0.5 to 2 nmol of glutathione. The assay system presented here can detect total glutathione levels, in a 100 μ l reaction volume using 10 μ l of sample, at about 0.01 nmol glutathione. This assay is a micro-version of the enzymatic assay presented by Tietze, 1969.

PRINCIPLE

The total glutathione level was quantitated by monitoring the rate of reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) by NADPH catalyzed by GSH or GSSG and glutathione reductase. The rate of the reaction was linearly proportional to the glutathione concentration (2 μ M) and could be measured spectrophotometrically at 412 nm. Instead of using the conventional spectrophotometer, with 1 ml sample volume, the 96 well plate reader with a reaction volume of 100 μ l was used. The difference in the physics of the two systems is the light path. Where as the light path in a 1 ml cuvette was 1 cm, the light path in the 100 μ l reaction volume in a well of the 96 well plate was 0.3 cm (Pick et al., 1981) i.e. in going from the 1 ml reaction system to 100 μ l reaction system we had to compensate for this difference in the extinction coefficient which instead of being in μ M⁻¹ cm⁻¹ would now be μ M⁻¹ (0.3 cm)⁻¹. But as we were measuring the reaction rate, which was dependent only on the concentration of total glutathione, it being an intrinsic variable, was independent of the volume of the reaction. Thus we had increased the sensitivity of measuring total glutathione ten fold in very small samples (10 μ l volume). This assay is quick, easy and highly reproducible. We can handle large amount of samples and quantitate their total glutathione levels quickly and accurately using only 1/10th the amount of reagents used in a conventional assay system.

MATERIALS

Buffer used was 0.1 M potassium phosphate (kPi) with 1 mM EDTA pH 7.0, NADPH, used at a concentration of 1.0 mg/ml in 0.5% NaHCO₃, prepared fresh. DTNB (0.3 mg/ml) was prepared in 0.5%

NaHCO₃. Glutathione reductase (Sigma Chemical Co., MO) was diluted in the buffer to give 0.6 units/ml. Standards of oxidized glutathione (Sigma Chemical Co., MO) were made at 1.25, 2.5, 5.0, 10.0, 20.0 and 40.0 μM in buffer.

PROCEDURE

A reaction mixture (for 200 assay wells) of 8 ml of 0.1 M kPi, 1 mM EDTA pH 7.0, 4 ml of (1 mg/ml) NADPH, 2 ml of (0.3 mg/ml) DTNB and 4 ml of (0.6 units/ml) glutathione reductase was made. We quantitate total glutathione in a 96 well plate reader (Molecular Devices) using SOFTmax software for MAXline microplate reader. The instrument parameters are set as follows:

Instrument set up screen and enter the following parameters were selected as follows:

Mode: Kinetic L₁

Wavelength: 412 nm

Ab. Limit: 0.125

Data: Raw

Lag Time: 0.00

Run Time: 5 minutes

Automix: Once

Autoprint: On

to 90 μl reaction mixture in six replicates in six rows of a 96 well plate 10 μl standard/well were added. Standards ranged from 1.25 mM (row A₁ . . .A₆) to 40 μM (row F₁ . . .F₆). A standard curve of concentration of oxidized glutathione and the m OD/min was plotted. Then using 90 μl of cocktail and 10 μl of sample: sample of 10 μl should contain tissue equivalent of at least 10⁴ cells (in six replicates) the m OD/min for the unknown samples were determined. With the help of the standard curve (or the equation for the standard curve) the total glutathione in 10 μl of sample could be estimated.

Knowing the protein content of the sample the data could be presented as total glutathione/mg protein. We presented the rates of reduction of oxidized glutathione in Figure 1 and TABLE 1. The standard curve was plotted as in Figure 3. The total glutathione content per mg protein of three different cell lines were presented in TABLE 2.

COMMENTS

This assay is very simple, quick, sensitive way to determine the total glutathione levels in tissue culture samples. This is because, the above assay system allows one to determine total glutathione level in typically 10^4 cells in a 96 well plate.

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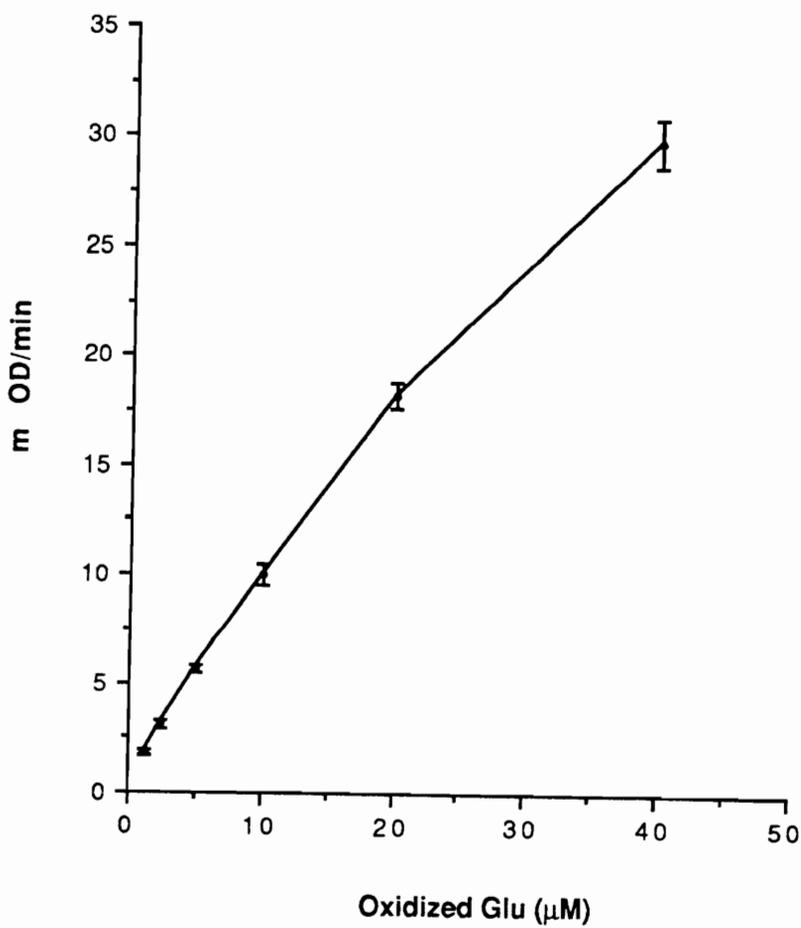


Figure 1. A Standard curve For Oxidized Glutathione (in m OD/min) at 412 nm. The results are represented as mean \pm S.D .

TABLE 1

Determination of total glutathione under A-MuLV transformation.

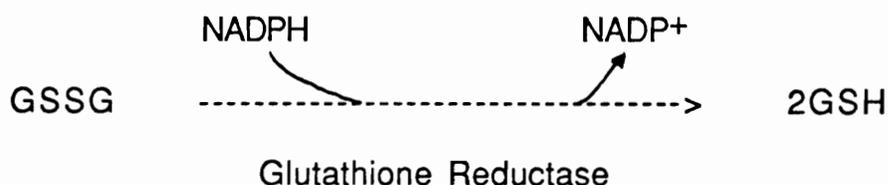
| Cell Line | g glutathione*/mg protein |
|--------------------|---------------------------|
| BALB/c CL-7 | 37.8 ± 3.2 |
| BALB/c A-MuLV 3R-1 | 76.8 ± 6.1 |
| BALB/c A-MuLV GR-1 | 25.6 ± 4.5 |

APPENDIX - 6**A MICROTITER ASSAY FOR GLUTATHIONE REDUCTASE****ABSTRACT**

The glutathione reductase was measured spectrophotometrically at 340 nm by monitoring the oxidation of NADPH in a microtiter assay system. The assay system presented here is convenient, rapid, and highly reproducible to quantitate glutathione reductase levels in cells.

INTRODUCTION

Reduced glutathione (GSH) is a major low molecular weight antioxidant in cells (Reed, 1985). It helps in reducing highly reactive species like H_2O_2 , $\cdot\text{OH}$, $^1\text{O}_2$ and even various lipid peroxides to less reactive electrophiles (Chance, et al. 1979). During this process GSH gets converted to oxidized glutathione (GSSG). Since GSH is necessary for the prevention of lipid peroxidation in the cell, replenishment of this antioxidant is important for proper functioning of the cell. This is achieved by the enzyme glutathione reductase which catalyzes the reaction:



Glutathione reductase reduces GSSG to restore intracellular concentration of GSH, using NADPH as a cofactor (Rall, et. al. 1952). Glutathione reductase has also been known to avidly reduce lipid hydroperoxides in the cytosol (Flohe, et. al. 1970). It is a key antioxidant enzyme found in the cytosol of normal cells but is however known to be found in low quantities in tumorigenic cells (Misdale et. al. 1983).

The enzyme can be assayed spectrophotometrically at 340 nm by monitoring the oxidation of NADPH. The enzyme reduces GSSG to produce a molecule of GSH utilizing NADPH as an electron source. Several assay methods are available to monitor glutathione reductase enzyme levels. The most popular assay system for glutathione reductase is a spectrometric assay system presented by Sies, et. al. (1984). All published assays however suffer from their insensitivity to report glutathione reductase levels in small tissue culture samples. Most assays are performed in a 1 ml cuvette and detect changes in NADPH concentrations. When the tissue sample is very small and/or when glutathione reductase levels in the samples is low it is important to increase the sensitivity of the reaction system. We present here a method to detect the enzyme activity using a microtiter assay. This was achieved simply by reducing the reaction volume from 1 ml to 100 μ l and using a 96 well plate reader rather than a conventional spectrophotometer. This method is not only sensitive and reproducible but also convenient in assaying great number of samples.

PRINCIPLE

The glutathione reductase in samples is determined from the rate of reduction of NADPH monitored at 340 nm, where the enzyme transfers two electrons from NADPH to GSSG to form a molecule of GSH. We define :

$$\text{Units/reaction volume} = \frac{dA}{dt} \times \frac{\text{sample dilution} \times \text{reaction volume}}{\text{sample volume} \times 3.3 \times e}$$

where e (micromolar extinction coefficient) of NADPH at 340 nm equals $6.22 \times 10^{-3} \mu\text{M}^{-1} \text{cm}^{-1}$ (Sies, et al. 1984). In a typical reaction system sample volume was 10 μ l, used as a 1: 4 dilution of samples with protein values in the range of 2 to 12 mg/ml. The total reaction volume was fixed at 100 μ l. The micromolar extinction coefficient was multiplied by 3.3 as the light path in microtiter plate reader (for 100 μ l reaction volume) is 0.3 cm (Pick, 1981). One unit of enzyme per reaction volume is defined as the amount of enzyme activity that brings about a change of 0.5 milli OD per minute (m OD/min). This rate was derived by taking a reciprocal of the calculated value in the above equation.

MATERIALS

The stock solutions of the reagents were : NADPH (1mM), BSA (1%), GSSG (20 mg/ml) desolved in 0.05 M potassium phosphate buffer, containing 0.05 % BSA were prepared fresh. All reagents were procured from Sigma Chemical Co, MD.

PROCEDURE

A reaction mixture containing 6 ml of buffer, 1 ml of NADPH (1 mM), 1 ml of BSA (1%) and 1 ml of GSSG was prepared fresh. Glutathione reductase levels were determined for normal and tumorigenic cell lines procured from American type culture collection (ATCC), Bethesda, MD. These cell lines include the normal murine embryonic fibroblast BALB/c CL.7 and two of its leukemia virus transformed clones BALB/c A-MuLV 3R.1 and BALB/c A-MuLV 6R.1. The cells were cultured in 75 mm flasks in Dulbecco's modified Eagles medium with 10% FCS. Confluent cultures were washed with Hank's balanced salt solution (HBSS) without phenol red. A cell scraper was used to transfer cells from the culture flask to a 15 ml centrifuge tube. The cell suspension was washed 2 x with HBSS, by centrifuging at 600 rpm for 10 minutes, and resuspended in 600 µl of buffer. A 1:4 dilution of the stock sample was done in the dilution buffer (0.05% BSA) for glutathione reductase assay and 1:4 dilution of the same was done in assay buffer (without BSA) for protein determination. Prior to the assay the samples were freeze thawed three times in liquid nitrogen and homogenized by repeat pipeting.

The 96 well plate reader (Molecular Devices Inc.) was used to assay for glutathione reductase activity using SOFT max software for MAX line microplate reader. The instrument parameters are set as follows:

| | | | |
|-----------|---------------------------------|-------------|-----------|
| Mode: | Negative kinetic L ₁ | Run time: | 3:00 |
| minutes | | | |
| Lag time: | 0:00 minutes | Wavelength: | 340 |
| nm | | | |
| Automix: | Once | OD Limit: | 0 to -0.1 |

Auto print: On

Calibration: On

10 μ l of (1:4 diluted) sample was added to 90 μ l of reaction mixture in six replicates in the 96 well plate. The kinetics of the reaction was determined at 25 °C to be linear for 180 seconds of the reaction time as shown in Figure 1. The specific activity of glutathione reductase in the normal and leukemic cell lines, calculated as described in the methods section, are presented in TABLE - 1. The protein determination was performed according to the dye binding method of Bradford (1976) using bovine serum albumin as a standard.

COMMENTS

The assay presented here is convenient, rapid, and highly reproducible to quantitate glutathione reductase levels in cells. This method will be useful to quantitate glutathione reductase when multiple assays are needed in a relatively small number of cells.

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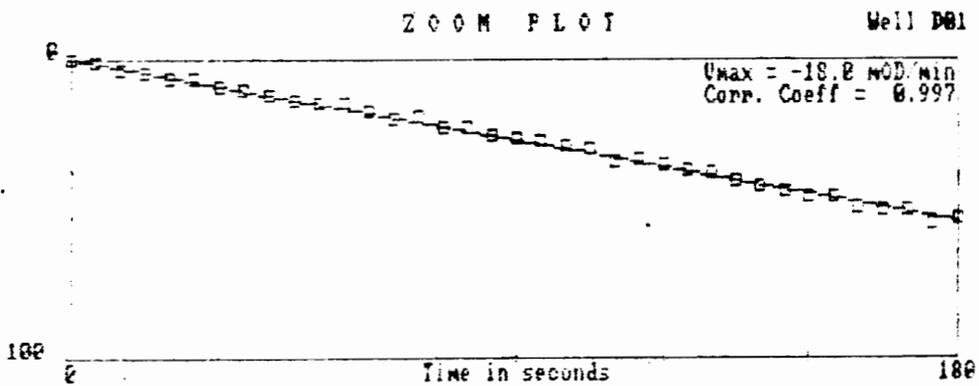


Figure 1 : Initial velocity of glutathione reductase as monitored by microtiter plate assay. The reaction was monitored for 3 minutes at 340 nm at an O.D limit of -0.1. 10 μ l of the sample was a 1 :4 dilution of a 11.7 mg/ml stock.

TABLE - 1**Quantitation Of Glutathione Reductase Using Microtiter Plate Assay**

| Cell Lines | Protein (mg/ml) | Specific Activity |
|--------------|-----------------|-------------------|
| BALB/c CL.7 | 8.76 ± 0.32 | 1510 ± 85 |
| A- MuLV 3R.1 | 2.14 ± 0.02 | 2877 ± 673 |
| A-MuLV 6R.1 | 11.76 ± 0.5 | 2898 ± 106 |

10 µl of cell sample homogenate used per well corresponds to 10⁴ cells. Protein was assayed by the dye binding method of Bradford (1979) using BSA as standard.

APPENDIX - 7**MICROTITER ASSAY FOR GLUTATHIONE PEROXIDASE****ABSTRACT**

Glutathione peroxidase is a key defense enzyme which protects the cells from oxidative damage. We present a very sensitive and reproducible assay for this enzyme by using a microtiter plate assay system as a modification of the macro assay published by Flohe et. al. 1984 and use it to determine the glutathione peroxidase levels in normal (CL.7), and A-MuLV transformed cells.

INTRODUCTION

Glutathione peroxidase (GSH-Px), EC1.11.1.9 catalyzes the reduction of hydroperoxides with glutathione as a reductant.



GSH-Px utilizes the reducing equivalents of GSH for reducing lipid hydroperoxides as well as hydrogen peroxides (Little, et al, 1969). It therefore could serve as a major mechanism for protection against the deleterious effect of hydroperoxides. Selenium is known to be an integral part of this enzyme (Flohe, et. al. 1973).

Many assays for glutathione peroxidase have been published (reviewed by Flohe et. al. 1984), including fixed time assay which measures GSH consumption by polarography as well as continuous monitoring of GSSG formation. In the later assay, although the reaction rate of the enzyme is dependent on the steady state level of GSH, the simplicity of the reaction system makes it suitable for measuring glutathione peroxidase activity in biological systems. The published assay (Flohe et. al. 1984), however, has a very large reaction volume (1 ml) and is unsuitable for monitoring glutathione peroxidase activity in small tissue samples of cell culture origin. We propose a modified version of the above assay in a 96 well plate

reader instead of a conventional spectrophotometer.

PRINCIPLE

The reactions involved in this assay are as follows:



GSSG formed in the GSH-Px reaction is continuously reduced, by the action of glutathione reductase (GSH-Rx) activity, back to GSH. As GSH is continuously regenerated by GSH-Rx the concentration of GSH in the assay is maintained at the initial level. The regeneration of GSH is associated with a concomitant oxidation of NADPH to NADP⁺. The decrease in the concentration of NADPH can be monitored spectrophotometrically at 340 nm.

In going from a conventional, 1 ml reaction volume, spectrophotometer to a 96 well, to a 100 µl reaction volume, plate reader, if the concentration terms are maintained constant the only change is in the light path. The light path in a spectrophotometer is 1 cm whereas in a plate reader it is 0.3 cm (Pick, et. al., 1981). Therefore in the absorbance equation the extinction coefficient instead of being µM⁻¹ cm⁻¹ will be in µM⁻¹ (0.3 cm)⁻¹. This decrease in NADPH concentration is calculated from the linear slopes of decreasing absorbance using the appropriate extinction coefficient.

For $\epsilon = 6.22 \times 10^{-3} \mu\text{M}^{-1} \text{cm}^{-1}$

Sample dilution = 1:2 (predetermined)

Reaction volume = 100 µl

Sample volume = 12 µl (predetermined)

Then Units/reaction volume = $812 \times \frac{dA}{dt}$

Thus one unit of enzyme activity per reaction volume for a fixed

amount of GSH and GSH-Rx is defined as the amount of sample that gives a change of 1.2 m OD/minute (reciprocal of 812). Further knowing the protein content in mg/ml and realizing that # Units/ml = 10. # Units/reaction volume

We get the specific activity of glutathione peroxidase as

$$\text{units/mg protein} = \frac{\text{Units/ml}}{\text{mg/ml}}$$

MATERIALS

The stock solutions of all the reagents were prepared in 50 mM Tris-HCl pH 7.6 with 0.1 mM EDTA. A prereaction mixture (0.25 mM GSH, 0.12 mM NADPH and 1 Unit GSH-Rx/ml cocktail) was prepared fresh. A stock solution of 0.5 mg/ml of cumen hydroperoxide was prepared in 200 proof ethyl alcohol.

PROCEDURE

Glutathione peroxidase levels were quantitated using a 96 well plate reader (Molecular Devices) using SOFTmax software for MAX line microplate reader. The instrument parameters were set as follows:

Select instrument set up screen and enter the following parameters:

Mode: Negative kinetic L₁

Wavelength: 340 nm

OD Limit: -0.1

Calibration: On

Run time: 3:00 minutes

Lag time: 0.00 minutes

Automix: Once

Auto print: On

Glutathione peroxidase levels were determined for three different cell lines procured from American Type Culture Collection (ATCC) Bethesda, MD. They are, the normal murine embryonic fibroblast BALB/c CL.7, and two of its leukemia virus transformed clones BALB/c A-MuLV 3R.1 and BALB/c A-MuLV 6R.1. The cells were cultured in 75 mm flasks in Dulbeccos modified Eagle's medium with 10 % fetal calf serum (FCS), pH 7.4. Confluent cultures were washed with Hank's balanced salt solution (HBSS) without phenol red. Using a cell scraper, cells were transferred to a 15 ml centrifuge tube and washed 2x with HBSS, by centrifuging at 600 rpm for 10 minutes and resuspended in 600 μ l of buffer (60 mM Tris-HCl pH 7.6 with 0.1 mM EDTA). A 1:2 dilution of this sample was used as a stock sample solution. Prior to the assay, the sample was freeze thawed in liquid nitrogen and homogenized by repeat pipeting. 82 μ l of cocktail + 12 μ l of sample were delivered in six replicates in a 96 well plate. The reaction was started by delivering 6 μ l of 0.5 mg/ml cumen hydroperoxide and the plate read at 340 nm for three minutes.

The kinetic rates (V_{max}) for the three cell lines were presented in Figure 1. The protein content of the samples as determined by the dye binding method of Bradford (1976). Specific activity of glutathione reductase levels in the three cell lines were calculated as given in methods section and were presented in TABLE-1.

COMMENTS

The assay is a rapid, easy and highly reproducible for small samples of cell culture origin. Typically homogenate equivalent of 10^4 to 10^5 cells/well can be used to quantitate the glutathione peroxidase very accurately. This method of determination of GSH-Px retains all the features of the original assay with the modification to handle very small cell culture samples.

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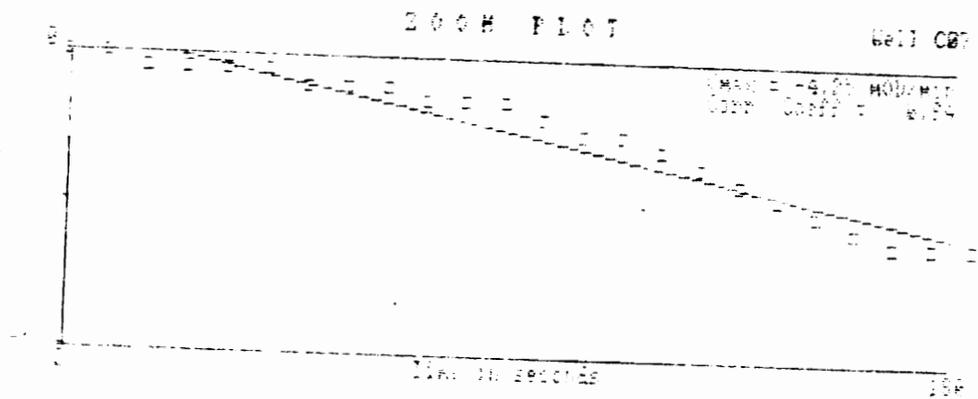


Figure 1. Negative V max in m OD/min of Glutathione Peroxidase Activity in CL.7 Cell Line. The reaction was carried out as described in the methods section.

TABLE 1

Specific activity of GSH-Px under A-MuLV transformation

| <u>Cell Line</u> | <u>GSH-Px Units/mg Protein</u> |
|------------------|--------------------------------|
| BALBc CL.7 | 4,390 ± 630 |
| A-MuLV 3R.1 | 33,370 ± 1910 |
| A-MuLV 6R.1 | 8,210 ± 480 |

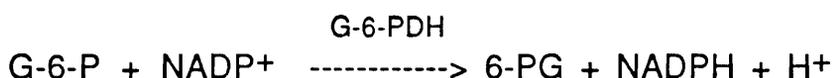
12 μ l of cell sample homogenate per well corresponds to 3×10^5 cells.

APPENDIX - 8**A MICROTITER ASSAY FOR GLUCOSE - 6 - PHOSPHATE
DEHYDROGENASE (G-6-PDH)****ABSTRACT**

The reaction catalyzed by G-6-PDH is proportional to the rate of formation of NADPH monitored at 340 nm. The published assays of G-6-PDH measure G-6-PDH levels in tissue samples with a reaction volume of 1 ml. With very minute samples of tissue culture origin it is important to convert the conventional spectrophotometric assay to a microtiter plate reader assay. We present here the microtiter plate assay version of the spectrophotometric procedure accompanying the G-6-PDH Sigma diagnostic kit.

INTRODUCTION

G-6-PDH (EC1.1.1.49) is responsible for catalyzing the first reaction in the pentose phosphate shunt. Glucose-6-phosphate (G-6-P) is oxidized to 6-phosphogluconate (6-PG) with a concomitant reduction of NADP to NADPH. The enzyme catalyzes the following reaction:



such that the rate of production of NADPH is proportional to the sample G-6-PDH activity and is measured spectrophotometrically as an increase in absorbance at 340 nm. The enzyme 6-phosphogluconate dehydrogenase (6-PGDH) competes for NADP⁺ substrate according to the reaction:



To measure only the G-6-PDH activity it is important to inhibit the 6-PGDH reaction. This is done by use of maleimide, an inhibitor of 6-PGDH. The G-6-PDH assay was performed using a

modified version of the Sigma diagnostic kit (Sigma Chemical Co., MO, Procedure #345-UV). The kit assay is adapted from Kornberg et. al., 1955 and Lohr, et. al., 1974. The published assays of G-6-PDH (Bishop,1966; Lohr et. al. 1974; Echler,1983) have been given for measurement of G-6-PDH levels in tissue samples with a reaction volume of 1 ml. With very minute samples of tissue culture origin it is important to convert the conventional spectrophotometric assay to a microtiter plate reader assay. We present here the microtiter plate assay version of the spectrophotometric procedure accompanying the G-6-PDH Sigma diagnostic kit.

PRINCIPLE

The reaction catalyzed by G-6-PDH has previously been described where the activity of G-6-PDH is proportional to the rate of formation of NADPH monitored at 340 nm. The proportionality constant apart from containing dilution factors also has the extinction coefficient. The extinction coefficient for a spectrophotometric assay (with 1 cm light path) is expressed in $\mu\text{M}^{-1}\text{cm}^{-1}$. But for a 96 well plate system, where the final reaction volume is 100 μl the light path is 0.3 cm (Pick, 1981). Therefore the extinction coefficient instead of being in $\mu\text{M}^{-1}\text{cm}^{-1}$, if the concentration terms are kept equivalent will be in $\mu\text{M}^{-1}\text{cm}^{-1}$. Thus increase in NADPH concentration is calculated from the linear slope of increasing absorbance using the appropriate extinction coefficient as given by the equation:

$$\text{Units/reaction volume} = \frac{dA/dt \times \text{dilution} \times \text{reaction volume}}{3.3 \epsilon \quad \text{sample volume}}$$

for predetermined dilution factor (1:16) with reaction volume = 100 μl and sample volume = 40 μl with $\epsilon = 6.22 \times 10^{-3} \mu\text{M}^{-1}\text{cm}^{-1}$ we get

$$\text{Units/reaction volume} = 1948.7 \frac{dA}{dt}$$

Therefore one unit of enzyme per reaction volume is the amount of sample that gives a change of 0.5 mOD/minute. Further, knowing the protein content in mg/ml and realizing that # units/ml = 10 #units/reaction volume we get the specific activity of G-6-PDH as

$$\text{units/mg protein} = \frac{\text{units/ml}}{\text{mg/ml}}$$

MATERIALS

G-6-PDH assay solution is prepared by reconstitution of G-6-PDH reagent (Sigma Chemical Co., MO, Catalog # 345-5). The assay solution contains NADP⁺ at 1.5 mM and maleimide at 12 mM concentrations. The G-6-PDH substrate solution is provided with the G-6-PDH assay kit.

PROCEDURE

G-6-PDH levels were determined for three different cell lines from American Type Culture Collection (ATCC) Bethesda, MD and were quantitated using a 96 well plate reader (Molecular Devices) using SOFT max software for MAX line microplate reader. The instrument parameters are set on the instrument set up screen as follows:

Mode: End point
wavelength: 340nm
Automix: On
Calibration: On

The normal murine embryonic fibroblast BALB/c CL.7 and two of its leukemia virus transformed clones BALB/c A-MuLV 3R.1 and BALB/c A-MuLV 6R.1 were grown to confluency in 75 cc flasks in Dulbecco's modified Eagle's medium with 10% FCS pH 7.4. Cells were washed with 10 μ l of Hanks balanced salt solution (HBSS) without phenol red, transferred to a 15 ml centrifuge tube in 10 μ l of HBSS using a cell scraper. Subsequently the cells were spun at 600 rpm for 10 minutes. The pellets were resuspended in 600 μ l of HBSS (final volume). 100 μ l of this pellet was freeze thawed 3 x with liquid nitrogen, homogenized by repeat pipeting and kept on ice. This stock sample was diluted 1:4 in G-6-PDH assay solution. This diluted stock was used to make a sample cocktail. The sample cocktail was made (for 6 replicates) by mixing 210 μ l of assay reagent with 70

μl of (1:4 diluted) sample. In a 96 well plate, dispense 60 μl (6 replicates) of G-6-PDH substrate solution (kept on ice). To the above is added 40 μl of cocktail and the plate is incubated at 37°C for two minutes. Absorbance read at 340 nm is called Ab(i). Incubate at 37°C for a further period of five minutes and again read absorbance at 340 nm and designate it as Ab(f).

$$\frac{dA}{dt} = \frac{Ab(f) - Ab(i)}{5}$$

Knowing the extinction coefficient and the protein content of the sample one can determine the specific activity of G-6-PDH in the sample. A preliminary experiment was conducted to determine the sample dilution that gave an appreciable absorbance (340 nm). The dilution curve for BALB/c CL.7 cells is presented in Figure-1. A stock dilution of 1:4 in G-6-PDH assay reagent was considered appropriate for the assay. This dilution was fixed for all the three cell lines for subsequent preparation of sample cocktail. A time incubation of the G-6-PDH reaction is shown in Figure-2 for the BALB/c CL.7 cell homogenate. The figure showed a saturation kinetics for the G-6-PDH reaction. The specific activity of G-6-PDH for the three different cell lines is calculated from the equation given in the materials section and is tabulated (TABLE-1).

COMMENTS

Using a single G-6-PDH assay kit now we can run 10 times more samples than before. The microtiter assay developed here is highly reproducible, easy and quick to perform.

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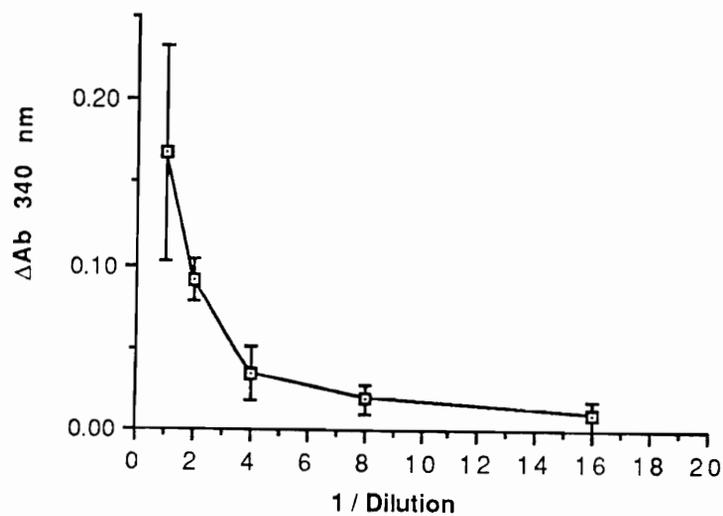


Figure 1. Differential NADPH production in G-6-PDH Assay System (monitored at 340 nm). The stock sample was at a protein concentration of 2.2 mg/ml. The data points were representative of six replicates presented as mean \pm S.D.

TABLE 1

Specific Activity of G-6-PDH in units/mg protein

| Cell Line | units/mg protein |
|-------------|------------------|
| BALBc CL.7 | 445.0 ± 2.14 |
| A-MuLV 3R.1 | 801.0 ± 38.4 |
| A-MuLV 6R.1 | 355.0 ± 19.0 |

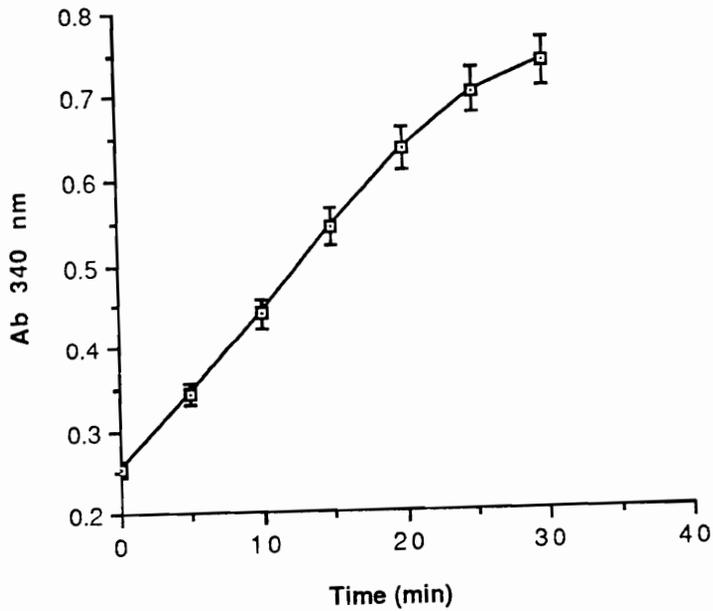


Figure 2. The kinetics of NADPH Production (in 1:4 diluted stock of CL.7 as monitored at 340 nm). The data points represent the average of six replicates ± S.D

APPENDIX - 9**A MICROTITER PLATE ASSAY FOR CYTOCHROME
P-450 REDUCTASE****ABSTRACT**

We present here a 96 well microtiter assay system, capable of measuring cytochrome P-450 reductase, in small tissue culture samples. The present assay is adapted from Dallner et. al. 1963 and is measured by its NADPH-cytochrome C reductase activity. The assay was used to obtain kinetic V_{max} (mOD/min) for cytochrome P-450 reductase activity in the three cell lines BALB/c CL.7, A-MuLV 3R.1 and A-MuLV 6R.1.

INTRODUCTION

Cytochrome P-450 enzymes catalyze the oxidation of a wide variety of steroids, fatty acids, eicosanoids, drugs, alkanes, carcinogens and pesticides (Guengurich, 1987). The P-450 enzymes act as sequential electron transfer oxidants where the oxidation would proceed via three major mechanistic processes namely, hydrogen abstraction, electron transfer and radicaloid addition to unsaturated molecules. Generally speaking the P-450 enzyme system are monooxygenase hemin containing enzymes with overlapping specificities. But the cytochrome P-450 are only active in association with another enzyme, an NADPH-cytochrome P-450 reductase, which is a flavoprotein necessary for transfer of electrons from NADPH to the monooxygenase:

NADPH-cytochrome P-450 reductase is conveniently measured by its NADPH-cytochrome C reductase activity (Phillip et. al. 1962, Dallner, 1963). The reduction of many compounds like dichlorophenolindophenol, ferricyanide (Vermilion, 1978) or a tetrazolium dye (Roerig, 1972) have been used to assay NADPH-cytochrome P-450 activity.

We present here a 96 well microtiter assay system, capable of measuring cytochrome P-450 reductase, in as small a volume as 100 μ l. This assay allows us to quantitate cytochrome P-450 reductase in very small samples of cell culture origin. The present assay is adapted from Dallner et. al. 1963. For a 100 μ l reaction volume the light path is 0.3 cm instead of being 1.0 cm (Pick, 1981) and the extinction coefficient reads $\mu\text{M}^{-1}(0.3 \text{ cm})^{-1}$ rather than $\mu\text{M}^{-1} \text{ cm}^{-1}$.

PRINCIPLE

The reaction catalyzed by NADPH-cytochrome P-450 reductase transfers two e- from NADPH singly to cytochrome P-450 monooxygenase system. The above reductase enzyme also possesses the ability to reduce cytochrome C(Fe^{3+}) to cytochrome C (Fe^{2+}). This reduction of ferri-cytochrome C can be monitored at 550 nm in a 96 well microtiter plate reader. Thus, this assay is an indirect measure of NADPH-cytochrome P-450 reductase activity. One unit of enzyme activity is defined from the following equation:

$$\text{Units/reaction volume} = \frac{\text{OD}}{\text{min}} \times \text{dilution factor} \times \frac{\text{Reaction volume}}{\text{sample volume}}$$

$$= \frac{\text{OD}}{\text{min}} \times \frac{100 \mu\text{l}}{2.5 \mu\text{l}} \times 3.33 \epsilon$$

Also $\epsilon = 21.1 \times 10^{-3} \mu\text{M}^{-1} \text{ cm}^{-1}$; the reaction volume = 100 μ l, sample volume = 2.5 μ l (pre-determined), dilution = 1:1

$$\text{Units/reaction volume} = 569.3 \frac{\text{OD}}{\text{min}}$$

Therefore one unit of enzyme activity per reaction volume is defined as the amount of sample that gives a change of 1.8 m OD/min for the above-mentioned fixed concentration of substrate. Further, with the knowledge of the protein content in mg/ml and realizing that

units/ml = 10. # units/reaction volume we get the specific activity of G-6-PDH as units/mg protein = # units/ml.

mg/ml

MATERIALS

Buffer used for the assay was 0.05 M KPi pH 7.5. We prepared a buffer cocktail mix for the reaction containing 74.2 ml of buffer, 3.3 ml of 10 mM KCN (32.5 mg in 50 ml kPi) and 5 ml of 0.2mM dicoumarol. KCN was added to inhibit cytochrome oxidase and dicoumarol inhibits diaphorase activity. Prior to the reaction, prepared a sample cocktail consisting of 25 μ l sample and 50 μ l of 1 mM cytochrome-C was prepared. 1mM NADPH was made in the above buffer.

PROCEDURE

Samples, whose cytochrome P-450 reductase activity were tested, were from three cell lines. Namely normal mouse embryonic fibroblast BALB/c CL.7 and two of it leukemia virus transformed clones BALB/c A-MuLV 3R.1 and BALB/c A-MuLV 6R.1. The cells were grown in 75 cc flasks in Dulbecco's modified Eagle's medium in 10% fetal calf serum (FCS) pH 7.6. Cells were washed with 10 ml of Hanks balanced salt solution (HBSS) without phenol red, transferred to a 15 ml centrifuge tube in 10 ml of HBSS using a cell scraper. Subsequently the cells were centrifuged at 600 rpm for 10 minutes. The pellets were resuspended in 600 μ l of HBSS (final volume). 100 μ l of this pellet was freeze thawed 3 x with liquid nitrogen, homogenized by repeat pipeting and kept on ice. 82.5 μ l of buffer cocktail and 7.5 μ l of sample cocktail were put in six replicates in 96 well plate. The reaction for cytochrome P-450 reductase was started by dispensing 10 μ l of 1 mM NADPH to the wells. The reaction kinetics was monitored in a 96 well plate reader (Molecular Devices) at 550 nm by using SOFTmax software. The instrument parameter were set as follows:

Mode: Kinetics L₁

Wave length: 550 nm

OD Limit: 0.01
Run time: 2:00 minutes
Lag time: 0:00 minutes

Raw kinetic Vmax (MOD/min) data for cytochrome P-450 reductase activity in the three cell lines BALB/c CL.7, A-MuLV 3R.1 and A-MuLV 6R.1 are given in TABLES 1-(a) thru 1(c). The kinetic graphics of the reaction rates are depicted in Figures 1-(a) thru 1(c). The protein content of the samples as determined by the dye binding method of Bradford (1976) is presented in TABLE-2. Specific activity of cytochrome P-450 reductase levels as calculated by the formula in the principle section is presented in table-3.

COMMENTS

The assay is a rapid, economical, easy to reproduce and highly sensitive method to determine cytochrome P-450 reductase levels in small tissue samples of cell culture origin. Typically homogenate equivalent of $\sim 10^4$ cells can be used to quantitate the cytochrome P-450 reductase by using a 96 well plate reader.

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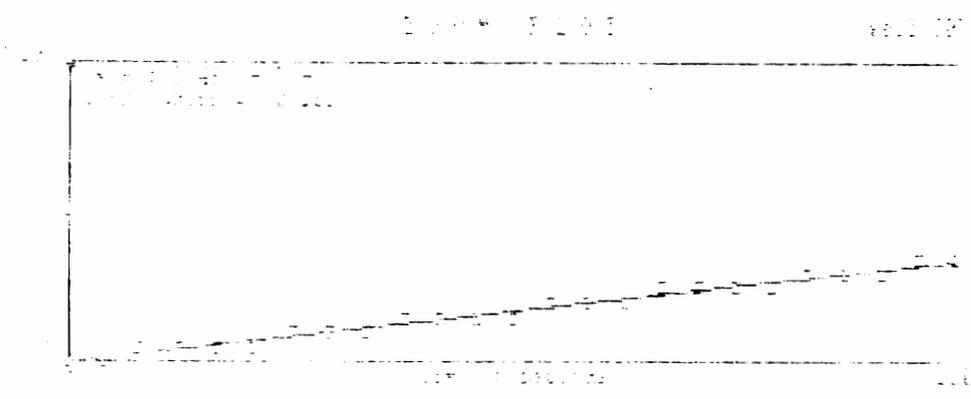


Figure 1. Kinetics of Cytochrome P450 Reductase Activity in CL.7 cells in m OD/min. The plot is a representative kinetic of six replicate readings.

TABLE 1.

Specific Activity of Cytochrome P 450 Reductase

| <u>Cell Line</u> | <u>units/mg protein</u> |
|------------------|-------------------------|
| BALBc CL.7 | 8.76 ± 0.32 |
| A-MuLV 3R.1 | 2.14 ± 0.02 |
| A-MuLV 6R.1 | 11.76 ± 0.5 |

20 μ l (1:4) sample + 80 μ l dH₂O was incubated with 5 ml of dye binding reagent for five minutes and protein concentration of the sample determined against a protein standard wave, using BSA, at 595 nm in a conventional spectrophotometer.

SUMMARY

Tumor necrosis factor- α (TNF- α), a 17kd multifunctional polypeptide, is secreted by activated macrophages and elicits hemorrhagic necrosis of certain kinds of tumors *in vivo* and is cytotoxic to several murine and human cell lines *in vitro*. TNF- α has also been shown to be growth promoting in normal human fibroblasts. It has been used in Phase I and Phase II clinical trials as an anticancer drug. Administration of TNF- α to cancer patients not only mediates hemorrhagic necrosis of some tumors but also potentiates the deleterious effects of endotoxic shock. TNF- α is, therefore, administered at a suboptimal dose in conjunction with other anti-tumorogenic cytokines like γ -interferon to reduce its toxic side effects. To understand the diversity in the mechanisms of TNF- α action in normal and transformed cells, it is important to establish that a normal cell line, formerly insensitive to TNF- α , exhibits altered TNF- α cytotoxicity when it is malignantly transformed. Abelson murine leukemia virus (A-MuLV), a replication defective retrovirus, induces *in vivo* transformation of B cells and potentiates *in vitro* transformation of fibroblasts in culture. A-MuLV-induced transformation was used to investigate the mechanism of the *in vitro* differential TNF- α cytotoxicity.

In vitro cytotoxicity of TNF- α to normal murine embryonic fibroblasts (BALB/c CL.7), and two of its Abelson-murine leukemia virus (A-MuLV) transformed clones (BALB/c A-MuLV 3R.1 and BALB/c A-MuLV 6R.1) were studied. The 6R.1 clone was found to be significantly more sensitive ($p < 0.05$) to TNF- α cytolysis than the CL.7 and 3R.1 cells. Thus, the % cytotoxicity of TNF- α at 100 units/ml in an 18 hr assay for CL.7, 3R.1, and 6R.1 cell lines were ($14.4 \pm 0.5\%$), ($24.1 \pm 3.5\%$), and ($49.4 \pm 2.9\%$) respectively. The murine TNF- α used in the investigation was obtained by immunopurification of *E Coli* lipopolysaccharide (LPS) activated RAW 264.7 macrophage supernatant over an anti-TNF- α mAb hydrazide column. We conclude that transformation of a TNF- α insensitive clone with the retrovirus A-MuLV can potentially alter

the susceptibility of the transformed clone to TNF- α . The following studies were performed to elucidate the mechanism of the differential susceptibility of these three cell lines to TNF- α .

(1) A protein factor, designated as cytotoxin inhibiting factor (CIF), was recently shown to inhibit TNF- α production from lipopolysaccharide (LPS) activated macrophage supernatants. The detection of such protein factor(s) present in the extracellular environment of CL.7, 3R.1, and 6R.1, as well as L929 (a TNF- α sensitive cell line) in modulating their susceptibility to the cytotoxic action of TNF- α was investigated. Initially the cell culture supernatants (CCS) from L929, CL.7, 3R.1, and 6R.1 confluent cultures, were tested for their ability to modulate the production of TNF- α in LPS activated rat alveolar macrophage cultures as determined by a 20 % SDS-PAGE. The CCS from CL.7 as well as 3R.1 was found to inhibit the TNF- α production in macrophage cultures whereas the CCS from 6R.1 did not do so. The CCS derived from TNF- α insensitive CL.7 clone at 1:2 dilution inhibited the cytotoxicity of 50 units/ml of TNF- α in TNF- α -sensitive L929 as well as 6R.1 clones ($p < 0.05$). Heat denatured CCS from CL.7 clone however did not show any significant protection. CCS from 3R.1, and 6R.1 transformed clones showed little cytoprotective effects in both native and heat denatured forms to L929 and 6R.1 against the toxic effects of TNF- α . Thus it appears that the differential cytotoxicity of TNF- α under A-MuLV transformation (Chapter1) could, in part, be due to the loss of production of cytoprotective protein factors from the transformed clones.

(2) At the level of the cell membrane of a TNF- α sensitive cell line, TNF- α affects the membrane permeability and the cell surface morphology. Therefore, the differential TNF- α cytotoxicity observed earlier in both the parent and transformed cell lines could be manifest as a differential change in membrane surface morphology and membrane permeability in these cells. Flow cytometric forward angle light scatter (FALS) and 90° light scatter (LI90) single and double parameter statistics were used to distinguish between CL.7, 3R.1, and 6R.1 clones. The A-MuLV transformed cells were distinctively smaller (as determined by FALS) and have smaller nuclear to cytoplasmic volume ratios (as determined by LI90) than

the untransformed CL.7 cells. A rapid flow cytometric assay was developed to monitor TNF- α cytotoxicity on a cell by cell basis on the above clones. The assay was established in the TNF- α sensitive murine L929 fibrosarcoma cell line. DNA bound propidium iodide (PI) fluorescence intensity, a measure of cell permeability, were plotted on a single parameter histogram for different TNF- α incubation times. The cell numbers in the fluorescence channels, represented in terms of the area under the PI fluorescence curve, was indicative of the cytotoxic effects of TNF- α . Incubation time with TNF- α , increased the cellular permeability for PI leading to an increase in DNA bound PI red fluorescence. The % cytotoxicity indices of CL.7 and the two transformed clones as determined by the flow cytometric assay were found to be comparable to those obtained by the standard tetrazolium dye reduction assay.

As TNF- α is known to alter the surface morphology of some cell types, especially endothelial cells, an investigation was performed to determine whether the differential cytotoxicity of TNF- α under A-MuLV transformation was reflective in the alterations of the surface morphology of the normal (untransformed) and the A-MuLV clones in presence of TNF- α . An electron microscopic investigation was carried out to observe changes in surface characteristics under the differential cytotoxicity of TNF- α . The scanning electron microscope (SEM) results suggest that TNF- α sensitive cells (L929 and 6R.1) tend to loose their surface morphology (blebs, microspikes, and lamellipodia) as compared to the TNF- α insensitive CL.7 and 3R.1 cells when treated with 100 units/ml of TNF- α for 18 hr. Thus it appears that the differential cellular permeability and the selective changes in the cellular morphology of the normal and the A-MuLV transformed clones under TNF- α cytotoxicity are signatures of the differential susceptibility of these clones to TNF- α .

(3) Cell surface expression of the TNF-receptor (TNF-R) is essential for binding and eliciting of TNF- α induced cytotoxic response. However, the constitutive TNF-R levels have not been correlated with the biological responsiveness of TNF- α . Whether the differential responsiveness of normal and A-MuLV transformed cells to TNF- α (Chapter 1) could, in part, be due to a differential

expression of the cell surface TNF-R was investigated. This objective was based on the hypothesis that the modulation of TNF-R by the viral transformation may lead to differential TNF- α cytotoxicity at the level of TNF- α uptake. The TNF- α binding and internalization assays were performed by using radio-labeled recombinant human (rh) TNF- α at a specific activity of 5.6×10^4 cpm/ng protein. The quantitative binding assay was performed at 4 $^{\circ}$ C for 2 hr. The internalization of TNF- α was monitored at 37 $^{\circ}$ C for 0 to 5 hrs. The total TNF- α binding and internalization kinetics in CL.7 and 6R.1 were found to be identical. The TNF- α insensitive A-MuLV clone 3R.1 however had a higher TNF-R content than the CL.7 or the 6R.1 clone ($p < 0.05$). The result of this study indicated that the differential cytotoxicity to TNF- α observed earlier in these three cell lines did not correlate with the TNF-R. Interestingly the binding of TNF- α to TNF-R on 3R.1 cells resulted in an increase in 3R.1 cell surface TNF-R content. The TNF-R induction further increased the kinetic of TNF- α uptake and internalization at 37 $^{\circ}$ C. The TNF- α induced cell surface TNF-R modulation has been known to be operative for macrophages. This is the first report where TNF- α induced TNF-R modulation has been shown for a fibroblastoid cell line.

(4) TNF- α is known to be a modulator of epidermal growth factor receptor (EGF-R) expression in normal human fibroblasts. The current investigation was based on the hypothesis that the TNF- α differential cytotoxicity observed in CL.7, and its two transformed cell lines 3R.1 and 6R.1 could have been due to differential TNF- α response in modulation of EGF-R. Transformation with A-MuLV altered the expression of EGF-R. The A-MuLV transformation not only suppressed the EGF-R levels for the 3R.1 clone, it also induced the expression of EGF-R in the case of 6R.1 cell line. Our results are in partial contradiction of the results of Blomberg et al. (1980). These authors claim that the transformation with A-MuLV leads to a loss of EGF-binding sites. EGF-R levels were determined as cpm 125 I-EGF bound/ μ g protein in six replicates for L929 fibrosarcoma, CL.7 fibroblast, and two of its transformed clones 3R.1, and 6R.1. The clones CL.7 and 3R.1 had lower levels of EGF-R, the clone 6R.1 expressed a significantly higher level of the receptor ($p < 0.05$). The

rates of induction of CL.7 and 3R.1 clonal EGF-R levels with TNF- α incubation time were found to be similar in the range of 0 to 17 hrs. The induction of EGF-R for 6R.1 cell line, however, was significantly higher than the CL.7 or the 3R.1 clones ($p < 0.001$). The 6R.1 clone thus not only possessed a high level of EGF-R but also had a greater potential to further its EGF-R induction in the presence of TNF- α . The induction of EGF-R for 3R.1 and 6R.1 with TNF- α was responsive to TNF- α concentration. The EGF-R content of the clones appeared to be associated with their susceptibility to TNF- α . The transformation with A-MuLV may be different from transformation by RNA sarcoma viruses with respect to the abolition of EGF-binding sites. Our results indicated that the TNF- α insensitive clones (CL.7 & 3R.1) had a lesser content of EGF-R than the clone (6R.1) with relatively higher TNF- α sensitivity.

(5) The biochemical events modulating TNF- α and EGF binding to their respective cell surface receptors were investigated. The binding and internalization were studied with radio-labeled TNF- α and EGF. The effect of modulators of the arachidonic acid cascade and protein kinase-C (PK-C) in governing the uptake and internalization of TNF- α and binding of EGF under the leukemia virus transformation was investigated. The EGF binding to EGF-R were inhibited by dexamethasone (phospholipase A_2 inhibitor), indomethacin (cyclooxygenase inhibitor), SKF-525A (cytochrome P450 inhibitor) and PMA (protein kinase C activator) for 6R.1 cell line indicating that phospholipase A_2 activated cyclooxygenase pathway, an active cytochrome P450, and protein kinase C all have the capability to modulate the binding of EGF to its receptor. SKF-525A and PMA were found to inhibit EGF binding for all clones. TNF- α binding to its receptor is significantly enhanced by the inhibitors of the cyclooxygenase pathway for all the cell lines ($p < 0.05$). Indomethacin as well as dexamethasone inhibited TNF- α binding to TNF-R for L929 cells. The inhibitors of the arachidonate pathway, known to reduce TNF- α cytotoxicity, were shown to modulate TNF- α action at the level of binding of the ligand to its receptor. The activators of PK-C are known to mediate down regulation of TNF- α binding capacity in both normal and malignant cells. In the leukemic transformation model only PMA had a non specific suppression of

TNF- α binding in CL.7, 3R.1, and 6R.1 clones. Oleyl acyl glycol (OAG), a PK-C activator, suppressed TNF- α binding for CL.7 but significantly enhanced its binding for 3R.1 and 6R.1 clones ($p < 0.05$). This differential TNF- α binding could possibly be due to the difference in PK-C activation of the normal and leukemia virus transformed clones. PMA induced suppression of TNF- α binding was accompanied by an enhanced TNF- α internalization for L 929 cells. PMA-activated PK-C, inhibited the EGF binding to EGF-R as well as TNF- α binding to its cell surface receptor in all the cell lines studied. On the contrary OAG-activated PK-C showed little inhibitory effect on TNF- α binding to TNF-R. These results indicated that the modulation of TNF- α and EGF binding to their cell surface receptors via PK-C activation need not be a general cellular growth surveillance property. SKF-525A inhibited the binding of EGF as well as TNF- α to all the cells. Thus it is possible that the inhibition of TNF- α cytotoxicity by SKF-525A could be at the level of TNF- α internalization. The early membrane events, revealed by the use of inhibitors of the arachidonic acid cascade and modulators of PK-C, could influence the binding and internalization of the ligands EGF and TNF- α , thereby controlling the physiologic future of the cell.

(6) The lipid soluble vitamins such as α -tocopherol (vitamin E) and β -carotene (vitamin A) are known to be potent anti-cancer agents. These vitamins are also known to be potent antioxidants. The ability of α -tocopherol, β -carotene, and dimethylsulfoxide (DMSO) to modulate the TNF- α inducible EGF-R expression in a malignant transformation system was investigated. The EGF-R was expressed in terms of the amount of radio-labelled EGF retained on the cells as cpm/ μ g protein. Vitamin A (1 mM) and Vitamin E (0.1 mM and 1.0 mM) reduced the TNF- α cytotoxicity to L929 cells ($p < 0.05$). Vitamin A (1.0 mM) suppressed the EGF-R expression both in presence and absence of TNF- α for CL.7 and 3R.1 clones ($p < 0.05$). In the 6R.1 clone vitamin E (1 mM) enhanced the TNF- α inducible EGF-R expression ($p < 0.05$). This enhancement was synergistic with TNF- α induction of EGF-R. Vitamin A suppressed the TNF- α inducible 6R.1 EGF-R ($p < 0.05$). These results indicated that vitamin A elicited an EGF-R suppression, independent of the EGF-R modulation by transforming agents like A-MuLV and ligands like TNF- α . This finding is

promising as it establishes the potential of vitamin A, under a complex interactive system, as an anticancer agent. Vitamin E, on the other hand facilitated the binding of EGF to EGF-R. Although vitamin A and vitamin E have previously been shown to be potential anticancer agents, vitamin A may be a better choice for cancers of epidermoid origin which have been known to possess a higher level of EGF-R levels. The modulation of EGF-R in A-MuLV transformed clonal fibroblasts by vitamin A and vitamin E suggests a potential role of such lipid soluble antioxidants in preventing progression of forms of cancers that depend on a highly expressed EGF-R levels on their cell surface.

(7) *V-abl*, an oncogene contained in the replication-defective transformation inducing retrovirus A-MuLV codes for the transmembrane protein p 120 with tyrosine kinase activity known to be essential for the transforming capacity of the virus. TNF- α has recently been shown to modulate the tyrosine phosphorylation of the transmembrane EGF-R (Bird et al, 1989). The overall objective of the current investigation was to determine whether the differential toxicity of TNF- α to the A-MuLV transformed clones was due to differences in the content of the viral antigen p120 on the cell surface and whether TNF- α elicits it's response by a differential transmodulation of tyrosine phosphorylation of oncogenic protein p120. Binding of control, anti-p15, anti-p30 and anti-A-MuLV (triton-disrupted) goat antibodies to CL.7, 3R.1 and 6R.1 cells grown on glass coverslips showed that the binding of anti-triton-disrupted A-MuLV antibody was higher for the transformed cells than for the untransformed cells ($p < 0.05$). Cellular phosphotyrosine levels were determined by use of radio labelled anti-phosphotyrosine antibody. Quantitation of phosphotyrosine levels for CL.7, 3R.1 and 6R.1 cells with incubation of TNF- α shows that CL.7 and 3R.1 had non-inducible phosphotyrosine levels, whereas cellular phosphotyrosine levels for the TNF- α sensitive cell line (6R.1) were inducible with the incubation time of TNF- α maximally induced around 8 hours. There was no significant change in the expression of A-MuLV gene product with incubation time of TNF- α . The expression of an equivalent amount of A-MuLV surface antigen when its non inducibility with TNF- α for 3R.1 and 6R.1 cell lines are added to the fact that cellular phosphotyrosine levels were inducible only in the 6R.1 cell line, a

likely possibility exists that 3R.1 would be a p120 kinase mutant whereas 6R.1 has a p120 kinase activity. p120 specific kinase activity was determined by an antigen capture assay. The inducibility of p120 phosphotyrosine was determined with TNF- α incubation time. 6R.1 clone possessed inducible phosphotyrosine levels, whereas 3R.1 had a transient induction. CL.7 and 3R.1, not sensitive to TNF- α , had low, hardly inducible p120 phosphotyrosine, whereas 6R.1 (the TNF- α sensitive cell line) has an inducible phosphotyrosine level which peaked around 8hr with TNF- α incubation. The p120 tyrosine phosphorylation, not known to be sensitive to any ligand, was shown to be sensitive to the cytokine TNF- α .

(8) In L929 cells the mitochondria were one of the earliest organelles to be affected by TNF- α (Matthews et al 1983). The mitochondrial toxicity affects the electron transport chain of the inner mitochondrial membrane which results in the accumulation of reducing equivalents. Potentially any such accumulation could reduce a cationic species, provided that the redox potentials were favorable. The TNF- α induced mitochondrial toxicity was investigated by monitoring the mitochondrial reduction of EPR signal height of a cationic spin probe 4-(N,N-dimethyl-N-hexadecyl) ammonium 2,2,6,6-tetramethyl piperidine-1-oxyl, iodide (CAT 16). Reduction of CAT 16 was used to investigate whether differential cytotoxicity to TNF- α under A-MuLV transformation in clones CL.7, 3R.1 and 6R.1 was manifest at the level of the toxicity to the mitochondria. The hyperfine splitting of the paramagnetic species CAT 16 gave rise to a 1:1:1 triplet EPR signal in an aqueous system. The spin label was found to be heavily quenched in presence of normal L929 mitochondria. Thus the mitochondria of the L929 cells incubated with TNF- α , gradually lost the ability to inhibit the CAT 16 signal with time. The decay of CAT 16 EPR signal with mitochondrial pellet from BALB/c CL.7, and two of its A-MuLV transformed clones 3R.1 and 6R.1 subjected to TNF- α quenched at different rates for different cell lines. When the slope of the decay of CAT 16 EPR signal was plotted against percent cytotoxicity for the same cell lines (MTT reduction assay) it was found that the susceptibility of a cell line to TNF- α did indeed correlated with the rate of quenching of the cationic paramagnetic CAT 16 signals.

Thus, the normal cell line CL.7 and one of its A-MuLV transformed clone found to be less sensitive to TNF- α had a higher ability to quench CAT 16 with TNF- α incubation time than the transformed clone 6R.1 which is more susceptible to TNF- α . We conclude that if a cell line is susceptible to TNF- α , its mitochondria loses ability to quench CAT 16 indicating mitochondrial toxicity. The quenching of CAT 16 EPR signal could be used as a sensitive method to compare relative TNF- α toxicity in cell culture systems.

(9) TNF- α may elicit its cytotoxic effect via an oxidative process. The reactive oxidants could manifested at the organelle level by inducing mitochondrial toxicity, fragmentation of cellular DNA, release of lysosomal enzymes and peroxidation of membrane lipids. Cells have evolved an array of defense enzymes to guard against this oxidative stress. We have investigated the antioxidant defenses in normal (CL.7) and A-MuLV transformed clones (3R.1 and 6R.1) under the action of TNF- α . The cellular antioxidants such as Cu/Zn- SOD, Mn-SOD, GSH-Px, GSH-Rx, total glutathione (GSH), G-6-PDH, and Cytochrome P450 were monitored. We have also determined the alterations in their antioxidant levels in response to the action of TNF- α as a function of time. We have developed sensitive microtiter assays for several of these enzymes to measure their levels in a smaller reaction volume. The 3R.1 clone had a significantly higher ($p < 0.05$) TNF- α noninducible Cu/Zn-SOD and Mn-SOD levels than the CL.7 and the 6R.1 clones. The Cu/Zn-SOD and Mn-SOD in CL.7 clone were however inducible with TNF- α incubation time. The constitutive cytochrome P450 levels were found to be higher in 3R.1 clone than in CL.7 or 6R.1 clones. The cytochrome P450 in 3R.1 and CL.7 clones unlike in 6R.1 were inducible with TNF- α incubation time. The A-MuLV transformed clones, unlike CL.7, do not induce their glutathione reductase levels with TNF- α incubation time. GSH-Px was significantly higher in 3R.1 than in CL.7 or 6R.1 clones. The GSH-Px was inducible with TNF- α incubation time in CL.7 as well as 3R.1 cell lines. From the above results we concluded that CL.7 protects itself from TNF- α cytotoxicity by inducing its defense enzymes when cells are incubated with TNF- α . Although the 3R.1 clone does not induce its defense enzymes upon TNF- α action is able to protect itself from a TNF- α mediated oxidative damage via a high

level of constitutive defense status. The 6R.1 clone on the other hand neither has high levels of antioxidant enzymes, nor are the enzymes inducible with TNF- α incubation. Thus 6R.1 clone is not prepared to cope with the oxidative stress induced by TNF- α .

(10) Recent investigations have demonstrated the generation of reactive species of oxygen, in particular hydroxyl radicals, by the action of TNF- α on TNF- α sensitive cell lines, using the production of methane in presence of dimethylsulfoxide (DMSO). These authors (Yamauchi, et al. 1989) failed to demonstrate the primary reactive species in this reaction. We quantitated the radical species produced under the action of TNF- α by using spin trapping in conjunction with electron paramagnetic resonance (EPR) spectrometry. L929 fibrosarcoma cells were incubated for a fixed time with and without 100 units/ml TNF- α . PBN, a spin trapping agent, was added at a final concentration of 14 mM, 1/2 hour prior to the incubation period. Incubation with TNF- α produced a three line spectrum with hyperfine splitting constants of $a_N = 14.75\text{G}$ and $a_H^{\beta} = 2.75\text{ G}$ characteristic of PBN-OOH adduct. Cytotoxicity of TNF- α for L929 cells has been known to be inhibited significantly in the presence of 70 μM desferrioxamine-MnO₂ complex (known as a superoxide dismutase mimic). In our system, incubation of cells with 10 μM desferrioxamine-MnO₂ complex inhibited the formation of PBN-OOH adduct by 30% indicating that superoxide (O₂⁻) radicals are produced during TNF- α action.

(11) The generation of other stable reactive species such as H₂O₂ during TNF- α action in a TNF- α susceptible cell line was investigated. The use of 2',7'- dichlorofluorescin (DCFH) is one of the most convenient ways by which intracellular H₂O₂ can be measured. Non-fluorescent DCFH was first loaded into the vital cells by enzymatic cleavage of the membrane-permeable 2',7'-dichlorofluorescin diacetate (DCFDA). Once DCFDA entered the cell, it was cleaved by an intracellular nonspecific esterase to form intracellular DCFH. Intracellular DCFH was oxidized to fluorescent 2',7'- dichlorofluorescin (DCF) upon reaction with intracellular H₂O₂, and the green fluorescence of DCF measured at emission around 520 nm in conjunction with flow cytometry, allowing for the quantitation of intracellular H₂O₂ on a cell by cell basis. TNF- α

sensitive L 929 cell samples were analyzed by a two parameter plot of forward angle light scatter (linear scale) and 90° light scatter (log scale) in a flow cytometer. The linear-logarithmic plot showed two distinct populations namely small cells (MAP 1) and the larger cells (MAP 2) which were analyzed separately and jointly for green fluorescence of DCF. MAP 2 cells were found to be a viable subpopulation of L929 cells and MAP 1 cells were non viable cells as determined by direct quantitation of the cells under the two subpopulations under TNF- α cytotoxicity. Unlike the MAP 1 cells, the MAP 2 cell number decreased with TNF- α incubation time. H_2O_2 production in MAP 2 cells increased sharply with concentration as well as time incubation of TNF- α . The total cell population did not show any significant increase in H_2O_2 production perhaps because the enhanced production of H_2O_2 by the MAP 2 cells may have compensated for the loss of H_2O_2 production by MAP 1 cells. We concluded therefore that H_2O_2 was one of the reactive species of oxygen produced when L 929 cells were incubated with TNF- α . We investigated the effect of various lipid soluble antioxidants on the H_2O_2 production. DMSO, at 500 mM, increased the H_2O_2 production in all the cell subpopulations. Vitamin E at both 0.1 and 1.0 mM concentrations decreased the production of TNF- α induced H_2O_2 production in MAP 2 and MAP 3 cells. Thus the status of vitamin E in inhibiting the peroxidative chain reaction could not only be in nonspecific termination of lipid peroxidation but also an inhibition of H_2O_2 production. Vitamin A at 0.1 mM had no effect on H_2O_2 production and at 1.0 mM was found to be synergistic with TNF- α induced H_2O_2 production. The increased H_2O_2 production could explain the causes of cellular toxicity by vitamin A at a high enough concentration. It is possible that the generation of O_2^- and H_2O_2 could, in part, mediate the cytotoxic response of TNF- α in these cells.

(12) The oxidative damage to cells caused by TNF- α could result in peroxidation of membrane lipids. We demonstrated that the TNF- α -induced peroxidative event could be monitored by measuring the differential quenching of the spin label 2, 2, 6, 6-tetramethyl-1-piperidine-n-oxyl (TEMPO) signal in TNF- α sensitive L929 cells using EPR spectroscopy. The cell-induced loss of EPR signal of

nitroxide spin label was shown to depend on TNF- α concentration, time of incubation of TNF- α and time of incubation of TEMPO with cells. Decrease in the rate of reduction of TEMPO with time of incubation of TNF- α correlated with time kinetics and concentration of TNF- α cytotoxicity respectively. The loss of TEMPO EPR signal under the action of TNF- α was determined to be towards the extracellular domain of the lipid bilayer as elucidated by the quenching and broadening of the TEMPO signal in presence of NiCl₂. From the above data, we concluded that the rate of quenching of a spin label spectra could be indicative of the peroxidative stress on a cell. Not only can spin labels inhibit lipid peroxidation but also, in comparison to normal cells, get reduced differentially by cells under peroxidative chain reactions. Thus reduction of a lipid soluble spin label like TEMPO as monitored by EPR could be an useful tool to study TNF- α cytotoxicity.

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 - 7664 (Off)**BIRTH :** July 30, 1961, Orissa, India **HEALTH :** Excellent**CITIZENSHIP :** India **VISA STATUS :** F-1 **MARITAL STATUS :** Single**EDUCATION :****(1) Doctoral Degree**

| | |
|-------------------------------|---|
| <i>Name of Degree :</i> | Ph.D |
| <i>Date Expected :</i> | April 1991 |
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| <i>Major field of study :</i> | Biochemistry |
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| <i>Dissertation :</i> | Mechanism of TNF- alpha Cytotoxicity In a Retroviral Transformation Model. |
| <i>Major Advisor :</i> | Dr. Hara, P. Misra, Chairman and Head of Department, Department of Biomedical Sciences VMRCVM, Virginia Tech |
| <i>External Examiner :</i> | Dr. Phillip H. Pekala, Department of Biochemistry, East Carolina School of Medicine, Greenville, NC |

(2) Master's Degree

Name of degree : M. Sc
Date conferred : April 1982
Granting institution : Department of Physics,
Delhi University, New Delhi, India
Major field of study : Theoretical Physics
Minor field of study : General Relativity, Quantum Field
Theory, and Particle Physics
University Rank : Fourth position in the University

(3) Bachelor's Degree

Name of degree : B. Sc
Date conferred : April 1980
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Major field of study : Physics
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Chemistry
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(4) Higher Secondary Education

Name of degree : A. I. H. S.
Date Conferred : April 1977
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Major field of study : Science
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PROFESSIONAL EXPERIENCE :

Honors/Awards :

| | |
|--|---------|
| School Captain | 1977 |
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| Secretary, Physics Society, St. Stephen's College, Delhi University | 1979 |
| National Science Scholarship 1980-82 | |
| Department of Atomic Energy Fellowship Bhaba Atomic Research Center | 1983-85 |
| Postgraduate Research Assistantship | 1986-91 |
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| Member Indian Student's Association | 1986-91 |

Professional Society Membership :

American Association For Advancement of Science
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Phi Sigma Society (Alpha Psi Chapter)
Association of Scientists of Indian Origin

Teaching Experience:

Lecturer, Department of Physics,
Hindu College, Delhi University 1982-83
Courses Taught :Advanced Mathematical
Physics (third year under graduates);
Advanced Mathematical Physics
(second year undergraduates);
Thermal Physics (Second year
undergraduates); Physics Laboratory
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Lecturer, Department of Physics,
St. Stephen's College, Delhi University. 1983-84
Courses Taught :Statistical Mechanics
(Third year Undergraduates); Advanced
Mathematical Physics (Second year
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(First Year Undergraduates)

Graduate Teaching Assistant, 1990-91
Courses Taught : Medical Biochemistry
(Molecular Cell Biology, First year students
Department of Biomedical Sciences, VMRCVM.);
and Membrane Physiology (EPR, and Flow Cytometric
Techniques; Biomedical Science Graduate Students)

Research Experience :

Graduate research assistant,
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VMRCVM; Virginia Tech, Blacksburg 1986- 91

Involvement In Funded Research Projects :

- i) Adriamycin induced cardiotoxicity (1986-87)
- ii) Microwave Effects on Biomembranes (1986-87)

- iii) Role of free radicals in lung reperfusion injury (1988-89)
- iv) Antioxidant properties of Ridogrel (1989-90)
- v) Treatment of bovine brucellosis by anti-TNF-alpha antibodies (Extramural funding).

PUBLICATIONS :

Refereed Journals :

(1) Mishra, S. (1991) Mechanism of TNF- alpha Cytotoxicity In a Retroviral Transformation Model Ph.D Thesis, Virginia Polytechnic Institute and State University.

Manuscripts Submitted ** / Completed * / Under Preparation :

(1) Mishra, S and Hara, P. Misra. (1991) Modulation of epidermal growth factor- receptor expression in AbL transformation, and its induction by tumor necrosis factor-alpha. (**, Submitted to Nature).

(2) Mishra, S and Hara, P. Misra. Monitoring TNF-alpha induced mitochondrial cytotoxicity by CAT 16 quenching using electron paramagnetic resonance (EPR) spectrometry. (*)

(3) Mishra, S and Hara, P. Misra. Generation of free radicals of oxygen under TNF cytotoxicity : an EPR study. (*)

(4) Mishra, S and Hara, P. Misra. Hydrogen peroxide production under TNF-alpha cytotoxicity : a flow cytometric approach. (*)

(5) Mishra, S and Hara, P. Misra. Differential cytotoxicity of TNF-alpha under A-MuLV induced leukemic transformation.(*)

(6) Mishra, S and Hara, P. Misra. Induction of tumor necrosis factor receptor (TNF-R) and epidermal growth factor receptor (EGF-R) under A-MuLV transformation.

(7) Mishra, S and Hara, P. Misra. TNF-alpha modulates tyrosine phosphorylation of V-*abl* oncogene protein product p120. (*)

(8) Mishra, S and Hara, P. Misra. Microtiter assay systems for cellular defense enzymes.

(9) Mishra, S and Hara, P. Misra. Use of TEMPO in monitoring TNF-alpha cytotoxicity in L929 cells by EPR technique. (*)

Abstracts published :

- (1) Mishra, S and Hara, P. Misra. (1991). Tumor necrosis factor modulates tyrosine phosphorylation of A-MuLV specific cell surface antigen (7251). The FASEB J. 5 (6) : 1619.
- (2). Mishra, S and Hara, P. Misra. (1991). EPR observations of TEMPO quenching in monitoring TNF-alpha cytotoxicity in L929 cells. in Rocky mountain conference on analytical chemistry. Conference Proceedings.
- (3) Deborah Ho, R. Lance , Johanson, Mishra, S and Hara, P. Misra. (1990). The role of free radicals during reperfusion injury in isolated rat lungs. American Physiological Society Conference proceedings.
- (4) Mishra, S and Hara, P. Misra. (1990). Reversal of superoxide dismutase reaction as detected by EPR spin trapping techniques. 2115, in Conference proceedings of Federation of American Society of Experimental Biology.
- (5) Mishra, S and Hara, P. Misra. (1990). TNF-alpha cytotoxicity and EGF-R Expression Under a Retroviral Transformation, in *Growth factor receptors in cancer* Conference proceedings.
- (6) Mishra, S and Hara, P. Misra. (1990). Effect of alpha-tocopherol and beta-carotene in EGF-R expression and differential TNF-alpha cytotoxicity under A-MuLV transformation. American Institute of Cancer Research, First annual conference of Cancer.
- (7) Melendez, J. A, Mishra, S., and Hara, P. Misra. (1989). Regulation of manganese superoxide dismutase in endothelial cells. J. Cell Biol 107, 217a.
- (8) Mishra, S. J. A. Melendez and Hara, P. Misra (1989). Mechanism of microwave-induced membrane damage in human erythrocytes. J cell biol 107, 188a.
- (9) Mishra, S and Hara, P. Misra. (1987). Broken symmetries of biomolecules. Biophysical J. 51, 96a.

AID IN WRITING GRANTS :

- (1) Treatment of bovine brucellosis by anti-TNF-alpha antibodies (Intramural funding).
- (2) Ameolioration of Feline leukemia by TNF-alpha (Submitted).

FUTURE RESEARCH OBJECTIVES :

My general research goals are in understanding of the genetic, biochemical, and pharmacological basis of the effect of immunotherapeutic proteins like tumor necrosis factor (TNF) in fight against human cancers. I am very much interested in investigating the effect of TNF and other anti-cancer cytokines in expression and regulation of cellular and viral oncogenes and their protein products. The regulation of epidermal growth factor receptor by TNF as well as by translation products of tumor supressor genes and by anti onco-fetal genes are specifically interesting to me.